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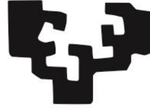

Universidad del País Vasco Euskal Herriko Unibertsitatea

CONTRIBUTION OF X-LINKED GENES TO THE ORIGIN AND DEVELOPMENT OF INTELLECTUAL DISABILITY IN PATIENTS FROM THE SPANISH BASQUE COUNTRY

Doctoral thesis

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“Erokeriaren bat, inozokeriaren bat,
egin beharko zenuke: zoriontsu izan
edo holako zeozer”

Gorka Urbizu. Berri Txarrak.

Aitari eta amari,

Nire ahizpa Aiorari,

Koldori

Hace ya más de cuatro años que llegué a Cruces y comencé esta aventura “¿Qué es una aventura? un mal rato que acaba bien”. Estos cuatro años han sido un viaje en una montaña rusa y este último año nada fácil. Pero estoy orgullosa de haber llegado hasta el final. Estos años me han servido mucho de aprendizaje tanto en lo profesional como en lo personal y quisiera dar las gracias a toda la gente que durante estos años ha sido parte de esta aventura y haberme ayudado de una manera u otra.

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Bereziki eskertu nahi dut nire alboan egon den eta dagoen jende guztia, beraiek gabe ez baitzen posible izango gaur honaino iristea. Ez gaude ohituak beti hor dagoen jende horri hitz batzuk eskaintzera eta une hau aprobetxatu nahi dut horretarako. Merezi dabenengatik, gora kopak!

Nire betiko lagun eta herriko kuadrilla, txikitatik alboan izan ditudanak. Koldoren kuadrillakoei eta neskei, kuadrillako beste bat banitz bezala sentiarazi nauzuelako.Eta inongo kuadrillaren parte izan ez arren, bidean ezagutu ditudan lagunei.

Koldoren familiari, Evari eta Jesus Mariri etxean bezala hartu nautelako. Pili eta Pedrori martitzenetako afari goxoengatik.

Aiora eta Olatz lehengusinen arteko bidaiengatik. Europan zehar Londres, Dublin, Belfast, Amsterdam … pasa ditugun momentuengatik eta bota ditugun barreengatik.

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Nire aitxitxe Albertori, berandu. Faltan botatzen ditut “Argiñanoren” sukaldeko menuak eta elkarren arteko zirikatzeak. Faltan botatzen dut zu hemen gurekin egotea eta nirekin amaiera hau ospatzea “Maria Cristinan” edo herriko tabernan. Sentitzen dut denbora gehiago zurekin pasatu ez izana. Zauden lekuan zaudela espero dut ni zutaz bezain harro sentitzea gutaz.

Nire ahizpa Aiorari, beti txakur eta katuak lez ibili izan bagara ere beti elkarrentzat hor gaudelako. Eta elkar lotzen gaituen hori irudikatzeko tatuajerik behar ez dudalako.

Aitari eta amari, inoiz hitzez gauzak adierazten ez ditugun arren asko maite zaituztedalako. Niretzako beti hor egon zaretelako, askatasuna eman didazuelako nire erabakiak hartzeko eta beti babestu izan nauzuelako.

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ABBREVIATIONS

ABC	ATP-binding cassette
aCGH	Array Comparative Genomic Hybridization
ACMG	American College of Medical Genetics
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole acid
AR	Androgen Receptor
Arf	ADP rybosilation factor
ASD	Autism Spectrum Disorder
CAM	Cell Adhesion Molecule
CC	Coiled coil
CCDS1	Cerebral Creatine Deficiency Syndrome 1
CLS	Coffin-Lowry Syndrome
CDKL5	Cyclin-Dependent Kinase-Like 5
CNV	Copy Number Variant
CRTR or CT1	Creatine Transporter
EGF	Epidermal Growth Factor
EJC	Exon-Junction Complex
ESHG	European Society of Human Genetics
Euro-MRX	European Mental Retardation X Consortium
EVS	Exome Variant Server
FGS1	Opitz-Kaveggia Syndrome or FG Syndrome 1
FISH	Fluorescent In Situ Hybridization
FMR1	Fragile Mental Retardation 1
FXPOI	Fragile X-associated Primary Ovarian Insufficiency
FXS	Fragile X Syndrome
FXTAS	Fragile X-associated Tremor/Ataxia Syndrome
GDD	Global Developmental Delay
GEF	Guanine Nucleotide Exchange Factor
HGMD	Human Gene Mutation Database
H-MRS	Proton Magnetic Resonance Spectroscopy
ID	Intellectual Disability
Ion PGM™	Ion Personal Genome Machine™
IPSC	Induced Pluripotent Stem Cell
IQ	Intelligence Quotient
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MAF	Minor Allele Frequency
MAGUK	Membrane Associated Guanylate Kinases
MECP2	Methyl CpG binding protein 2
MLPA	Multiple Ligation-dependent Probe Amplification

NGS	Next Generation Sequencing
NHGRI	National Human Genome Research Institute
NHLBI	National Heart, Lung and Blood Institute
NMD	Nonsense Mediated mRNA Decay
NMDA	N-methyl-D-aspartate
NS-ID	Non-Syndromic/Specific Intellectual Disability
NS-XLID	Non-Syndromic/Specific X-linked Intellectual Disability
PCR	Polymerase Chain Reaction
REN3B	Regulator of nonsense mediated mRNA decay 3B
PQL domain	Proline-Glutamine and Leucine rich domain
RSK	Ribosomal S6 Kinase
SHH	Sonic Hedgehog
S-ID	Syndromic Intellectual Disability
SLC	Solute carrier
SNV	Single Nucleotide Variant
S-XLID	Syndromic- X-linked Intellectual Disability
UCSC	University of California Santa Cruz
VUS	Variant of Unknown/Uncertain Significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
XLID	X-linked Intellectual Disability

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- **Ibarluzea** N, de la Hoz AB, Villate O, Llano I, Ocio I, Martí I, Guitart M, Gabau E, Andrade F, Gener B, Tejada MI. Targeted Next-Generation Sequencing in Patients with Suggestive X-Linked Intellectual Disability. Genes (Basel). 2020;11(1):51. doi:10.3390/genes11010051.

- Tejada MI, **Ibarluzea N**. Non-syndromic X linked intellectual disability: Current knowledge in light of the recent advances in molecular and functional studies. Clin Genet. 2020:1-11. doi:10.1111/cge.13698.

- Tejada MI, Elcoroaristizabal X, **Ibarluzea N**, Botella MP, de la Hoz AB, Ocio I. A novel nonsense homozygous variant in the NLGN1 gene found in a pair of monozygotic twin brothers with intellectual disability and autism. Clin Genet. 2019;95(2):339-340. doi:10.1111/cge.13466.

- Aguilera-Albesa S, de la Hoz AB, **Ibarluzea N**, Ordoñez-Castillo AR, Busto-Crespo O, Villate O, Ibiricu-Yanguas MA, Yoldi-Petri MP, García de Gurtubay I, Perez de Nanclares G, Pereda A, Tejada MI. Hereditary spastic paraplegia and intellectual disability: clinicogenetic lessons from a family suggesting a dual genetics diagnosis. Front Neurol. 2020;11:41. doi:10.3389/fneur.2020.00041.

INTRODUCTION

The 1.7. section has been published as an "Invited Review":

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1. INTELLECTUAL DISABILITY

1.1. Definition

Intellectual disability (ID) is an early childhood neurodevelopmental disorder and is defined by impaired intellectual functioning and adaptive behaviour with an onset before 18 years old (Schalock *et al.*, 2010). Adaptive behaviour encompasses a set of conceptual, social, and practical abilities that are acquired and performed by people in their daily routine (Schalock *et al.*, 2010; Moeschler and Shevell, 2014). In order to make a diagnosis, the Intelligence Quotient (IQ) test is usually performed. The IQ test evaluates skills like learning, reasoning, and problem solving and the degree of intellectual impairment can be classified into mild, moderate, severe or profound (Table 1). However, this test or other tests that measure developmental skills cannot be performed until the child is older than 5 years as they are not reliable and valid before that age. Instead, the term Global Developmental Delay (GDD) is used in younger children (Schalock *et al.*, 2010; Moeschler and Shevell, 2014). GDD is defined as having significant delay in achieving 2 or more of the developmental milestones such as speech/language, cognition, motor and social skills at the expected age. This diagnosis may serve to later predict ID, but mild forms might be temporary and will therefore lack of predictive ability for ID or other developmental delays (Moeschler and Shevell, 2014).

Table 1. Classification of Intellectual Disability based on the IQ score.

Category	IQ score
Mild	50-55 to approx. 70
Moderate	35-40 to 50-55
Severe	20-25 to 35-40
Profound	<20-25

ID co-occurs with dysmorphic features or other neurological disorders such as epilepsy, autism spectrum disorders (ASD) and sensory impairments many times. The disorder could also comprise the impairment of other organs and presence of malformations.

1.2. Epidemiology

A recent population meta-analysis estimated that prevalence of ID worldwide is about 1% (Maulik *et al.*, 2011) and consequently places a serious burden on families, society and medical care.

The prevalence varies from one population to the other being nearly twice higher in lower-middle income countries (Maulik *et al.*, 2011). This could be easily explained by the deficits in prenatal screening methods or health care, poverty or food security. Indeed, birth injury, asphyxia, intra-uterine growth retardation and infections are common perinatal causes.

Consanguinity also contributes to a higher prevalence of ID owing to the recessive forms. Actually, prevalence of ID has been shown to be highly correlated with frequency and degree of parental consanguinity, the prevalence being 2-3 times higher in inbred populations (Morton, 1978; Bundey and Alam, 1993; Fareed and Afzal, 2014; Jamra, 2018).

It has also been observed that prevalence for mild ID is higher (85%), being lower in moderate (10%), severe (4%) and profound (2%) ID. The prevalence of mild ID is also more variable while the prevalence of severe ID remains constant. This could be attributed to either methodological issues or environmental factors (Roeleveld *et al*., 1997; Leonard and Wen, 2002; Maulik *et al.*, 2011).

Likewise, the prevalence of ID is higher in males (Roeleveld *et al*., 1997; Leonard and Wen, 2002; Maulik *et al.*, 2011; McKenzie *et al.*, 2016) mainly due to higher prevalence in mild ID, as the male to female ratio decreases as IQ value decreases (Maulik *et al.*, 2011). Obviously, X-linked conditions contribute significantly to the higher prevalence in males (Roeleveld *et al*., 1997; Stevenson and Schwartz, 2009).

Despite many population studies being carried out to assess the prevalence of ID, these studies lack consistency due to variations in methodology such as case ascertainment method, data source and study design. Indeed, A more recent meta-analysis published by Mckenzie *et al*. (2016) intended to give an update on the epidemiology of ID but failed in corroborating the results reported by Maulik *et al*. (2011) due to the variability of study design of the prevalence and incidence studies. However, this study suggested that the prevalence of ID might be lower than 1% now. For sure, improvements in health care and prenatal screening programs must have contributed to a decreased number of patients with ID. Nevertheless, robust epidemiological studies are needed to evaluate the effect of any of these factors.

1.3. Clinical evaluation

Intellectual Disability is comprised by extremely heterogeneous set of rare diseases and can be caused by environmental factors during perinatal, prenatal or postnatal period or genetic alterations, or often combined with each other in milder and more complex forms of ID. Environmental causes include maternal exposure to toxic substances (i.e. chemicals, drugs or alcohol) and radiation during pregnancy, prenatal infections and vascular accidents and birth complications like asphyxia. Similarly, genetic factors are estimated to contribute to up to 50-60% of ID (Willemsen and Kleefstra, 2014). These include chromosomal aberrations,

imprinting/epigenetic disorders and monogenic disorders which include inborn errors of metabolism. This heterogeneity makes a good clinical evaluation necessary.

ID/GDD is the most common reason for referral to medical genetics clinic and comprehensive clinical evaluation is required to assess these patients. This process involves different health care specialist such as primary care paediatrician, paediatric neurologist and medical geneticist.

If the patient is a child, it is the primary care paediatrician's responsibility to supervise their development and identify any delays in acquiring any of the developmental milestones. If any developmental impairments are identified the child should be referred to the appropriate consultant to determine the type of developmental delay (Moeschler and Shevell, 2006, 2014).

The main goal of the clinical evaluation of a child with ID or GDD is to identify the underlying cause. To that end, it is important to identify the type of developmental delay as not all developmental delays lead to ID and different approaches might be taken depending on each case (Moeschler and Shevell, 2006, 2014). The Bayley Scale of Infant and Toddler Development (3rd edition) (Michalec, 2011) enables an early identification and quantification of developmental delay in infants between 1-42 months by measuring the child's development in five areas: 1) Cognitive 2) Language and 3) Motor scales and caregiver ratings of 4) Social-Emotional and 5) Adaptive behaviour. However, Bayley scale might not adequately predict cognitive impairments (Spencer-Smith *et al.*, 2015; Anderson and Burnett, 2017). Therefore, cognitive function should be assessed in children with developmental delays in order to determine intellectual disability and assess its severity. The Wechsler Intelligence Scale is the most widely used cognitive test and includes three different scales of intelligence: The Wechsler Preschool and Primary Scale of Intelligence (4th edition) (2.6–7.7 years) (Wechsler, 2012), The Wechsler Intelligence Scale for Children (5th edition) (Wechsler, 2014) (6.0–16.11 years) and The Wechsler Adult Intelligence Scale (4th edition) (Wechsler, 2008) (16.0–90.11 years). The last scale is very important when the evaluation is not carried out on a child but on an adult patient. All of these tests provide an estimate of global intellectual functioning (Full Scale IQ) by evaluating verbal comprehension, perceptual reasoning, working memory and processing speed.

Obtaining an etiologic diagnosis has been demonstrated to be of great benefit for both the family and medical care. Indeed, it can help in predicting the clinical course and prognosis of the disease and also evaluating the treatment options available. Knowing the exact genetic cause also enables to assess the family for recurrence risk. Moreover, families can benefit from specific support and research treatment protocols to guarantee best health, social and educational services for both the child and family (Moeschler and Shevell, 2006, 2014).

An optimal clinical evaluation of a child with ID/GDD should include a family pedigree of at least 3 generations, with relevant family history of ID/GDD, congenital malformations, miscarriages, stillbirths, early childhood death and other psychiatric conditions (Moeschler and Shevell, 2006, 2014). A thorough physical examination could also be crucial to identify dysmorphic features that fit a syndrome and should involve an experienced clinical geneticist. Neurological abnormalities should also be taken into account at the physical examination

(Moeschler and Shevell, 2006, 2014). Environmental factors should also be excluded before proceeding with the genetic evaluation.

1.4. Genetic evaluation

If a specific condition is suspected on the initial clinical investigations specific tests should be performed in order to confirm the diagnosis. Some clinical presentations are associated with a specific gene or genetic alteration and are straightforward to assess by either single gene testing or chromosome analysis. Clinical conditions that show some clinical and genetic heterogeneity however are tested through disease or phenotype specific gene panel sequencing (i.e. epilepsy; rasopathies…) (Xue *et al.*, 2015).

On the contrary, if the patient shows an unspecific phenotype, basic or first tier genetic tests should be undertaken; and if no diagnosis is stablished, second tier tests should be followed. A possible clinical genetic evaluation protocol is displayed in Figure 1.

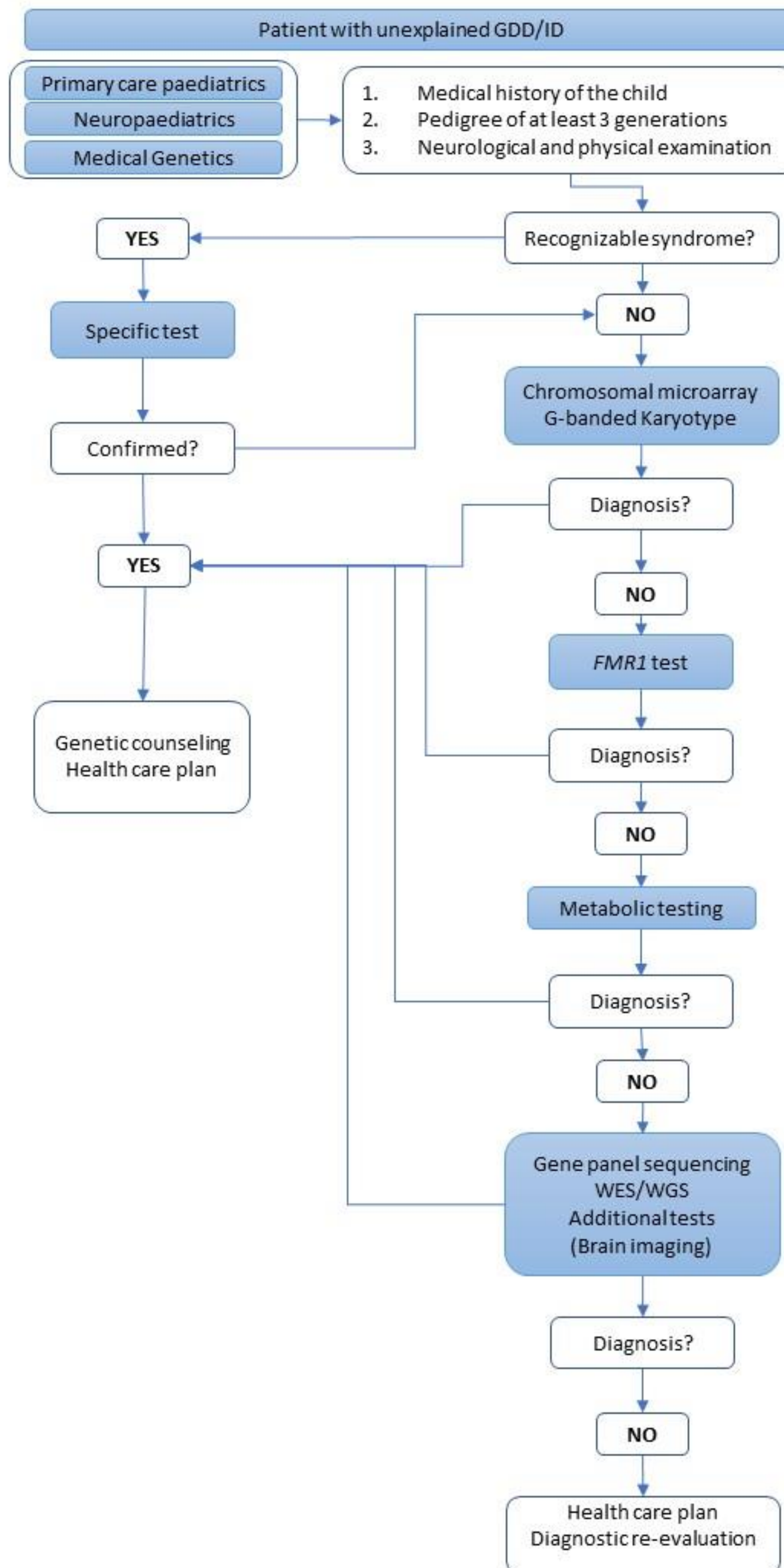


Figure 1. Suggested clinical genetic evaluation approach in patients with unexplained ID/GDD. Adapted from Moeschler and Shevell (2014) and Vasudevan and Suri (2017).

1.4.1. G-banded karyotype and chromosomal microarray

Chromosomal abnormalities are known to contribute to 25% ID (Willemsen and Kleefstra, 2014). Chromosomal aneuploidies which are detectable by conventional G-banded karyotype are responsible for 15% of ID (van Karnebeek *et al.*, 2005; Michelson *et al.*, 2011; Willemsen and Kleefstra, 2014) and Down syndrome or trisomy 21, is the most common form.

Although G-banded karyotypes were routinely analyzed for years, chromosome microarrays such as array comparative genomic hybridization (aCGH) have now replaced the conventional karyotype in patients with ID/GDD, ASD or congenital anomalies (Miller *et al.*, 2010; Flore and Milunsky, 2012; Moeschler and Shevell, 2014). This method has a much higher resolution and is also able to detect mosaicism at the same level as the conventional karyotype (Waggoner *et al.*, 2018). It is already known that submicroscopic chromosomal microdeletions and microduplications like Angelman and Prader Willi syndrome together with other small copy number variants (CNV) such as subtelomeric rearrangements detected by chromosome microarray techniques or by Multiplex Ligation-dependent Probe Amplification (MLPA) account for 10% of ID cases (Michelson *et al.*, 2011; Willemsen and Kleefstra, 2014). Altogether, aCGH has a higher diagnostic yield (15-20%) compared to the conventional karyotype (3% approx. excluding recognizable chromosomal syndromes such as Down syndrome) (Miller *et al.*, 2010).

Nevertheless, chromosome microarrays have limitations in detecting balanced rearrangements. Indeed, a recent literature review estimated that 0.78-1.3% of the patients with normal chromosomal microarray show balanced rearrangements by G-banded karyotype analysis or Fluorescent In Situ Hybridization (FISH); and 0.56-0.91% show disruptive breakpoints (gene or regulatory domain disruptions) explaining the patient’s phenotype (Waggoner *et al.*, 2018). Anyway, given the low prevalence, they concluded that it might not be cost effective to do a G-banded karyotype after a chromosome microarray.

Furthermore, recurrent microduplication and microdeletions with variable penetrance might be detected as well as variants of unknown/uncertain significance (VUS) of which the interpretation might be difficult. Therefore it is important for the molecular diagnostic laboratory to work close to clinical geneticists when interpreting the results of chromosome microarrays (Moeschler and Shevell, 2014).

Even so, conventional karyotype is still the recommended baseline test if a chromosomal syndrome such as trisomy 21 is suspected or FISH in patients in which microduplication or microdeletion syndromes are suspected (Flore and Milunsky, 2012).

1.4.2. *FMR1* test

Monogenic forms of ID generally show Mendelian inheritance and can be divided into autosomal dominant, autosomal recessive and X-linked forms. All in all, today more than 1200 genes both autosomal and X-linked are known to be involved in the aetiology of ID (SysID database) (Kochinke *et al.*, 2016). As Fragile X Syndrome (FXS) is the most common monogenic form of ID, analysis of the *FMR1* (Fragile Mental Retardation 1) CGG triplet repeat is considered to be a first line test in the evaluation of a patient, either male or female, with

unexplained ID/GDD as it has been shown that it has approximately 2% diagnostic yield in males and females. However, unless there is a strong evidence either X-linked family history or clinical features, molecular karyotype analysis is performed first since it has a higher diagnostic yield (Flore and Milunsky, 2012).

1.4.3. Metabolic testing

Recommendations on ID/GDD evaluation are based on frequencies of single disease conditions and diagnostic method yields. Despite inborn errors of metabolism having a low prevalence, metabolic screening should also be considered in patients with unknown aetiology of ID as specific clinical symptoms of metabolic diseases might not appear at the initial evaluation of ID/GDD and they hold great potential for treatment after diagnosis (van Karnebeek and Stockler, 2012; van Karnebeek *et al.*, 2014). In total, 89 inborn errors of metabolism that have ID as the main feature have been identified and most of them are treatable and can be systematically screened by urine and blood metabolic tests (van Karnebeek *et al.*, 2014).

Many countries have already introduced newborn screening methods for treatable metabolic disorders. However, these are quite recent and usually include a few conditions, and normal newborn screening results in a patient with GDD/ID do not completely rule out inborn errors of metabolism. Therefore, metabolic screening in blood and urine should still be applied as first line tests (van Karnebeek *et al.*, 2014).

1.4.4. Additional tests: Next Generation Sequencing

After baseline investigations are carried out, candidate genes might be tested to try to uncover the origin of ID/GDD. Several gene panels are offered in diagnostic laboratories to specific subtypes of ID/GDD such as X-linked gene panels or clinical features such as epilepsy gene panels (Flore and Milunsky, 2012). In fact, X-linked gene panel testing in males, particularly in those who have X-linked ID inheritance pattern is suggested (Flore and Milunsky, 2012; Moeschler and Shevell, 2014). In case a specific syndrome is suspected disease specific gene panels are also available for genetically heterogeneous syndromes such as Cornelia de Lange. Indeed, it is necessary to know the exact mutation for both, carrier testing and genetic counselling even if there is a definite clinical diagnosis (Moeschler and Shevell, 2014). Moreover, the advances in DNA sequencing and its decrease in price, have now allowed molecular diagnostic laboratories to move to genome-wide analysis such as whole exome sequencing (WES) or whole genome sequencing (WGS) (See Section 2).

1.5. Autosomal dominant intellectual disability

Autosomal dominant ID is known to be the most common cause of ID in Western societies and almost always occurs due to *de novo* mutations (Vissers *et al.*, 2010). It is already known that the mutation rate per generation is high in humans (Lynch, 2010) what could explain why the prevalence of severe developmental disorders such as intellectual disability remains constant in the population.

Although *de novo* occurring aneuploidies and copy number variants were known to cause ID, other genetic causes of autosomal dominant ID were hardly studied due to technical

limitations. High throughput technologies like NGS and aCGH have clearly facilitated the discovery of autosomal dominant genes (Vissers *et al.*, 2010).

Trio sequencing has been the preferred approach to identify *de novo* mutations. Vissers *et al.*, (2010) first took this approach to identify *de novo* mutations in 10 families by whole exome sequencing and confirmed the burden of sporadic mutations in ID. Several other studies have been carried out following the same strategy in larger cohorts and obtaining varying diagnostic yields (de Ligt *et al.*, 2012; Rauch *et al.*, 2012; Fitzgerald *et al.*, 2015). Other exome sequencing studies have also been performed in individuals with severe ID obtaining similar diagnostic yield of 30% approximately (Hamdan *et al.*, 2014).

More recently, genome sequencing studies have demonstrated that the diagnostic yield could be increased up to 60% in sporadic cases of ID identifying both *de novo* point mutations and copy number variants that were missed in previous exome studies (Gilissen *et al*., 2014). Reanalysis of previously sequenced exomes has also improved the diagnostic yield (Lelieveld *et al.*, 2016; McRae *et al.*, 2017).

All in all, sequencing studies have identified more than 400 autosomal dominant ID genes to date (Vissers *et al*., 2015; Wieczorek, 2018).

1.6. Autosomal recessive intellectual disability

While autosomal recessive ID is known to account for 10-20% of ID in outbred populations, it is the main genetic cause in inbred populations (Musante and Ropers, 2014). Indeed, prevalence of ID has been shown to be highly correlated with frequency and degree of parental consanguinity, the prevalence being 2-3 times higher in inbred populations (Morton, 1978; Bundey and Alam, 1993; Fareed and Afzal, 2014; Jamra, 2018). Hence, homozygosity mapping in consanguineous families has been the preferred strategy to analyse recessive disorders (Lander and Botstein, 1987).

Yet, little was known about autosomal recessive forms of ID until 2002 when homozygosity mapping of large consanguineous families started taking place and led to the identification of several autosomal recessive genes (Najmabadi *et al.*, 2007; Abou Jamra *et al.*, 2011; Kuss *et al.*, 2011). Homozygous intervals in consanguineous families are large and many genes need to be screened by Sanger sequencing to identify de causal mutation and therefore it is quite a tedious work. Moreover, these studies demonstrated that autosomal recessive forms of ID are extremely heterogeneous (Musante and Ropers, 2014).

Next generation sequencing has clearly eased and accelerated the identification of autosomal recessive genes in consanguineous families (Caliskan *et al.*, 2011; Najmabadi *et al.*, 2011).To date more than 600 genes are known to contribute to autosomal recessive ID (Musante and Ropers, 2014; Vissers et al., 2015; Jamra, 2018) and it is estimated that there are still more than 2000 genes to uncover (Musante and Ropers, 2014; Jamra, 2018). Recently autosomal recessive forms of ID have gained popularity and many sequencing studies are being carried out in inbred populations to elucidate its genetic origin (Alazami *et al.*, 2015; Anazi *et al.*, 2016; Harripaul *et al.*, 2017; Reuter *et al.*, 2017; Riazuddin *et al.*, 2017; Hu *et al.*, 2018; Santos-Cortez *et al.*, 2018).

1.7. X-linked intellectual disability

In line with different population-based studies, it has always been stated that there is an unbalanced sex ratio in patients affected with ID, the prevalence being 30% higher in males than females (Maulik *et al.*, 2011). It has been suggested that this might be due to the fact that the X chromosome is enriched with genes related to cognition and thus, variants in the X chromosome greatly contribute to ID in males (Gécz *et al*., 2009; Lubs *et al*., 2012). Even so, X-linked conditions do not fully explain the male excess.

X-linked intellectual disability (XLID) generally occurs with a recessive inheritance pattern and could be transmitted from one generation to the other by unaffected female carriers *(Gécz et al*., 2009; Stevenson and Schwartz, 2009). For this reason, it has been possible to study large families with XLID. Nevertheless, it has been reported that female carriers could also be affected depending on the inactivation of the X-chromosome. Furthermore, some XLID conditions are dominant with variable penetrance (i.e. FXS) and could affect females; and others are dominant and mostly affect females (i.e. Rett Syndrome) since they are considered to be lethal for hemizygous males (Gécz *et al*., 2009; Stevenson and Schwartz, 2009). The latter dominant XLID generally occurs *de novo*, although parental germline mutations could also contribute.

The most common and the first X-linked Intellectual Disability condition is FXS, a dominant condition with incomplete penetrance affecting approximately 1/3717 to 1/8918 Caucasian males (Crawford *et al.*, 2001). It was initially mapped to a chromatid constriction marker on the distal long arm on the X chromosome (named marker X chromosome) that was detectable under the microscope (Lubs, 1969; Harrison *et al.*, 1983; Tejada *et al*., 1983). The *FMR1* gene responsible for this syndrome was identified in 1991 (Kremer *et al.*, 1991; Oberlé *et al.*, 1991; Verkerk *et al.*, 1991) and revealed a novel mutational mechanism at the time: the anomalous expansion of the CGG trinucleotide repeat (Bell *et al.*, 1991; Kremer *et al.*, 1991; Oberlé *et al.*, 1991).This unstable trinucleotide repeat region is located in the 5'UTR of the *FMR1* gene and when the number of trinucleotide repeats exceeds 200 (full mutation), the adjacent CpG island becomes hypermethylated leading to the absence of the production of the fragile mental retardation protein and consequently produce FXS. It has been stablished that the normal CGG repeat number is below 45 while those that fall in between are premutation (55-200 repeats) or intermediate alleles (45-54 repeats) and have been associated either to Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) or Fragile X-associated Primary Ovarian Insufficiency (FXPOI) (Lozano *et al.*, 2014).

After the identification of *FMR1*, plenty of families presenting with ID and X-linked inheritance pattern were screened for this gene (Tejada *et al.*, 1992) but most of XLID patients were not positive for this CGG expansion. This fact, together with the observation of the male excess in ID, led to large scale collaborative studies worldwide aiming to uncover the origin of XLID (Stevenson and Schwartz, 2009; Lubs *et al.*, 2012). Indeed, these global collaborative networks like the European X-linked mental retardation Consortium (Euro-MRX) (de Brouwer *et al.*, 2007) have been the key in unravelling genes that cause XLID. Several techniques have been applied for this purpose in families with clear XLID inheritance. Among these, chromosomal microarray technologies and linkage analysis followed by candidate gene testing have being

the most successful approaches (Stevenson and Schwartz, 2009; Lubs *et al.*, 2012)(Stevenson and Schwartz, 2009; Lubs, Stevenson and Schwartz, 2012). In the past decade, thanks to the Human Genome Project (2004), NGS has clearly accelerated the identification of new loci (Figure 2).

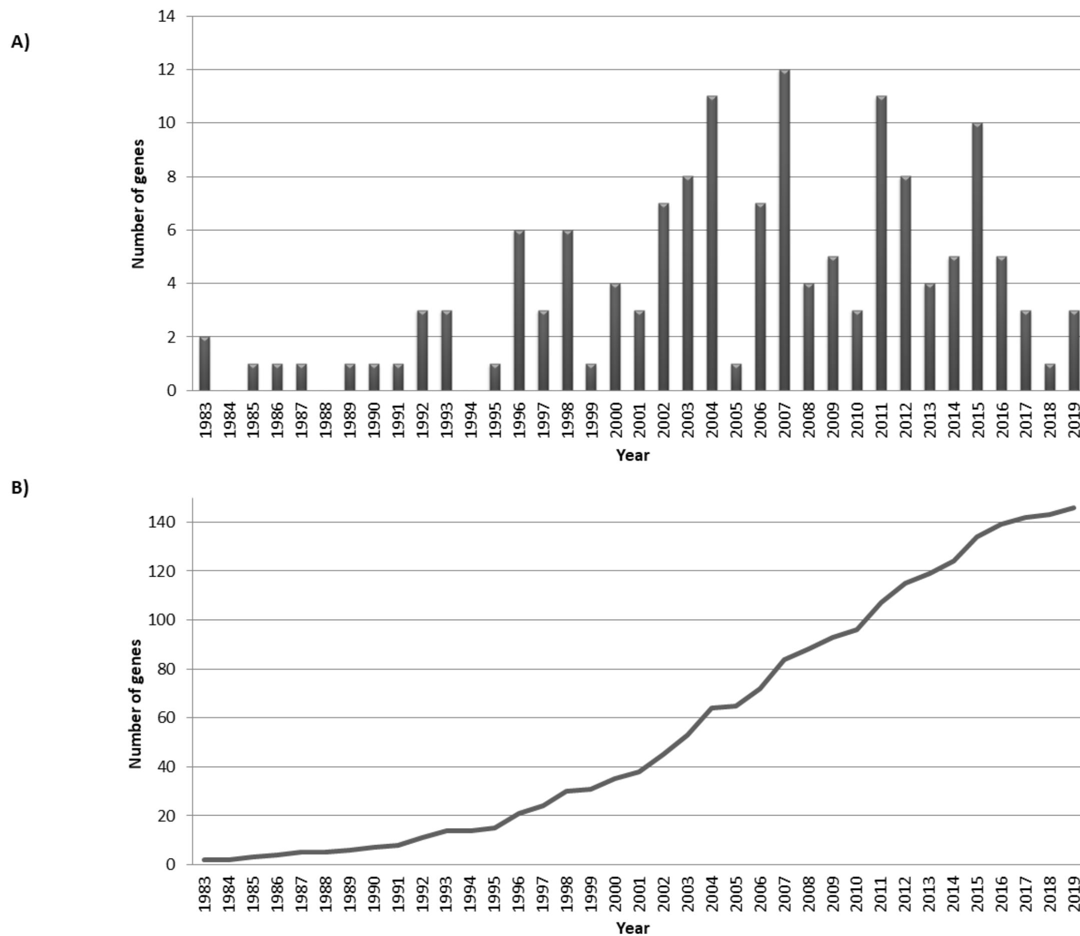


Figure 2. Number of genes associated with X-linked Intellectual Disability throughout the past years. In total, 146 genes have been reported to be related to X-linked Intellectual Disability. **A)** Number of genes identified each year and **B)** cumulative number of genes identified. Gene list updated from *Neri et al.* (2018).

The last XLID update published (Neri *et al.*, 2018) estimated that 141 genes are associated with XLID. Since then, more genes have been associated to XLID such as *CXorf56* (Verkerk *et al.*, 2018), *HS6ST2* (Paganini *et al.*, 2019), *NAA15* (Cheng *et al.*, 2018), *POLA1* (van Esch *et al.*, 2019) an *SLC9A7* (Khayat *et al.*, 2019) (Figure 2). However, the link of some of these genes is still questionable (Piton *et al*., 2013). These authors evaluated the implication of variants and genes (106 XLID genes at that moment) linked to XLID in light of the Exome Variant Server (EVS), an exome sequencing project carried out by the National Heat Lung and Blood Institute (NHLBI). This server included exomes of 6500 individuals with cardiac, lung or metabolic disorders, but not cognitive disorders. Comparing the XLID variants to these data they questioned the implication of 28 genes mainly because: (a) they found truncating mutations in the EVS in genes previously implicated in XLID; (b) the frequency of the variant was high in the

EVS or (c) only one or few variants have been reported and validation is needed (Piton *et al*., 2013). Some of those genes that were pending for validation have already been confirmed while others (*AGTR2*, *ARHGEF6*, *CLIC2*, *IGBP1*, *KLF8*, *MTM1*, *NXF5*, *SIZN1*, *SRPX2*, *ZDHHC15*, *ZMYM3*, *ZNF41*, *ZNF674*, *ZNF81*) have not been reported any more in XLID.

Despite resolving the genetic origin of many XLID patients and families, there are many X-linked conditions without known causative genetic defects. It is likely that other mechanism such as epigenetic modifications or regulatory elements will also contribute to the development of ID, which is a field that it is still in its early stages.

1.7.1. Copy Number Variants on the X chromosome

Chromosome microarray techniques have enabled to detect chromosomal microduplications and microdeletions on the X chromosome. Indeed, it has been observed that there are some duplication (Xq28 including *MECP2*, Xp11.22 including *HUWE1* and Xq27.1 including *SOX3*) and deletion hotspots (Xp22.3, Xp11.4 and Xp11.3) (Gécz *et al*., 2009).

Genomic duplications are an important cause of XLID (Stevenson and Schwartz, 2009). These duplications always include known XLID genes and range from kilobases to megabases. XLID duplications have recently been reviewed by Neri *et al*. (2018). XLID duplications may either have the same phenotypical consequence as deletions or loss of function mutations or a completely different phenotype (Tejada *et al.*, 2011). The latter duplication syndromes were defined after molecular diagnosis since patients generally presented with non-specific intellectual disability. Therefore, molecular diagnosis helped in defining the phenotype of these patients and delineating these syndromes. For example, the following are two of the most common XLID duplications.

1. <u>*MECP2* duplication syndrome (OMIM#300260)</u>
   Deletion or loss of function mutations of *MECP2* cause Rett syndrome in females while duplications of *MECP2* lead to severe ID, autistic features, absent or limited speech, mild dysmorphic features, infantile hypotonia, progressive spasticity, seizures and recurrent respiratory infections mainly in males (Ramocki *et al.*, 2012).

2. <u>Chromosome Xp11.22 duplication syndrome (OMIM#300705)</u>
   Patients with *HUWE1* duplications show moderate to severe ID, limited speech or dysarthria, mild dysmorphic facial features and normal growth (Froyen *et al.*, 2012). These duplications also include *HSD17B10* gene, however increased dosage of *HUWE1* is thought to be responsible for the phenotype (Froyen *et al.*, 2012). Conversely, point mutations in both genes lead to distinct syndromic phenotype. Indeed, sequence variants in the *HUWE1* gene lead to Turner-type syndrome which shows a wide phenotypic spectrum.

1.7.2. Non-syndromic X-linked intellectual disability

For clinical purposes it has historically been very useful to divide ID into two other categories (Stevenson and Schwartz, 2002): 1) Syndromic Intellectual Disability (S-ID) in which ID presents with generally consistent dysmorphic, neurological or systemic features that should be

recognized by clinical geneticists; and 2) Non-Specific or Non-Syndromic Intellectual Disability (NS-ID) where the only common feature is ID, although patients may individually present with other neurological or dysmorphic features. However, these additional features are never consistent from one patient to the other and therefore, there is a high phenotypic heterogeneity.

Despite this classification, the rapid discovery of novel variants in genes already known to cause a syndrome or the overlapping phenotypes observed have made this classification no longer adequate (Renieri *et al.*, 2005), obscure (Ropers, 2006) and sometimes arbitrary (Neri *et al.*, 2018). In this way, the same mutation in the family may cause both forms (Tarpey *et al.*, 2007; Tejada *et al.*, 2019) showing the importance of environmental factors or other genetic factors. Clinical differences might also result from affecting different domains, or the genetic background. Genes involved in the same pathways may also lead to similar phenotypes and molecular testing of XLID patients has helped in grouping them, as well as splitting other entities previously considered the same (Lubs *et al*., 2012). As a result, throughout these years, certain genes have been associated with NS-XLID as well as S-XLID.

According to Neri *et al*., (2018) 56 XLID genes have been implicated in NS-XLID of which 28 have also been reported as responsible for syndromic forms (Figure 3). Genes that were questioned by Piton *et al*., (2013) have been excluded, as well as genes that generally do not involve ID such as the Duchenne muscular dystrophy gene. Table 2 shows 44 genes of which 25 were reported as responsible for both syndromic and non-syndromic forms.

Investigating the current knowledge of some of these genes with the emphasis on their pathogenic mechanism and functional studies may allow elucidating how they can give rise to syndromic and non-syndromic phenotypes or if they can be considered as a continuum of phenotypes because the causative genes are interconnected in the same pathway.

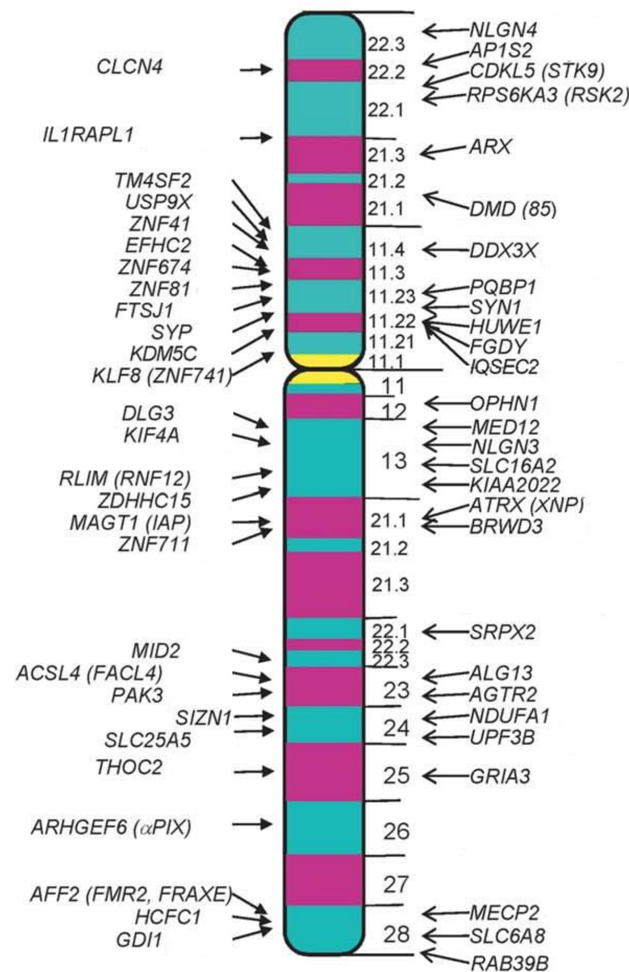


Figure 3. Genes on the X chromosome that have been implicated in non-syndromic ID according to Neri *et al*. (2018). On the left, **g**enes implicated only in non-syndromic ID have been underlined. On the right, genes implicated both in syndromic and non-syndromic forms.

Molecular and cellular pathways

Focusing on NS-ID and compiling the current updates where the emphasis was made in the causal genes, processes involved in cognitive function can be identified because patients with NS-ID present cognitive impairment as main feature (Kaufman *et a*l., 2010). The proteins encoded by NS-ID genes play a role in one or more shared molecular and cellular pathways either through direct interactions or as part of more complex interaction networks (Chelly *et al.*, 2006; Vissers *et al*., 2015). By identifying such genetic networks in NS-ID, finding treatments for relieving symptoms and uncovering other candidate genes will be easier in the future (Kaufman *et al*., 2010).

While cellular processes such as neurogenesis and neuronal migration are mainly altered by variants in genes that results in specific syndromes, it seems that NS-ID genes are mainly involved in regulation of synaptic function (Kleefstra *et al.*, 2014). Although the grouping of NS-XLID genes could be arbitrary because the function of most known genes is not fully understood (Vissers *et al.*, 2015), after a careful examination of the genes shown in Figure 3, several molecular and cellular mechanisms can be highlighted (Table 2).

Table 2. Molecular and cellular functions and pathways of genes involved in non-syndromic X-linked intellectual disability grouped in major processes. Genes are in alphabetical order.

Gene	Gene function	Syndromic and Non-syndromic ID	OMIM
1. Transcription and translation regulation			
AFF2 (FMR2)	RNA binding protein. Alternative splicing regulation		300806
ARX	Transcription factor	x	300382
ATRX	ATP dependent DNA helicase. SWI/SNF family. Chromatin remodelling	x	300032
BRWD3	Chromatin remodelling	x	300553
DDX3X	ATP dependent RNA/DNA helicase. DEAD-box family.	x	300160
FTSJ1	Processing and modification of ribosomal RNA		300499
HCFC1	DNA-binding protein. Transcriptional co-regulator		300019
KDM5C	Chromatin modifications. Histone demethylase		314690
MECP2	DNA methylation	x	300005
MED12	Transcription regulation through RNA polymerase II	x	300188
PQBP1	Transcription regulation and mRNA splicing	x	300463
RPS6KA3 (RSK2)	Serine/Threonine protein kinase	x	300075
THOC2	mRNA nuclear export		300395
UPF3B	mRNA nuclear export and surveillance by nonsense mediated decay	x	300298
ZNF711	DNA-binding protein. Transcription factor		314990
2. Ubiquitination			
HUWE1	Ubiquitin ligase	x	300697
MID2	Ubiquitin ligase		300204
RLIM	Ubiquitin ligase		300379
USP9X	Deubiquitinase		300072
3. Synaptic function			
DLG3	Organization of NMDA receptors in signaling pathways within the postsynaptic excitatory synapses		300189
FGD1	Rho GTPase regulation. Actin cytoskeleton regulation	x	300546
GDI1	Rab GTPase regulation. Vesicle trafficking		300104
GRIA3	Postsynaptic receptor. AMPA glutamate receptor signaling.	x	305915
IL1RAPL1	Presynaptic neurotransmitter release. Regulation of calcium dependent vesicle secretion		300206
IQSEC2	ARF GTP-binding protein regulator. Cytoeskeletal organization	x	300522
KIAA2022	Regulates cell adhesion	x	300524
NLGN3	Postsynaptic transmembrane protein (Cell adhesion molecule)	x	300336
NLGN4	Postsynaptic transmembrane protein (Cell adhesion molecule)	x	300427
OPHN1	Rho GTPase activating protein	x	300127
PAK3	Rho GTPase signaling. Actin cytoskeleton regulation		300142
RAB39B	Rab GTPase. Vesicle trafficking	x	300774
SYP	Integral synaptic vesicle membrane protein that regulates synaptic vesicle endocytosis		313475
SYN1	Synapse vesicle associated protein. Neurotransmitter release in presynaptic vesicle trafficking	x	313440
TSPAN7	Integrin binding		300096

4. Other Functions			
ACSL4	Lipid biosynthesis and fatty acid degradation		300157
ALG13	N-Glycosylation	x	300776
AP1S2	Recruits clathrin to vesicular membranes	x	300629
CDKL5	Serine/Threonine protein kinase	x	300203
CLCN4	Chloride transport		302910
KIF4A	Motor protein. Microtubule binding		300521
NDUFA1	Mitochondrial membrane respiratory chain	x	300078
SLC16A2	Thyroid hormone transport	x	300095
SLC25A5	Mitochondirial ADP/ATP transporter		300150
SLC6A8	Creatine transport	x	300036

1. Transcription and translation regulation

Gene expression and protein synthesis are necessary for synaptic plasticity (van Bokhoven, 2011). Neurons are highly specialized cells and alterations in the mechanisms implicated in gene expression will lead to defective brain function and consequently ID. There are many XLID genes that code for proteins involved in these processes: Table 1 shows 15 genes, nine of which have been reported to be responsible for syndromic and non-syndromic forms. Of these 15 genes, some are involved in transcriptional activation/silencing, regulation of RNA splicing, export and degradation (Vaillend *et al.*, 2008). Genes implicated in chromatin structure changes or chromatin remodelling like *ATRX* also influence transcription (Vaillend *et al.*, 2008; van Bokhoven, 2011) together with genes involved in DNA methylation like *MECP2* which have been associated with gene silencing at promoter regions and tissue specific regulation (Kleefstra *et al.*, 2014). Furthermore, chromatin structure is determined by epigenetic marks like DNA methylation or histone modifications that shape the gene expression profile of each cell. Modifications of histone proteins like acetylation, methylation, phosphorylation, and rarely ubiquitination, sumoylation, and ADP-ribosylation contribute to gene expression regulation by regulating accessibility of the transcription machinery (van Bokhoven, 2011; Kleefstra *et al.*, 2014). Among the genes involved in histone modifications *KDM5C* and *RPS6KA3 (*RSK2) can be highlighted. These chromatin signatures are then recognized by other transcription regulator proteins like *HCFC1* that give access to the transcription machinery (Kleefstra *et al.*, 2014). The disruption of these gene expression processes by *de novo* and /or somatic mutations is also related to carcinogenesis.

ATRX (OMIM*300032) is a typical example of a XLID gene that shows great phenotypic variability. Pathogenic variants in this gene have been related to several syndromes in addition to the best known X-linked Alpha Thalassemia syndrome (OMIM 301040): Carpenter-Waziri syndrome, Holmes-Gang syndrome, Chudley-Lowry syndrome and Arch fingerprints–hypotonia syndrome. Other syndromes such as XLID with spastic paraplegia and XLID with epilepsy as well as non-syndromic phenotypes have also been associated with *ATRX* variants. Thus, all of these phenotypic entities should be considered as part of the ATRX spectrum (Stevenson, 2000).

MECP2 (OMIM*300005) is one of the most important genes contributing to the spectrum of neurodevelopmental phenotypes. It codes for a methyl CpG-binding protein first identified in the cell nucleus as a transcriptional repressor that recognizes DNA methylation marks. Recent

studies, however, have revealed that MeCP2 plays a more complex roles than previously thought and it is now considered as a global chromatin organizer (Gulmez Karaca *et al*., 2019).

Mutations in *MECP2,* mostly *de novo,* are associated with Rett syndrome (OMIM #312750) in females. In spite of that, the pathogenicity of the *MECP2* mutations depends on the type of mutation and in which domain of the gene they are in. In fact, different types of mutations having been found in this gene (deletions, duplications, frameshift, nonsense and missense mutations), which have shown that *MECP2* not only is involved in Rett syndrome but also in a broad range of other neurological disorders in females and in males: severe encephalopathy, autism, progressive spasticity, and NS-XLID (Maortua *et al.*, 2013). Duplications of chromosome Xq28, including the entire *MECP2* gene have been associated with severe XLID with recurrent infections in males (Bijlsma *et al.*, 2012).

The *RPS6KA3* (OMIM*300203) gene encodes a member of the RSK (ribosomal S6 kinase) family of growth factor-regulated serine/threonine kinases, known also as p90 (rsk). RSK2 phosphorylates histone H3 which likely affects transcription and chromatin structure. Furthermore, RSKs appear to have important roles in cell cycle progression, differentiation, and cell survival. Mutations in *RPS6KA3* are associated with Coffin-Lowry syndrome (CLS; OMIM #303600), although they have also been identified in families with NS-XLID and none of the clinical features of CLS (review by Marques Pereira *et al*., (2010)). Microduplications at Xp22.12 including *RPS6KA3* have also been associated with mild XLID (Tejada *et al.*, 2011).

It is also important to mention *MED12* and *UPF3B* as an example of genes causing syndromic and non-syndromic phenotypes and their overlap in the associated phenotypes.

The *MED12* gene (OMIM*300188) encodes the Mediator complex that regulates gene expression through the RNA polymerase II. The Mediator complex is comprised of 30 different subunits approximately that are grouped into four distinct modules (Head, Middle, Tail and Kinase); subunit 12 is part of the kinase module. The kinase module has been linked to different developmental and oncogenic signalling pathways like Wnt, Sonic Hedgehog (SHH), Epidermal Growth Factor (EGF) and Notch pathways (Clark *et al.*, 2015). Dysfunction of transcription machinery components, including *MED12*, has been shown to dysregulate gene expression. Germline defects in *MED12* have been reported to disrupt GLI3 dependent SHH signalling pathway (Zhou *et al.*, 2012; Srivastava *et al.*, 2019), as well as REST dependent epigenetic signalling (Vulto-van Silfhout *et al.*, 2013) and immediate early gene expression (Donnio *et al.*, 2017).

Germline mutations in the *MED12* gene were first identified in a families diagnosed of Opitz-Kaveggia syndrome, also known as FG syndrome 1 (FGS1: OMIM # 305450), which is characterized by ID, hypotonia, distinct facial features, relative macrocephaly, broad thumbs and halluces, corpus callosum abnormalities and gastro-intestinal complications (Risheg *et al.*, 2007). Mutations in *MED12* were also reported in patients with Lujan-Fryns syndrome (OMIM # 309520). The phenotype of this syndrome overlaps with FGS1 since both are characterized by ID, relative macrocephaly, hypotonia and corpus callosum abnormalities (Schwartz *et al.*, 2007). Distinct features of Lujan-Fryns syndrome include tall stature, a hypernasal voice and hyperextensible digits. A third clinical phenotype was also associated with *MED12* mutations named X-linked Ohdo syndrome (OMIM # 300895) (Vulto-van Silfhout *et al.*, 2013). In contrast

to both FGS1 and Lujan-Fryns syndrome, patient with Ohdo syndrome have some distinguishable features such as ptosis and blepharophimosis. However, in the last years, thanks to the massive use of next generation sequencing techniques (NGS), more *MED12* variants were identified, broadening the phenotypic spectrum, making it difficult to fit the description of any of the syndromes described above in the majority of cases. Therefore, it has been suggested to define as *MED12*-related disorders rather than attributing a syndrome (Charzewska *et al.*, 2018). To date, 16 variants have been identified, most of which are missense variants (Rubinato *et al.*, 2019).

UPF3B (OMIM*300298), like *MED12,* was also described as responsible for a syndromic condition similar to FGS1 and Lujan-Fryns syndromes (Tarpey *et al*., 2007). *UPF3B* encodes the Regulator of nonsense mediated mRNA decay 3B (REN3B) protein initially identified as a one of the components of an exon-junction complex (EJC) that promotes nonsense-mediated mRNA decay (NMD). NMD represents a key mechanism to control the expression of wild-type and aberrant mRNAs. Two EJC components -hUpf3a and hUpf3b- serve a dual function: promote NMD, and regulate translation efficiency (Kunz *et al.*, 2006; Ivanov *et al.*, 2008).

The implication of *UPF3B* in ID was first described by Tarpey *et al*., (2007) who reported hemizygous variants in *UPF3B* in affected males of four unrelated families with a variable phenotype, including mild to severe ID and autistic features. While three of these families had originally been diagnosed as having FGS1 and Lujan-Fryns syndromes, the fourth was described as having NS-XLID. A recent report on large NS-XLID family (Tejada *et al.*, 2019) with a nonsense variation in *UPF3B* also included an update of the variants found so far: a total of 22 variants, 18 of them pathogenic. Although the clinical characteristics of most patients were not described in the respective reports, the majority of these *UPF3B* variants could be considered to be responsible for a non-specific phenotype as they all entered NGS studies because they did not have a recognizable syndrome.

2. *Ubiquitination*

Ubiquitination has recently been recognized as a biological process of interest in the field of ID because some authors have reported its implication in neurological disorders that include neurodegenerative and neurodevelopmental disorders (George *et al.*, 2018). Ubiquitination is a posttranslational modification that involves the addition of a small polypeptide called ubiquitin to target proteins. Neuronal maintenance of protein homeostasis is important for synaptic plasticity and requires a precise control of processes like protein synthesis, folding and degradation mainly by the ubiquitin proteasome system or autophagy (Osinalde *et al.*, 2019). The ubiquitin proteasome system targets ubiquitinated proteins for proteasomal degradation and it is also involved in signal transduction.

The ubiquitination process is achieved by 3 different types of enzymes: E1 ubiquitin activation enzyme, E2 ubiquitin conjugation enzyme and E3 ubiquitin ligases. Deubiquitinating enzymes, on the other hand, are responsible for removing ubiquitin molecules from target proteins. E3 ligases provide target specificity, and can be classified in 3 classes: RING type, HECT type and U-box type E3 ligases. Mutation in E3 ligases either reduce or disrupt the activity of these enzymes resulting in altered ubiquitination of their substrates.

Although little is known about E3 ligase substrates and function and their effect at cellular level in neurodevelopmental disorders, some of XLID genes have already been implicated in this process (Table 2).

HUWE1 (OMIM*300697) encodes a HECT type E3 ligase and it is known to regulate neural differentiation and proliferation through poly-ubiquitination of specific target proteins like MYCN, p53 and CDC6 for subsequent proteasomal degradation (George *et al.*, 2018). *HUWE1* is known to be critical in development, since its loss leads to embryonic lethality in mice (D'Arca *et al.*, 2010). Studies in mice have also revealed its importance in neuronal development and differentiation. However, as a gene related to proliferation, it was first discovered in oncogenesis.

Microduplications and mutations in *HUWE1* are now recognised as responsible for ID. Microduplications in *HUWE1* are associated to mild to moderate NS-XLID (Froyen *et al.*, 2008, 2012) and missense variants were found in moderate to profound NS-ID (Froyen *et al.*, 2008) mainly clustering to the HECT domain. These mutations have been shown to alter expression of the HUWE1 protein and its downstream targets (Friez *et al.*, 2016; Bosshard *et al.*, 2017). Remarkably, no truncating variants nor deletions have been reported supporting what has been demonstrated in mice studies (D'Arca *et al.*, 2010). Although mutations in this gene cause a wide phenotypic spectrum (Moortgat *et al.*, 2018), a syndromic form has also been described. Indeed, a recurrent mutation in the HECT domain was associated with Juberg-Marsidi and Brooks-Wisniewski-Brown syndrome (OMIM# 309590) (Juberg and Marsidi, 1980; Brooks, Wisniewski and Brown, 1994; Friez *et al.*, 2016). This syndrome is characterized by ID, poor or absent speech, short stature and microcephaly. Dysmorphic features include deep-set eyes, prominent nose and blepharophimosis. As in most of the X-linked disorders, males are mainly affected, although affected females have also been reported (Fitzgerald *et al.*, 2015; Moortgat *et al.*, 2018).

3. *Synaptic function*

Chemical synapses are the main synapses in the central nervous system and respond to different stimuli by releasing neurotransmitters like glutamate in excitatory synapses (van Bokhoven, 2011). Synapses consist of presynaptic axon terminals with synaptic vesicles and a postsynaptic region on dendrites with neurotransmitters receptors. Activation of genes encoding synaptic proteins and synaptic vesicle generation, aggregation and trafficking is required for the formation of presynaptic boutons and postsynaptic differentiation (Figure 4). Synapse development then leads to the conformation of synaptic junctions (Vaillend *et al.*, 2008).

Table 2 shows 15 XLID genes implicated in synaptic function, nine of which have been described as responsible for both syndromic and non-syndromic forms.

- Pre-synaptic vesicle trafficking

The pre-synaptic compartment holds a large number of vesicles containing neurotransmitters that are released in response to stimuli by exocytosis (Humeau *et al.*, 2009; van Bokhoven, 2011). The process of synaptic vesicle trafficking and release is tightly regulated. Rab proteins,

small GTPases that cover synaptic vesicles, are regulated by GDP/GTP dissociation factors such as *GDI1* (OMIM*300104) (Vaillend *et al*., 2008; van Bokhoven, 2011). Other X-linked genes associated with vesicle trafficking and neurotransmitter release are: *IL1RAPL1* (OMIM*300206) whose protein regulates calcium dependent vesicle secretion, *SYN1* (OMIM*313440) encoding a synapse vesicle associated protein and *SYP* (OMIM*313475) which codes for an integral synaptic vesicle membrane protein that regulates synaptic vesicle endocytosis (Vaillend, Poirier and Laroche, 2008; Humeau *et al.*, 2009).

Presynaptic and postsynaptic compartments are separated by the synaptic cleft where cell adhesion molecules (CAMs) hold axon and dendrite together. CAMs are composed by diverse proteins that have large extracellular domains and are crucial in synaptic signalling (Forrest, Parnell and Penzes, 2018). Neurexins and neuroligins are well known CAMs. While neurexins, encoded by autosomal genes locate to the pre-synaptic compartment, X-linked neuroligin genes (*NLGN3, NLGN4*) code for postsynaptic cell adhesion molecules (Vaillend *et al*., 2008; Forrest *et al*., 2018).

- <u>Post synaptic signalling</u>

Postsynaptic signalling pathways contribute to synaptic plasticity at excitatory glutamatergic synapses (Forrest *et al*., 2018). Postsynaptic compartments like dendritic spines receive chemical signals from pre-synaptic compartments (Humeau *et al.*, 2009). Postsynaptic neuroligins are associated with scaffolding MAGUK (Membrane Associated Guanylate Kinases) proteins such as DLG3 (OMIM*300189) that regulates N-methyl-D-aspartate (NMDA) glutamate receptor. GRIA3 (OMIM*305915), on the other hand, is part of the α-amino-3-hydroxy-5-methyl-4-isoxazole acid (AMPA) glutamate receptor. These scaffolding proteins are the core of the synapse since they translate upstream signals from CAM and receptors to changes in gene expression or cytoskeleton organization (Forrest *et al*., 2018; Zamboni *et al.*, 2018).

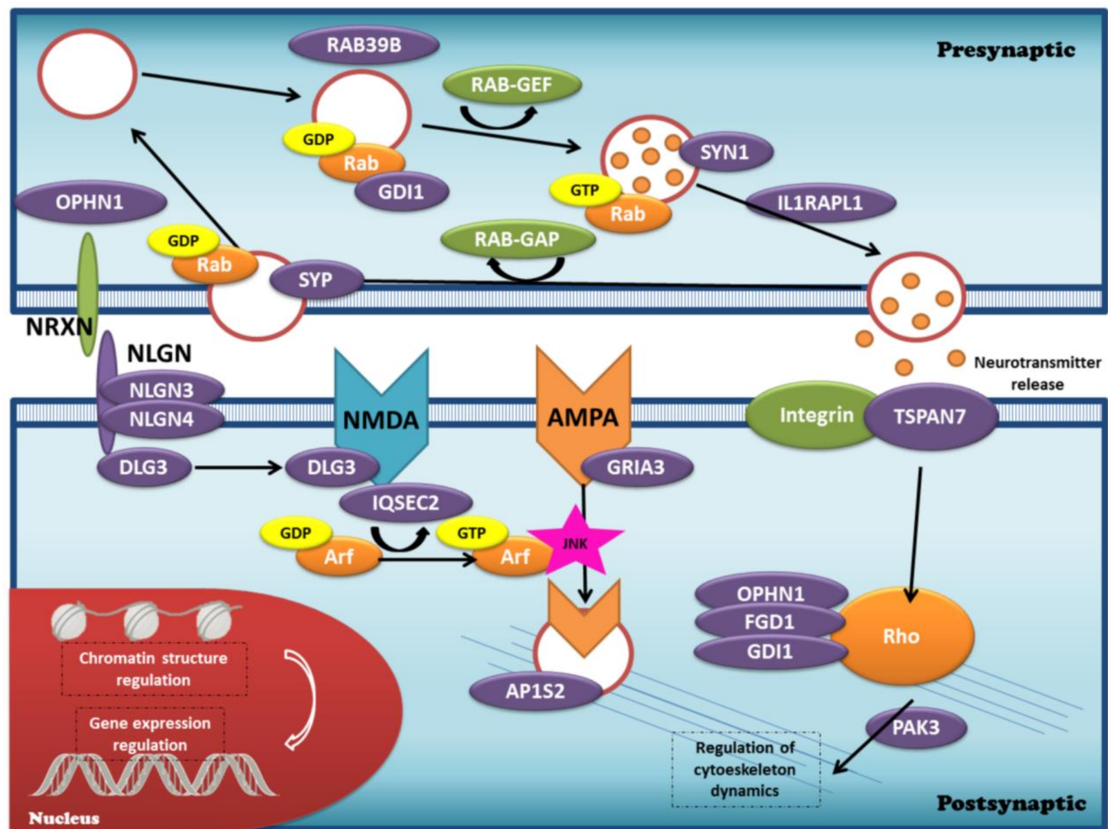


Figure 4. Presynaptic and postsynaptic signalling pathways in non-syndromic X-linked intellectual disability. Schematic representation of the major presynaptic and postsynaptic signaling pathways in which NS-XLID genes take part: presynaptic vesicle trafficking and neurotransmitter release, organization of the postsynaptic density, cytoskeleton dynamics, and gene expression regulation. NS-XLID genes are shown in purple. It can be seen that vesicles containing neurotransmitters are released by exocytosis from the presynaptic compartment. This process is controlled by the Rab proteins which are also regulated by positive and negative regulators. Chemical signals are then received at the postsynaptic compartment through receptors and cell adhesion molecules and transduced by the scaffolding proteins to translate them into changes in gene expression or cytoskeleton organization which contribute to synaptic plasticity.

- Cytoskeleton dynamics

Cytoskeleton dynamics have a great influence on the structure and function of dendrites and spines. Rho family of small GTPases are key regulators of actin and microtubule activity for synapse development and synaptic plasticity (Vaillend *et al*., 2008; Kaufman *et al*., 2010; Ba, van der Raadt and Nadif Kasri, 2013). Rho GTPase activity is regulated by positive regulator Guanine nucleotide Exchange factors (GEF) and negative regulators like the protein encoded by *FGD1* (OMIM*300546) (Kaufman *et al*., 2010; Ba *et al*., 2013). Other genes involved in Rho GTPase pathway and cytoskeleton organization include *PAK3* (OMIM*300142) and *OPHN1* (OMIM *300127) (Ba *et al*., 2013).

IQSEQ2 is another member of the GEF family that activates specific Arf (ADP ribosylation factor) targets instead to regulate actin dynamics and is encoded by the *IQSEC2* (OMIM*300522) gene (Shoubridge et al., 2010). This protein is localized at the post-synaptic

density of excitatory synapses and it is known to be part of the NMDA complex contributing to the dendritic spine formation and synaptic plasticity (Petersen *et al*., 2018; Levy *et al.*, 2019). Arfs are members or Ras superfamily of small G proteins and are known to regulate actin dynamics and membrane trafficking in dendritic spines. *In vitro* experiments have shown evidence that IQSEC2 binds to Arf6 which is known to regulate trafficking between cell membrane and endocytotic membrane via actin cytoskeleton regulation (Petersen *et al*., 2018; Levy *et al.*, 2019). It has also been demonstrated that IQSEC2 regulates AMPA receptor trafficking via Arf6 demonstrating its implication learning and memory processes. Indeed, alterations in AMPA receptors have been associated with cognitive impairments and social behavioural abnormalities (Petersen *et al*., 2018; Levy *et al.*, 2019).

IQSEC2 mutations were first described in families with non-syndromic XLID (Shoubridge *et al.*, 2010). The four families showed moderate to severe ID in affected males, seizures, autistic traits and psychiatric problems were also reported in an inconsistent manner. To date, more than 70 mutations have been reported in *IQSEC2* leading to moderate to severe intellectual disability with variable seizures and autistic traits (Rogers *et al.*, 2019; Shoubridge *et al*., 2019). Although this gene has been included in Figure 2 as possibly responsible for syndromic forms according to Neri *et al.*, (2018), this review has failed in finding syndromic forms. Furthermore, there is not even genotype –phenotype correlation to explain how its mutations can affect IQSEC2 function and influence cognition and social behaviour. Some of these mutations are clustered in functional domains like IQ and Sec7 domains, providing a possible mechanism of disease. IQSEC2 has been shown to bind calmodulin in a calcium dependent manner through the IQ domain. Rogers *et al*., (2019) demonstrated that missense mutations on the IQ domain lead to impaired calmodulin binding, increase IQSEC2 GEF activity and lead to decreased AMPA receptor in brains of mice. These mice show abnormal behavioural phenotypes with increased locomotion, abnormal social interactions and decreased learning. Mutations in SeC7 domain, on the other hand, decrease Arf6 GEF activity (Shoubridge *et al.*, 2010; Kalscheuer *et al.*, 2016).

IQSEC2 is known to escape X inactivation, although there must be another compensatory mechanism since expression levels in females and males are similar. As in other X-linked disorders, affected females have also been identified. Pathogenic variants found in females are mainly truncating, whereas males also have missense and truncating variants. Truncating mutations in males and females are associated with severe neurodevelopmental disorder, females being globally less severely affected (Mignot *et al.*, 2019). Missense variants altering functional domains IQ and Sec7 are better tolerated in females and therefore can be inherited over several generations (Mignot *et al.*, 2019).

4. Other functions

There are other 10 X-linked genes in Table 1 that do not fit the previous 3 classifications and could not be grouped in any other major group. Among them, there are genes coding for membrane transporters, proteins that manage the transport of different small molecules in and/or out of the cell. These small molecules can be sugars, vitamins, amino acids, bile acids and hormones. In the human genome there are two superfamilies that encode for membrane transporters: ATP-binding cassette (ABC) superfamily and the solute carrier (SLC) superfamily.

Whereas ABC transporters get molecules out of the cell using energy from ATP hydrolysis, SLC transporters are mainly involved in the uptake of small molecules into cells.

The most important XLID gene coding for SLC transporters is *SLC6A8* (OMIM*300036) which encodes for a creatine transporter also known as CRTR or CT1. Creatine regulates the storage and delivery of intracellular energy and therefore plays a key role in energy homeostasis, particularly in brain. Therefore, dysfunction of this membrane transporter leads to creatine deficiency due to impaired creatine uptake. The first patient with creatine deficiency and a mutation in *SLC6A8* gene was described by Salomons *et al*., (2001). Since then, more families have been described. Indeed, the prevalence of *SLC6A8* mutations in males with ID is estimated to be about 1% (Clark *et al.*, 2006). Moreover, it is thought that creatine deficiency might be under-diagnosed due to its unspecific phenotype. In fact, the diagnosis is usually suspected by elevated creatine/creatinine ratios in urine, as plasma creatine levels might be normal. Then, the diagnosis is usually confirmed by molecular testing. Special imaging analyses such as Proton Magnetic Resonance Spectroscopy (H-MRS) may also be applied to detect creatine deficiency in the brain. *In vitro* functional assays to assess transporter activity are also performed in cultured fibroblasts.

The main clinical features of patients with creatine deficiency are moderate to severe intellectual disability and severe language delay. They may also show behavioural disturbances and epilepsy. As in many X-linked disorders, female carriers are usually asymptomatic although they may have some mild features (van de Kamp *et al.*, 2013).

Another gene that should be highlighted in this group encodes the protein kinase cyclin-dependent kinase-like 5 gene (*CDKL5*).

CDKL5 (OMIM*300203) was found to be associated with atypical Rett syndrome with infantile spasms or early seizures starting in the first postnatal months (Maortua *et al.*, 2012). *CDKL5* mRNA is highly expressed in the adult human brain, which is indicative of its importance in neuronal function and development (Chen *et al.*, 2010). CDKL5 possesses kinase activity and is able to autophosphorylate as well as to mediate MeCP2 phosphorylation, suggesting that CDKL5 and MeCP2 may belong to the same molecular pathway (Mari *et al.*, 2005). As with *MECP2*, it seems that the nature of the mutations and their location result in phenotypic heterogeneity mainly in females, although there are also boys with an early onset, severe epileptic encephalopathy described. In any case, the vast majority of cases are *de novo*.

2. NEXT GENERATION SEQUENCING

Sanger sequencing was developed in the late 1970s by Sanger and colleagues (Sanger *et al.*, 1977) and has been the prevailing DNA sequencing technique in genetic diagnostic laboratories for the past 30 years. Although it initially was a labour intensive technique, improvements in the sequencing methodology, automation and its commercialization have clearly eased the process. Nowadays, DNA sequencing machines are extensively used. These sequencers are able to generate 600-1000 base length accurate sequences and require low DNA input. This method is commonly used to sequence regions of interest that are previously amplified using specific DNA primer or templates by conventional PCR. It can also be used to analyse DNA fragment size using fluorescently labelled templates. However, it also enables doing large scale sequencing studies as they can handle 96-384 reactions in parallel. Indeed, the first human genome was completed in 2004 by this method (International Human Genome Sequencing Consortium, 2004). However, it required an enormous amount of time and resources and the limitation of this technique in high throughput sequencing became noticeable. Therefore, the National Human Genome Research institute (NHGRI) started a funding program to accelerate the development of DNA sequencing techniques with the aim to reduce the cost of human genome sequencing to US$1000 in ten years (Schloss, 2008). The challenge resulted in the development of massively parallel sequencing also called next generation sequencing (NGS) or second generation sequencing. NGS technologies are able to generate hundreds of megabases to gigabases of DNA sequence in a single run depending on the sequencing platform. Several sequencing platforms have been developed these years (reviewed in (van Dijk *et al.*, 2014; Slatko *et al.*, 2018). The first sequencing platform, which has been discontinued, was launched by 454 Life Sciences (now Roche) and was based on the pyrosequencing method. Later on, Solexa (now Illumina) sequencing platforms were commercialized. These are based on the detection of fluorescently labeled reversible terminators. In 2010, Ion Torrent (now Life Technologies) technology was launched. Despite Illumina being the current leader on next generation sequencing platforms, we will focus on Ion Torrent technology since this is the sequencing technology that has been applied in this study.

2.1. Ion Torrent Technology

Ion Torrent (now Life technologies) chip technology is based on sequencing by synthesis. The principle of its technology is the electrochemical detection of nucleotide incorporation by massively parallel sensor arrays on a semiconductor chip (Merriman *et al.*, 2012). When the correct nucleotide is incorporated into the growing strand a hydrogen ion (H+) is released giving rise to a change in local pH. Changes in pH can be recorded as a voltage change by a pH sensitive sensor (Figure 5). Despite the sequencing chemistry being similar to 454 Life Sciences

(now Roche) pyrosequencing system, the Ion Torrent system enables direct detection of the signal via the sensor as the semiconductor chip has an incorporated hardware to process the received signals and produce base calls. This makes Ion Torrent technology to be fast and simple.

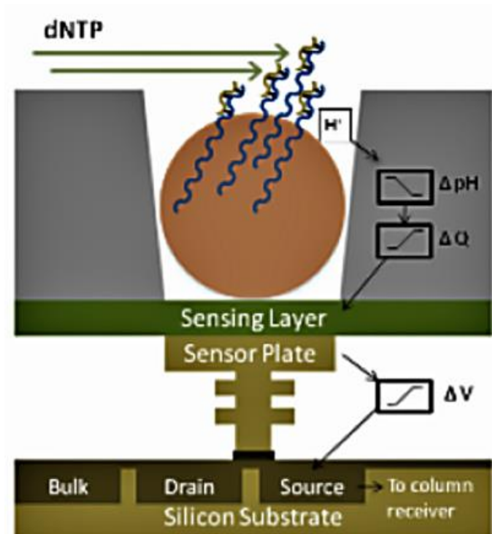


Figure 5. Schematic representation of a single well of the Ion semiconductor chip. DNA template is sequenced by synthesis. When a nucleotide is incorporated to the on the growing DNA strand, a proton is released changing local pH. A sensing layer detects pH changes and translates chemical signal to digital signal.

Currently, there are several Ion Torrent sequencing platforms that offer different throughputs: Ion Personal Genome Machine^{TM} (PGM^{TM}) system, Ion Proton^{TM} system, Ion S5 system and Ion S5 XL system.

A general workflow is followed in all next generation sequencing studies: 1) Library preparation, 2) Template preparation, 3) Sequencing and 4) Data analysis. DNA needs to be pre-processed for sequencing, and hence, DNA libraries are constructed. DNA library preparation is carried out by DNA fragmentation into short fragments and ligation of adapters. Sequencing templates are then generated by DNA library amplification. These are after sequenced and sequencing data is finally analyzed (Figure 6).

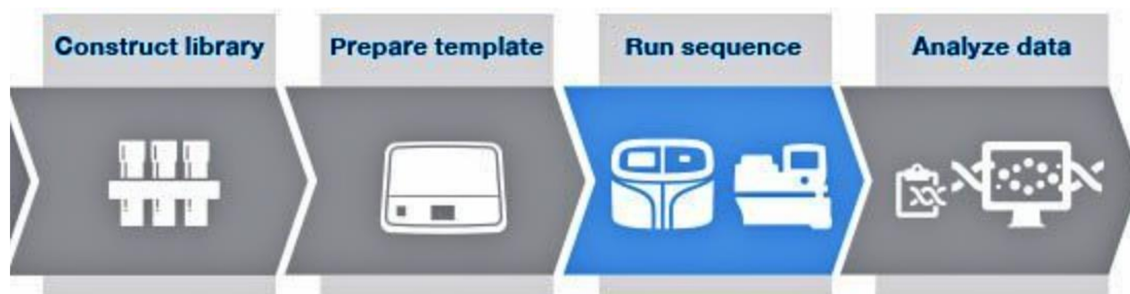


Figure 6. Ion Torrent sequencing workflow.

Ion Torrent has automated most of the next generation sequencing workflow and offers solutions to any of the steps. Indeed, for library building and targeted sequencing approaches Ion Torrent launched Ampliseq^{TM} for rapid target enrichment by multiplex PCR amplification using minimal DNA input. Briefly, a set of primers is designed using Ion Ampliseq^{TM} designer platform and target regions are amplified. Primers are then digested and adaptors and sample specific barcodes are ligated. Sequencing templates are then generated by emulsion PCR using the Ion One Touch^{TM} System. DNA library fragments are ligated onto the Ion Sphere^{TM} particles and are clonally amplified. The Ion Sphere^{TM} particles are then deposited on to the Ion semiconductor chip for sequencing. And once sequencing sequence data is generated, it is automatically transferred to the Ion Torrent Server for analysis. The analysed data can then be imported into NGS data analysis solutions like Ion Reporter^{TM}.

Ion Torrent technology offers an all to one solution for NGS making it attractive. Moreover, it is also fast and simple. The only drawback is the error in sequencing homopolymer regions.

2.2. Data analysis

Next generation sequencing generates loads of sequence data and therefore it is important to synthesize these data in order to interpret it. In this context, bioinformaticians role has become crucial in developing advanced computational tools for sequencing data analysis. Briefly, a general workflow should is followed in next generation data analysis in order to identify the sequence variant of interest (reviewed in (Nielsen *et al.*, 2011; Wang and Xing, 2013; Oliver *et al.*, 2015). Initially, raw signals generated on the sequencing platforms are translated into bases with quality scores and short sequence reads. This process is called “base calling” and is platform specific. Indeed, it has become integrated on the sequencing platforms and associated software. After generating sequence reads these are aligned to a reference genome. This step is crucial for variant detection and it is important that the algorithms applied are able to cope with sequencing errors and polymorphisms in the reference genome. When reads are mapped to a reference genome variants are called (“variant calling”), meaning that the reference genome and the DNA sequence of interest are compared and differences reported. Variant calling can identify hundreds to millions of sequence variants depending on the enrichment method used and identifying the causal might therefore be like “finding a needle in a haystack”. Hence, variant priorization pipelines are necessary and should involve high quality databases for variant annotation to determine the biological significance and to evaluate its pathogenicity.

While the effects of many deleterious variants like large insertions and deletions or protein truncating variants are simple to explain, the interpretation of other genetic variations like amino acid changes or missense variants is not as straightforward (Niroula and Vihinen, 2016). Ideally all variants should be experimentally tested, but it is often costly and requires time. For this reason, computational tools have been developed for the variant priorization. Checking if a variant is present in population databases might be useful to determine if a variant is benign or not. *In silico* pathogenicity predictors based on phylogenetic conservation, protein function and structure can assess the effect of resulting amino acid changes and potential effects on splicing might also be assessed (Niroula and Vihinen, 2016).

Despite all these tools are helpful for variant priorization, additional information is needed to define if a variant is pathogenic or not (Niroula and Vihinen, 2016). Clinical information of the patient as well as segregation analyses give further information on the variant pathogenicity. Moreover, segregation analysis also determines if the variant occurred *de novo* or was inherited which is valuable in genetic counselling. If the identified variant is novel prior analyses might have not been conclusive in determining its pathogenicity. In this line, collaborative networks have demonstrated to be useful in bringing together similar cases and Matchmaker Exchange (Philippakis *et al.*, 2015) is one such platform.

Once all possible analyses have been carried out and all the evidence gathered, variants can be classified into 5 categories: Pathogenic, Likely pathogenic, Variant of uncertain significance, Likely benign and Benign. The American College of Medical Genetics (ACMG) has developed guidelines for genetic variant classification (Richards *et al.*, 2015).

2.3. Gene panel vs Exome

The diagnosis of monogenic disorders has jumped from single gene testing to gene panels o genome-wide screenings. Many disorders like intellectual disability have greatly benefited from this due to its heterogeneous nature both clinically and genetically. Next generation sequencing technologies have enabled testing multiple genes at the same time in order to find the origin of a disorder and due to its cost-effectiveness next generation technologies are increasingly applied in clinical laboratories. NGS technologies, however, cannot currently detect all type of genetic alterations (i.e. triplet repeat changes). Therefore, it is essential to understand the strengths and limitations of molecular tests in order to choose the appropriate test in each case. NGS technologies allow sequencing specific set of genes (targeted or gene panel sequencing), the entire genome (whole genome sequencing-WGS) or the coding part (whole exome sequencing-WES). One approach or the other is undertaken depending on the disease in question, preferences of the clinical laboratory and available resources.

Gene panels or targeted sequencing became popular with the introduction of NGS in clinical laboratories and the number of disease specific multigene panels increased dramatically. Gene panel testing is regarded as the most appropriate first tier test because it is fast and inexpensive (Meienberg *et al.*, 2016; Caspar *et al.*, 2018). Gene panels are especially suitable for disorders that show clinical and genetic heterogeneity, disorders with overlapping phenotypes, or disorders that share a clinical feature (i.e. epilepsy) or are associated with genes involved in the same molecular pathway (i.e. Rasopathies) (Xue *et al.*, 2015). The

number of genes included in each gene panel varies among different laboratories. Some laboratories may include genes that have somehow been associated with a specific clinical indication with the hope of increasing diagnostic yield while others only include genes that have a strong evidence of association with the disorder. However, more is not always better and appropriate gene selection is required in order to satisfy the needs of each specific clinical setting. Further, it is important for a gene panel to be cost and time effective and the inclusion of genes with weak association with the disease might result in variants of uncertain significance limiting its utility in the clinical setting (Xue *et al.*, 2015). ACMG recommendations are that only genes that have a supported role should be included in gene panels (Rehm *et al.*, 2013). It is obvious that gene panels will require updates as novel genes are identified in association with a clinical condition (Caspar *et al.*, 2018). Finally, negative results in gene panel testing are not conclusive (Caspar *et al.*, 2018) and second tier test like whole exome sequencing might be needed.

Whole exome sequencing, however, has recently become the most widely used first tier diagnostic approach in Mendelian disorders due to its decrease in price and improvements in sequencing technology and data analysis (Monroe *et al.*, 2016). Whole exome sequencing has indeed enabled the identification of novel disease causing genes and consequently has increased the diagnostic yield (Bamshad *et al.*, 2011; Timal *et al.*, 2012). In addition, WES does not require a previous knowledge of the genetic condition and could be useful when the patient does not show specific signs. However, clinical evaluation of the patient is always essential and can be of great value in variant interpretation. In this line, collaboration between physicians and diagnostic laboratories becomes important. Conversely, if the patient presents with a specific phenotype, WES analysis can also be directed to a specific subset of genes or in silico gene panels (Caspar *et al.*, 2018).

Variant interpretation has become the biggest issue in NGS, and especially in WES. WES detects between 20.000-50.000 variants per exome and even after variant priorization around 100 variants could remain for analysis (Xue *et al.*, 2015). This could be eased by following a trio based approach (Caspar *et al.*, 2018). Trio analysis has indeed been demonstrated to increase the diagnostic yield in WES (Retterer *et al.*, 2016). On the other hand, gene panel variant interpretation is more straightforward as the analysis is directed to genes already known to be associated with the disease (Caspar *et al.*, 2018). In any case, interpretation and reporting of the variants found by NGS in the disease context is still a tedious work in diagnostic laboratories. Indeed, the more variants are identified the more is the number of VUS and thus can be of concern in WES (Xue *et al.*, 2015). VUS generally include novel or rare missense variants that have not been reported in any databases and are of limited clinical utility. For the interpretation of these variants, additional analyses are needed.

Moreover, the chance of incidental findings in WES is another challenge (Xue *et al.*, 2015; Caspar *et al.*, 2018). Incidental findings are findings unrelated to the clinical indication of the patient but of medical value for patient care. These may also have implications in unaffected patients (i.e. trio base approach). Which incidental findings to report and how is important and also controversial. ACMG have developed some recommendations for reporting incidental findings (Green *et al.*, 2013; Hehir-Kwa *et al.*, 2015).

Anyways, both gene panel sequencing and WES involve capture methods which have implications on target region coverage. Neither of the approaches can offer complete coverage of all the regions of interest, since both sequencing technologies have difficulties in amplifying sequence homology regions or pseudogenes, GC-rich regions, highly repetitive regions and other sequence complexities (Meienberg *et al.*, 2016). In spite of this, targeted gene panels generally offer a higher coverage of the target regions leading to better variant identification (Jones *et al.*, 2013; Valencia *et al.*, 2013; Saudi Mendeliome Group, 2015).

WGS offers uniform genome-wide coverage allowing reliable detection of copy number variants and other structural variants (Sun *et al.*, 2015; Meienberg *et al.*, 2016). Furthermore, WGS has demonstrated to increase diagnostic yield compared to WES (Gilissen *et al.*, 2014; Belkadi *et al.*, 2015) and promises to be a comprehensive second tier test and an alternative to WES. Although WGS is still expensive and analysis time consuming (Sun *et al.*, 2015) it may become first tier test in the future not only by replacing WES but also aCGH (Meienberg *et al.*, 2016).

All in all, gene panel sequencing could be still useful as first tier in specific clinical conditions as it offers high coverage and variant interpretation straightforward. Nevertheless, it is important for gene panels to be inexpensive and not surpass WES in price. On the other hand, WGS seems to be the most comprehensive approach as a second tier test. Nevertheless, its high cost is still a limitation for routine diagnostic implementation. Hence, WES is still the most appropriate second tier test or even first tier test due to its cost-effectiveness. WES analysis can either be restricted to gene panels making analysis simpler and reducing the chance of incidental findings or not. Indeed, WES analysis enables deciphering novel disease genes and finding novel disease-gene associations.

HYPOTHESIS AND AIMS

It is already known that X-linked conditions are an important cause of ID in males. NGS has significantly contributed to the research in this field deciphering new genes and mutations responsible for ID. Our hypothesis is that by reassessing and molecularly studying patients without diagnosis, we will contribute to the research in this field evaluating the contribution of the X chromosome in our cohort of patients.

The main objective of this doctoral thesis has been to identify the contribution of X-linked genes in a retrospective cohort of male patients with ID/GDD with family history of intellectual disability.

In order to answer the initial hypothesis the main objective has been divided into three specific aims:

- **Aim 1**. To retrospectively review the cohort of male patients with family history of intellectual disability referred to our laboratory since 1991 for Fragile X Syndrome testing (Chapter 1).

    - To report the diagnostic yield obtained through the last 25 years.
    - To identify patients with unexplained intellectual disability and suggestive X-linked intellectual disability.

- **Aim 2.** To study patients with suggestive X-linked intellectual disability by targeted next generation sequencing (Chapter 2).

    - To design a X-linked intellectual disability gene panel.
    - To analyse the variants identified by this panel.

- **Aim 3.** To evaluate the contribution of the X chromosome in male patients with intellectual disability and affected brothers (Chapter 3).

    - To validate the targeted X-linked intellectual disability gene panel for XLID testing by comparing it with whole exome sequencing.
    - To determine the contribution of the X chromosome in patients with affected brothers.

CHAPTER 1:
Molecular genetic diagnosis in male patients with intellectual disability and family history: 25 years of history

This chapter was presented as a poster in "I. Congreso Interdisciplinar en Genética Humana" (25-28 April 2017, Madrid, Spain): "Diagnóstico genético molecular en varones con Discapacidad Intelectual e historia familiar: 25 años de historia" (Supplementary data_6)

1. INTRODUCTION

Intellectual disability constitutes a major problem in public health, family and society. It is estimated that up to 50% of the causes of ID are of genetic origin. The excess of males with ID and the observation of large pedigrees with X-linked inheritance of syndromic intellectual disability have served as a prelude to X-linked gene discovery (Lubs *et al*., 2012). Linkage analysis followed by molecular analysis of the candidate genes was the most popular strategy in XLID gene identification. Despite not being as productive as linkage analysis, other strategies have also contributed to X-linked gene discovery (Figure 1). Analysis of known molecular and metabolic pathways has helped in identifying many genes, and investigations of chromosome rearrangement break regions have also given significant results. Since the conclusion of first human genome, genomic microarray and next generation sequencing technologies have become the most favourite approaches.

Retrospective studies have demonstrated to be of great value in genetic testing of patients with unresolved ID, by screening these patients in light of novel gene identifications or development of new genetic diagnostic tools. Before genome-wide technologies became available, single gene testing was performed in unresolved patients that showed a similar phenotype or biomarker or in patients with unspecific ID. Indeed, as soon as the *FMR1* gene was discovered, many families with XLID were screened for this gene (Tejada *et al.*, 1992). In the same way, male patients with unexplained NS-XLID were tested for the *SLC6A8* gene (Rosenberg *et al.*, 2004). In addition, patients presenting with similar phenotypic features were also analysed for candidate genes. For instance, after *PHF8* gene was identified in a male patient with Siderius syndrome (Laumonnier *et al.*, 2005), many male patients presenting with ID and cleft lip or palate were tested for this gene (Abidi *et al.*, 2007; Koivisto *et al.*, 2007). Similarly, *MECP2* screenings were performed in females with Rett-like features or even unspecific ID (Tejada *et al.*, 2006). The introduction of next generation technologies has now substantially increased the diagnostic yield in patients with unexplained ID together with identifying novel genes responsible for ID (Tarpey *et al.*, 2009; Athanasakis *et al.*, 2014; Fitzgerald *et al.*, 2015; Grozeva *et al.*, 2015; Hu *et al.*, 2016).

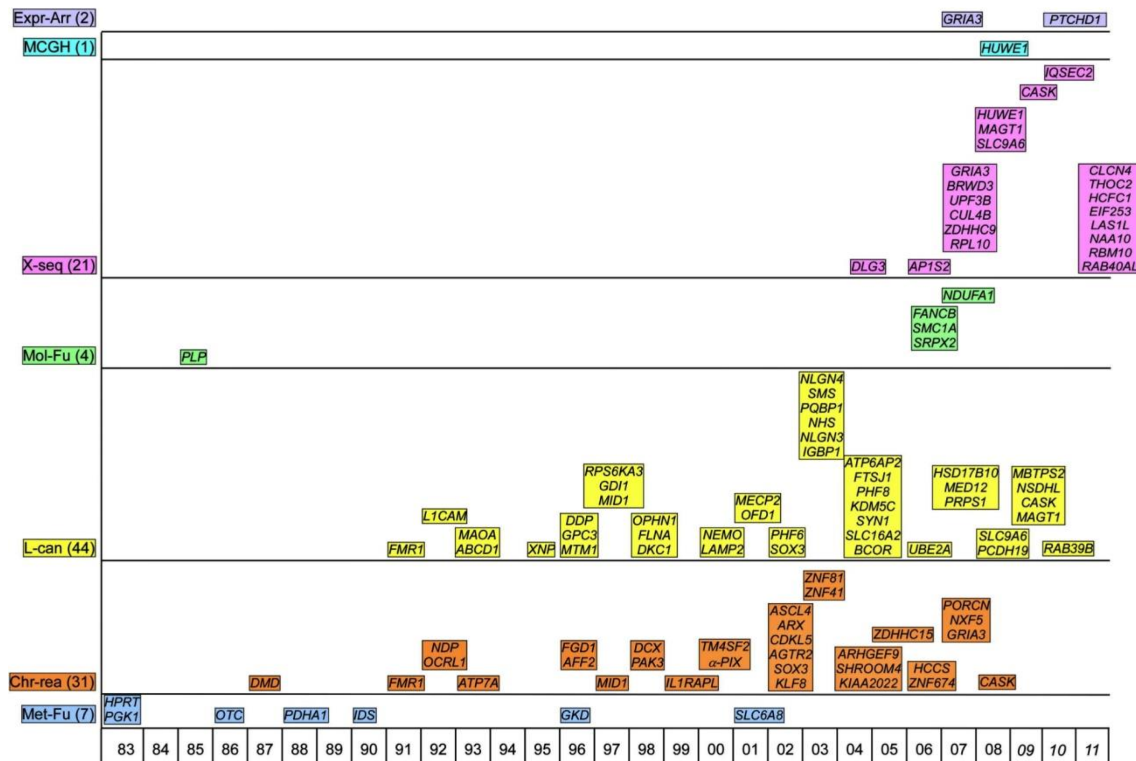


Figure 1. Techniques applied in X-linked intellectual disability gene discovery. X-axis indicates the year of gene discovery. Figure taken from Lubs *et al*., 2012.

Met-Fu: follow-up of a known metabolic pathway, Chr-rea: chromosome rearrangement analysis, L-can: linkage analysis followed by candidate gene testing, Mol-Fu: follow-up of known molecular pathway, X-seq: gene sequencing, MCGH: genomic microarray, Exp-Arr: expression microarray.

Chromosomal aberrations are known to be common in ID and have been tested using different molecular techniques. Prior to the development of genomic microarray, karyotype analysis was the gold standard technique for detecting gross chromosomal aberrations and other techniques like MLPA were developed in order to detect recurrent submicroscopic copy number variants. MLPA testing for subtelomeric regions (Koolen *et al.*, 2004) as wells as the X chromosome (Madrigal *et al.*, 2007) have indeed been useful in elucidating the origin of many patients with unexplained ID and have identified the genetic origin in about 5% of the patients tested. Genomic microarray techniques have now replaced the standard karyotype and other tests like MLPA due to its higher resolution and diagnostic yield, and is routinely performed increasing the diagnostic yield in unresolved patients with ID up to 20% (Miller *et al.*, 2010; Vissers *et al.*, 2010).

The molecular genetics laboratory in which this work has been carried out, has developed all these different molecular techniques over the years to try to uncover the genetic origin of patients with unexplained ID. An extensive review of all male patients that were referred for *FMR1* testing from 1991 to 2015 has been made and the contribution of family history has been analysed, especially X-linked family history, in order to identify the number of patients with suggestive X-linked intellectual disability. At the same time, the diagnoses obtained during this period have also been collected.

2. MATERIAL AND METHODS

Since the discovery of the *FMR1* gene in 1991 the laboratory leaded by Dr. Tejada has been the centre of reference for FXS and has tested loads of DNA samples of patients presenting with GDD or ID. DNA samples of all these index patients as well as their mothers have been routinely stored and they currently are in the Genetics Service at Cruces University Hospital.

Patients mainly came from paediatric neurology units, neurologists, psychiatrists or medical geneticists of the four different public hospitals in the Spanish Basque Country. All patients had a normal G-banded karyotype and were generally evaluated using a standardized questionnaire before *FMR1* testing (Supplementary data_1). The questionnaire briefly included information on clinical and family history and phenotype. Patients that are generally referred for FXS testing show a non-syndromic or unspecific phenotype, meaning that they do not show apparent dysmorphic features that could be recognised as a syndrome by medical geneticists. In spite of that, they may show subtle dysmorphic features or other neurological features like epilepsy or ASD besides intellectual disability.

An overview on clinical data of male patients with family history of ID that were referred for Fragile-X testing to the molecular genetics laboratory during the period of 1991-2015 was performed through a codified dataset. This dataset includes information on gender, age at referral, degree of ID, family history of intellectual disability and relevant clinical data on other comorbidities like epilepsy, ASD or dysmorphic features. The dataset also includes information on molecular genetic techniques applied to each sample through these years and the diagnosis obtained. For the purpose of this study, the dataset was classified based on inheritance of ID and the cohort of index patients was divided into 6 different categories: 1) affected brothers 2) affected brothers and sisters 3) affected brother and other family history of ID 4) affected brother and X-linked family history of ID 5) X-linked family history of ID 6) Others and 7) Not available . X-linked family history of ID was defined as having an affected half-brother and/or maternal uncle/nephew and/or maternal male cousin.

3. RESULTS

3.1. Family History

In total 1909 males were referred for *FMR1* testing during the period of 1991-2015 and 230 of these males (12%) have family history of ID (categories 1 to 6). Of these, about 40% (93/230) show X-linked family history of ID (Figure 2). These include patients that were classified in categories 4 (affected brother and X-linked family history) and 5 (X-linked family history).

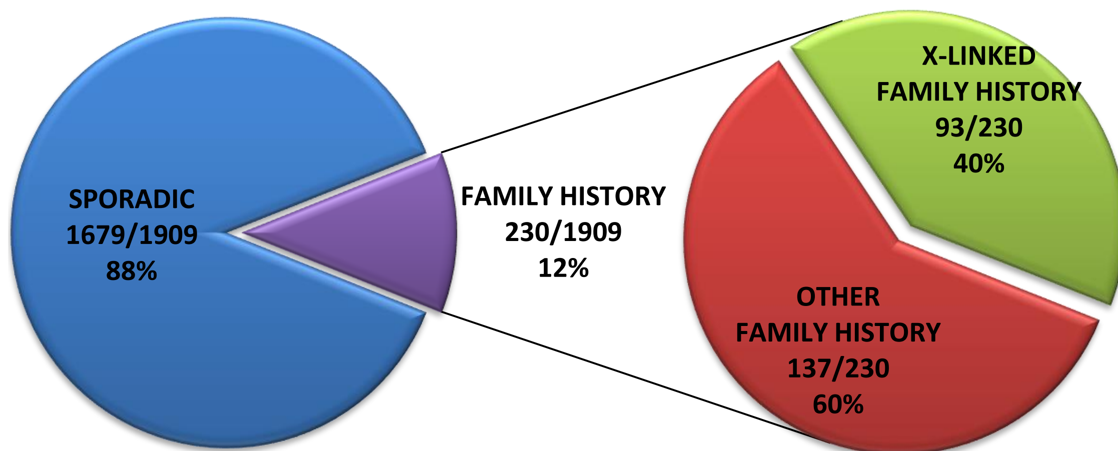


Figure 2. Inheritance of intellectual disability. Percentage of sporadic cases of intellectual disability and those with family history of intellectual disability is represented. Percentage X-linked family history of intellectual disability is also represented.

3.2. Diagnosis

Throughout these years a definite molecular diagnosis has been obtained in 25.6% (59/230) of the male patients with family history of ID thanks to the molecular diagnostic techniques developed (Figure 3). Fragile X syndrome diagnosis was the most frequent diagnosis made (43/230=18.7%).

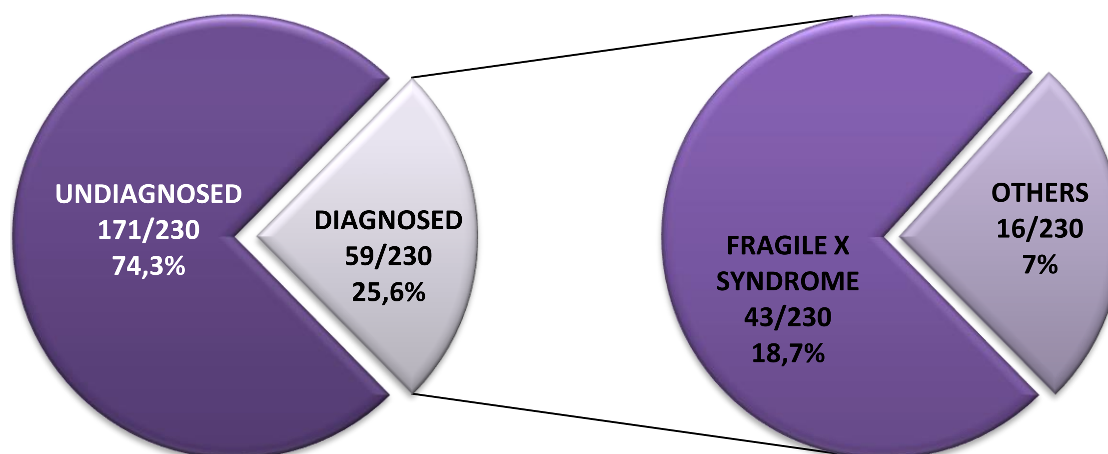


Figure 3. Genetic causes of intellectual disability in male patients with intellectual disability and family history. Genetic causes have been identified in about 26% patients, Fragile X Syndrome being the most common diagnosis.

Among the other patients with family history of ID and cytogenetic marker Xq28, one patient with FRAXE mental retardation syndrome (OMIM#309548) was identified, having an anomalous CGG expansion in the AFF2 gene.

The development of MLPA techniques followed by aCGH has clearly helped in detecting copy number variants. Altogether, copy number variants have been detected in 11 patients (11/230=4.78%) either through MLPA or aCGH (Table 1). Of these 6 are microdeletions and microduplications of the X chromosome.

Table 1. Copy number variants identified in male patients with family history of intellectual disability.

Region	Type	Inheritance	Technique
Xp11.22 (*HUWE1*)	duplication	X-linked	XLID MLPA
Xp22.12 (*RPS6KA3*)*	duplication	X-linked	XLID MLPA
Xq28ter (*MECP2*)**	duplication	X-linked	XLID MLPA
Xq28ter (*MECP2*)	duplication	X-linked	XLID MLPA
OPHN1 c.169-?_239+?	deletion	X-linked	XLID MLPA
IL1RAPL1 c.178-?_249+?	deletion	X-linked	XLID MLPA
der(6)t(5;6)(qter;qter)	translocation	NA	subtelomeric MLPA
der(3)t(3;8)(pter;qter)	translocation	paternal	subtelomeric MLPA
der(3)t(3;5)(p26;q35)	translocation	maternal	aCGH
dup(1)(pter)	duplication	*de novo*	subtelomeric MLPA
17p13.1	duplication	*de novo*	aCGH

*Tejada *et al.*, 2011

**Madrigal *et al.*, 2007

The laboratory has also participated and contributed to NGS collaborative studies. As a result, other 4 diagnoses were obtained. Three of them were truncating variants: *UPF3B* (c.118C>T; p.Gln40*) (Martínez *et al.*, 2004; Tejada *et al.*, 2019), *SYP* (c.829_832delGACT; p.Asp277fs*36)

(Tarpey *et al.*, 2009) and *AGA* (c. 503G>A; p.Trp168*) (Grozeva *et al.*, 2015); and the other a missense variant in *KRAS* (c.40G>A; p.Val14Ile) (Grozeva *et al.*, 2015).

If we focus on the X chromosome, 52 molecular diagnoses were X-linked (43 FXS; 1 FRAXE; 6 CNVs and 2 SNV) (Figure 4) and constitute the majority of the diagnoses obtained.

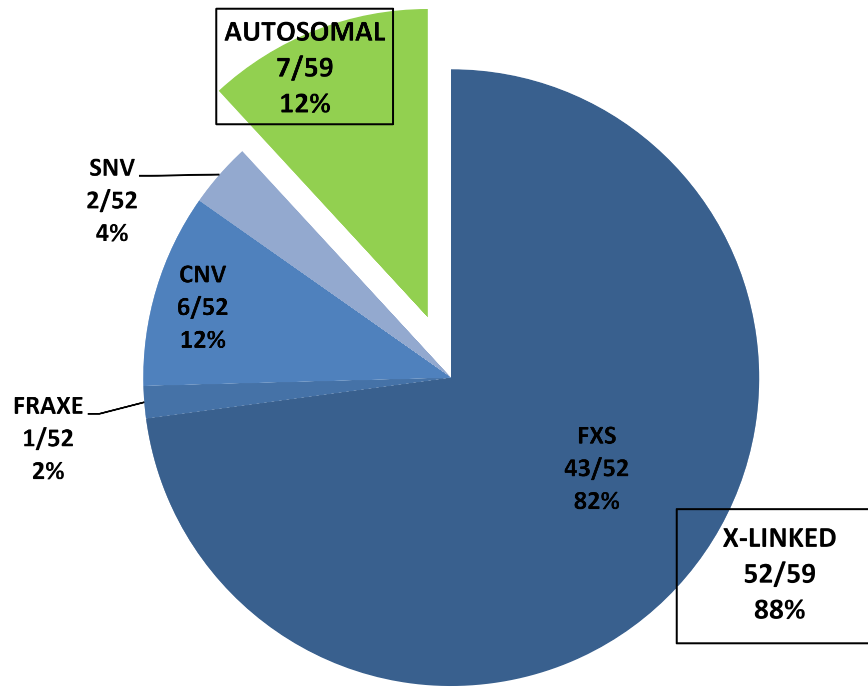


Figure 4. X-linked versus autosomal origin in male patients with intellectual disability and family history. Most of the diagnoses made in male patients with family history have their origin on the X chromosome (52/59).

Nevertheless, 171 male patients with family history of intellectual disability still remained unsolved at the time (Figure 3).

4. DISCUSSION

We have reviewed our cohort of male patients referred for *FMR1* testing between 1991 and 2015 and analysed deeper those having family history of intellectual disability identifying the number of patients with X-linked family history and reporting the diagnosis obtained.

In our cohort of male patients, 88% did not refer family history of ID suggesting that they are sporadic cases (Figure 2). As it was stated in the Introduction, ID is extremely heterogeneous and can be caused by environmental and socioeconomic factors or genetic alterations or both, and many sporadic cases may be due to both causes. As for genetic alterations, it is said that *de novo* occurring mutations are important cause of ID in outbred populations. The high mutation rate per generation in humans (Lynch, 2010) also supports the *de novo* paradigm. McRae *et al*., (2017) estimated that 42% of the intellectual disability could be explained by *de novo* mutations.The occurrence of *de novo* mutations is higher in severe ID and could explain the origin of ID in up to 60% of the patients (Gilissen *et al.*, 2014). Finally, other genetic factors could also lead to sporadic ID. Actually, most of the patients with FXS and a full mutation occur due to the anomalous CGG repeat expansion of premutation alleles on their mothers and are therefore sporadic cases (Biancalana *et al.*, 2004).

On the other hand, ID can be inherited and therefore occur in families. Inherited forms of ID are generally associated with milder phenotypes. In our male cohort of patients 12% of the patients showed family history of ID and 40% of them show X-linked inheritance of ID (Figure 2). During the past two decades, great effort has been done in our laboratory in elucidating the genetic origin of ID, being XLID the main focus of our research. The different technologies that have been developed in our laboratory these years succeeded in making a definite diagnosis in 25.6% of male patients with family history of ID (Figure 3). Among the diagnosis made, it is worth noting that Fragile X Syndrome was obviously the most common (43/230=18.7%).

Copy number variants have also been identified in about 5% (11/230) of the reviewed cases. It has been estimated that CNVs can explain up to 20% of ID overall *(Miller et al., 2010;* Vissers *et al*., 2010). However, most of the copy number variants occur *de novo* (Vissers *et al*., 2010) and hence, our percentage is lower in inherited ID. Moreover, aCGH has not been routinely performed in our laboratory until recently. Therefore, many of the patients in our cohort have only been assessed by subtelomeric and/or XLID MLPA.

On the other hand, single nucleotide variants (SNV) have only been identified in 1.7% (4/230) of the patients. This is due to the lack of resources to test this type of variants at that time. Next generation technologies have now enabled testing of multiple genes at the same time and our laboratory also applied this technology through collaborative studies. In this way,

single nucleotide variants reported here were identified in in these collaborative studies (Tarpey *et al.*, 2009; Grozeva *et al.*, 2015) and previously mapped linkage regions (Martínez *et al.*, 2004; Tejada *et al.*, 2019).

Overall, the diagnosis obtained suggests that X chromosome greatly contributes to the origin of ID since nearly 90% (52/59) of the diagnoses obtained are X-linked conditions (Figure 4). These represent about 23% (52/230) of whole cohort of male patients with family history of ID. As it was already stated on the Introduction, genetic defects on X chromosome substantially contribute to the origin of ID in males. Nevertheless, these results might be biased due the effort made in our laboratory in the diagnosis of FXS. Thus, the results of 43/59 (73%) cases with FXS is a high number and it is noteworthy that excluding Fragile X, 7 of the other diagnosis were autosomal and 9 were X-linked. This would imply that genes involved in ID are just as likely to be on the X as it to be on one of the autosomes. But as it has been said, most of our patients had neither aCGH nor NGS at the time this review was done.

Anyway, 171 male patients with family history of ID still remain without molecular diagnosis. In line with what has been reviewed, we believe that the contribution of X-linked genes should be significant in these patients, especially in those that have X-linked family history of ID. Therefore, sequencing of XLID genes on these patients could help in revealing the genetic origin of ID.

CHAPTER 2:
Targeted next generation sequencing in patients with suggestive X-linked intellectual disability

This chapter has been published and presented as a poster:

- ✓ *Poster presented in the "19th International Workshop on Fragile X and other Neurodevelopmental Disorders" (18-21 September2019, Sorrento-Italy): Targeted next generation sequencing in patients with possible X-linked intellectual disability (Supplementary data_7)*
- ✓ *Ibarluzea N, de la Hoz AB, Villate O, Llano I, Ocio I, Martí I, Guitart M, Gabau E, Andrade F, Gener B, Tejada MI. Targeted Next-Generation Sequencing in Patients with Suggestive X-Linked Intellectual Disability. Genes (Basel). 2020;11(1):51. doi:10.3390/genes11010051.*

1. INTRODUCTION

Intellectual disability is the most common neurodevelopmental disorder with a worldwide prevalence of about 1% (Maulik *et al.*, 2011; McKenzie *et al.*, 2016) and is defined by impaired cognitive functioning and adaptive behaviour arising before the age of 18 (Schalock *et al.*, 2010). Its severity is usually measured by the intelligence quotient score and classified as mild (IQ 55–70), moderate (IQ 40–55), severe (IQ 25–40), or profound (IQ < 25). Clinically, it has also been useful to divide into two other categories: Syndromic and non-syndromic or unspecific intellectual disability (Stevenson and Schwartz, 2002). Syndromic forms of ID show dysmorphic, neurological, or systemic features that are recognizable by medical geneticists. Conversely, non-syndromic or unspecific forms only show ID as a common feature although they may individually show additional clinical features. Yet, the distinction between the two categories is obscure and sometimes arbitrary (Ropers and Hamel, 2005; Ropers, 2006; Stevenson and Schwartz, 2009).

X-linked intellectual disability has captured great attention due to the overrepresentation of males in intellectual disability (Maulik *et al.*, 2011). Scientists have spent a good deal of energy in deciphering the genetic origin of XLID by applying different techniques. Linkage analysis followed by candidate gene testing has been a popular technique in screening large families with X-linked inheritance of ID (Stevenson and Schwartz, 2009; Lubs *et al*., 2012). Over the past decade, sequencing studies have clearly stimulated the gene discovery process (Vissers *et al*., 2015). Currently, roughly 141 genes are known to be associated with XLID (Neri *et al.*, 2018). Regardless, the aberrant expansion of the CGG trinucleotide repeat at the *FMR1* gene that causes Fragile X Syndrome is still the most common condition in XLID (Crawford *et al.*, 2001).

Owing to the genetic heterogeneity of XLID, next-generation sequencing technologies have become of great benefit in this field. Tarpey *et al*., 2009 set the grounds for next-generation sequencing in XLID by sequencing all coding exons of the X-chromosome and reaching a diagnostic yield of 25%. Both XLID gene panel (Tzschach *et al.*, 2015) or X-exome sequencing strategies (Philips *et al.*, 2014; Niranjan *et al.*, 2015; Hu *et al.*, 2016) have been applied since then in families with suggestive X-linked inheritance of ID (Tarpey *et al.*, 2009; Philips *et al.*, 2014; Hu *et al.*, 2016) and affected male sib pairs or sporadic cases (Niranjan *et al.*, 2015; Tzschach *et al.*, 2015) obtaining a similar diagnostic yield. However, many of the XLID families tested through X-exome or gene panels remained without diagnosis. Whole-exome sequencing of patients with suggestive XLID has demonstrated that ID could be of autosomal origin in these patients and occur de novo despite family history (Sanchis-Juan *et al.*, 2019). Indeed, exome sequencing has been demonstrated to increase the diagnostic yield in unresolved patients with ID, highlighting the importance of *de novo* mutations (Vissers *et al.*, 2010; Vissers

et al., 2015). Despite the popularity of whole-exome sequencing in intellectual disability, gene panels are still useful in clinical practice.

In this study, we have targeted 82 XLID genes by next-generation sequencing in a cohort of 61 patients with suggestive non-syndromic XLID to try to elucidate the genetic origin of ID.

2. MATERIAL AND METHODS

2.1. Patient cohort

Patients' samples have been received since 1991 in our molecular genetics laboratory for Fragile X Syndrome testing from paediatric neurology units, neurologists, or medical geneticists, mostly of the four public hospitals of the Basque Autonomous Community (Spain) because they had been diagnosed with global developmental delay or unspecific intellectual disability. Clinical data for *FMR1* testing were generally collected using a standardized questionnaire that briefly included information on clinical and family history and phenotype. Written informed consent (Supplementary data_2) was obtained from patients' parents or legal representatives prior to genetic testing and this study was approved by the Research Ethics Committee at Cruces University Hospital (CEIC: E18/07).

Male patients with possible non-syndromic XLID were selected from the initial database (see Chapter 1) based on the X-linked family history of ID, meaning that they had affected half-brother and/or uncle/nephew and/or maternal male cousin. Moreover, index males with ID and affected brothers were also selected based on the mother's skewed X-inactivation pattern. Furthermore, male patients that have been referred to our laboratory since 2015 and fulfill the criteria for this study have also been included. The initial cohort comprised 54 index male patients with suggestive X-linked inheritance of ID and 62 index male patients with affected brothers.

2.2. X inactivation

X chromosome inactivation on peripheral blood was assessed on the available samples of the selected patients' mothers by genotyping the highly polymorphic small tandem repeat within the 5'UTR of the human androgen receptor (AR) gene following the protocol described by Allen *et al*., 1992. Inactivation was considered to be random when the ratio of active to inactive X was less than 75:25. Extreme skewing of X inactivation was defined as the preferential inactivation of one X chromosome in 90–95% of cells (Ørstavik, 2009). However, skewing of X inactivation of women aged above 55 was considered to be uninformative, because skewed X inactivation is known to be age-related (Ørstavik, 2009).

2.3. Multiple Ligation Probe Amplification

As most patients were selected from our laboratory database (see chapter 1), many of them lack chromosomal microarray studies. Therefore, a specific MLPA analysis for the X chromosome was performed on the selected samples to discard any common microduplications and microdeletions. MLPA was performed using the P106-C1 kit (MRC-

Holland, the Netherlands) and following the manufacturer's recommendations. The kit contains probes that are specific for 16 known X-linked intellectual disability genes: *RPS6KA3, ARX, IL1RAPL1, TSPAN7, PQBP1, HUWE1, OPHN1, ACSL4, PAK3, DCX, AGTR2, ARHGEF6, FMR1, AFF2* (*FMR2*), SLC6A8 and *GDI1*. Electrophoresis was performed with Rox 500 size standard using the ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA) and visualized using GeneMapper software (Applied Biosystems, Foster City, CA). GeneMapper data were exported to an Excel file to perform the analysis as suggested by the manufacturer.

2.4. Targeted Next Generation Sequencing

To create a cost-efficient gene panel that could be transferable to XLID diagnosis in the clinic, we selected 82 X-linked intellectual disability genes based on the available literature at that moment (in 2015, see references in Table 1). The selected gene panel includes genes that can lead to either non-syndromic XLID or high phenotypic variability in males and candidate genes that have been reported in XLID but have not yet been established as XLID genes (see Table 1). Genes leading to syndromic forms that show identifiable clinical features were excluded since they are generally identifiable by medical geneticists. The selected genes are the following: *ACSL4*, *AFF2*, *AGTR2*, *ALG13*, *AP1S2*, *ARHGEF9*, *ARX*, *ATP6AP2*, *ATP7A*, *ATRX*, *BRWD3*, *CASK*, *CCDC22*, *CLIC2*, *CUL4B*, *DCX*, *DLG3*, *DMD*, *FAM120C*, *FLNA*, *FMR1*, *FTSJ1*, *GDI1*, *GPC3*, *GPC4*, *GRIA3*, *HCFC1*, *HDAC8*, *HSD17B10*, *HUWE1*, *IGBP1*, *IL1RAPL1*, *IQSEC2*, *KAL1*, *KDM5C*, *KDM6A*, *KIAA2022*, *L1CAM*, *MAOA*, *MECP2*, *MED12*, *MID1*, *NAA10*, *NDP*, *NHS*, *NLGN3*, *NLGN4X*, *NSDHL*, *OFD1*, *OPHN1*, *PAK3*, *PHF6*, *PHF8*, *PLP1*, *PQBP1*, *PRPS1*, *PTCHD1*, *RAB39B*, *RBM10*, *RBMX*, *RNF113A*, *RPL10*, *RPS6KA3*, *SLC16A2*, *SLC6A8*, *SLC9A6*, *SMARCA1*, *SMC1A*, *SMS*, *SRPX2*, *SYN1*, *SYP*, *TAF1*, *TSPAN7*, *UBE2A*, *UPF3B*, *USP9X*, *WDR45*, *ZC4H2*, *ZDHHC15*, *ZDHHC9*, *ZNF711.*

Table 1. List of the 82 genes included in our XLID gene panel. All the phenotypes that have been associated with each gene are shown. These phenotypes might include ID or not. Moreover, references for which these genes were included in our gene panel are also listed.

Gene symbol	OMIM	Associated phenotype	References
ACSL4	300157	Mental retardation, X-linked 63 (OMIM#300387)	(Meloni *et al.*, 2002; Longo *et al.*, 2003)
AFF2	300806	Mental retardation, X-linked, FRAXE type (OMIM#309548)	(Stettner *et al.*, 2011; Mondal *et al.*, 2012)
AGTR2	300034	Severe mental retardation, pervasive developmental disorder, and epilepsy	(Takeshita *et al.*, 2012)
ALG13	300776	Congenital disorder of glycosylation, type Is; Epileptic encephalopathy, early infantile, 36 (OMIM#300884)	(Bissar-Tadmouri *et al.*, 2014)
AP1S2	300629	Mental retardation, X-linked syndromic 5 (OMIM#304340)	(Tarpey *et al.*, 2006)
ARHGEF9	300429	Epileptic encephalopathy, early infantile, 8 (OMIM#300607)	(Harvey *et al.*, 2004; Shimojima *et al.*, 2011)
ARX	300382	Epileptic encephalopathy, early infantile, 1 (OMIM#308350)	(Shoubridge *et al.*, 2010)
		Hydranencephaly with abnormal genitalia Lissencephaly, X-linked 2 (OMIM#300215)	
		Mental retardation, X-linked 29 and others (OMIM#300419)	
		Partington syndrome (OMIM#309510)	
		Proud syndrome (OMIM#300004)	
ATP6AP2	300556	Parkinsonism with spasticity, X-linked (OMIM#300911)	(Gupta *et al.*, 2015)
		Mental retardation, X-linked, syndromic, Hedera type (OMIM#300423)	
ATP7A	300011	Menkes disease (OMIM#309400)	(Kaler *et al.*, 1994; Tümer, 2013)
		Occipital horn syndrome (OMIM#304150)	
		Spinal muscular atrophy, distal, X-linked 3 (OMIM#300489)	
ATRX	300032	Alpha-thalassemia myelodysplasia syndrome, somatic (OMIM#300448)	(Yntema *et al.*, 2002; Moncini *et al.*, 2013)
		Alpha-thalassemia/mental retardation syndrome (OMIM#301040)	
		Mental retardation-hypotonic facies syndrome, X-linked (OMIM#309580)	
BRWD3	300553	Mental retardation, X-linked 93 (OMIM#300659)	(Field *et al.*, 2007)
CASK	300172	Mental retardation, with or without nystagmus; FG syndrome 4 (OMIM#300422)	(Hackett *et al.*, 2010)
		Mental retardation and microcephaly with pontine and cerebellar hypoplasia (OMIM#300749)	
CCDC22	300859	Ritscher-Schinzel syndrome 2 (OMIM#300963)	(Voineagu *et al.*, 2012)

CLIC2	300138	Mental retardation, X-linked, syndromic 32 (OMIM#300886)	(Witham *et al.*, 2011; Takano *et al.*, 2012)
CUL4B	300304	Mental retardation, X-linked, syndromic 15 (Cabezas type) (OMIM#300354)	(Tarpey *et al.*, 2007)
DCX	300121	Lissencephaly, X-linked; Subcortical laminal heterotopia, X-linked (OMIM#300067)	(Guerrini *et al.*, 2003)
DLG3	300189	Mental retardation, X-linked 90 (OMIM#300850)	(Tarpey *et al.*, 2004; Zanni *et al.*, 2010)
DMD	300377	Becker muscular dystrophy (OMIM#300376)	(de Brouwer et al., 2014; Srour et al., 2008)
		Cardiomyopathy, dilated, 3B (OMIM#302045)	
		Duchenne muscular dystrophy (OMIM#310200)	
FAM120C	300741	Autism spectrum disorder	(de Wolf *et al.*, 2014)
FLNA	300017	FG syndrome 2 (OMIM#300321)	(Robertson, 2005)
		Cardiac valvular dysplasia, X-linked (OMIM#314400)	
		Congenital short bowel syndrome (OMIM#300048)	
		Frontometaphyseal dysplasia 1 (OMIM# 305620)	
		Heterotopia, periventricular, 1 (OMIM#300049)	
		Intestinal pseudoobstruction, neuronal (OMIM#300048)	
		Melnick-Needles syndrome (OMIM#309350)	
		Otopalatodigital syndrome, type I (OMIM#311300)	
		Otopalatodigital syndrome, type II (OMIM#304120)	
		Terminal osseous dysplasia (OMIM#300244)	
FMR1	309550	Fragile X syndrome (OMIM#300624)	(Collins *et al.*, 2010; Myrick *et al.*, 2014, 2015)
		Fragile X tremor/ataxia syndrome (OMIM#300623)	
		Premature ovarian failure 1 (OMIM#311360)	
FTSJ1	300499	Mental retardation, X-linked 9/44 (OMIM#309549)	(Freude *et al.*, 2004)
GDI1	300104	Mental retardation, X-linked 41 (OMIM#300849)	(D'Adamo *et al.*, 1998)
GPC3	300037	Simpson-Golabi-Behmel syndrome, type 1 (OMIM#312870)	(Veugelers *et al.*, 2000; Cottereau *et al.*, 2013)
		Wilms tumor, somatic (OMIM#194070)	
GPC4	300168	Simpson-Golabi-Behmel syndrome type 1 (OMIM#312870)	(Waterson *et al.*, 2010; Cottereau *et al.*, 2013)
GRIA3	305915	Mental retardation, X-linked 94 (OMIM#300699)	(Wu *et al.*, 2007; Philips *et al.*, 2014)
HCFC1	300019	Mental retardation, X-linked 3 (methylmalonic acidemia and homocysteinemia, cblX type)	(Huang *et al.*, 2012)

		(OMIM#309541)	
HDAC8	300269	Cornelia de Lange syndrome 5 (OMIM#300882)	(Kaiser *et al.*, 2014)
HSD17B10	300256	HSD10 mitochondrial disease (OMIM#300438)	(Lenski *et al.*, 2007; Froyen *et al.*, 2008)
HUWE1	300697	Mental retardation, X-linked syndromic, Turner type (OMIM#309590)	(Froyen *et al.*, 2008)
IGBP1	300139	Corpus callosum, agenesis of, with mental retardation, ocular coloboma and micrognathia (OMIM#300472)	(Graham *et al.*, 2003)
IL1RAPL1	300206	Mental retardation, X-linked 21/34 (OMIM#300143)	(Carrié *et al.*, 1999)
IQSEC2	300522	Mental retardation, X-linked 1/78 (OMIM#309530)	(Gandomi *et al.*, 2014; Shoubridge *et al.*, 2010)
KAL1	300836	Mild intelectual disability, hyperosmia, ectrodactyly	(Sowińska-Seidler *et al.*, 2015)
KDM5C	314690	Mental retardation, X-linked, syndromic, Claes-Jensen type (OMIM#300534)	(Adegbola *et al.*, 2008; Jensen *et al.*, 2005; Tzschach *et al.*, 2006)
KDM6A	300128	Kabuki syndrome 2 (OMIM#300867)	(Lederer *et al.*, 2012; Miyake *et al.*, 2013)
KIAA2022	300524	Mental retardation, X-linked 98 (OMIM#300912)	(van Maldergem *et al.*, 2013; Kuroda *et al.*, 2015)
L1CAM	308840	Corpus callosum, partial agenesis of (OMIM#304100)	(Rosenthal *et al.*, 1992; Jouet *et al.*, 1994)
		CRASH syndrome; MASA syndrome (OMIM#303350)	
		Hydrocephalus due to aqueductal stenosis; Hydrocephalus with congenital idiopathic intestinal pseudoobstruction; Hydrocephalus with Hirschsprung disease (OMIM#307000)	
MAOA	309850	Brunner syndrome; Antisocial behavior (OMIM#300615)	(Brunner et al., 1993; Piton et al., 2014)
MECP2	300005	Encephalopathy, neonatal severe (OMIM#300673)	(Orrico *et al.*, 2000; Villard, 2007)
		Mental retardation, X-linked syndromic, Lubs type (OMIM#300260)	
		Mental retardation, X-linked, syndromic 13 (OMIM#300055)	
		Rett syndrome; Rett syndrome, atypical; Rett syndrome, preserved speech variant (OMIM#312750)	
		Autism susceptibility, X-linked 3 (OMIM#300496)	
MED12	300188	Lujan-Fryns syndrome (OMIM# 309520)	(Lesca *et al.*, 2013)
		Ohdo syndrome, X-linked (OMIM#300895)	
		Opitz-Kaveggia syndrome (OMIM#305450)	
MID1	300552	Opitz GBBB syndrome, type I (OMIM#300000)	(Quaderi *et al.*, 1997)
NAA10	300013	Microphthalmia, syndromic 1 (OMIM#309800)	(Casey *et al.*, 2015; Popp *et al.*, 2015)

		Ogden syndrome (OMIM#300855)	
NDP	300658	Exudative vitreoretinopathy 2, X-linked (OMIM#305390)	(Berger *et al.*, 1992)
		Norrie disease (OMIM#310600)	
NHS	300457	Cataract 40, X-linked (OMIM#302200)	(Burdon *et al.*, 2003)
		Nance-Horan syndrome (OMIM#302350)	
NLGN3	300336	Asperger syndrome susceptibility, X-linked 1 (OMIM#300494)	(Jamain *et al.*, 2003)
		Autism susceptibility, X-linked 1 (OMIM#300425)	
NLGN4X	300427	Mental retardation, X-linked; Autism susceptibility, X-linked 2 (OMIM#300495)	(Jamain *et al.*, 2003)
		Asperger syndrome susceptibility, X-linked 2 (OMIM#300497)	
NSDHL	300275	CHILD syndrome (OMIM#308050)	(König *et al.*, 2000; McLarren *et al.*, 2010)
		CK syndrome (OMIM#300831)	
OFD1	300170	Retinitis pigmentosa 23 (OMIM#300424)	(Ferrante *et al.*, 2001; Budny *et al.*, 2006; Coene *et al.*, 2009)
		Joubert syndrome 10 (OMIM#300804)	
		Orofaciodigital syndrome I (OMIM#311200)	
		Simpson-Golabi-Behmel syndrome, type 2 (OMIM#300209)	
OPHN1	300127	Mental retardation, X-linked, with cerebellar hypoplasia and distinctive facial appearance (OMIM#300486)	(Billuart *et al.*, 1998)
PAK3	300142	Mental retardation, X-linked 30/47 (OMIM#300558)	(Allen et al., 1998)
PHF6	300414	Borjeson-Forssman-Lehmann syndrome (OMIM#301900)	(Lower *et al.*, 2002)
PHF8	300560	Mental retardation syndrome, X-linked, Siderius type (OMIM#300263)	(Laumonnier *et al.*, 2005)
PLP1	300401	Pelizaeus-Merzbacher disease (OMIM#312080)	(Saugier-Veber *et al.*, 1994)
		Spastic paraplegia 2, X-linked (OMIM#312920)	
PQBP1	300463	Renpenning syndrome (OMIM#309500)	(Kalscheuer *et al.*, 2003; Lenski *et al.*, 2004)
PRPS1	311850	Arts syndrome (OMIM#301835)	(Mittal *et al.*, 2015)
		Charcot-Marie-Tooth disease, X-linked recessive, 5 (OMIM#311070)	
		Deafness, X-linked 1 (OMIM#304500)	
		Gout, PRPS-related; Phosphoribosylpyrophosphate synthetase superactivity (OMIM#300661)	
PTCHD1	300828	Autism, susceptibility to, X-linked 4 (OMIM#300830)	(Noor *et al.*, 2010; Chaudhry *et al.*, 2015)

RAB39B	300774	Mental retardation, X-linked 72 (OMIM#300271)	(Giannandrea *et al.*, 2010)
		Waisman syndrome (OMIM#311510)	
RBM10	300080	TARP syndrome (OMIM#311900)	(Johnston *et al.*, 2010, 2014)
RBMX	300199	Mental retardation, X-linked, syndromic 11, Shashi type (OMIM#300238)	(Shashi *et al.*, 2015)
RNF113A	300951	Trichothiodystrophy 5, nonphotosensitive (OMIM#300953)	(Corbett *et al.*, 2015)
RPL10	312173	Mental retardation, X-linked, syndromic, 35 (OMIM#300998)	(Klauck *et al.*, 2006; Thevenon *et al.*, 2015)
		Autism, susceptibility to, X-linked 5 (OMIM#300847)	
RPS6KA3	300075	Coffin-Lowry syndrome (OMIM#303600)	(Merienne *et al.*, 1999)
		Mental retardation, X-linked 19 (OMIM#300844)	
SLC16A2	300095	Allan-Herndon-Dudley syndrome (OMIM#300523)	(Dumitrescu *et al.*, 2004; Friesema *et al.*, 2004)
SLC6A8	300036	Cerebral creatine deficiency syndrome 1 (OMIM#300352)	(Salomons *et al.*, 2001)
SLC9A6	300231	Mental retardation, X-linked syndromic, Christianson type (OMIM#300243)	(Masurel-Paulet *et al.*, 2016)
SMARCA1	300012	Intellectual disability, microcephaly and spasticity (Coffin-Siris like phenotype)	(Karaca *et al.*, 2015)
SMC1A	300040	Cornelia de Lange syndrome 2 (OMIM#300590)	(Deardorff *et al.*, 2007)
SMS	300105	Mental retardation, X-linked, Snyder-Robinson type (OMIM#309583)	(Zhang *et al.*, 2013)
SRPX2	300642	Rolandic epilepsy, mental retardation, and speech dyspraxia (OMIM#300643)	(Roll *et al.*, 2006)
SYN1	313440	Epilepsy, X-linked, with variable learning disabilities and behavior disorders (OMIM#300491)	(Fassio *et al.*, 2011)
SYP	313475	Mental retardation, X-linked 96 (OMIM#300802)	(Tarpey et al., 2009)
TAF1	313650	Dystonia-Parkinsonism, X-linked (OMIM#314250)	(O'Rawe *et al.*, 2015)
		Mental retardation, X-linked, syndromic 33 (OMIM#300966)	
TSPAN7	300096	Mental retardation, X-linked 58 (OMIM#300210)	(Zemni *et al.*, 2000)
UBE2A	312180	Mental retardation, X-linked syndromic, Nascimento-type (OMIM#300860)	(Nascimento *et al.*, 2006)
UPF3B	300298	Mental retardation, X-linked, syndromic 14 (OMIM#300676)	(Tarpey *et al.*, 2007;)
USP9X	300072	Mental retardation, X-linked 99 (OMIM#300919)	(Homan *et al.*, 2014)
		Mental retardation, X-linked 99, syndromic, female-restricted (OMIM#300968)	
WDR45	300526	Neurodegeneration with brain iron accumulation 5 (OMIM#300894)	(Hoffjan *et al.*, 2016)
ZC4H2	300897	Wieacker-Wolff syndrome (OMIM#314580)	(Hirata *et al.*, 2013)

ZDHHC15	300576	Severe non-syndromic intellectual disability (female)	(Mansouri *et al.*, 2005; Moysés-Oliveira *et al.*, 2015)
ZDHHC9	300646	Mental retardation, X-linked syndromic, Raymond type (OMIM#300799)	(Masurel-paulet *et al.*, 2013)
ZNF711	314990	Mental retardation, X-linked 97 (OMIM#300803)	(Tarpey *et al.*, 2009)

Genomic DNA target enrichment was performed by PCR based amplification using the custom X-linked Intellectual Disability gene panel. Ion Ampliseq™ Designer (Life Technologies, Foster City, CA, USA) was used to design primer pairs to amplify all exons and flanking regions of the selected 82 genes. The designed gene panel generates 2471 amplicons of 125–375 bp resulting in 98.71% in silico coverage of all the regions of interest. DNA library was prepared by multiplex PCR, using the Ion Ampliseq™ Library kit v2.0 kit (Life Technologies, Foster City, CA, USA). Sequencing templates were built using Ion One Touch™ 2 System (Life Technologies, Foster City, CA, USA) and then sequenced using the Ion PGM™ System (Life Technologies, Foster City, CA, USA) with Hi-Q View chemistry. Torrent Suite™ Software (Life Technologies, Foster City, CA, USA) was used for sequencing raw data analysis, alignment to the human reference genome (hg19/GRCh37), and variant calling (Torrent Variant Caller plug-in). Variants were also called, annotated, filtered, and analyzed on the Ion Reporter™ Software version 5.2 (Life Technologies, Foster City, CA, USA).

Variants below10x coverage and 30 Phred score were excluded due to quality reasons. Furthermore, common variants and variants with a >0.005 minor allele frequency (MAF) were filtered out. Variants that appeared more than 2 times in our cohort were also excluded. Finally, non-synonymous variants in exonic and splice site regions were also prioritized. In order to predict the pathogenicity of the genetic variants found, different in silico predictors such as SIFT (Kumar *et al*., 2009), Polyphen-2 (Adzhubei *et al.*, 2010), and MutationTaster-2 (Schwarz *et al.*, 2014) were used. CADD scores (Kircher *et al.*, 2014) were also calculated for every variant. Variants in splice-site regions were evaluated using in silico predictors like Human Splicing Finder (Desmet *et al.*, 2009) and NNSplice (Reese *et al.*, 1997).

Candidate variants were then validated by conventional Sanger sequencing (Primer sequences available on request) and were then classified following the ACMG guidelines (Richards *et al.*, 2015) and using the InterVar software (Li and Wang, 2017).

Novel sequence variants have been deposited in GenBank. Accession numbers are provided on the results section.

2.5. Variant segregation and additional analysis

Variant segregation of the identified candidate variants was performed when possible. For this purpose, families were re-contacted. Variants were confirmed by conventional Sanger sequencing, as previously described.

Putative splicing mutations were also tested using blood RNA. Briefly, RNA was extracted from peripheral blood and cDNA was obtained using Superscript RT II enzyme (Invitrogen, Carlsbad, CA, USA) from 1 ug of total RNA extracted in a volume of 20 ul. cDNA was then amplified and sequenced to identify potential splicing variants.

Additional biochemical studies were also performed when required. In this way, plasma creatine, creatinine, and guanidoacetate levels were measured by Liquid chromatography—tandem mass spectrometry (LC-MS/MS) following the protocol described by Bodamer *et al*., (2001) to determine creatine deficiency syndrome.

3. RESULTS

3.1. Patient cohort

Out of the initial 54 unresolved male patients with possible non-syndromic XLID, 47 were studied in this work based on the X-linked family history of ID; the rest were excluded due to poor quality of DNA or previous diagnosis. Indeed, a microduplication of the Xp11.22 region, which includes *HUWE1*, was identified in one of the patients prior to XLID gene panel testing. Furthermore, out of the initial 62 index males with ID and affected brothers, 14 were also selected for the X-panel study based on the mother's skewed X-inactivation pattern (Table 2).

Table 2. X inactivation results on mothers of index male patients. The results have been divided into two groups: Index males that have suggestive X-linked inheritance and index males with affected brothers. Skewed inactivation values (>75%) are highlighted.

	X-linked	Male siblings
Total	**54**	**62**
>90%	3	6
>80%	6	6
>75%	4	2
Random	25	27
Homozygous	6	8
Uninformative		3
NP	10	10

Uninformative: mothers aged >55; NP: not performed, because there was no sample available

All 61 index male patients (47 with suggestive XLID and 14 with affected brothers) had normal G-banded karyotype and showed normal *FMR1* trinucleotide repeat numbers. Additional molecular techniques have also been applied in some of them and yielded normal results (Table 3).

According to the clinical data collected for the *FMR1* test, the probands were aged 2–63 in the XLID group and 2–24 in the male siblings group, 55.32% and 64.29% of the patients being under 10 at the time of referral in both groups, respectively. IQ evaluation had not been performed in 42.55% and 50% of the patients or was unknown, and those that already had an IQ evaluation mainly showed mild to borderline intellectual disability (34.04% and 35.71%,

respectively). Most of the selected patients did not have relevant dysmorphic features, confirming that they show an unspecific phenotype. However, autism spectrum disorders were present in nearly 30% of them. Almost 15% of the patients in the X-linked group also reported having epilepsy. Table 3 summarizes all of these data.

Table 3. Description of the selected cohort of 61 patients with suggestive X-linked intellectual disability. The cohort has been divided into two groups: Index males that have suggestive X-linked inheritance (X-linked) and index males with affected brothers (Siblings).

	X Linked		Siblings	
Total	*n* = 47		*n* = 14	
Age range	2–63		2–24	
0–10	26	55.32%	9	64.29%
10–20	10	21.28%	4	28.57%
>20	11	23.40%	1	7.14%
Intellectual disability				
Mild/Borderline	16	34.04%	5	35.71%
Moderate	6	12.77%	1	7.14%
Severe	3	6.38%	1	7.14%
Profound	2	4.26%	0	
Unknown	20	42.55%	7	50%
Comorbidity				
Macrocephaly	1	2.13%	0	
Microcephaly	1	2.13%	1	7.14%
Autism Spectrum Disorder	14	29.79%	4	28.57%
Hypotonia	1	2.13%	0	
Epilepsy	7	14.89%	0	
Behavioural disturbances	5	10.64%	2	14.29%
Previous studies				
Karyotype	47	100%	14	100%
Fragile-X	47	100%	14	100%
MLPA-X	42	89.36%	14	100%
aCGH	15	31.91%	14	100%

3.2. Targeted next generation sequencing

Targeted sequencing generated a mean of 788.702 reads per sample with a 255 bp mean read length, which covered 96.31% of the target regions with a 281.46 mean read depth, and 97% of the sequenced regions were covered at least 20x (Detailed data on technical sequencing is included on Table 4). Not covered regions mainly include GC-rich regions like 3' or 5'UTR regions, which are already known to be a burden in next-generation sequencing.

Table 4. Technical sequencing data.

	Bases	>Q20	Reads	Mean Read Length (bp)	Mapped Reads	On Target (%)	Mean read Depth	Uniformity (%)	1x	20x	100x
ID1010	172.336.456	150.381.667	707.609	243	706.532	97,53	243,60	94,11	99,01	96,55	87,29
ID1011	173.654.359	151.361.505	720.241	241	719.198	96,91	244,40	94,20	99,18	96,76	87,27
ID1012	182.762.521	160.227.812	745.785	245	744.697	96,47	256,40	94,65	99,13	96,79	89,55
ID1013	189.606.935	165.318.041	788.709	240	787.504	96,08	265,10	94,59	99,08	96,73	89,85
ID1014	182.873.265	160.110.795	751.080	243	749.791	96,60	256,90	94,67	99,14	96,68	89,20
ID1015	202.276.662	177.111.831	826.559	244	825.458	96,82	284,50	94,55	99,21	96,91	90,61
ID1016	190.270.717	16.258.545	787.297	241	786.004	96,16	266,40	94,43	99,13	96,75	89,37
ID1121	181.024.729	163.690.936	690.857	262	689.382	96,30	253,70	94,41	99,16	97,26	86,73
ID1122	196.671.250	178.399.923	743.869	264	742.496	96,17	275,40	94,88	99,15	97,42	89,61
ID1123	188.994.839	170.565.341	721.035	262	719.776	96,39	264,90	95,04	99,19	97,46	89,62
ID1124	183.474.581	165.691.233	699.832	262	698.591	96,29	257,00	95,06	99,32	97,46	88,86
ID1125	204.477.709	184.626.622	776.880	263	775.480	96,52	286,70	94,79	99,20	97,42	90,06
ID1126	200.027.452	180.238.028	767.504	260	765.898	96,71	281,50	94,15	99,20	97,27	87,89
ID1127	194.151.975	175.578.728	741.095	261	739.779	96,17	271,60	94,84	99,22	97,38	89,85
ID1128	226.380.173	203.784.703	852.605	265	851.508	96,79	318,80	94,75	99,21	97,54	91,80
ID1129	138.782.591	125.493.142	528.309	262	527.220	96,55	195,00	94,79	99,19	96,77	81,36
ID1210	212.969.472	188.801.480	834.085	255	832.110	96,21	298,20	94,25	99,22	97,44	89,32
ID1203	153.959.274	137.398.085	592.561	259	591.498	95,42	214,10	94,99	99,09	96,96	84,20
ID1204	187.145.229	166.149.146	734.483	254	732.916	95,63	260,70	95,08	99,31	97,32	89,14
ID1205	192.693.038	170.668.010	756.428	254	754.897	95,38	268,40	95,13	99,22	97,44	89,93
ID1206	192.591.059	171.731.541	745.192	258	743.813	95,53	268,10	95,09	99,23	97,32	90,11
ID1207	172.091.117	153.045.049	674.996	254	673.552	95,56	239,80	94,86	99,19	97,14	87,88
ID1208	214.838.518	189.829.745	852.449	252	850.553	95,57	299,20	94,81	99,18	97,51	90,72

ID1209	222.570.770	197.170.231	875.273	257	873.533	95,91	310,50	94,30	99,23	97,49	89,94
ID1301	134.513.017	121.686.559	538.597	249	537.426	95,67	188,00	93,00	99,08	95,64	76,27
ID1302	181.004.063	165.026.183	688.960	262	687.605	95,61	252,70	95,00	99,25	97,40	87,71
ID1303	164.147.174	150.243.280	611.968	268	611.026	96,00	229,60	95,09	99,17	97,28	86,48
ID1304	200.431.561	182.589.436	750.351	267	749.293	95,94	280,10	95,10	99,31	97,67	90,31
ID1305	203.873.194	185.310.210	775.110	263	773.839	95,02	282,80	95,25	99,41	97,76	91,47
ID1306	213.353.669	195.139.902	794.718	268	793.470	95,55	297,60	95,34	99,35	97,78	91,63
ID1307	207.092.403	189.183.619	774.531	267	773.182	96,12	289,90	94,56	99,23	97,48	89,31
ID1308	238.768.282	216.804.170	907.496	263	905.598	95,98	334,50	94,93	99,33	97,85	92,30
ID1309	151.599.911	138.158.919	573.465	264	572.330	95,60	211,70	95,13	99,25	97,23	84,47
ID1401	203.840.979	182.807.972	764.907	266	764.212	97,03	287,60	93,52	99,18	97,43	87,14
ID1402	197.007.741	177.222.986	740.589	266	739.717	96,44	276,50	94,40	99,28	97,29	88,10
ID1403	188.534.597	170.391.797	707.718	266	706.819	96,48	254,90	94,54	99,25	97,31	88,11
ID1404	245.362.497	220.512.343	922.456	265	921.413	96,32	344,40	94,73	99,26	97,71	92,30
ID1405	255.159.169	229.055.358	964.750	264	963.625	96,45	358,60	94,46	99,32	97,74	92,25
ID1406	232.202.677	209.908.493	864.299	268	863.443	96,32	326,00	94,04	99,31	97,51	90,60
ID1407	246.784.204	222.589.160	925.500	266	924.546	96,44	346,70	94,01	99,30	97,50	90,94
ID0214	215.496.766	185.704.605	1.002.191	215	996.809	95,89	301,60	91,66	98,87	95,35	86,07
ID0216	243.264.109	209.386.549	1.126.580	216	1.121.391	96,07	340,40	91,20	98,76	95,54	87,47
ID0318	177.975.033	154.813.416	772.924	230	770.401	94,49	246,70	93,52	98,99	96,13	85,73
ID0319	216.366.107	188.673.741	934.264	232	931.338	96,96	305,60	93,57	99,11	96,59	88,54
ID0320	236.867.002	205.674.956	1.027.427	231	1.024.562	97,51	336,20	92,96	99,04	96,53	89,10
ID0321	192.787.153	168.382.244	829.280	232	826.301	97,63	274,10	92,02	99,06	96,14	85,08
ID0517	174.896.087	151.037.659	784.295	223	779.641	95,94	246,30	92,32	99,04	96,02	83,64
ID0606	150.904.964	126.651.801	727.143	208	723.138	95,47	211,90	92,13	98,93	95,37	79,82
ID0706	230.802.725	212.713.526	859.860	268	859.037	97,12	326,70	94,48	99,11	97,01	92,13
ID0707	241.283.155	222.702.886	903.017	267	902.121	97,67	342,80	93,91	99,15	97,03	91,07
ID0810	167.874.019	151.182.915	635.360	264	634.407	96,64	236,20	93,43	98,95	96,45	85,63

ID0811	169.365.936	152.456.671	645.692	262	644.685	96,71	238,50	93,93	99,13	96,65	87,61
ID0812	168.529.620	152.704.110	636.772	264	635.951	96,05	236,00	94,61	99,12	96,62	88,21
ID0813	211.898.868	190.966.206	807.244	262	805.862	96,19	297,50	93,78	99,17	96,71	89,24
ID0814	201.872.893	226.380.173	763.368	264	762.228	95,63	281,90	93,52	99,02	96,69	88,54
ID0808	162.661.167	145.968.914	623.171	261	622.209	96,59	229,00	93,68	99,16	96,31	86,35
ID0919	222.648.667	198.859.731	859.060	259	857.811	97,22	314,40	93,72	99,18	97,09	89,98
ID0921	250.040.631	224.325.707	958.266	260	956.565	97,12	352,80	94,06	99,20	97,07	91,50
ID0922	291.221.340	261.731.357	1.102.581	264	1.100.866	97,06	411,10	94,43	99,09	97,37	93,32
ID0924	214.538.756	192.045.948	823.137	260	822.020	97,34	303,40	94,17	99,18	96,88	90,40
ID0925	279.242.559	250.388.307	1.069.056	261	1.067.239	96,91	393,50	94,19	99,19	97,10	93,00
MEAN	200.669.465	177.590.393	788.702	255	787.120	96,31	281,46	94,21	99,17	97,00	88,56
MIN	134.513.017	16.258.545	528.309	208	527.220	94,49	188,00	91,20	98,76	95,35	76,27
MAX	291.221.340	261.731.357	1.126.580	268	1.121.391	97,67	411,10	95,34	99,41	97,85	93,32

A mean of 188 variants per patient were annotated. Variants were then filtered and prioritized as described before. In total, 17 variants were prioritized as candidate (Table 5), 12 of them were identified in 11 patients that showed suggestive X-linked inheritance (11/47=23.4%) and 5 in male sib-pairs (5/14=35.7%). These include 14 missense variants, 1 nonsense, 1 splice-site variant, and an in-frame deletion. All of the variants had been inherited from the mother and most of them were not annotated in any of the population databases like GnomAD, although others were. Indeed, missense variants in *UPF3B* (c.1118G>A; p.Arg373His); *DLG3* (c.1424C>T; p.Ser475Leu), *FMR1* (c.1816C>T; p.Arg606Cys); *HUWE1* (c.1125G>T; p.Met375Ile) and *CCDC22* (c.1388C>G; p.Ala463Gly) have been identified in hemizygous males in GnomAD and dbSNP (Table 5) and the variant c.1118G>A in *UPF3B* has already been reported in ClinVar as VUS. Although there is no detailed information on frequency data, variants in *MAOA* (c.617G>A; p.Arg206Gln), *PRPS1* (c.611G>A; p.Arg204His), and *SYN1* (c.796G>A; p.Val266Met) have also been reported in dbSNP (rs1218703391, rs1169615098, rs1327735600).

On the other hand, two possible truncating variants have been identified and classified as pathogenic: *PHF8* (c.252C>A; p.Tyr84*) and *UPF3B* (c.371-1G>C). An in-frame deletion in *SLC6A8* (c.1390_1392delGAT; p.Asp464del) has also been identified and classified as likely pathogenic. The other six missense variants found have been classified as VUS since they have not previously been reported in the literature and more information on the function or segregation analysis is needed. This is why we made an effort in re-contacting the families.

Accession numbers of the novel sequence variants that have been analyzed in this study (Table 5) are the following: *PHF8* (c.252C>A; p.Tyr84*) MN817111, *UPF3B* (c.371-1G>C) MN817115, *SLC6A8* (c.1390_1392del GAT; p.Asp464del) MN817113, *IQSEC2* (c.128G>C; p.Arg43Pro) MN817117, *SLC9A6* (c.316A>G; p.Met106Val) MN817110, *NHS* (c.1270A>G; p.Arg424Gly) MN817112, *CASK* (c.490G>A; p.Gly164Arg) MN817116, *HUWE1* (c.12209C>G; p.Ser4070Cys) MN817114, and *MED12* (c.5009C>T; p.Ser1670Phe) MN817118.

Table 5. Variants identified in our 61-patient cohort. Variants were initially classified using InterVar software and this classification was manually adjusted after segregation analysis based on the results obtained.

Patient ID	Gene	Variant	Inheritance [1]	Family History	GnomAD Allele Freq males	dbSNP	ClinVar	CADD [2]	InterVar [3]	InterVar (Manually Adjusted)
Pathogenic/likely pathogenic variants										
ID0707	*PHF8*	NM_001184896.1: c.252C>A; p.Tyr84*	Maternal (97.04%)	Sib-pair				36	Pathogenic (PVS1, PM2, PP3)	Pathogenic (PVS1, PM2, PP3)
ID1204	*UPF3B*	NM_080632.2: c.371-1G>C	Maternal (80.49%)	Sib-pair					Pathogenic (PVS1, PM2, PP3)	
ID1122	*SLC6A8*	NM_005629.3: c.1390_1392del GAT; p.Asp464del	Maternal (79.37%)	X- linked					Likely Pathogenic (PVS1, PM2)	Pathogenic (PS3, PM2, PM4, PP1, PP3)
ID1208	*IQSEC2*	NM_001111125.2: c.128G>C; p.Arg43Pro	Maternal (53.7%)	X- linked				26.2	VUS (PM2)	Likely Pathogenic (PM2, PP1, PP2, PP3, PP4)
Variants of unknown significance										
ID0216	*SLC9A6*	NM_001042537.1: c.316A>G; p.Met106Val	Maternal (57.09%)	X- linked				23.1	VUS (PM1, PM2, BP1)	VUS (PM1, PM2, PP1, BP1)
ID0919	*UPF3B*	NM_080632.2: c.1118G>A; p.Arg373His	Maternal (uninformative)	X- linked	2/66856	rs146785878	VUS	26.7	VUS (PM2, PP3)	
ID1010	*DLG3*	NM_021120.3: c.1424C>T; p.Ser475Leu	Maternal (91.97%)	Sib-pair	1/75937	rs953325312		31	VUS (PM1, PM2, BP1)	

ID1011	*NHS*	NM_198270.3: c.1270A>G; p.Arg424Gly	Maternal (uninformative)	X- linked			23.7	VUS (PM2, BP1)	
ID1125	*FMR1*	NM_002024.5: c.1816C>T; p.Arg606Cys	Maternal (83.18%)	X- linked	1/67871	rs782778170	34	VUS (PM1, PM2)	
ID1128	*HUWE1*	NM_031407.6: c.1125G>T; p.Met375Ile	Maternal (86.55%)	Sib-pair	0/41548	rs1043071474	22.6	VUS (PM1, PM2)	VUS (PM1, PM2)
ID1205	*CASK*	NM_003688.3: c.490G>A; p.Gly164Arg	Maternal (74.26%)	X- linked			32	VUS (PM1, PM2, PP3)	
ID1206	*HUWE1*	NM_031407.6: c.12209C>G; p.Ser4070Cys	Maternal (97.31%)	Sib-pair			24	VUS (PM1, PM2)	
ID1304	*CCDC22*	NM_014008.4: c.1388C>G; p.Ala463Gly	Maternal (54.33%)	X- linked	1/73246	rs782691732	26.9	VUS (PM2)	
ID1307	*PRPS1*	NM_002764.3: c.611G>A; p.Arg204His	Maternal (uninformative)	X- linked		rs1169615098	24	VUS (PM1, PM2, PP2)	
ID1402	*SYN1*	NM_006950.3: c.796G>A; p.Val266Met	Maternal (69.45%)	X- linked		rs1327735600	26.4	VUS (PM1, PM2)	VUS (PM1, PM2, PP3)
ID1405	*MED12*	NM_005120.2: c.5009C>T; p.Ser1670Phe	Maternal (95.36%)	X- linked			33	VUS (PM2)	VUS (PM2, PP1, PP3)
				Benign/likely benign variants					
ID1208	*MAOA*	NM_000240.3: c.617G>A; p.Arg206Gln	Maternal (53.7%)	X- linked		rs1218703391	31	VUS (PM1, PM2, PP3)	Likely benign (PM1, PM2, PP3, BS2, BP5)

[1] Parenthesis indicates mothers' X-inactivation; [2] CADD scores are Phred-scaled and range from 1 to 99. Higher values are more likely to indicate deleterious effects; [3] Parenthesis indicates ACMG criteria applied for molecular variant classification using InterVar software; VUS: Variant of uncertain significance; PVS: Very strong criteria for pathogenic/likely pathogenic; PS: Strong criteria for pathogenic/likely pathogenic; PM: Moderate criteria for pathogenic/likely pathogenic; PP: Supporting criteria for pathogenic/likely pathogenic; BP: Supporting criteria for benign/likely benign.

3.3. Segregation analysis and genotype-phenotype correlation

Segregation analysis was performed on seven families and variants were reclassified based on the results obtained. Thanks to updating the pedigree and the clinical data of the patients, a genotype–phenotype correlation has been possible.

3.3.1. Pathogenic/Likely Pathogenic Variants

Family ID0707

The proband is an eight-year-old male, second child of healthy non-consanguineous parents. He was first referred at four years of age to our laboratory because of developmental delay and autistic features and an older brother presenting with global developmental delay. He had no oral speech and communicated with signs, had no sphincter control, and did not tolerate solid food. He had minor dysmorphic features such as borderline low-set ears, thick and abundant hair, broad eyebrows with a medial eyebrow flare, small nose because of small nares, and no cleft palate or nasal voice. He had a history of repetitive otitis that caused unilateral hearing loss. Likewise, he had distal pectus excavatum, no lumbar lordosis or scoliosis, and normal genitalia. aCGH results were normal.

We identified a nonsense variant in the *PHF8* gene (NM_001184896.1: c.252C>A; p. Tyr84*). This variant was not present in any population databases and has neither been reported in the literature. The variant was inherited from his mother who had a skewed X-inactivation and was not present on the brother (Figure 1A). Despite the brother being reported as having global developmental delay in his early childhood, he eventually did not develop ID and currently has a normal development. There was no other information on the maternal family as she had been adopted. Mutations in *PHF8* were first described by Laumonnier *et al.*, 2005 and are known to cause Siderius syndrome (first described by Siderius *et al.*, 1999). Siderius syndrome (OMIM#300263) is characterized by mild to borderline intellectual disability associated with cleft lip/palate. Moreover, patients with this syndrome may also present with minor dysmorphic features including preaxial polydactyly, large hands, and cryptorchidism. In total, 12 truncating variants, either nonsense, frameshift, or splicing variants, have been reported in this gene in human gene mutation database (HGMD) (Figure 1B) (Abidi *et al.*, 2007; Koivisto *et al.*, 2007; Redin *et al.*, 2014; Fitzgerald *et al.*, 2015; Retterer *et al.*, 2016; Posey *et al.*, 2017; Faundes *et al.*, 2018; Hu *et al.*, 2018). The present proband does not present cleft lip or palate or any of the classical features observed in Siderius syndrome. Still, it is important to mention that there are just a few clinical reports on the identified *PHF8* variants apart from the initial reports that described the syndrome. Although the proband does not match the initial phenotype described, the identification of an early truncating variant on the proband suggests that it is a pathogenic variant (PVS1, PM2, PP3).

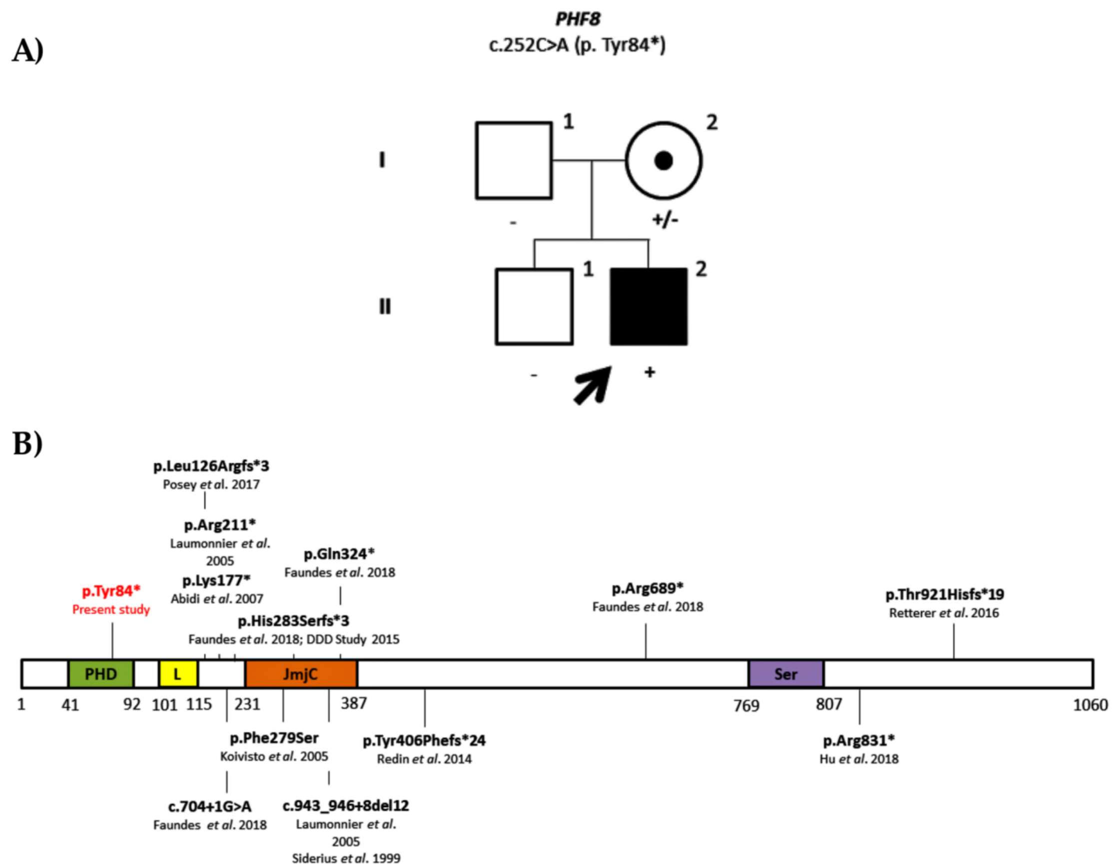


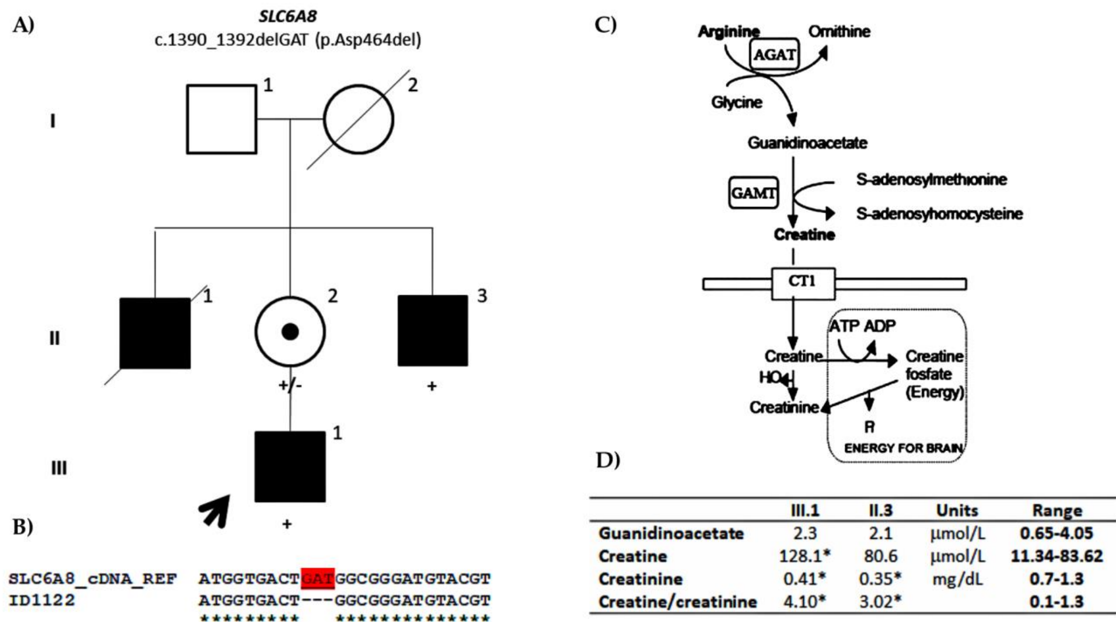
Figure 1. (**A**) Family pedigree of the ID0707 proband. Segregation analysis of the nonsense variant in *PHF8* gene (NM_001184896.1: c.252C>A; p. Tyr84*). (**B**) Histone lysine demethylase PHF8 protein. Protein domains are shown in color: In green, plant homeodomain (PHD) finger domain; in yellow, linker region (L); in orange, jumonji-C (JmjC) domain; and in purple, serine-rich domain (Ser). All the mutations identified to date are also shown with the respective references. The present variant is shown in red.

Family ID1122

The proband is a 19-year-old male with intellectual disability, autistic features, behavioral disturbances, and generalized epilepsy. He was first referred to our laboratory at two years of age with global developmental delay and family history of X-linked intellectual disability because he had two maternal uncles affected with severe intellectual disability and epilepsy. X-linked intellectual disability MLPA and aCGH results were normal.

We identified an in-frame deletion in the *SLC6A8* gene (NM_005629.3: c.1390_1392delGAT; p.Asp464del) that is located to the ninth transmembrane domain of the protein. The variant is not present in any of the population databases or literature and is predicted to be pathogenic. The variant also segregated with the phenotype. Indeed, the mother and affected maternal uncle were carriers of the variant (Figure 2A). The mother showed skewed X-inactivation. Being an in-frame variant located in a splicing region, it was predicted to cause changes in splicing. Nevertheless, it was confirmed by cDNA sequencing that there were no changes in splicing (Figure 2B). Anyway, the decreased plasma creatinine levels confirmed the pathogenicity (Figure 2D). Therefore, the variant was reclassified as pathogenic (PS3, PM2, PM4, PP1, PP3). Indeed, mutations in *SLC6A8* cause cerebral creatine deficiency syndrome (CCDS1: OMIM#300352). The first patient with CCD1 and a mutation in *SLC6A8* gene was

described by Salomons et al., 2001 presenting with a similar phenotype as our proband, and since then, many families have been described, some of them with in-frame deletions as our family. The prevalence of *SLC6A8* mutations in males with ID has been estimated to be about 1% (Clark *et al*., 2006), but it could be higher. It is interesting to mention that our patients showed normal low levels of plasma creatine but not outside the normal range, so the clinicians never suspected this syndrome until the variant was found and more accurate creatine/creatinine measures were performed.



	III.1	II.3	Units	Range
Guanidinoacetate	2.3	2.1	µmol/L	**0.65-4.05**
Creatine	128.1*	80.6	µmol/L	**11.34-83.62**
Creatinine	0.41*	0.35*	mg/dL	**0.7-1.3**
Creatine/creatinine	4.10*	3.02*		**0.1-1.3**

Figure 2. (**A**) Family pedigree of the ID1122 proband. Segregation of the c.1390_1392delGAT in-frame deletion in *SLC6A8* (NM_005629.3) is shown. (**B**) Partial cDNA sequence of the *SLC6A8* gene. cDNA sequence of the proband aligned to the reference *SLC6A8* cDNA sequence is shown. The deleted nucleotides are shown in red. (**C**) Creatine metabolism pathway (derived from Andrade et al., 2008). The protein encoded by *SLC6A8* is named creatine transporter (CT1) and it is a transmembrane protein that is responsible for the creatine uptake. (**D**) Plasma levels of guanidinoacetate, creatine, and creatinine in the proband (III.1) and affected maternal uncle (II.3) are shown together with the creatine/creatinine ratio. The asterisk indicates the values that fall out of the reference range values.

Family ID1208

The proband is a 30-year-old male who was referred to our laboratory at five years of age because he had global developmental delay, autistic features, and epilepsy. He also had X-linked family history of intellectual disability meaning that his half-brother and two maternal uncles of the mother were also affected with intellectual disability. Results for *MECP2, CDKL5,* and *ARX* screenings as well as MLPA for X-linked intellectual disability and subtelomeric regions were normal.

We identified two candidate variants: *IQSEC2* (NM_001111125.2) c.128G>C (p.Arg43Pro) and *MAOA* (NM_000240.3) c. 617G>A (p. Arg206Gln). While *IQSEC2* variant is a novel variant and therefore is not present in any population databases, *MAOA* variant was found in dbSNP (rs1218703391). Both variants are located in conserved amino acid residues located and are

predicted to be likely pathogenic by in silico predictors (*IQSEC2*: SIFT 0.0, Polyphen-2 0.999 and MutationTaster 0.89; and *MAOA*: SIFT 0.06, Polyphen-2 0.999 and MutationTaster 0.99). *IQSEC2* and *MAOA* missense variants are both present on the half-brother as well as on the healthy mother, who has random X inactivation. While *IQSEC2* variant occurred de novo on the mother, the *MAOA* variant had been inherited from the healthy maternal grandfather, suggesting that it is a likely benign variant (PM1, PM2, PP3, BS2, BP5). None of the healthy maternal uncles or grandmother are carriers of any of these variants (Figure 3A). The obtained results are clearly reflected on the phenotypes observed. Both the proband and his half-brother share the same phenotype: Severe to profound intellectual disability, no speech or language, autistic features, and generalized epilepsy. The proband shows minor dysmorphic features that include synophrys, everted lower lip vermilion, and kyphoscoliosis, and his brother also shows scoliosis besides unspecific dysplastic features in the facies and feet. Mutations in *IQSEC2* were first described in families with non-syndromic XLID *(Shoubridge et al.*, 2010) and affected males showed moderate to severe intellectual disability, seizures, autistic traits, and behavioral disturbances as our patients do. To date, more than 70 mutations have been reported in *IQSEC2* leading to a similar phenotype (Rogers *et al.*, 2019; Shoubridge *et al*., 2019). Some of these mutations are clustered in functional domains like IQ and Sec7 domains, which have been demonstrated to impair GEF activity (Shoubridge *et al.*, 2010; Kalscheuer *et al.*, 2016; Rogers *et al.*, 2019). The present variant is located to the N terminal coiled coil (CC) domain of the protein where no pathogenic missense variants have been reported so far (Figure 3B). This CC domain is thought to promote self-assembly and its disruption is likely to influence interactions with other proteins like calmodulin or PDZ containing proteins. Knockout of this domain alters its accumulation at the post synaptic density (Myers *et al.*, 2012; Petersen *et al.*, 2018; Levy *et al.*, 2019). All in all, the segregation of the variant with the phenotype together with the correlation with the phenotypes observed in patients with *IQSEC2* variants suggests that this variant is likely pathogenic (PM2, PP1, PP2, PP3, PP4). However, as it is a missense variant and no other pathogenic missense variants have been reported at the CC domain, functional studies would be needed to establish its pathogenicity and understand its disease mechanism.

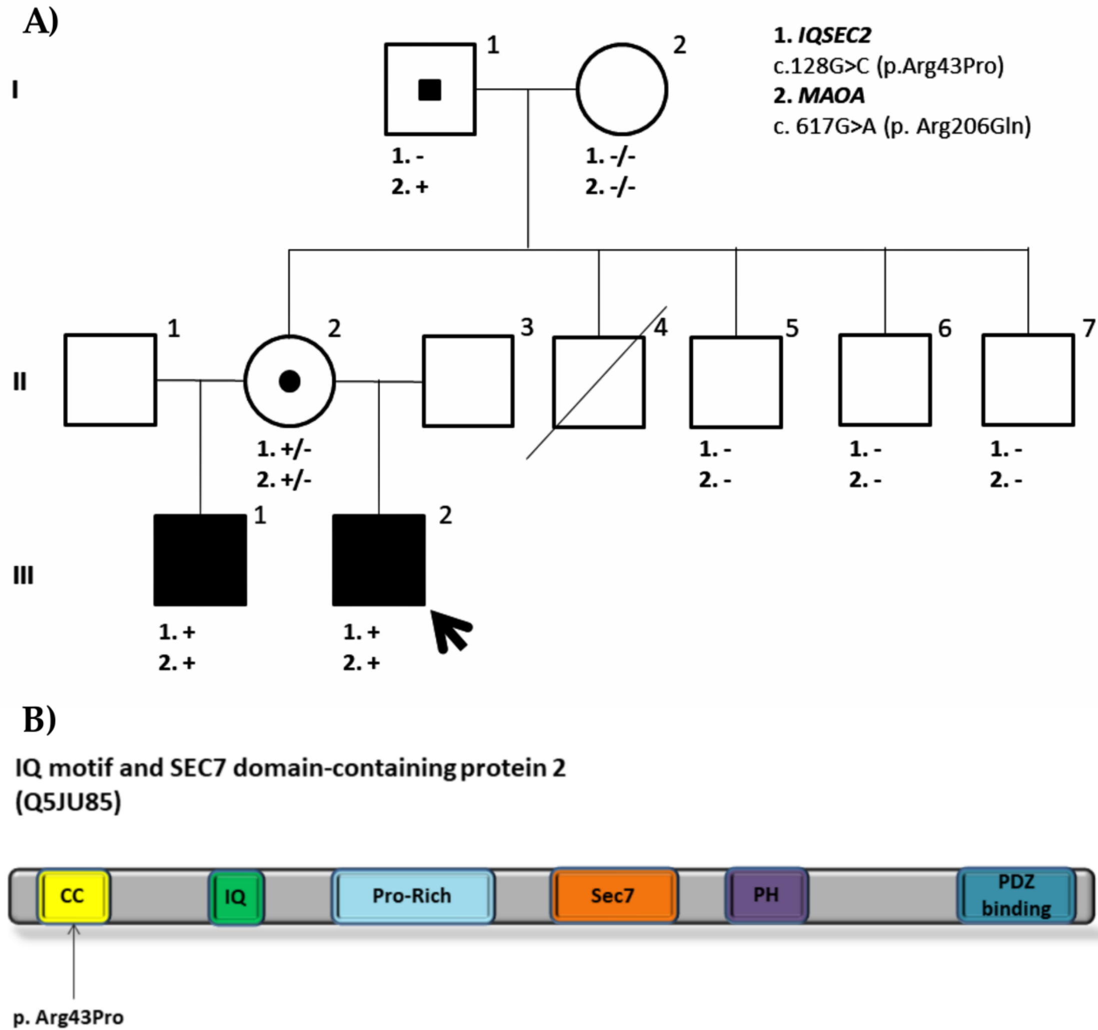


Figure 3. (**A**) Family pedigree of the ID1208 proband. Segregation analysis of both missense variants found in *IQSEC2* (NM_001111125.2: c.128G>C; p.Arg43Pro) and *MAOA* (NM_000240.3: c. 617G>A; p. Arg206Gln) is shown. (**B**) IQ motif and SEC7 domain-containing protein 2. Protein domains are shown in color: In yellow, coiled coil domain (CC); in green, IQ domain (IQ); in light blue, proline-rich (Pro-Rich) motif; in orange, Sec7 domain (Sec 7); in purple, Pleckstrin homology (PH) domain; and in blue, PDZ binding domain (PDZ binding). The missense variant found and located to the CC domain is also represented.

3.3.2. Variants of Unknown Significance

Family ID0216

The proband is a 27-year-old male with intellectual disability and severe behavioral disturbances. He was first referred to the molecular genetics service at six years of age because he presented with global developmental delay. He is the first child of heathy non-consanguineous parents and has a younger brother and sister, both of them healthy. He has two maternal uncles affected with mild ID. aCGH results were normal.

A missense variant in the *SLC9A6* gene (NM_001042537.1: c.316A>G; p.Met106Val) was identified in this study. The variant was inherited from the mother who presented with random X-inactivation and was also present in both maternal uncles having mild ID (Figure 4).

Therefore, the variant seems to segregate with the disease. Moreover, the variant has not been reported in population databases and is located on a conserved residue in the second transmembrane domain of the protein and could affect its structure and, consequently, function. Nevertheless, the in silico variant predictors do not consistently agree on its pathogenicity (SIFT 0.36, Polyphen-2 0.097 and Mutation taster 0.99). Mutations in *SLC9A6* have been associated with Christianson syndrome (OMIM#300243) (Gilfillan *et al.*, 2008), which is mainly characterized by severe intellectual disability, no speech, postnatal microcephaly, early onset seizures, ataxia, and hyperactivity (Pescosolido *et al.*, 2014). To date, more than 50 causative variants that mainly include nonsense, frameshift, splicing, and indels have been reported in HGMD. However, patients showing mild ID have also been reported (Masurel-Paulet *et al.*, 2016), as it is the case of the present patient. Altogether, although the segregation of the variants suggests that the variant might be pathogenic, it would still be a VUS according to the ACMG classification criteria (PM1, PM2, PP1, BP1) until more information is available or functional assays are performed like others that have already been performed in *SLC9A6* (Ilie *et al.*, 2019; Ouyang *et al.*, 2019).

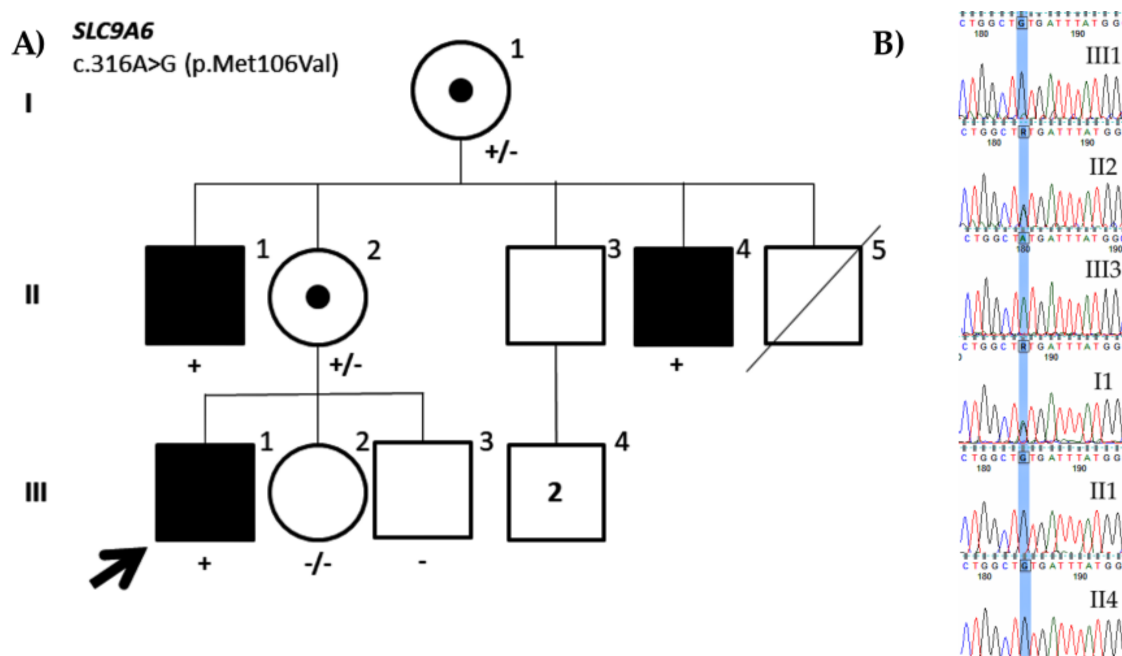


Figure 4. (**A**) Family pedigree of the ID0216 proband. Segregation of the missense variant identified in *SLC9A6* (NM_001042537.1: c.316A>G; p.Met106Val). (**B**) Partial DNA sequence of the SLC9A6 gene. The identified nucleotide change is highlighted.

Family ID1128

The proband is a 33-year-old male with moderate to severe intellectual disability, no speech, and behavioral disturbances including aggressive behavior who was first referred to the molecular genetics laboratory at three years of age. He has an affected monozygotic twin who has mild intellectual disability and aggressive behavior, and a healthy brother and sister. Their healthy parents are consanguineous (first cousins). A cousin of the parents, who died at 18 years of age, was also reported to have severe intellectual disability and seizures (Figure 5). Molecular studies including X-linked intellectual disability MLPA and aCGH results were

normal. As parents are consanguineous, whole-exome sequencing was also performed to exclude pathogenic homozygous variants.

The only candidate variant identified either in the exome or gene panel is a missense variant in *HUWE1* gene (NM_031407.6: c.1125G>T; p.Met375Ile). The variant has already been reported in population databases like GnomAD with very low overall frequency (0.000008010) and two other variants have also been reported at the same protein residue also with a low frequency, suggesting that it could be a rare benign variant. Although it is in a conserved residue, in silico predictors suggest that it might be tolerated (SIFT 0.02, Polyphen-2 0.77 and MutationTaster 0.99). As expected, the affected monozygotic twin is also carrier of the variant, as well as the mother—who shows skewed X-inactivation—and his healthy sister. The normal brother does not carry the variant (Figure 5). No more segregation analysis was performed due to the lack of collaboration of the family. *HUWE1* missense variants were first identified in patients with moderate to profound non-syndromic ID (Froyen *et al.*, 2008). Since then, missense variants mainly clustering to the HECT domain have been reported in patients with ID. These mutations have been shown to alter expression of HUWE1 protein and its downstream targets (Friez *et al.*, 2016; Bosshard *et al.*, 2017). Although mutations in this gene cause a wide phenotypic spectrum (Moortgat *et al.*, 2018), a syndromic form has been described. A recurrent mutation in the HECT domain was associated with Juberg Marsidi and Brooks syndrome (OMIM#309590) (Juberg and Marsidi, 1980; Brooks *et al.*, 1994; Friez *et al.*, 2016), which is characterized by intellectual disability, poor or absent speech, short stature, and microcephaly. Dysmorphic features include deep-set eyes, prominent nose, and blepharophimosis. Despite *HUWE1* being highly intolerant to missense variants (Z = 8.87), the present variant is not located in any of the functional domains described. The variable phenotype observed in patients with *HUWE1* variants does not help in determining its pathogenicity neither. Therefore, although exome sequencing was performed and no other candidate variant was found, this variant would still be a VUS (PM1, PM2) since more evidence like a broader segregation analysis or functional assays are needed to claim its pathogenicity.

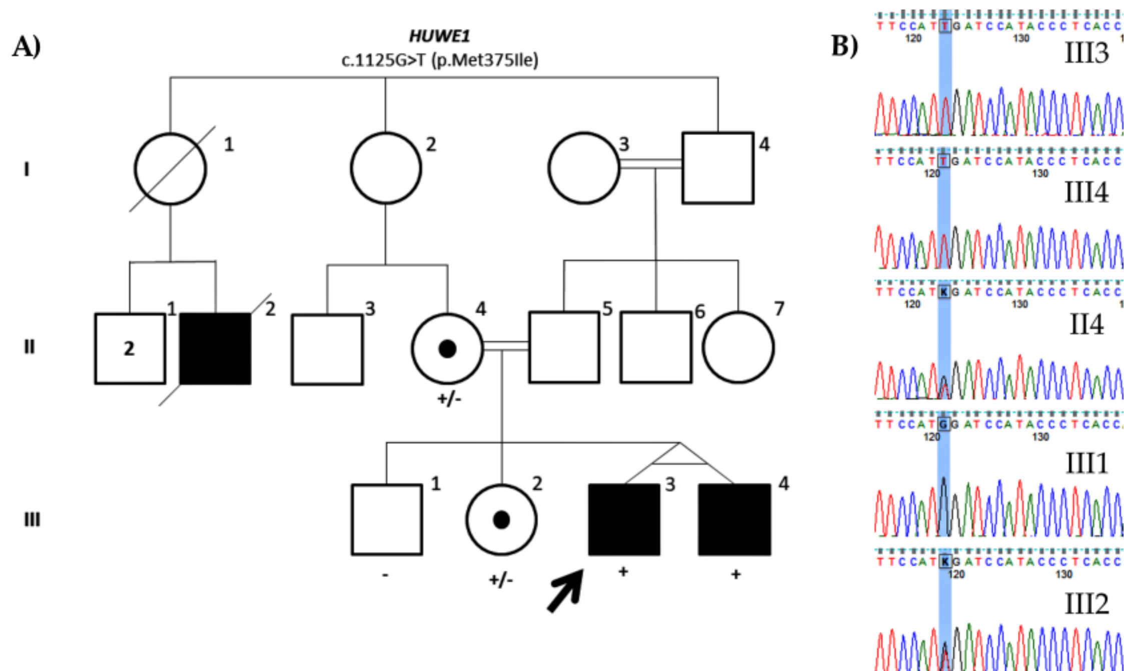


Figure 5. (**A**) Family pedigree of the ID1128 proband. Segregation of the missense variant found in *HUWE1* (NM_031407.6: c.1125G>T; p.Met375Ile). (**B**) Partial DNA sequence of the *HUWE1* gene. The identified nucleotide change is highlighted.

Family ID1402

The proband is a 21-year-old male who was referred to our laboratory at five years of age with mild intellectual disability and autistic features. He was born to healthy non-consanguineous parents and has two younger sisters. He has a maternal uncle who had school and language delay in childhood and now suffers from paranoid schizophrenia.

We identified a missense variant in *SYN1* (NM_006950.3: c.796G>A; p.Val266Met). The variant was inherited from the mother and was also present on the maternal uncle affected with schizophrenia, but not on the healthy maternal uncle. The two younger sisters and maternal aunt were also carriers of the variant (Figure 6). Although this variant has already been reported (rs1327735600), no hemizygous variants have been identified. The variant is located on a conserved amino acid residue at the actin and synaptic vesicle binding region of the protein and is predicted to be deleterious (SIFT 0.0, Polyphen-2 1.0 and Mutation Taster 0.99). Variants in *SYN1* have been associated with learning difficulties, epilepsy, and aggressive behavior (Garcia *et al.*, 2004). Fassio *et al.*, (2011) identified more variants both truncating and missense associated with ASD and/or epilepsy. The absence of any other members affected with ID in the family makes it difficult to assess the pathogenicity of the variant as the carrier uncle seems to show a different phenotype (low but normal IQ and schizophrenia) (Figure 6). Therefore, it should be still considered a VUS (PM1, PM2, PP3) until functional studies are performed or more information on the variant is obtained.

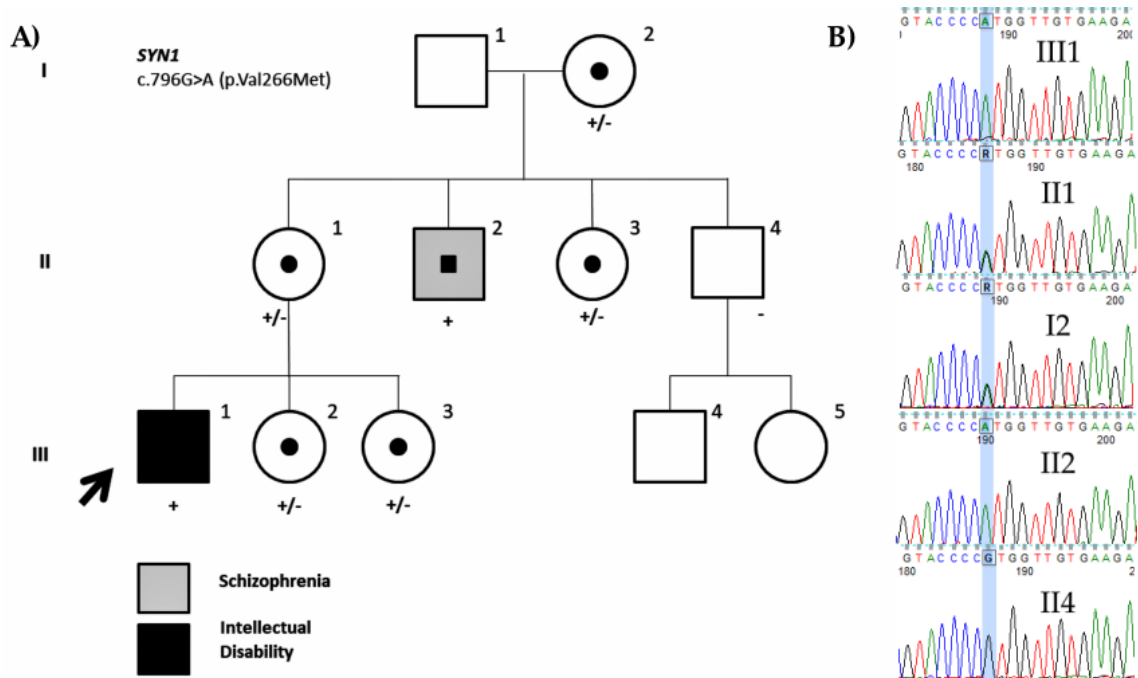


Figure 6. Family pedigree of the ID1402 proband. (**A**) Segregation of the missense variant in *SYN1* (NM_006950.3: c.796G>A; p.Val266Met) is shown. The black color indicates intellectual disability and the grey indicates schizophrenia. (**B**) Partial DNA sequence of the *SYN1* gene. The identified nucleotide change is highlighted.

Family ID1405

The proband is a 42-year-old male referred to our laboratory for X-linked intellectual disability testing. He presents with intellectual disability, autistic features, schizophrenia, and behavioral disturbances. He has a nephew with global developmental delay and autistic features and no other family history of intellectual disability. aCGH results were normal.

We identified a missense variant in *MED12* (NM_005120.2: c.5009C>T; p.Ser1670Phe) located on the PQL domain of the protein. The variant was inherited from the mother and was also present on the affected nephew whose mother was also a carrier (Figure 7). It is a novel variant as it has not been reported in population databases or literature and is predicted to be likely pathogenic (SIFT 0.0, Polyphen-2 0.995 and Mutation Taster 0.99). Germline mutations in the *MED12* gene have been associated with Opitz–Kaveggia syndrome, also known as FG syndrome 1 (OMIM#305450) (Risheg *et al.*, 2007), Lujan–Fryns syndrome (OMIM#309520) (Schwartz *et al.*, 2007), which overlaps with FG syndrome 1, and X-linked Ohdo syndrome (OMIM#300895) (Vulto-van Silfhout *et al.*, 2013). However, the more genetic variants identified, the broader is the phenotypic spectrum observed in patients with *MED12* variants, making it difficult to fit any of the syndromes described to date in the majority of cases. Therefore, it has been suggested to define it as *MED12*-related disorders rather than attributing a syndrome (Charzewska *et al.*, 2018). To date, 28 variants have been identified in HGMD, of which most of them are missense variants. The patient ID1405 shares many of the clinical features reported in *MED12*-related disorders and the segregation of the variant with ID supports the pathogenicity. Nevertheless, according to the ACMG classification criteria, this variant is still a VUS (PM2, PP1, PP3) until some functional assay is performed to assess MED12 function.

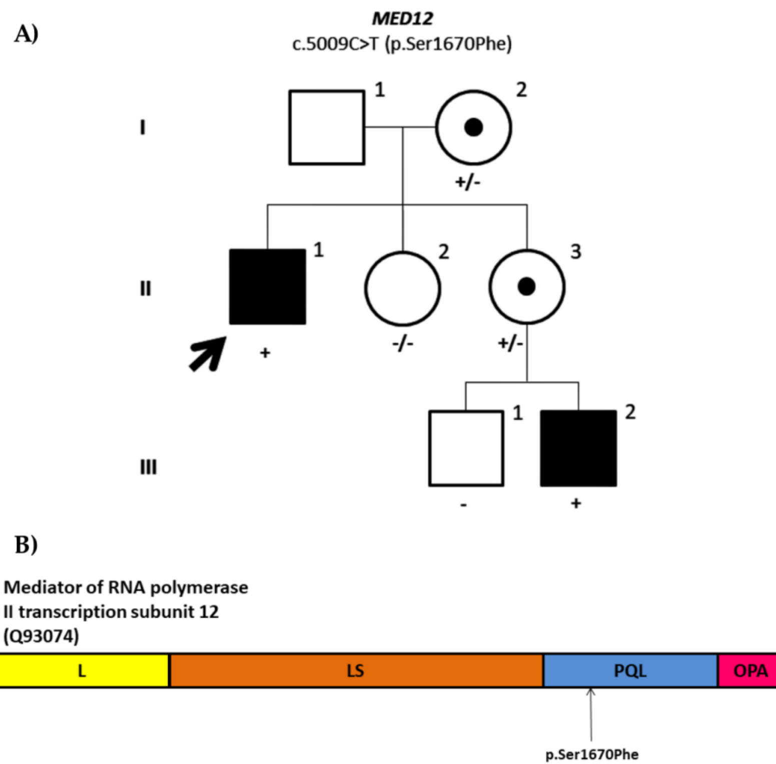


Figure 7. (**A**) Family pedigree of the ID1405 proband. Segregation analysis of the missense variant in *MED12* (NM_05120.2: c.5009C>T; p.Ser1670Phe). (**B**) Mediator of RNA polymerase II transcription subunit 12. The subunits of the protein encoded by *MED12* include leucine-rich domain (L) in yellow, leucine serine-rich domain (LS) in orange, proline glutamine and leucine-rich domain (PQL) in blue, and glutamine-rich domain (OPA) in pink. The variant identified in ID1405 is located on the PQL domain as shown in the figure.

4. DISCUSSION

In this study, we have analyzed a cohort of 61 patients with suggestive non-syndromic XLID through next-generation sequencing using a gene panel of 82 XLID genes. We have identified 17 variants in 16 of the probands analyzed (Table 5) and we have been able to perform segregation analysis of eight variants in seven families.

The cohort had been selected specifically for XLID, as there was a suspicion of X-linked family history of ID in 47 of the probands. Candidate variants -namely those variants that could be causative of ID- were found in 11 probands out of 47 (Table 5) and therefore, the contribution of the X chromosome has been 23% (11/47) in these patients. Tzschach *et al.*, 2015 followed a similar approach and sequenced 107 XLID genes in a cohort of 150 male patients that included 50 patients with suggestive XLID (affected brother or male maternal relatives) and sporadic male patients obtaining a diagnostic yield of 26% in the XLID group. Our cohort also included 14 male patients with ID with affected brothers that were selected based on their mothers' skewed X inactivation. Five candidate variants were found (5/14 = 35%) (Table 5). Overall, we have identified candidate variants in 26% (16/61) of our cohort, similar to other targeted sequencing studies in XLID (Tarpey *et al.*, 2009; Philips *et al.*, 2014; Tzschach *et al.*, 2015; Hu *et al.*, 2016).

More candidate variants have been identified in index males with affected brothers (35%) than in patients with suggestive X-linked inheritance of ID (23%) probably due to the fact that they were selected based on skewed X-inactivation. Similar to our study, Giorgio *et al.*, 2017 selected eight males with suggestive XLID based on their mothers' skewed X-inactivation (>80%) and performed whole-exome sequencing identifying XLID variants in 50% of the patients. In the same line, in our cohort of 61 patients, mothers of 21 showed >80% of skewing of X-inactivation (12 on the male siblings group and 9 on the X-linked group) and candidate variants were found in 7 patients (33%). X-inactivation is a gene dosage compensatory mechanism that occurs randomly at early embryonic stage in females in most of the X-linked genes and skewed X-inactivation is known to be indicative of genomic alterations and less consistently genetic mutations. Indeed, it is quite a common feature in X-linked disorders (Plenge *et al.*, 2002; Ørstavik, 2009; Stevenson and Schwartz, 2009) although some X-linked genes like *IQSEC2* are known to escape this mechanism. Skewed X-inactivation has been proposed as a protective mechanism against the mutant X chromosome and the varying degrees of X-inactivation have been attributed to milder manifestations of the disease in females and so it has been shown that there is a female phenotype in most of the XLID conditions, although X-inactivation does not always correlate with the female phenotype (Ziats *et al.*, 2019).

In spite of the absence of more candidate variants for the remaining families, XLID cannot be ruled out since other genes that have not been included in this gene panel might be the cause of XLID. Indeed, since this gene panel was designed, more genes have been reported to be associated with non-syndromic XLID (Neri *et al.*, 2018). This is one of the pitfalls of targeted sequencing that could be overcome by actively modifying gene panels in the basis of the current knowledge. Actually, gene panels should be dynamic and therefore should be evaluated every now and then to add new genes or remove others.

On the other hand, despite having indications such as X-linked family history that suggests XLID, ID might be of a different origin as it has been demonstrated by Sanchis-Juan et al., 2019. The family of our patient ID1208 reported that two maternal uncles of the mother had ID besides his half-brother. Nevertheless, as the grandmother is not the carrier of the *IQSEC2* missense variant, the ID of these relatives should be of different origin. Due to the ascertainment method applied, the collected clinical data are scarce and might not be as accurate. As it was shown in Table 3, most of the patients were referred before 10 years of age and had no IQ evaluation or was unknown. This could mean that they presented with global developmental delay but have not necessarily develop ID later. In line with this, not many details on comorbidities that could help in variant assessment were reported. Family history of ID was mostly based on what the parents or legal representatives of the patients reported, and no assessment was performed to determine if there were any similarities on the phenotypes they present. Being aware of this, re-contacting has been necessary to evaluate the pathogenicity of the candidate variants found and it has been possible in nearly half of them (7/16).

Segregation analysis together with RNA analysis and biochemical tests has helped in establishing the pathogenicity of the *SLC6A8* in-frame deletion. Segregation analysis itself has also been useful in excluding variants as causative -like the missense variant in *MAOA*- or identifying a plausible cause of XLID due to genotype–phenotype correlation as it has been the case of *IQSEC2* missense variant. Nevertheless, segregation analysis and genotype phenotype correlations have not been enough in determining the pathogenicity of missense variants in *SLC9A6*, *HUWE1*, *SYN1*, and *MED12*, although variants in *SLC9A6* and *MED12* seem likely pathogenic due to their co-segregation in the families. Moreover, the phenotype in ID and, consequently, XLID is highly variable and makes diagnosis difficult. Indeed, despite the nonsense variant identified in *PHF8* being pathogenic, the proband does not resemble any phenotypical features of Siderius syndrome, which makes us suspect that the phenotypical spectrum of *PHF8* variants must be expanded. To our knowledge, this case would be the first described without Siderius syndrome.

With reference to the families that could not be re-contacted, it is interesting to discuss family ID1204 because the splicing variant found (c.371-1G>C) has been classified as pathogenic (Table 5). In fact, samples of the proband and his brother got to the lab years ago from an institution for disabled people to exclude FXS. The collected clinical data indicated that they were 30 and 32 years old and had non-specific mild ID without autism. Furthermore, both had dolichocephaly and abnormal dental implantation. *UPF3B* is a gene previously implicated in XLID that encodes a protein involved in nonsense-mediated mRNA decay. The UPF3B protein is an important component of the nonsense-mediated mRNA decay surveillance machinery and

it has been proposed that it may have a potential function in the regulation of the expression and degradation of various mRNAs present at the synapse (Laumonnier *et al.*, 2010). A recent article (Tejada et al., 2019) has added new insights into the wide variability between and within families that had been previously described. Therefore, this new family adds even more to the growing evidence of the clinical and genetic variability in neurodevelopmental disorders. As for the remaining missense candidate variants that could not further analyzed (Table 5), four of the missense variants identified in ID0919, ID1010, ID1125, and ID1304 are present in hemizygous males in population databases like GnomAD, although at very low frequency, and this could indicate that these are rare benign variants. Nevertheless, having such a low frequency, they cannot be excluded as candidate variants and were indeed prioritized using our variant filtering criteria. As for the missense variant identified in ID1011 in the *NHS* gene, despite that the variant has not been reported in population databases or the literature and is predicted to be possibly damaging by in silico predictors (SIFT 0.0, Polyphen-2 0.925 and MutationTaster 0.99), mainly truncating mutations have been reported in HGMD. Mutation in *NHS* lead to either Nance Hooran Syndrome (NHS: OMIM#302350) or congenital cataracts (CTRCT40: OMIM#302200). NHS syndrome is characterized by dental anomalies, dysmorphic features, and in some cases, intellectual disability besides congenital cataracts. Our patient has severe ID, autism, epilepsy, and obesity, but cataracts have not been reported, which is typical in patients with *NHS* mutations. Similarly, a missense variant was identified in *PRPS1* gene in ID1307. This variant falls in a highly conserved residue in the ribose-phosphate diphosphokinase domain of the protein. To date, 34 missense variants have been reported in HGMD leading to four distinct syndromes: PRPS1 superactivity (OMIM#300661), X-linked Charcot-Marie-Tooth disease-5 (CMTX5: OMIM#311070), Arts syndrome (OMIM#301835), and isolated X-linked sensorineural deafness (DFNX1: OMIM#304500). All of these phenotypes described include sensoneural deafness, which was not reported in our proband that was only reported to have ID and X-linked family history. On the other hand, missense variants in *HUWE1* and *CASK* could possibly be pathogenic as the molecular evidence suggests. The variant in *HUWE1* gene is in a highly conserved residue and is predicted to be deleterious by in silico predictors (SIFT 0.0, Polyphen-2 1.0 and MutationTaster 0.99). Moreover, it is located to the HECT domain where most of the mutations are clustered, which suggests that it is a likely pathogenic variant. The proband ID1206 was referred at 30 years of age with mild ID and behavioral disturbances and has a similar affected brother. Moreover, his mother has an extreme skewing X-inactivation. The missense variant in *CASK* is also in a highly conserved residue and predicted to be deleterious by in silico predictors (SIFT 0.0, Polyphen-2 1.0 and MutationTaster 0.99). Furthermore, it is located on the protein kinase domain, which suggests that it is a likely pathogenic variant. In this case, the proband was referred at 27 years of age with non-syndromic mild ID and has a similar affected brother.

In conclusion, targeted next-generation sequencing of 82 XLID genes on 61 male patients with suggestive non-syndromic XLID has demonstrated to be useful in elucidating the genetic basis of ID in some of the cases, especially in retrospective cases in which exome sequencing cannot be properly evaluated. It is important to highlight that patient and family re-contacting together with variant segregation, a more accurate description of the phenotype, and the additional tests performed have helped in reclassifying the identified variants. Indeed, *SLC6A8* in-frame variant has been reclassified as pathogenic, *IQSEC2* missense variant as likely

pathogenic, and *MAOA* missense variant as likely benign. Despite variants in *MED12* and *SLC9A6* remain classified as VUS according to the ACMG criteria applied, we believe that these variants are likely pathogenic because the segregation analysis and phenotype suggest so.

With the advent of next-generation sequencing, more and more missense VUS are identified in patients with ID and XLID and therefore there is an urge to assess the pathogenicity of these variants. Phenotype correlation is not straightforward since it is highly variable in patients with ID. Therefore, functional assays are needed to assess if these variants have any impact in the protein function and consequently on the patient's phenotype.

CHAPTER 3:

Contribution of genes on the X chromosome versus autosomes in male siblings with unexplained intellectual disability

The 3.5. section of this chapter has been presented as a poster in "II. Congreso Interdisciplinar en Genética Humana" (3-5 April 2019, Madrid, Spain): "Estudios genéticos moleculares en hermanos varones con discapacidad intelectual en el País Vasco" (Supplementary data_8)

1. INTRODUCTION

X-linked conditions have classically been considered when there are several maternally linked affected males in different sibships. Yet, with the decline in birth rate, families have become smaller, and families with several affected patients tend to be an exception. Actually, it has been hypothesized that the contribution of X-linked genes in families where there are two affected brothers with intellectual disability of unknown origin and no other family history could be up to 40% (de Brouwer *et al.*, 2007). The European mental retardation X (Euro-MRX) Consortium has analysed index male patients with affected brothers as part of XLID cohort studies. In 2007, they sequenced coding regions of 90 known and candidate XLID genes identifying causal variants on the X chromosome in 17% males with affected brothers (de Brouwer *et al.*, 2007). Similarly, Tzschach *et al.*, 2015 sequenced 107 XLID genes by next generation sequencing in 50 male patients with family history suggestive of XLID -including affected brothers- and identified pathogenic variants in 13 patients (26%) of which 6 were found in male patients with affected brothers. On the other hand, whole exome sequencing of 19 small non-consanguineous families with S-ID or NS-ID and two to five affected siblings, both brothers and sisters, identified three pathogenic variants (16%) and five likely pathogenic variants. This study only included three families with affected brother-brother pairs, and two pathogenic and one likely pathogenic X-linked variants were identified in them highlighting the impact of X-linked genes (Schuurs-Hoeijmakers *et al.*, 2013).

Due to the drop in price of whole exome sequencing, this technology has been easily implemented into research and diagnostic settings, and has demonstrated to be effective in identifying causative variants in ID and in elucidating novel ID genes (Bamshad *et al.*, 2011; Gilissen *et al.*, 2012; Willemsen and Kleefstra, 2014). WES technology enables the detection of variants all over the coding regions of known genes in the genome, and hence, can identify variants of either autosomal (recessive or dominant) or X-linked origin. On the other hand, family based trio (patient, mother and father) WES has been powerful in identifying *de novo* mutations in intellectual disability which are known to account for most of the cases (Vissers *et al.*, 2010; de Ligt *et al.*, 2012; Rauch *et al.*, 2012). Trio-based exome sequencing also facilitates recessive or X-linked variant identification making WES variant interpretation simpler.

In the evaluation of a patient with unexplained ID a general diagnostic approach is undertaken (Introduction-Figure 1). When no diagnosis is obtained after baseline analyses (Introduction: 1.4.1 to 1.4.3) NGS is nowadays applied. Next generation technologies enable screening of multiple genes or the entire exome or genome. The decision on the approach to be taken usually depends on the available resources on each genetic laboratory. With this in mind, we wanted to know which is the best diagnostic approach to take in families with two affected brothers with ID for its best handling on prognosis, genetic counselling an future treatments. In

this context, we began a trio-based exome sequencing study to identify pathogenic variants in index male patients with ID and an affected brother to evaluate the contribution of X-linked genes. To compare efficiency, sensitivity, specificity and reliability in detecting variants on the X chromosome, targeted sequencing of 82 X-linked genes was also performed in index male patients.

2. MATERIAL AND METHODS

2.1. Patients

Our initial dataset (Chapter 1) was thoroughly reviewed in order to identify index male patients with unexplained intellectual disability and affected brothers. Additionally, patients that were referred after 2015 were also included in this study.

As informed consent was required for trio whole exome sequencing and targeted sequencing studies, as well as DNA samples from the affected brother and father, it was necessary to recontact patients for this study. In total, we managed to recruit 13 families (named TRIO1-TRIO13).

All patients had previously been evaluated either by medical geneticist or paediatric neurologist and syndromic forms of ID were excluded. Karyotype and *FMR1* CGG trinucleotide repeat numbers were normal in all of them. aCGH was also a prerequisite for next generation sequencing studies. Written informed consent (Supplementary data_3) was obtained from the parents and peripheral blood samples were collected and DNA was extracted using standard procedures from both brothers and parents for NGS studies and segregation analysis. These studies were performed in the context of a research project (PI14/00321) approved by the Ethics Committee for clinical research of Euskadi-Basque Country (CEIC-E: PI2014192).

2.2. Array Comparative Genomic Hybridization

Despite aCGH being a prerequisite, not all of the patients had it. Therefore, aCGH screening was performed in those patients. Briefly, DNA samples were tested against normal DNA (Agilent Reference DNA) using qChip Post® Postnatal Research Microarray 8x60K, which has been designed and optimized by qGenomics-Genomics for Human Health Laboratories (Barcelona, Spain). Both, labelling and hybridization were performed following the standard operating procedures and the process was subject to internal quality control. Feature extraction software version 11.0.1.1 was used to extract and normalize data from microarray image files (TIFF file) of scanned microarrays. Quality control metrics were within the normal ranges. Data were processed using Cytogenomics software v4.0 (Agilent Technologies), with the statistical algorithm ADM-2, sensitivity threshold 5 and at least 3 consecutive aberrant probes.

2.3. Whole exome sequencing

Whole Exome sequencing was performed on Genetracer Biotech (Santander, Spain) using the Ion Torrent™ technology (Life technologies, Foster City, CA, USA). Genomic DNA from the index patient and parents (father and mother) was enriched using the Ampliseq Exome kit™

(Life technologies, Foster City, CA, USA) which targets about 33 Mb covering more than 97% of coding regions of known genes in the human genome. DNA libraries were amplified by emulsion PCR, using the Ion Chef™ Instrument (Life technologies, Foster City, CA, USA) and Hi-Q chemistry. Templates were then sequenced on the Ion Proton™ sequencer (Life technologies, Foster City, CA, USA) with the 200 bp chemistry. Torrent Suite™ software (version 4.4.3) (Life technologies, Foster City, CA, USA) and the TMAP software were used to align reads to the UCSC hg19 reference genome. Variants were called and analysed on the Ion Reporter™ software (version 4.4.2) (Life technologies, Foster City, CA, USA) following the "AmpliSeq Exome trio" protocol.

Additional variant analyses were also performed in our laboratory using the Ion Reporter™ software.

2.4. Targeted sequencing of X-linked intellectual disability genes

Targeted next generation sequencing of 82 XLID genes was performed and genetic data analysed as previously described in Chapter 2.

2.5. Variant segregation

Candidate variants were validated by conventional Sanger sequencing (Primer sequences available on request) as well as segregated within the family.

2.6. Whole exome sequencing vs targeted sequencing

Whole exome sequencing and targeted sequencing were compared by comparing the variant detection on regions covered by both capture methods. Despite the same Ampliseq™ amplicon-based capture system was used in both approaches, they differ on the design. In order to compare both methods, regions amplified in both designed have been identified by intersecting and merging both design files. This has been done using "intersect" and "merge" tools in the Galaxy platform (Afgan *et al.*, 2016) (Figure 1). The intersect tool returns overlapping regions between the first and second dataset and merge tool unifies contiguous regions. The generated file was then verified on the UCSC Genome Browser (Kent *et al.*, 2002; Karolchik *et al.*, 2004) by visualizing it together with the original design files (Figure 2).

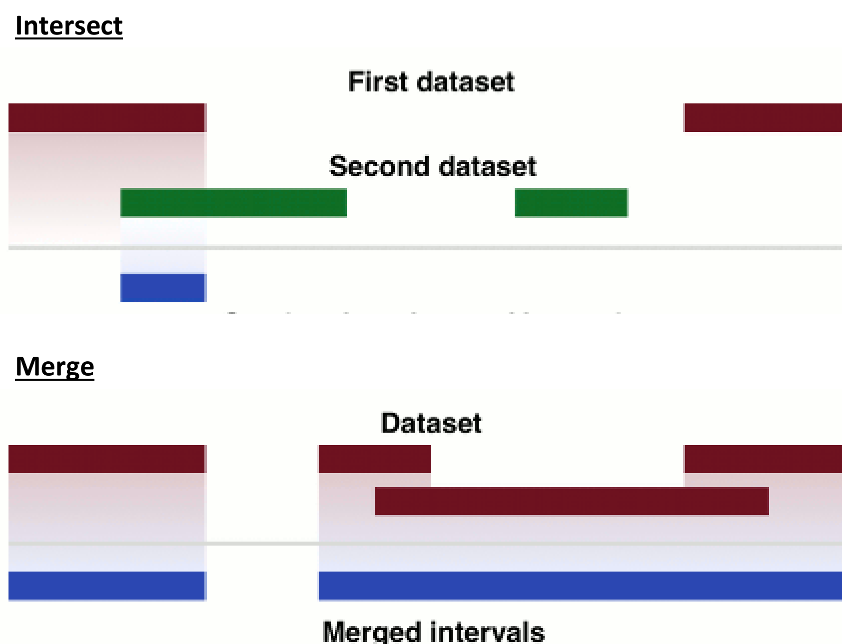


Figure 1. Graphical presentation of the "intersect" and "merge" functions on Galaxy.

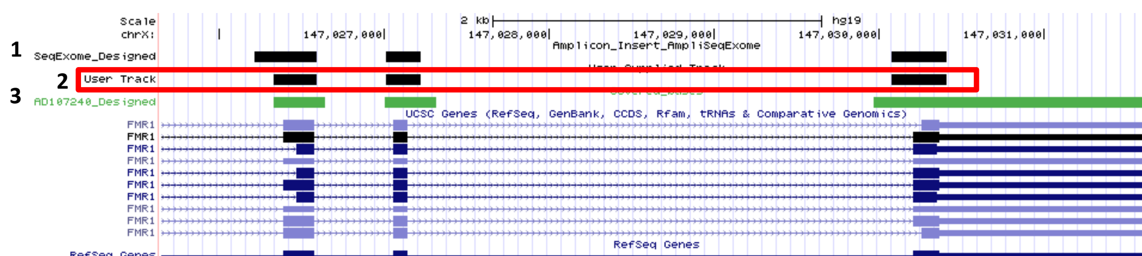


Figure 2. Visualization of the original files together with the generated file (in red) in UCSC Genome Browser. File 1: Ampliseq^{TM} Exome, File 3: Targeted X-linked intellectual disability gene panel and File 2: Common regions between file 1 and file 3.

After generating the file containing common regions, WES and targeted sequencing data were analysed using this file on the Ion Reporter^{TM} software (version 5.4) and variants within these regions were identified and differences in variant identification and consequently coverage were assessed. In order to compare variant identification, quality filters were applied (p value <0.001 and homozygosis).

3. RESULTS

3.1. aCGH results

Twelve of the thirteen families had normal aCGH results. Only one family (TRIO8) revealed a duplication in the chromosomal region 15q11.2-q13.1 by aCGH analysis and consequently this aberration was further studied in the brother, sister and mother as well. The segregation analyses showed that the affected brother as well as his heathy sister and mother were carriers of the duplication (Aguilera-Albesa *et al.*, 2020). Despite the extension of the duplication showed certain degree of size variability, the break-points are in accordance with those reported as “Type II” by (Roberts *et al.*, 2002) going from the breakpoint BP2 to BP3 and including the Prader-Willi/Angelman critical region.

3.2. Whole exome sequencing

Whole exome sequencing generated a mean of 29.363.610 reads per sample which covered 94,81% of the target regions with an average uniformity of 92,60% and 88,90 mean read depth, and 92,53%of the sequenced regions were covered at least 20x (Supplementary data_4). Trio based variant analysis identified candidate variants in about half of the cases (7/13). Supplementary data_5 reports these variants as well as some clinical data of the patients.

After segregation analyses were performed, a possible diagnosis was obtained in 4 out of 13 (30.77%) affected male brothers:

- A novel nonsense homozygous variant in *NLGN1* (c.74T>A; p.Leu25*) (NM_014932.3), was identified in TRIO1. This variant segregated with ID and was considered to be pathogenic because it is a truncating variant and both affected brothers fit the phenotype observed in patients with *NLGN1* mutations (Tejada *et al.*, 2019).
- A missense variant in *USP9X* (c.4841G>A; p.Arg1614Lys) (NM_001039590.2) was found in TRIO3. Despite it was not present on the affected brother it was considered likely pathogenic due to the *in silico* evidence and its association with ASD (Homan *et al.*, 2014). Yet, as no segregation analyses were performed on the maternal family it is still a VUS. The brother, who was not carrier of this variant, was diagnosed with another *de novo* variant in *ZMYND11* (c.1798T>C; p.Arg600Trp) that has already been reported in the literature (Cobben *et al.*, 2014). Indeed, it was later on known that both brothers presented with a different phenotype.
- Reanalyses of WES in TRIO8 revealed a candidate missense variant in *REEP1* (NM_022912.2) gene: c.73A>G; p.Lys25Glu (Aguilera-Albesa *et al.*, 2020). Despite this male sib pair yielded pathogenic aCGH results, it was also subject to WES studies

because spastic paraplegia could not be explained by the 15q duplication. Nevertheless, this variant has not been included in our final results because *REEP1* is not an ID gene.

- A *PDZD4* missense variant (c.1442C>T; p.Pro481Leu) (NM_032512.2) in TRIO12 was also considered likely pathogenic as it segregated with the phenotype (ID and autism). Nevertheless, no mutations have been reported in *PDZD4* in patients with ID and the gene has not been related to any disorder yet, and so, it is a candidate gene for ID.

3.3. Targeted next generation sequencing

Targeted sequencing generated a mean of 719.971 reads per sample with a 227bp mean read length, which covered 96,25% of the target regions with an average uniformity of 92,10% and 230,90 mean read depth, and 95,50% of the sequenced regions were covered at least 20x (Table 1).

After variant analysis, only one candidate variant was identified in patient ID0516 (TRIO3): *USP9X* (NM_001039590.2) c.4841G>A (p.Arg1614Lys). This variant had already been reported on the trio WES study (Supplementary data_5).

3.4. Whole exome sequencing vs targeted sequencing

A mean of 75 variants were identified on WES data by analysing common region on the 82 XLID genes included in the gene panel. However, about 8.6% of these variants were covered <10x and 24.4% <20x. On the other hand, while a mean of 79 variants were identified by gene panel sequencing, 4.5% of the variants being covered <10x and 7.3% <20x (Table 2).

In total, a mean of about 77 unique variants were detected. 77.2% of these variants were detected by both technologies. Yet, 13.9% more variants were detected by gene panel sequencing while 8.85% were missed by gene panel sequencing and were detected by WES (Table 3).

Table 1. Technical sequencing data of targeted gene panel sequencing.

WES ID	Patient ID	Bases	>Q20	Reads	Mean Read Length (bp)	Mapped Reads	On Target	Mean Depth	Uniformity	1x	20x	100x
TRIO1	**ID0514**	155.630.324	134.306.593	694.821	224	690.817	96,82%	219,70	91,98%	99,04%	95,51%	79,69%
TRIO2	**ID0515**	221.572.965	191.027.541	992.936	223	987.511	96,13%	312,10	92,55%	99,09%	96,59%	87,65%
TRIO3	**ID0516**	189.925.851	163.551.375	852.792	223	847.536	96,75%	268,40	91,59%	98,94%	95,73%	83,44%
TRIO4	**ID0517**	174.896.087	151.037.659	784.295	223	779.641	95,94%	246,30	92,32%	99,04%	96,02%	83,64%
TRIO5	**ID0518**	145.116.535	125.935.842	639.812	227	637.268	97,14%	205,40	90,70%	98,94%	95,21%	70,97%
TRIO6	**ID0601**	128.689.268	107.243.244	627.739	205	625.101	96,04%	180,90	90,55%	98,63%	94,10%	70,85%
TRIO7	**ID0602**	120.969.223	101.295.609	581.004	208	578.624	96,79%	170,80	92,04%	98,72%	94,67%	72,79%
TRIO8	**ID0701**	212.127.817	194.838.161	796.725	266	795.945	97,34%	301,00	92,88%	99,10%	96,60%	87,32%
TRIO9	**ID0603**	109.385.612	91.988.934	521.938	210	519.647	96,43%	154,30	91,94%	98,79%	94,29%	67,98%
TRIO10	**ID0604**	127.328.650	106.672.739	616.383	207	613.902	96,42%	179,50	91,13%	98,72%	94,22%	70,85%
TRIO11	**ID0605**	168.846.985	140.884.320	835.914	202	832.033	92,37%	230,40	91,83%	98,86%	95,44%	80,66%
TRIO12	**ID0702**	184.351.467	169.708.466	697.623	264	696.808	95,31%	257,40	93,95%	99,08%	96,58%	87,07%
TRIO13	**ID0703**	193.697.452	178.765.295	717.638	270	716.973	97,81%	275,50	93,78%	99,06%	96,51%	88,49%
MEAN		164.041.403	142.865.829	719.971	227	717.062	96,25%	230,90	92,10%	98,92%	95,50%	79,34%
MIN		109.385.612	91.988.934	521.938	202	519.647	92,37%	154,30	90,55%	98,63%	94,10%	67,98%
MAX		221.572.965	194.838.161	992.936	270	987.511	97,81%	312,10	93,95%	99,10%	96,60%	88,49%

WES ID: whole exome sequencing identification code; Patient ID: Patient identification code

Table 2. Variants detected on commonly covered regions on both capture methods: whole exome sequencing and gene panel sequencing.

	TRIO 1	TRIO 2	TRIO 3	TRIO 4	TRIO 5	TRIO 6	TRIO 7	TRIO 8	TRIO 9	TRIO 10	TRIO 11	TRIO 12	TRIO 13	MEAN
WES														
VARIANTS	76	69	81	80	92	81	59	79	72	63	77	78	64	74,69
<10x	15,79%	7,25%	8,64%	8,75%	11,96%	11,11%	8,47%	8,86%	5,56%	6,35%	3,90%	3,85%	10,94%	8,57%
<20x	31,58%	30,43%	25,93%	25,00%	31,52%	24,69%	16,95%	31,65%	19,44%	17,46%	18,18%	20,51%	23,44%	24,37%
GENE PANEL														
VARIANTS	76	77	90	86	90	92	64	76	77	69	87	76	69	79,15
<10x	2,63%	2,60%	7,78%	3,49%	3,33%	6,52%	3,13%	0,00%	3,90%	10,14%	8,05%	2,63%	4,35%	4,50%
<20x	7,89%	7,79%	11,11%	4,65%	5,56%	13,04%	6,25%	1,32%	5,19%	14,49%	14,94%	2,63%	4,35%	7,63%

Table 3. Comparison between whole exome sequencing and targeted sequencing in variant identification.

	TRIO 1	TRIO 2	TRIO 3	TRIO 4	TRIO 5	TRIO 6	TRIO 7	TRIO 8	TRIO 9	TRIO 10	TRIO 11	TRIO 12	TRIO 13	MEAN
TOTAL VARIANTS	85	83	99	94	102	102	71	84	83	77	96	81	73	86,92
COMMON	78,82%	75,90%	72,73%	76,60%	78,43%	69,61%	73,24%	84,52%	79,52%	71,43%	70,83%	90,12%	82,19%	77,23%
WES	10,59%	7,23%	9,09%	8,51%	11,76%	9,80%	9,86%	9,52%	7,23%	10,39%	9,38%	6,17%	5,48%	8,85%
GENE PANEL	10,59%	16,87%	18,18%	14,89%	9,80%	20,59%	16,90%	5,95%	13,25%	18,18%	19,79%	3,70%	12,33%	13,93%

3.5. Final results

In order to determine the total molecular diagnosis made in male patients with intellectual disability and an affected brother, trio based WES results (named TRIO) were compiled with the ones we had previously (named IDSP) (see Chapter 1 and 2). Moreover, male sib pairs that were referred after 2015 were also included.

As the dataset includes patients that were referred since 1991 and many of them have not been evaluated by the actual genetic diagnostic approach (Introduction-Figure 1), only patients that had been tested for the following diagnosis techniques were considered: *FMR1* test, MLPA (subtelomeric regions, XLID or autism), aCGH and next generation sequencing studies (gene panel or WES).

In total, 45 male sib pairs were selected (Figure 3). Of these, a diagnosis was obtained in 42.22% (19/45): 8 (17.77%) had been positive for FXS; copy number variants were detected in 3 (6.67%) either by aCGH or MLPA and single nucleotide variants by NGS in 8 (17.77%) of the sib pairs (Table 4). Interestingly, after all analyses were performed, we knew that TRIO1 and IDSP8 were monozygotic twins respectively. Besides, discrepancies were noted on two of the male sib pairs: the second brother in sib pair IPSP7 was healthy although initially they were both referred as two affected brothers with ID; and different molecular diagnoses were obtained in TRIO3 after performing another WES in the second brother. In the reevaluation of both brothers, it was seen that they also show a different phenotype. Further, a double genetic diagnosis was made in TRIO8, one of them for ID.

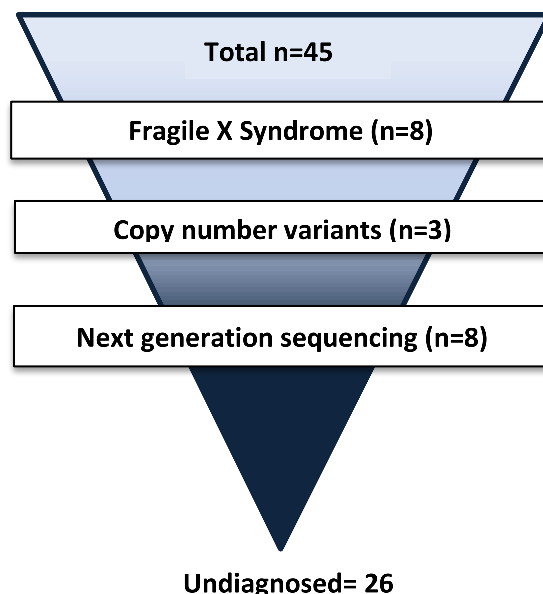


Figure 3. Molecular diagnosis in male patients with intellectual disability and an affected brother. In total 45 male sib pairs have been studied and a diagnosis was obtained in 19 of them (8 Fragile X Syndrome, 3 copy number variants and 8 single nucleotide variants identified by next generation sequencing studies). 26 male patients with affected brother still remain without diagnosis.

Table 4. Diagnoses made in male sib pairs with intellectual disability. Copy number variants and single nucleotide variants identified are shown.

Patient Code	Variant	OMIM	Inheritance	Classification	1st brother	2nd brother
			CNV			
IDSP2	*OPHN1* c.169-?_239+?del	300127	mat-XL	Pathogenic	+	+
IDSP5	der(3)t(3;5)(p26;q35)		mat-t(3;5)	Pathogenic	+	+
TRIO8	dup(15)(q11.2-q13.1)	608636	mat-met	Pathogenic	+	+
			SNV			
IDSP6	*AGA* (NM_000027.3): c. 503G>A; p.Trp168*	613228	AR	Pathogenic	+	+
IDSP7	*PHF8* (NM_001184896.1): c.252C>A; p.Tyr84*	300560	mat-XL	Pathogenic	-	+
IDSP8	*HUWE1*(NM_031407.6): c.1125G>T; p.Met375Ile	300697	mat-XL	VUS	+	+
IDSP9	*UPF3B* (NM_080632.2): c.398-1G>C	300298	mat-XL	Likely pathogenic	+	NE
IDSP10	*HUWE1* (NM_031407.6): c.12209C>G; p.Ser4070Cys	300697	mat-XL	VUS	+	NE
TRIO1	*NLGN1* (NM_014932.3): c.74 T>A; p.Leu25*	600568	AR	Pathogenic	+	+
TRIO3	*USP9X* (NM_001039590.2): c.4841G>A; p.Arg1614Lys	300072	mat-XL	VUS	+	-
	ZMYND11 (NM_006624.5): c.1798T>C; p.Arg600Trp	608668	*de novo*	Pathogenic	-	+
TRIO12	*PDZD4* (NM_032512.2): c.1442C>T; p.Pro481Leu	300634	mat-XL	VUS	+	+

mat: maternal, inherited from the mother; XL: X-linked; AR: autosomal recessive; AD: autosomal dominant; NE: not evaluated

Among the diagnoses made, most of them (15/19) were variants identified on the X chromosome; and hence the contribution of the X chromosome is of 33.33% (15/45) in male sib pairs that have been studied. Focusing on variants detected by next generation studies, 8 variants were identified in 8 affected brothers (the missense variant in *ZMYND11* was not found with our methodology, but with an additional WES study). Of these, 75% (6/8) variants were variants with X-linked inheritance and 25% (2/8) autosomal recessive. These results highlight once again the relevance of X-linked genes on the origin of ID in male patients including male sib pairs.

4. DISCUSSION

13 index male patients with an affected brother have been studied by trio based whole exome sequencing and targeted next generation sequencing of 82 XLID genes and a comparison between the NGS approaches in detecting variants on the X chromosome has been made. Finally, a snapshot of the molecular diagnosis obtained in male patients with ID and affected brothers has been given.

Although we expected to analyse more patients for this study, eventually we were able to study 13 families. This was because we encountered difficulties in recruiting patients for genetic studies. Intellectual disability is generally untreatable and symptomatic treatments are the only solution for these patients to date becoming burdensome for families. Therefore, some families might not be interested in collaborating in genetic studies as they see no major benefit. Similarly, some clinicians do not see any benefit for patient management or they no longer see these patients. Nevertheless, genetic studies are always of especial interest when there are putative female carriers, as they could provide information for family planning. Beunders *et al*., (2018) recontacted families either by phone or letter for re-evaluation due to the new technologies and had a response rate of 36% and 4% respectively. This demonstrates the difficulties in patient recontacting.

Discrepancies observed in Table 4 and described in the Final Results section, show the importance of patient reevaluation and recontact. Patient IDSP7, who is carrier of a truncating *PHF8* variant, had been initially referred as a having a brother with GDD. However, the brother -who is not carrier of the variant- did not develop ID and had a normal development. In addition, although the splicing variant in *UPF3B* is a likely pathogenic variant, the family could not be recontacted for segregation analyses. Therefore, it has not been possible to determine the pathogenicity of the variant. Finally, the second brother in TRIO3 was reevaluated by a clinical geneticist posterior to exome sequencing studies, and showed a distinct phenotype to his brother. Therefore, other sequencing studies were performed and a pathogenic variant in *ZMYND11* was identified. Similar discrepancies have already been observed in other sequencing studies (Sanchis-Juan *et al.*, 2019).

All in all, we have identified a possible genetic cause of ID in 4 of the families (4/13=30.77%): Trio based exome sequencing has elucidated a possible genetic origin in three families (3/12=25%) and in one family we have found a duplication on the 15q11.2-q13.1 region and a candidate missense variant for spastic paraplegia in *REEP1* gene. Bearing in mind that patients with severe ID, syndromic forms or with other associated clinical features yield an increased diagnostic rate and our patients have non syndromic forms of ID, our results are comparable to other trio based WES studies (de Ligt *et al.*, 2012; Rauch *et al.*, 2012; Helsmoortel *et al.*, 2015; Retterer *et al.*, 2016; Carneiro *et al.*, 2018). Athanasakis *et al*., (2014) also followed trio based

WES in nine patients with non-syndromic ID and identified a possible genetic origin in three of them (3/9=33.33%), and they also found three other new candidate genes for ID. This is possible because WES interrogates all known coding sequences on the genome and it enables the identification of novel disease related genes. In our study, we have identified *PDZD4* as a candidate gene for ID and it is now being assessed functionally through collaboration via GeneMatcher (Sobreira *et al.*, 2015) and Matchmaker Exchange (Philippakis *et al.*, 2015).

On the other hand, targeted next generation of 82 XLID genes only identified one missense candidate variant in *USP9X*, which was also identified by trio WES (1/13=7.7%). *PDZD4* is also an X-linked gene, but it has not been related to ID yet and therefore, it was not included on the gene panel. All in all, 2/13 (15.4%) patients and their affected brothers could have an X-linked causative variant, a percentage that does not significantly differ from the 17% given by the Euro-MRX in males with affected brothers (de Brouwer *et al.*, 2007).

Targeted gene panel sequencing enables the identification of genetic variants in genes already known to be causative of a disease in a fast an inexpensive manner and is a suitable first tier genetic test in particular disorders (Xue *et al.*, 2015). Also, it enables to identify smaller number of candidate variants for a specific condition and makes follow up and segregation studies easier (Redin *et al.*, 2014). Nevertheless, it does not allow exploring novel disease phenotype associations or novel disease genes as WES does (Caspar *et al.*, 2018). Further, gene panels are disease specific and therefore the clinical condition of the patient should be defined in order to choose the most appropriate gene panel, while WES allows disease gene/variant identification without previous knowledge on the clinical condition and phenotype (Caspar *et al.*, 2018). If both technologies are compared in terms of coverage and variant identification targeted gene panel sequencing generally offers a higher coverage of the targeted regions leading to better variant identification (Jones *et al.*, 2013; Valencia *et al.*, 2013; Saudi Mendeliome Group, 2015). In our study, we have compared both capture methods by assessing variant identification on the commonly covered regions. Variant calling detected a mean of 77 unique variants on the targeted regions and 77.2% of these variants were detected by both technologies. Yet, 13.9% more variants were detected by gene panel sequencing while 8.85% were missed by gene panel sequencing and were detected by WES (Table 3). These results are in accordance with the previously mentioned studies. In any case, both gene panel sequencing and WES involve capturing of specific regions on the genome and hence, they both fail in providing complete coverage of all the regions of interest because of the difficulties in amplifying sequence homology regions or pseudogenes, GC-rich regions, highly repetitive regions and other sequence complexities (Meienberg *et al.*, 2016).

All in all, compiling previous and current molecular results a molecular diagnosis has been obtained in 19/45 (42.22%) being the most common ID condition FXS (8/45=17.77%). This demonstrates again the relevance of FXS testing in male patients with unexplained ID, also if they have affected brothers. Copy number variants have also been identified in three of the male sib pairs (3/45=6.67%). This together with the identification of a duplication in TRIO8 previous to NGS studies highlights the importance of aCGH studies.

Focusing on next generation sequencing studies, a possible genetic diagnosis has been obtained in 8 out of 34 (excluding FXS and CNVs) male patients with ID and affected brothers

(23.53%). Of these 6 are variants on the X chromosome (6/34= 17.65%) highlighting the relevance of X-linked conditions in male siblings. These results are in agreement with those already published (de Brouwer *et al.*, 2007). In this study causal variants were identified in 17% males with affected brothers by sequencing about 90 XLID genes.

In summary, genetic studies on male patients with affected brothers have corroborated the relevance of XLID genes. Therefore, going back to the initial research question of which diagnostic approach to take in families with two affected brothers, we believe that gene panel sequencing of XLID genes could be applied as an initial screening in male patients with ID and affected brothers as long as it is more cost effective than WES. If no diagnosis is obtained, WES studies could be applied. This could also be the most appropriate approach on the analysis of patients in which one of the parents is missing and in retrospective cases as WES usually requires parent's patient re-contacting and as this is not easy, as previously mentioned.

FINAL REMARKS

The present study highlights the contribution of X chromosome in male patients with non-syndromic ID and suggestive X-linked intellectual disability which include patients with X-linked inheritance of ID and patients with affected brothers.

The elucidation of the genetic origin of these patients has been achieved thanks to the continuous application of novel molecular genetic technologies and patient recontact. Next generation sequencing technologies have recently been introduced to molecular genetic diagnostic laboratories and therefore, analyses of former patients with recent technologies could provide novel diagnoses. Families might be recontacted in light of new genetic findings or to inform about novel diagnostic opportunities. Recontacting involves re-establishing contact with former patients and updating clinical information. Further, recontacting should always be for the benefit of the patient and hence provide the family with clinically or personally relevant information. Despite most of the families have positive feelings towards recontacting, it is only achieved in up to 36% of the cases (Beunders *et al.*, 2018) showing that recontacting former patients is usually complex. Therefore, European Society of Human Genetics (ESHG) has developed some recommendations on this issue (Carrieri *et al.*, 2019). In our study we have recontacted families both for reporting novel genetic findings or to perform novel genetic studies. We managed to recontact 7 families out of 16 for variant reporting (43.75%) and only 13 families with affected brother pairs were recruited for trio based whole exome sequencing studies out of about 45 (30% approx.) which shows the complexity of former patient recontacting. Nevertheless, clinical information updates have been of great value in both cases.

Our work demonstrates that recontacting is also necessary for the genetic variant interpretation. Next generation sequencing identifies lots of genetic variants and for the priorization and evaluation of these variants clinical information of the patient is required. While interpretation of some variants like truncating variants (i.e. nonsense, splicing, frameshift…) is simple in X-linked genes, NGS results in many variants of uncertain significance. Most of these VUS are missense variants. Variant identification in population databases might help in excluding these variants as pathogenic. In this context, it is important that researchers and clinicians report their genetic results either in the literature or public databases. Variant segregation analyses might also help in excluding one variant as pathogenic if a variant is detected on a healthy family member, or might help in determining its pathogenicity if the variant is detected in multiple affected members on the family and is absent in healthy family members. Actually, we were able to classify the *IQSEC2* variant as pathogenic thanks to the segregation analyses and genotype phenotype correlation. Although we also consider that missense variants in *SLC9A6* and *MED12* are likely pathogenic because of segregation analyses, these variants are still classified as VUS according to ACMG variant classification criteria (Richards *et al.*, 2015). However, we believe that ACMG variant classification guidelines are

important because they provide a homogenous variant classification and hence should be universally applied.

Segregation analyses are not always conclusive and further analyses should be undertaken. RNA analyses could help in evaluating the effect of splice variants, and biochemical tests could also help in determining the effects of genetic variants in some genes. Indeed, creatine deficiency syndrome was determined in one of the patients in our cohort by the identification of an inframe variant in *SLC6A8* and by measuring creatine/creatinine ratio in blood again with a new technology. Anyway, our results show that further analyses are needed either *in vitro* or *in vivo* assays to determine if these VUS are pathogenic or not.

Animal models, as mouse, zebrafish and fly models have widely been used in neurodevelopmental disorders like ASD and ID. These models have greatly contributed to the understanding of the neurobiological mechanisms of these pathologies. Nevertheless, they have not being successful in reproducing all clinical features that are typical to a particular disorder (Tamburini and Li, 2017). In the past decade, iPSC (induced Pluripotent Stem Cells) have emerged as a promising tool for disease modelling in neurodevelopmental disorders (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). These cell lines can be derived from adult somatic cells or can be patient specific cell lines that contain a genetic variant of interest. Furthermore, the possibility to generate different types of neuronal cell types makes this approach attractive to certain neuropathologies (Tamburini and Li, 2017).

Intellectual disability is a heterogeneous disorder both genetically and clinically and therefore genetic diagnosis becomes complex. Non-syndromic forms only show ID as main feature despite other subtle or variable features might occur, while syndromic forms show other consistent clinical features besides ID that should be recognized. In any case, the variability of the clinical features sometimes makes the distinction of these forms blurry. A comprehensive clinical examination on comparison of patients presenting with a similar genetic basis might help in better defining the boundaries between NS-ID and S-ID. This is not always possible due to the lack of multiple patients with a similar genetic aetiology. Our recent review (Tejada and Ibarluzea, 2020) intended to find the difference in functional pathways between the gene groups involved in syndromic XLID and non-syndromic XLID and concluded that XLID genes involved in some syndromic forms are also phenotypically variable and might also involve non-syndromic ID, suggesting that they cause a phenotypic spectrum rather than a syndrome. Further, we pointed out the need for functional studies investigate the individual effect of the genetic variants found. We believe that research on the molecular mechanism and associations with phenotypes might help in better understanding if there is a clear division between syndromic and non-syndromic forms. In this line, we identified genetic variants in genes known to cause syndromic forms in our cohort of patients with non-syndromic ID. A nonsense variant was identified in *PHF8* in a patient with non-syndromic ID. *PHF8* is known to cause Siderius syndrome which is characterized by ID and cleft lip or palate. Nevertheless, the few cases reported in the literature and the phenotypic variability among them suggests that phenotypic spectrum of patients with *PHF8* variants should be better defined. This could be achieved with the accumulation of more patients with a common genetic origin of ID in the literature (Aspromonte *et al.*, 2019).

Recently, different platforms like Matchmaker Exchange (Philippakis *et al.*, 2015) or GeneMatcher (Sobreira *et al.*, 2015) have been developed for data sharing between clinicians an researcher on rare diseases. These platforms facilitate the identification and gathering of patients with a similar phenotype and/or genetic basis and hence help defining either the phenotype of a genetic condition or assessing genotype phenotype correlation. Besides, collaborations through these platforms also enable molecular genetics laboratories to communicate with research groups working on, or interested in a specific gene. In this way, we have identified a candidate gene for ID (*PDZD4*) through trio based WES studies and have stablished collaboration via these platforms to assess the implication of this gene in ID.

Intellectual disability is also known to co-occur with other neuropsychiatric disorders like schizophrenia (Morgan *et al.*, 2008). This observation suggests that both conditions share the same molecular pathways and that mutations in the same gene might lead to different impairments in cognitive abilities (Fromer *et al.*, 2014). Dysregulation of synaptic vesicle trafficking has been associated with schizophrenia (Egbujo *et al.*, 2016). In particular, decreased expression of SYN2 and SYN3 synapsin proteins has been observed in patients with schizophrenia. *SYN1* also encodes a synapsin protein and is involved in synaptic vesicle trafficking. However, mutations in this gene have been associated with epilepsy with variable learning disabilities and behavioural disturbances (Fassio *et al.*, 2011). We have identified a missense mutation in *SYN1* in a patient with mild intellectual disability and an affected maternal uncle with schizophrenia who is also carrier of the variant and was diagnosed with GDD in his childhood. This suggests that *SYN1* might also be related to schizophrenia. Nevertheless, more evidence is needed both to support the pathogenicity of the missense variant and to determine its implication of in schizophrenia.

Next generation approaches have certainly increased the diagnostic yield in intellectual disability reaching a diagnostic yield of about 60% in severe and syndromic forms of intellectual disability (Gilissen *et al.*, 2014; Vissers *et al.*, 2015). On the other hand, we have performed next generation sequencing studies in patients with non-syndromic ID and family history. Despite our results are not comparable to the previously mentioned, we have also increased our diagnostic yield: initially 25.6% (59/230) of the patients with ID had been diagnosed and now we have identified possibly causative variants, including VUS, in 29.4% (72/245) of the patients. Targeted next generation sequencing or gene panel sequencing has proven to be useful in elucidating the origin of specific conditions like epilepsy or syndromic forms of ID that show genetic heterogeneity. Actually, our study has demonstrated that XLID gene panel sequencing is useful in patients with suggestive XLID. Interestingly, the detection rate with our panel (26.2%) is similar to that found with X-exome sequencing (28,5%)(Philips *et al.*, 2014), demonstrating the power of targeted gene panels. In this sense, Redin *et al.*, (2014) obtained a similar diagnostic rate (25%) with a panel of 217 ID genes (autosomes and X-linked).

Conversely, whole exome sequencing interrogates coding regions on the genome and does not require previous knowledge on the phenotype. Therefore, it enables novel disease gene identification or disease-phenotype associations. Indeed, we have identified *PDZD4* gene as a candidate for ID and autism. Furthermore, WES enables to identify other genes located on chromosomes other than the X, as it has been the case of TRIO 8 in which a duplication of 15q11 and a variation in *REEP1* was found (Aguilera-Albesa *et al.*, 2020). This family with a

possible dual genetic diagnosis points to the fact that, possibly, patients with ID could have two or more causative variants, something that has been reported for other pathologies in 3-7% of case series (Posey *et al.*, 2017). Obviously, this could not be detected with targeted panels.

Despite of the great advances on the field in the last decades a high percentage of ID remains undiagnosed, especially in mild and non-syndromic forms of ID. The reasons for this could be multiple, including the technical limitations of the sequencing techniques and the existence of yet unknown ID genes. Further, variants in non-coding regions of the genome or regulatory elements could also contribute to the origin of ID. In this line, whole genome sequencing holds great promise in decoding the origin of ID in patients with unexplained ID.

CONCLUSIONS

1. We made a retrospective review of male patients with family history of intellectual disability referred to our laboratory for Fragile X Syndrome testing between 1991 and 2015.
    1.1. A definite genetic diagnosis was made in 25.65% (59/230) of male patients. Fragile X Syndrome was the most frequent inherited condition with a frequency of 18.69% (43/230); copy number variants were also a significant cause with a frequency of 4.78% (11/230), while single nucleotide variants were identified in 1.74% (4/230) of the patients. An anomalous CCG expansion in the *AFF2* gene was also identified (1/230=0.43%). Overall, the diagnoses obtained suggest that X chromosome greatly contributes to the origin of ID since 88.13% (52/59) of the diagnoses obtained are X-linked conditions. These represent 22.60% (52/230) of the whole cohort of male patients reviewed.
    1.2. A total of 171 patients remained without genetic diagnosis (171/230=74.35%) and 19.13% of these patients (44/230) were reported to have suggestive X-linked inheritance of intellectual disability.

These findings highlight the need to reanalyse patients by applying new diagnostic techniques to try to achieve a higher diagnostic yield.

2. We analysed 61 male patients with suggestive X-linked intellectual disability by targeted next generation sequencing. These include patients with suggestive X-linked inheritance of intellectual disability and brother pairs selected based on their mothers' skewed X inactivation.
    2.1. We designed a gene panel that includes 82 X-linked genes that have been associated with either non-syndromic X-linked intellectual disability or high phenotypic variability in males and candidate genes that have not yet been established as X-linked intellectual disability genes.
    2.2. We identified 17 variants in 16 probands (26.22%) that could be the cause of their intellectual disability. These include 14 missense variants that were classified as VUS and one nonsense variant, one splice-site variant and an in-frame deletion that were classified as pathogenic/likely pathogenic. We further analysed eight variants in seven families recontacted and as a result two variants were reclassified, one as pathogenic/likely pathogenic and the other as benign. Overall, only four variants were classified as pathogenic/likely pathogenic (4/61=6.56%).

These results point out the utility of the X-linked gene panel and the limitations of phenotype correlation and segregation analysis in assessing the pathogenicity of missense VUS found, since the phenotype is highly variable in patients with ID. Therefore, our work reinforces the need for functional assays to assess if these variants have any impact in the protein function and consequently on the patients' phenotype.

3. We evaluated the contribution of the X chromosome in male patients with intellectual disability and affected brothers.
   3.1. We validated the targeted X-linked intellectual disability gene panel by comparing it with whole exome sequencing in 13 patients with intellectual disability and an affected brother. A mean of 77 unique variants were detected on the targeted regions and 77.2% of these variants were called by both technologies. Yet, 13.9% more variants were detected by gene panel sequencing while 8.85% were missed by gene panel sequencing and detected by whole exome sequencing. Nevertheless, trio based exome sequencing elucidated a possible genetic origin of ID in 3 families (3/12=25%), and targeted next generation of 82 XLID genes only identified one missense candidate variant in *USP9X*, which was also identified by trio WES (1/13=7.7%). Actually, WES enabled novel candidate disease gene identification as *PDZD4,* an X-linked gene not included on the gene panel.
   3.2. We have determined that the contribution of the X chromosome in patients with affected brothers is about 17% (2/12). In fact, next generation technologies have uncovered a possible genetic origin of ID in 8 out of 34 (excluding FXS and CNVs) male patients with ID and affected brothers (23.53%). Of these 6 are variants on the X chromosome (6/34= 17.65%) highlighting the relevance of X-linked conditions in male siblings.

These results show that gene panel sequencing of XLID genes could still be valuable as an initial screening in male patients with ID and affected brothers especially in retrospective cases, as WES usually requires re-contacting. If no diagnosis is obtained, WES studies could be applied.

As a general conclusion we have demonstrated the significant contribution of X-linked genes in a retrospective cohort of male patients with intellectual disability or global developmental delay and family history of ID. The contribution of X-linked genes is of about 25-30% in our cohort.

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SUPPLEMENTARY DATA

1. Questionnaire for Fragile X Syndrome testing (in Spanish).

Osakidetza
Servicio vasco de salud

Gurutzetako Ospitalea
Hospital de Cruces

Hospital de Cruces
Teléfono: 94 600 6000
Barakaldo - Bizkaia

Síndrome X Frágil (SXF): Protocolo clínico

A cumplimentar para envío de muestras de sangre (10-20cc en EDTA) de pacientes para estudio genético del **gen FMR1**. Si el paciente es varón, se ha de adjuntar asimismo muestras de **sangre de la madre** en las mismas condiciones. Si la paciente es niña o mujer, a poder ser se acompañará la petición con **muestras de sangre de la madre y el padre.**

Fecha de petición:

Nombre y apellidos del paciente:
Nº Historia (Sólo para H.Cruces): Fecha de nacimiento:
Nombre y apellidos de la madre: Fecha de nacimiento
Nombre y apellidos del padre: Fecha de nacimiento

Servicio y Centro de procedencia:
Doctor solicitante:
Teléfono de contacto: e-mail:

Motivo Principal de estudio (Contestar obligatoriamente **SI/NO**)

Retraso Mental (RM) ________________ C.I. ______________
Antecedentes familiares de RM (señalar cuales caso de SI) ______________
Fenotipo compatible SXF ______________________________
Comportamiento compatible SXF ______________________________

Antecedentes personales:

Gestación ______________________________
Peso y talla al nacer ______________________________
Edad de la marcha ______________________________
Edad 1as palabras ______________________________
Desarrollo psicomotor ______________________________
Desarrollo pondoestatural ______________________________

Comportamiento y status mental: (señalar si presente o pasado)

Autismo ______________________________
Crisis comiciales ______________________________
Comportamiento nervioso ______________________________

1

Hiperactividad ______________________
Déficit de atención ______________________
Lenguaje (ausencia o repetitivo) ______________________
Aleteo de manos ______________________
Timidez, fobia social ______________________
Otros aspectos a señalar ______________________

Fenotipo:

Orejas grandes y despegadas ______________________
Cara alargada, frente elevada ______________________
Perímetro cefálico ______________________
Hiperextensibilidad ______________________
Surco simiesco ______________________
Surco plantar ______________________
Macroorquidia (si hombre) ______________________
Menopausia precoz (si mujer) ______________________
Otros aspectos a reseñar: ______________________

En la medida de lo posible dibujar árbol familiar

2. Informed consent for the project 2017111017 (in Spanish).

HOJA DE INFORMACIÓN Y CONSENTIMIENTO INFORMADO PARA PADRES O TUTORES DE VARONES AFECTOS DE DISCAPACIDAD INTELECTUAL SIN FILIAR, PARA EL PROYECTO DE INVESTIGACIÓN QUE ABAJO SE INDICA Y QUE IMPLICA LA UTILIZACIÓN DE MUESTRAS BIOLÓGICAS.

TÍTULO DEL PROYECTO: Caracterización funcional de variantes de significado incierto como herramienta para aumentar la tasa de diagnóstico de la secuenciación masiva (NGS) en discapacidad intelectual.

INVESTIGADOR PRINCIPAL: Dra. Mª Isabel TEJADA

Centro/Hospital: Hospital Universitario CRUCES/Instituto de Investigación Sanitaria Biocruces.

Nº DE EXPEDIENTE: 2017111017

ENTIDAD FINANCIADORA: Departamento de Salud del Gobierno Vasco.

DURACIÓN: Años 2018 y 2019.

DESCRIPCIÓN GENERAL:

Considerando que en su familia hay uno o más chicos/varones con discapacidad intelectual sin filiar, les solicitamos su consentimiento para participar en un estudio del que les informamos a continuación. Antes de decidir si quieren participar o no, les rogamos lean detenidamente este documento que incluye la información sobre este proyecto. Pueden formular todas las preguntas que le surjan y solicitar cualquier aclaración sobre cualquier aspecto del mismo.

PROPÓSITO DEL ESTUDIO:

El objetivo final es el poder averiguar la causa de la discapacidad intelectual que padece el/los varones de su familia, con el fin de poder darles el tratamiento más adecuado para ellos, así como poder proporcionarles a ustedes y al resto de la familia el mejor consejo genético y reproductivo. Pretendemos sobre todo conocer si esa causa está ligada al cromosoma X o no y así poder asesorar sobre todo a las mujeres en edad reproductiva de su familia.

EXPLICACIÓN DEL ESTUDIO:

A uno de sus hijos (o hermano o familiar del que tiene la tutela) ya se le han realizado los estudios que están en nuestra práctica asistencial (Síndrome X Frágil, cariotipo, arrays-CGH, e incluso exoma) sin resultado positivo, es decir, sin haber encontrado una mutación causal de su patología. Las nuevas tecnologías, sobre todo la llamada secuenciación masiva (paneles, exoma, etc.), han permitido describir nuevos genes y nuevas mutaciones en muchos pacientes, llegando casi hasta el 62% de rendimiento diagnóstico. Pero a pesar de estos buenos resultados, más del 40% de los pacientes con discapacidad intelectual -entre ellos los varones de su familia- no han sido todavía diagnosticados. La razón de esto puede ser:

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1) Que a los varones de su familia no se les haya estudiado todavía con un panel de 82 genes del cromosoma X que hemos diseñado con un anterior Proyecto en el Instituto de Investigación BioCruces y que en este caso se les estudiaría o,

2) Que habiendo sido estudiados con este panel, hayamos encontrado una serie de variantes llamadas de "significado incierto" para las que necesitamos de técnicas complementarias en otras moléculas (ARN y proteína) para poder averiguar si son patogénicas o no.

Esto es lo que proponemos estudiar en su hijo/hermano/familiar. Para ello, necesitamos una muestra de sangre de ellos para obtener ADN. Es probable que incluso no necesitemos esta muestra por tenerla de los estudios realizados previamente.

Posteriormente, en los casos en los que se haya encontrado una variante de significado incierto, necesitaremos muestra de sangre de la madre –también para obtener ADN- y nueva muestra de los pacientes para la obtención del ARN y de la proteína. En este punto del estudio es muy probable que necesitemos muestras de más familiares, en cuyo caso será usted quien se lo deba de proponer. La razón de esto es porque, como ya hemos dicho, esta tecnología de secuenciación masiva proporciona el conocimiento de numerosas variantes en las que tenemos primero que ver si son heredadas y de quien, para estudiar la correspondencia entre la variante y la discapacidad intelectual –o no- del individuo que la lleva.

La información de este proyecto, su aceptación y la extracción de las muestras se realizarán en una visita inicial en nuestra Consulta de Consejo Genético o bien aprovechando el día que su hijo/hermano/familiar tenga algún control con su médico especialista correspondiente que colabora con nosotros y se lo explicaría. El resto de las visitas, caso de haber variantes para estudiar, se les llamará para acudir expresamente a nuestra Consulta de Consejo Genético o, como hemos dicho, sería el especialista correspondiente quien proporcionaría esta información.

Es importante señalar que no hay contraprestación económica de ningún tipo por la participación en este Proyecto.

MUESTRAS A RECOGER:

Como parte de este proyecto aprobado por el Comité Ético de Investigación Clínica de Cruces se les va a extraer (o se ha extraído ya con fines asistenciales) una muestra de sangre para utilizarla con fines de investigación, con objeto de aumentar los conocimientos sobre la patología o proceso objeto de estudio.

SANGRE: La extracción de sangre no conlleva más molestias que un simple pinchazo en la vena en el brazo. A veces, muy raramente, le puede ocasionar un pequeño hematoma o una leve inflamación que remitirán en pocos días.

De esas muestras de sangre se va a obtener ADN y, si fuera necesario, ARN y proteína.

TRATAMIENTO DE LOS DATOS Y CONFIDENCIALIDAD:

Se solicita su consentimiento para la utilización de los datos de la Historia Clínica de su hijo/hermano/familiar y de usted/ustedes (caso de ambos padres), así como de los datos obtenidos de las muestras para el desarrollo de este proyecto. Tanto los datos personales (edad, sexo, raza), como los datos de salud, como la muestra para investigación, se recogerán empleando un procedimiento de codificación. Sólo el investigador principal podrá relacionar estos datos

con ustedes, siendo responsable de custodiar el documento de consentimiento y garantizando el cumplimiento de su voluntad en relación al uso de las muestras biológicas que se ceden para investigación.

BENEFICIO Y ATENCIÓN MÉDICA:

Esperamos que ustedes y sus familiares con discapacidad intelectual puedan beneficiarse personalmente de este proyecto. Pero, aunque no fuera así, los datos recogidos en el mismo podrán derivar en un mayor conocimiento de su patología.

La participación en este estudio es completamente voluntaria: Si deciden no participar, sus familiares con discapacidad intelectual recibirán de igual forma todos los cuidados médicos que pudieran necesitar y su relación con el equipo médico que les atiende no se verá afectada.

La información será procesada durante el análisis de los resultados obtenidos y aparecerá en los informes finales.

En ningún caso será posible identificarle, garantizándole la confidencialidad de la información obtenida, en cumplimiento de la legislación vigente.

ACCESO A LAS MUESTRAS Y/O LA INFORMACIÓN:

Ustedes tienen derecho a conocer los datos genéticos clínicamente relevantes que se obtengan a partir del análisis de las muestras, siempre que así lo deseen y lo soliciten con la firma del consentimiento informado. La información que se obtenga podría ser relevante también para otros familiares. Si fuera este el caso, es decisión personal suya informar a dichos familiares -algo que nosotros le aconsejamos- con el fin de que, si ellos lo desean, puedan ser estudiados y valorar así cuál es su riesgo personal y sus opciones de salud en un futuro.

En todo caso, las muestras estarás disponibles si ustedes las requieren por motivos de salud.

REVOCACIÓN DEL CONSENTIMIENTO:

Pueden revocar en cualquier momento su participación sin necesidad de dar explicaciones. En este caso, no se recogerán nuevos datos después del abandono del estudio. Los derechos de acceso, rectificación, cancelación y oposición puede ejercitarlos ante el **Dr./Investigador/Clínico que le informa, cuyo lugar de trabajo es: (CUMPLIMENTAR)**________________________________

DESTINO DE LAS MUESTRAS TRAS SU UTILIZACIÓN EN ESTE PROYECTO DE INVESTIGACIÓN:

Una vez finalizada la investigación, es posible que existan muestras sobrantes. En relación a las mismas, se ofrecen las siguientes opciones:

A. La **destrucción** de las muestras sobrantes.

B. Su **utilización en futuros proyectos de investigación biomédica** relacionados con la patología de su hijo/hermano/familiar, o para cualquier fin de investigación relacionado con la discapacidad intelectual en general. A tal fin, se les ofrece la opción de donar las muestras excedentes a la colección de muestras del laboratorio de Genética Molecular del Servicio de Genética, centro referente para este tipo de patologías en el País Vasco, que tiene registrada su colección en el Instituto de Salud Carlos III con el nº C.0000398, teniendo como responsable a la IP de este Proyecto, la Dra. Mª Isabel Tejada.

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C. También pueden **donar las muestras al Biobanco Vasco** para la Investigación de la Fundación Vasca de Innovación e Investigación Sanitaria (BIOEF) con objeto de que pueda ser conservada y destinada a futuras investigaciones biomédicas. En este caso, se le dará se le dará una hoja de información y un consentimiento informado específico para esta opción a la finalización del proyecto, y si tenemos muestras sobrantes. Por ello, con la firma del consentimiento actual, ustedes pueden escoger las opciones B y C a la vez.

En ambas opciones, **la utilización de las muestras biológicas para una finalidad distinta** a la expresada habrá de ser expresamente autorizada por ustedes en un nuevo documento de consentimiento.

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CONSENTIMIENTO PARA LA REALIZACIÓN DEL PROYECTO DE INVESTIGACIÓN

TÍTULO DEL PROYECTO: Caracterización funcional de variantes de significado incierto como herramienta para aumentar la tasa de diagnóstico de la secuenciación masiva (NGS) en discapacidad intelectual.

Nosotros, (Nombre, apellidos y DNI)……………………………………………………………………………

Como padres/tutores del paciente……………………………………………………………………………

- Declaramos que hemos leído la Hoja de Información para el Proyecto de Investigación que se plantea realizar, de la que se nos ha entregado copia. Se nos han explicado las características del estudio, así como los posibles beneficios y riesgos que podemos esperar, los derechos que podemos ejercitar, y las previsiones sobre el tratamiento de datos y muestras. Hemos recibido suficiente información sobre el estudio, hemos comprendido la información recibida y hemos podido formular todas las preguntas que nos han parecido oportunas. Sabemos que se mantendrá en secreto la identidad y que se identificarán nuestras muestras con un sistema de codificación. Somos libres de revocar el consentimiento en cualquier momento y por cualquier motivo, sin tener que dar explicación y sin que repercuta negativamente sobre cualquier tratamiento médico presente o futuro.
- Damos el consentimiento para que se utilicen las muestras y los datos asociados como parte de este proyecto de investigación. Consentimos en participar voluntariamente.
- En el caso de identificarse variantes genéticas que pudieran ser responsables de la discapacidad intelectual o candidatas a estudio para averiguarlo, deseamos ser informados de la misma (circular) ↑SI ↑NO
- Si hubiera excedente de la muestra, afirmamos haber sido advertidos sobre las opciones de destino al finalizar el proyecto de investigación. En este sentido (circular):
  A) Solicitamos la destrucción de las muestras excedentes
  B) Solicitamos que se mantengan en una colección de muestras del Servicio de Genética (C.0000398)
  C) Solicitamos la incorporación del excedente en el Biobanco Vasco

Fecha …………………… Firma/firmas del padre/madre/tutor del paciente………………………………..

Yo, Investigador/Responsable clínico: Dr.. ____________________, del Hospital___________________, constato que he explicado las características del proyecto de investigación y las condiciones de conservación que se aplicarán a la muestra y a los datos conservados.

Fecha ……………………

Firma ……………………………

Versión 2: 08/03/2018

Osakidetza

GURUTZETAKO UNIBERTSITATE OSPITALEA
HOSPITAL UNIVERSITARIO CRUCES

ANEXO ACLARATORIO

SE GARANTIZA QUE LA REALIZACIÓN DE ESTE PROYECTO, EL TRATAMIENTO, ALMACENAMIENTO Y UTILIZACIÓN DE LAS MUESTRAS ALMACENADAS EN EL BIOBANCO CUMPLIRÁN CON LA **NORMATIVA APLICABLE**:

-- Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal. En observancia a esta ley los datos de carácter personal recogidos en este estudio pasarán a formar parte de un fichero automatizado que reúne las medidas de seguridad de nivel alto.
-- Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica
-- Ley 14/2007, de 3 de julio, de Investigación biomédica.
-- Reglamento (UE) 20161679 del Parlamento Europeo y del Consejo de 27 de abril de 2016 relativo a la protección de las personas físicas en lo que respecta al tratamiento de datos personales y a la libre circulación de estos datos, por el que se deroga la Directiva 94146/CE (Reglamento General de Protección de Datos).

- *muestra codificada: la muestra se identifica con un número que sólo su médico o el coordinador del Biobanco Vasco en su hospital podrá relacionarla con usted
- **muestra anonimizada es aquella no asociada con los datos identificativos

¿QUÉ ES UN BIOBANCO?

Un **biobanco** es un centro de conservación, en condiciones adecuadas, de muestras, tejidos, ADN y otros derivados, que representan un valioso instrumento con destino a la investigación de enfermedades y que puede permitir la obtención de conocimientos que sirvan para el desarrollo de nuevas estrategias y terapias aplicables a pacientes.

El Biobanco de BIOEF está constituido en nodos, uno de los cuales está ubicado en el Hospital ………………., en dondese almacenaría y conservaría su muestra

Los proyectos de investigación realizados con las muestras almacenadas en el Biobanco serán aprobados por un Comité deÉtica de la Investigación, y, si procede, autorizado por la autoridad sanitaria pertinente, previo vio informe favorable delos comités ético y científico externos del biobanco.

Tanto el Biobanco Vasco para la Investigación, como el investigador al que en un futuro se puedan ceder las muestras, son responsables del manejo de los Datos, conforme a la Ley orgánica 15/1999, de 13 de diciembre, sobre Protección de Datosde Carácter Personal. El Hospital _________________ garantiza que en ningún caso saldrá del centro dato alguno que le identifique personalmente.

Versión 2: 08/03/2018

3. Informed consent for the project PI14/00321 (in Spanish).

GURUTZETAKO UNIBERTSITATE OSPITALEA
HOSPITAL UNIVERSITARIO CRUCES

HOJA DE INFORMACIÓN PARA LOS PADRES DE DOS O MAS HIJOS VARONES AFECTOS DE DISCAPACIDAD INTELECTUAL SIN FILIAR, PARA EL PROYECTO DE INVESTIGACIÓN QUE ABAJO SE INDICA Y QUE IMPLICA LA UTILIZACIÓN DE MUESTRAS BIOLÓGICAS.

TÍTULO DEL PROYECTO: Contribución de las mutaciones de los genes del X versus mutaciones en autosomas en parejas de hermanos varones con discapacidad intelectual sin filiar y sin otros antecedentes familiares.

INVESTIGADOR PRINCIPAL: Dr./Dra. Mª Isabel TEJADA

Centro/Hospital: Universitario CRUCES.

ENTIDAD FINANCIADORA: Fondo de Investigación Sanitaria (FIS) del Instituto de Salud Carlos III.

DESCRIPCIÓN GENERAL:

Considerando que tienen dos (o más) hijos con discapacidad intelectual sin filiar, les solicitamos su consentimiento para participar en un estudio del que les informamos a continuación. Antes de decidir si quieren participar o no, les rogamos lean detenidamente este documento que incluye la información sobre este proyecto. Pueden formular todas las preguntas que le surjan y solicitar cualquier aclaración sobre cualquier aspecto del mismo.

PROPÓSITO DEL ESTUDIO:

El objetivo final es el poder averiguar la causa de la patología que padecen sus hijos, con el fin de poder darles el tratamiento más adecuado para ellos, así como poder proporcionarles a ustedes el mejor consejo genético y reproductivo. Además, pretendemos averiguar si esa causa está ligada al cromosoma X o no y así valorar la mejor oferta diagnóstica para otros pacientes en el futuro.

EXPLICACIÓN DEL ESTUDIO:

A uno de sus hijos (o a los dos) ya se les ha realizado los estudios que están en nuestra práctica asistencial (Síndrome X Frágil, cariotipo, arrays-CGH, etc.) sin resultado positivo. Pero sabemos que hay más de 100 genes en el cromosoma X que producen discapacidad intelectual y otros más de 400 en el resto de cromosomas. El estudio de todos ellos no es posible hoy día de forma asistencial, pero sí que disponemos de una potente tecnología, llamada secuenciación masiva, que permite este tipo de estudios. Hoy día, en el Instituto de investigación BioCruces tenemos un equipamiento con el que podemos estudiar los 100 genes del X y además, hemos firmado un acuerdo de colaboración con una empresa de Biotecnología de Cantabria- llamada Gentracer, que nos hará el resto del estudio de los otros genes del genoma.

Esto es lo que proponemos estudiar en sus hijos. Para ello, sólo necesitamos una muestra de sangre de ellos y de ustedes los padres biológicos para obtener ADN. Es probable que incluso no necesitemos muestra de su hijo/hijos por tenerla de los estudios realizados previamente. En este caso sólo necesitamos su consentimiento para utilizarla en este proyecto.

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La razón de necesitar muestra de ustedes -los padres- es porque esta tecnología de secuenciación masiva proporciona el conocimiento de numerosas variantes que no tienen por qué tener repercusión alguna en la salud. De ahí que sus muestras sirven para ver las que son heredadas de ustedes como variantes normales y así, poner de manifiesto las que de verdad pueden estar implicadas en la patología. Asimismo, caso de que sean variantes del cromosoma X, se puede valorar si se han heredado de la madre o son *de novo*.

También es posible que se pongan de manifiesto variantes inesperadas que tengan que ver con temas de salud, pero no con la patología de sus hijos y que ustedes puede elegir si desean o no que se les comunique, así como a sus familiares de primer grado.

La información de este proyecto, su aceptación y la extracción de las muestras se realizarán en una única visita que podría ser el día que su/sus hijos tengan algún control o bien se les llamará para acudir expresamente a su hospital.

En todo caso, no hay contraprestación económica de ningún tipo.

MUESTRAS A RECOGER:

Como parte de este proyecto aprobado por el Comité Ético de Investigación Clínica de Euskadi se les va a extraer (o se ha extraído ya con fines asistenciales) una muestra de sangre para utilizarla con fines de investigación, con objeto de aumentar los conocimientos sobre la patología o proceso objeto de estudio.

SANGRE: La extracción de sangre no conlleva más molestias que un simple pinchazo en la vena en el brazo. A veces, muy raramente, le puede ocasionar un pequeño hematoma o una leve inflamación que remitirán en pocos días.

De esas muestras de sangre se va a obtener ADN. Parte de esa muestra será enviada a la empresa Genetracer de forma completamente anonimizada. A la finalización del estudio si hay muestras sobrantes se nos devolverán.

TRATAMIENTO DE LOS DATOS Y CONFIDENCIALIDAD:

Se solicita su consentimiento para la utilización de los datos de la Historia Clínica de sus hijos y de ustedes, así como de los datos obtenidos de las muestras para el desarrollo de este proyecto. Tanto los datos personales (edad, sexo, raza), como los datos de salud, como la muestra para investigación, se recogerán empleando un procedimiento de codificación. Sólo el investigador principal y el investigador colaborador que le informa y que es el médico responsable de sus hijos podrá relacionar estos datos con ustedes, siendo responsable de custodiar el documento de consentimiento y garantizando el cumplimiento de su voluntad en relación al uso de las muestras biológicas que se ceden para investigación.

BENEFICIO Y ATENCIÓN MÉDICA:

Esperamos que ustedes y sus hijos puedan beneficiarse personalmente de este proyecto. Pero, aunque no fuera así, los datos recogidos en el mismo podrán derivar en un mayor conocimiento de su patología.

La participación en este estudio es completamente voluntaria: Si deciden no participar, sus hijos recibirán de igual forma todos los cuidados médicos que pudieran necesitar y su relación con el equipo médico que les atiende no se verá afectada.

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La información será procesada durante el análisis de los resultados obtenidos y aparecerá en los informes finales. En ningún caso será posible identificarle, garantizándole la confidencialidad de la información obtenida, en cumplimiento de la legislación vigente.

ACCESO A LAS MUESTRAS Y/O LA INFORMACIÓN:

Ustedes tienen derecho a conocer los datos genéticos clínicamente relevantes que se obtengan a partir del análisis de las muestras, siempre que así lo deseen y lo soliciten con la firma del consentimiento informado. La información que se obtenga podría ser relevante también para otros familiares. Si fuera este el caso, es decisión personal suya informar a dichos familiares -algo que nosotros le aconsejamos- con el fin de que, si ellos lo desean, puedan ser estudiados y valorar así cuál es su riesgo personal y sus opciones de salud en un futuro.

En todo caso, las muestras estarás disponibles si ustedes las requieren por motivos de salud.

REVOCACIÓN DEL CONSENTIMIENTO:

Pueden revocar en cualquier momento su participación sin necesidad de dar explicaciones. En este caso, no se recogerán nuevos datos después del abandono del estudio. Los derechos de acceso, rectificación, cancelación y oposición puede ejercitarlos ante el **Dr./Investigador/Clínico que le informa, cuyo lugar de trabajo es: (CUMPLIMENTAR)**________________________________

DESTINO DE LAS MUESTRAS TRAS SU UTILIZACIÓN EN ESTE PROYECTO DE INVESTIGACIÓN:

Una vez finalizada la investigación, es posible que existan muestras sobrantes. En relación a las mismas, se le ofrecen las siguientes opciones:

A. La **destrucción** de las muestras sobrantes.

B. Su **utilización en futuros proyectos de investigación biomédica** relacionados con la patología de sus hijos, o para cualquier fin de investigación relacionado con la discapacidad intelectual en general. A tal fin, se les ofrece la opción de donar las muestras excedentes a la colección de muestras del laboratorio de Genética Molecular del Servicio de Genética, centro referente para este tipo de patologías en el País Vasco, que tiene registrada su colección en el Instituto de Salud Carlos III con el nº C.0000398, teniendo como responsable a la IP de este Proyecto, la Dra. Mª Isabel Tejada.

C. También pueden **donar las muestras al Biobanco Vasco** para la Investigación de la Fundación Vasca de Innovación e Investigación Sanitaria (BIOEF) con objeto de que pueda ser conservada y destinada a futuras investigaciones biomédicas. En este caso, se le dará se le dará una hoja de información y un consentimiento informado específico para esta opción a la finalización del proyecto y si tenemos muestras sobrantes. Por ello, con la firma del consentimiento actual, ustedes pueden escoger las opciones B y C a la vez.

En ambas opciones, **la utilización de las muestras biológicas para una finalidad distinta** a la expresada habrá de ser expresamente autorizada por ustedes en un nuevo documento de consentimiento.

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CONSENTIMIENTO PARA LA REALIZACIÓN DEL PROYECTO DE INVESTIGACIÓN

Investigador/Responsable clínico: Dr.. ____________________, del Hospital____________________

TÍTULO DEL PROYECTO: Contribución de las mutaciones de los genes del X versus mutaciones en autosomas en parejas de hermanos varones con discapacidad intelectual sin filiar y sin otros antecedentes familiares.

Nosotros, (Nombre, apellidos y DNI)…………………………………………………………………………………

Como padres de los pacientes……………………………………………………………………………………

Declaramos que hemos leído la Hoja de Información para el Proyecto de Investigación que se plantea realizar, de la que se nos ha entregado copia. Se nos han explicado las características del estudio, así como los posibles beneficios y riesgos que podemos esperar, los derechos que podemos ejercitar, y las previsiones sobre el tratamiento de datos y muestras. Hemos recibido suficiente información sobre el estudio, hemos comprendido la información recibida y hemos podido formular todas las preguntas que nos han parecido oportunas. Sabemos que se mantendrá en secreto la identidad y que se identificarán nuestras muestras con un sistema de codificación. Somos libres de revocar el consentimiento en cualquier momento y por cualquier motivo, sin tener que dar explicación y sin que repercuta negativamente sobre cualquier tratamiento médico presente o futuro.

Nosotros damos el consentimiento para que se utilicen las muestras y los datos asociados como parte de este proyecto de investigación. Consentimos en participar voluntariamente.

En el caso de identificarse la alteración genética responsable del Síndrome o Enfermedad de nuestros hijos, deseamos ser informados de la misma (circular) SI NO

En el caso de que se obtengan resultados no previstos en este estudio, pero que pudieran tener importancia para mí o para otros miembros de mi familia deseo ser informado de la misma (circular) SI NO

Si hubiera excedente de la muestra, afirmamos haber sido advertidos sobre las opciones de destino al finalizar el proyecto de investigación.

En este sentido:

A) Solicitamos la destrucción de las muestras excedentes ☐

B) Solicitamos que se mantengan en una colección de muestras del Servicio de Genética (C.0000398) ☐

C) Solicitamos la incorporación del excedente en el Biobanco Vasco ☐

Fecha …………………… Firma del padre de los pacientes ………………………………..

Fecha…………………….. Firma de la madre de los pacientes………………………….

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Constato que he explicado las características del proyecto de investigación y las condiciones de conservación que se aplicarán a la muestra y a los datos conservados.

Nombre del Investigador colaborador/clínico responsable de haber proporcionado esta información: ……………………………………………………………..

Fecha ……………………

Firma ……………………………

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ANEXO ACLARATORIO

SE GARANTIZA QUE LA REALIZACIÓN DE ESTE PROYECTO, EL TRATAMIENTO, ALMACENAMIENTO Y UTILIZACIÓN DE LAS MUESTRAS ALMACENADAS EN EL BIOBANCO CUMPLIRÁN CON LA **NORMATIVA APLICABLE**:

Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal. En observancia a esta ley los datos de carácter personal recogidos en este estudio pasarán a formar parte de un fichero automatizado que reúne las medidas de seguridad de nivel alto.

Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica

Ley 14/2007, de 3 de julio, de Investigación biomédica.

- *muestra codificada: la muestra se identifica con un número que sólo su médico o el coordinador del Biobanco Vasco en su hospital podrá relacionarla con usted
- **muestra anonimizada es aquella no asociada con los datos identificativos

¿QUÉ ES UN BIOBANCO?

Un **biobanco** es un centro de conservación, en condiciones adecuadas, de muestras, tejidos, ADN y otros derivados, que representan un valioso instrumento con destino a la investigación de enfermedades y que puede permitir la obtención de conocimientos que sirvan para el desarrollo de nuevas estrategias y terapias aplicables a pacientes.

El Biobanco de BIOEF está constituido en nodos, uno de los cuales está ubicado en el Hospital ……………….., en donde se almacenaría y conservaría su muestra

Los proyectos de investigación realizados con las muestras almacenadas en el Biobanco serán aprobados por un Comité de Ética de la Investigación, y, si procede, autorizado por la autoridad sanitaria pertinente, previo vio informe favorable de los comités ético y científico externos del biobanco.

Tanto el Biobanco Vasco para la Investigación, como el investigador al que en un futuro se puedan ceder las muestras, son responsables del manejo de los Datos, conforme a la Ley orgánica 15/1999, de 13 de diciembre, sobre Protección de Datos de Carácter Personal. El Hospital __________________ garantiza que en ningún caso saldrá del centro dato alguno que le identifique personalmente.

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4. Technical sequencing details of whole exome sequencing studies.

	ID	Reads	On Target	Mean Depth Coverage[1]	Uniformity	Quality threshold[2]
TRIO1	Proband	27.957.848	96,49%	82,20	91,85%	91,45%
TRIO1	Mother	25.753.312	96,78%	76,22	91,65%	90,20%
TRIO1	Father	28.507.477	96,75%	84,73	91,17%	90,96%
TRIO2	Proband	28.290.198	96,86%	85,55	85,55%	91,76%
TRIO2	Mother	31.270.864	96,69%	94,28	92,47%	93,44%
TRIO2	Father	32.053.750	96,37%	96,52	91,37%	92,48%
TRIO3	Proband	32.467.700	96,45%	97,89	86,36%	87,18%
TRIO3	Mother	30.509.791	96,67%	91,95	87,86%	87,92%
TRIO3	Father	32.987.442	96,82%	100,90	88,55%	89,68%
TRIO4	Proband	26.979.365	95,84%	77,87	90,75%	89,37%
TRIO4	Mother	24.387.755	95,73%	69,78	91,00%	87,95%
TRIO4	Father	23.850.178	95,96%	69,41	92,27%	89,44%
TRIO5	Proband	31.590.567	96,19%	96,93	90,73%	91,41%
TRIO5	Mother	30.647.789	96,31%	94,30	91,86%	92,35%
TRIO5	Father	30.512.643	96,34%	93,59	91,93%	92,40%
TRIO6	Proband	29.580.266	95,95%	88,10	93,23%	93,72%
TRIO6	Mother	27.620.365	96,29%	82,93	92,75%	92,18%
TRIO6	Father	23.855.230	96,50%	70,89	91,97%	89,65%
TRIO7	Proband	34.889.128	95,34%	110,30	90,28%	91,87%
TRIO7	Mother	24.956.605	95,97%	79,70	89,01%	86,43%
TRIO7	Father	31.378.215	94,68%	97,71	93,12%	93,75%
TRIO8	Proband	25.130.285	94,09%	77,00	94,36%	92,75%
TRIO8	Mother	24.445.784	94,07%	75,20	93,25%	90,80%
TRIO8	Father	24.607.283	94,13%	75,30	94,12%	92,22%
TRIO9	Proband	31.257.750	95,83%	97,78	92,84%	93,34%
TRIO9	Mother	31.594.467	96,11%	99,19	95,19%	95,71%
TRIO9	Father	27.436.064	96,19%	87,02	95,09%	94,83%
TRIO10	Proband	32.037.518	91,25%	93,94	95,35%	95,91%
TRIO10	Mother	38.071.768	91,93%	113,00	95,63%	96,79%
TRIO10	Father	26.328.500	92,41%	78,94	95,52%	94,81%
TRIO11	Proband	31.226.996	91,11%	96,03	95,47%	95,18%
TRIO11	Mother	28.367.305	90,33%	83,67	95,70%	95,38%
TRIO11	Father	31.226.996	91,10%	92,93	94,94%	95,93%
TRIO12	Proband	30.908.824	93,30%	96,23	95,45%	95,73%
TRIO12	Mother	30.855.893	91,97%	94,72	95,24%	95,41%
TRIO12	Father	29.858.952	91,09%	89,77	95,25%	95,17%
TRIO13	Proband	31.023.832	93,61%	92,20	93,36%	93,77%
TRIO13	Mother	31.880.742	93,31%	94,78	93,40%	94,04%
TRIO13	Father	28.875.356	94,81%	87,61	95,34%	95,27%
MEAN		29.363.610	94,81%	88,90	92,60%	92,53%
MIN		23.850.178	90,33%	69,41	85,55%	86,43%
MAX		38.071.768	96,86%	113,00	95,70%	96,79%

[1] Mean depth of coverage refers to the sequence mean read depth across the targeted region, defined as coding exons and splice junctions of Ion Ampliseq^{TM} Exome kit targeted protein coding RefSeq genes.
[2] The quality threshold refers to the percentage of the defined target region where read depth was at least 20x coverage to permit high quality exome variant base calling, annotation and evaluation. Average quality thresholds may range from >90-95% of the targeted region, indicating a small portion of the target region may not be covered with sufficient depth or quality to confidently call variant positions.

5. Reported candidate variants on trio based whole exome sequencing studies.

	Phenotype	Gene	Type of variant	Variant	Inheritance	Segregation	Comments
TRIO1	GDD and megacisterna magna	*NLGN1* (NM_014932.3)	nonsense	c.74T>A; p.Leu25*	AR	Parents carriers and brother homozygous	
		BTD (NM_000060.3)	missense	c.1330G>C; p.Asp444His	AR	Parents carriers and brother homozygous	Biotinidase deficiency excluded
TRIO3	severe ID and autism	*USP9X* (NM_001039590.2)	missense	c.4841G>A; p.Arg614Lys	XL	Mother carrier not present on the brother	
		GAD2 (NM_001134366.1)	missense	C.683T>C; p.Ile228Thr	AR	Parents and brother carriers	
TRIO6	severe ID and spastic paraplegia	*KIF1A* (NM_001244008.1)	inframe deletion	c.2751_2753delGGA; p.Glu917del	AR	Parents and brother carriers	Polymorphism
		KIF1A (NM_001244008.1)	inframe deletion	c.2721_2723delGGA; p.Gly907del	AR	Parents and brother carriers	Polymorphism
TRIO10	mild ID and minor dysmorphic features	*B3GALTL* (NM_194318.3)	missense/ frameshift	c.77G>T, c.77_78insT; p.Gly26Val, p.Leu27fs	*de novo*	NP	Do not fit phenotype
		DNAH17 (NM_173628.3)	missense	c.13060G>C; p.Val4354Leu	*de novo*	Not present on the brother and parents	
		ITSN1 (NM_003024.2)	splice variant	c.3469+2T>C	*de novo*	Not present on the brother and parents	No collaboration with family
TRIO11	GDD	*DHX34* (NM_014681.5)	missense	c.2440G>A; p.Val814Ile	*de novo*	NP	Do not fit phenotype
		SMPD1 (NM_000543.4)	inframe deletion	c.108_113delGCTGGC, p.Leu37_Ala38del	AR	Brother and mother carries	Polymorphism
		TSPYL2 (NM_022117.3)	missense	c.302T>C; p.Ile101Thr	XL	Brother and mother carries. Also present on the maternal grandfather	

TRIO12	ID and autism	*PDZD4* (NM_032512.2)	missense	c.1442C>T; p.Pro481Leu	XL	Brother and mother carries
TRIO13	ID, microcephaly and other dyrmorphic features	*KIAA1244* (NM_020340.4)	missense	c.5860G>T; p.Gly1954Cys	AR	Parents carriers, not present on the mother
		COL7A1 (NM_000094.3)	missense	c.5086C>T; p.Arg1696Cys	AR	Parents and brother carriers

AR: autosomal recessive; XL: X-linked

6. Poster presented at "I. Congreso Interdisciplinar en Genética Humana" in Madrid (Spain) (in Spanish).

Diagnóstico genético molecular en varones con Discapacidad Intelectual e historia familiar: 25 años de historia

Ibarluzea, Nekane[1]; Martínez-Bouzas, Cristina[1,2,3]; Maortua, Hiart[1,2,3]; De la Hoz, Ana Belén[1,3]; López-Aríztegui, María-Asun[1,2,3]; García-Naveda, Laura[1,2,3]; Llano-Rivas, Isabel[1,2,3]; Gener, Blanca[1,2,3]; García-Barcina, María[4]; Botella, María-Pilar[5]; Ocio, Intzane[5]; Zubillaga, Paul[6]; Múgica, Iñaki[6]; Tejada, María-Isabel[1,2,3].

[1]Instituto de Investigación Sanitaria Biocruces, Barakaldo, Bizkaia
[2]Servicio de Genética, Hospital Universitario Cruces, Barakaldo, Bizkaia
[3]Grupo clínico vinculado (GCV04) del Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid
[4]Unidad de Genética, Hospital Universitario Basurto, Bilbao
[5]Servicio de Pediatría (Neuropediatría), Hospital Universitario Araba, Vitoria-Gasteiz
[6]Fundación Uliazpi, Gipuzkoa.

INTRODUCCIÓN:

El descubrimiento del gen *FMR1* (1991) supuso un hito en el diagnóstico genético molecular de la Discapacidad Intelectual (DI). Desde entonces numerosas herramientas moleculares han mejorado su diagnóstico, como el MLPA, los arrays-CGH, etc.. Recientemente, gracias a la secuenciación masiva el número de casos con DI de origen genético diagnosticados ha crecido exponencialmente. La DI es prevalente (1-3%) y muy heterogénea, estimándose que un 25-50% de los pacientes tienen un origen genético, y que un 10% tienen su origen en el cromosoma X (XLID). Esto explica la mayor proporción de varones con DI (1.4:1).

OBJETIVOS:

1) Averiguar la contribución de la historia familiar de DI entre los pacientes varones que llegan al laboratorio para diagnosticar el Síndrome del X Frágil (SXF) y entre ellos, los posibles ligados al X (XL).
2) Revisar los diagnósticos obtenidos y calcular su frecuencia.
3) Reportar las diferentes mutaciones halladas.

MATERIAL Y MÉTODOS:

Se ha realizado una revisión exhaustiva de los protocolos clínicos de todos los pacientes varones cuyas muestras llegaron al laboratorio para el estudio del gen *FMR1* durante 25 años (1991-2015). **En total 1909 pacientes.**

INDICACIONES PARA EL ESTUDIO DEL GEN *FMR1*

1- Cariotipo normal
2- No presentar ningún síndrome identificable
3- Sin dismorfología o dismorfología sutil

RESULTADOS:

1) El 12% de los pacientes (230/1909) tiene historia familiar de DI
2) Entre ellos, el 40,43% de los varones parece presentar herencia ligada al X
3) El 26% de los casos con historia familiar han sido diagnosticados

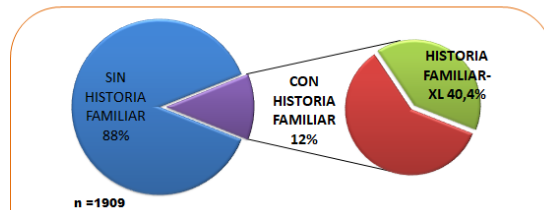

Figura 1. Clasificación de los casos. Se ha tomado como herencia ligada al X cuando al menos hay 2 varones afectos emparentados vía materna como puede ser 1) hermano con distinto padre 2) primo (hijo de hermana madre) y/o 3) tío materno., etc. Además, se han incluido los casos positivos SXF aunque no presenten árbol familiar XL.

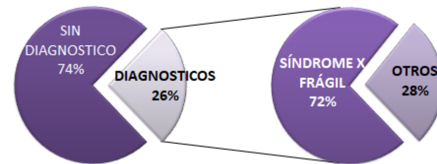

Figura 3. Porcentaje de casos diagnosticados en los pacientes con historia familiar y su relación con el SXF.

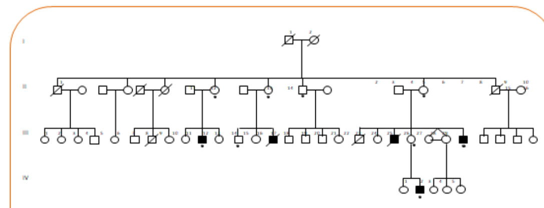

Figura 2. Ejemplo de árbol familiar de una familia que presenta herencia ligada al X.

Tabla 1. Casos no SXF diagnosticados.

	Ligada al X		Autosómico	
	Gen	**Variante**	**Gen**	**Variante**
Anomalías de tripletes	AFF2	>200 repeticiones CGG		
CNV	HUWE1	dup(X)(p11)		der(6)t(5;6)(qter;qter)
	MECP2	dup(X)(q28ter)		der(3)t(3;8)(pter;qter)
	RPS6KA3	dup(X)(p22.12)		der(3)t(3;5)(p26;q35)
	MECP2	dup(X)(q28ter)		dup(1)(pter)
	OPHN1	c.169-?_239+?del		
	IL1RAPL1	c.178-?_249+?del		
SNV	UPF3B	c.118C>T /p.Gln40*	AGA	c. 631G>A /p.Trp168*
	SYP	c.829_832delGACT /p.Asp277fs*29	KRAS	c.40G>A/p.Val14Ile
			NLGN1	c.74 T>A/p.Leu25*

DISCUSIÓN

Este estudio demuestra:

1) **La necesidad de estudios retrospectivos para valorar el porcentaje de diagnóstico**
2) **La importancia de la historia familiar en la DI, y en la XLID en concreto**
3) **La importancia de aplicar las diversas herramientas moleculares disponibles para llegar a un diagnóstico**

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Targeted next generation sequencing in patients with possible X-linked intellectual disability

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Introduction

X-linked intellectual disability (XLID) is known to contribute up to 10% of intellectual disability (ID) in males and could therefore explain the increased ratio of affected males to females observed in patients with ID. Great effort has been done in elucidating the genetic origin of ID and in the past decade next generation sequencing has clearly stimulated the gene discovery process.

Objectives

To uncover the genetic origin of ID in male patient cohort with suggestive XLID using a targeted sequencing approach.

1. Cohort selection

Patients were referred since 1992 to our molecular genetics laboratory for Fragile X Syndrome testing from paediatric neurology units, neurologists and medical geneticists of the 4 public hospitals of the Basque Autonomous Community (Spain). Unresolved patients were selected both retrospectively and prospectively based on the family history. The main selection criterion was the X-linked inheritance pattern meaning that they had half-brothers or maternal cousin or uncles affected. Besides, patients with affected brothers were also studied based on the mother's X-inactivation pattern.

All in all 61 patients with possible XLID were selected. These included 47 patients presenting with suggestive XLID inheritance and 14 male siblings whose mothers present with skewed X inactivation. Table 1 summarizes the cohort description.

Table 1. Description of the cohort of 61 patients with suggestive X-linked Intellectual disability.

	X linked		Siblings	
Total	n=47		n=14	
Age range	2-63		2-24	
0-10	26	54,17%	9	64,29%
10-20	10	20,83%	4	28,57%
>20	11	22,92%	1	7,14%
Intellectual disability				
Mild/Borderline	16	33,33%	5	35,71%
Moderate	6	12,50%	1	7,14%
Severe	3	6,25%	1	7,14%
Profound	2	4,17%	0	
Not evaluated	20	41,67%	7	50%
Comorbidity				
Macrocephaly	1	2,08%	0	
Microcephaly	1	2,08%	1	7,14%
Autism Spectrum Disorder	14	29,17%	4	28,57%
Hypotonia	1	2,08%	0	
Epilepsy	7	14,58%	0	
Behavioural disturbances	5	10,42%	2	14,29%
Previous studies				
Karyotype	48	100%	14	100%
Fragile-X	48	100%	14	100%
MLPA-X	42	87,50%	14	100%
array CGH	15	31,25%	14	100%

2. Targeted sequencing

A cost-efficient amplicon-based gene panel containing 82 XLID genes was designed. The 82 genes are mainly causative of non-specific ID or show high phenotypic variability including non-specific ID.

Targeted sequencing was performed on the Ion PGM™ Platform (Thermofisher Scientific) and data was analysed using the Torrent Suite™ Software (Thermofisher Scientific) followed by variant priorization on the Ion Reporter™ Software (Thermofisher Scientific).

Table 2. Summary of targeted sequencing results.

	Bases	>Q20	Reads	Mean Read Length (bp)	Mapped Reads	On Target (%)	Mean read Depth	Uniformity (%)	1x	20x	100x	Variants
MEAN	200.456.422	177.359.822	788.402	255	786.833	96,32	281,18	94,21	99,17	97,00	88,56	188
MIN	134.513.017	16.258.545	528.309	208	527.220	94,49	188,00	91,20	98,76	95,35	76,27	144
MAX	291.221.340	261.731.357	1.126.580	268	1.121.391	97,67	411,10	95,34	99,41	97,85	93,32	289

Of the detected variants, common variants and variants with a >0.005 minor allele frequency (MAF) were filtered out. Variants that were present more than 2 times in our cohort were also excluded. Non-synonymous variants in exonic and splice site regions were also prioritized.

After variant prioritization, 17 candidate variants in 16 patients were selected (Table 2) and validated by Sanger sequencing.

Table 2. Candidate variants found in our 61 patient cohort.

Patient	Gene	Variant	Inheritance*	Family history	GnomAD freq males	dbSNP	ClinVar	CADD	InterVar**
1208	MAOA	NM_000240.3: c.617G>A; p.Arg206Gln	Maternal (53,7%)	X-linked		rs1218703391		31	VUS (PM1, PM2, PP3)
	IQSEC2	NM_001111125.2: c.128G>C;p.Arg43Pro	Maternal (53,7%)	X-linked				26.2	VUS (PM2)
1206	HUWE1	NM_031407.6: c.12209C>G; p.Ser4070Cys	Maternal (97,31%)	Sib-pair				24	VUS (PM1, PM2)
1205	CASK	NM_003688.3: c.490G>A; p.Gly164Arg	Maternal (74,26%)	X-linked				32	VUS (PM1, PM2, PP3)
1204	UPF3B	NM_080632.2: c.371-1G>C	Maternal (80,49%)	Sib-pair					Pathogenic (PVS1, PM2, PP3)
1307	PRPS1	NM_002764.3: c.611G>A; p.Arg204His	Maternal (uninformative)	X-linked		rs1169615098		24	VUS (PM1, PM2, PP2)
1128	HUWE1	NM_031407.6: c.1125G>T; p.Met375Ile	Maternal (86,55%)	Sib-pair	0/41548	rs1043071474		22.6	VUS (PM1, PM2)
1125	FMR1	NM_002024.5: c.1816C>T; p.Arg606Cys	Maternal (83,18%)	X-linked	1/ 67871	rs782778170		34	VUS (PM1, PM2)
1304	CCDC22	NM_014008.4: c.1388C>G; p.Ala463Gly	Maternal (54,33%)	X-linked	1/73246	rs782691732		26.9	VUS (PM2)
1122	SLC6A8	NM_005629.3: c.1390_1392delGAT; p.Asp464del	Maternal (79,37%)	X-linked					Likely pathogenic (PVS1, PM2)
1402	SYN1	NM_006950.3: c.796G>A; p.Val266Met	Maternal (69,45%)	X-linked		rs1327735600		26.4	VUS (PM1, PM2)
1010	DLG3	NM_021120.3: c.1424C>T; p.Ser475Leu	Maternal (91,97%)	Sib-pair	1/75937	rs953325312		31	VUS (PM1, PM2, BP1)
1011	NHS	NM_198270.3: c.1270A>G; p.Arg424Gly	Maternal (uninformative)	X-linked				23.7	VUS (PM2, BP1)
0919	UPF3B	NM_080632.2: c.1118G>A; p.Arg373His	Maternal (uninformative)	X-linked	2/66856	rs146785878	VUS	26.7	VUS (PM2, PP3)
0707	PHF8	NM_001184896.1: c.252C>A;p.Tyr84*	Maternal (97,04%)	Sib-pair				36	Pathogenic (PVS1, PM2, PP3)
0216	SLC9A6	NM_001042537.1: c.316A>G; p.Met106Val	Maternal (57,09%)	X-linked				23.1	VUS (PM1, PM2, BP1)
1405	MED12	NM_005120.2: c.5009C>T; p.Ser1670Phe	Maternal (95,36%)	X-linked				33	VUS (PM2)

*Parenthesis indicate mother's X inactivation
**Parenthesis indicate ACMG/AMP 2015 criteria applied for molecular variant classification

3. Genotype/Phenotype correlation

7 families were re-contacted for segregation analysis of 8 genetic variants (*MAOA*, *IQSEC2*, *HUWE1*, *SLC6A8*, *SYN1*, *PHF8*, *SLC9A6* and *MED12*) and additional studies. As an example, Figure 1 shows variant segregation analysis on 3 of the families.

These studies have changed the molecular classification in the following variants: *SLC6A8* (Pathogenic), *MAOA* (Benign) and *IQSEC2*, *HUWE1* and *SLC9A6* (Likely pathogenic). The other variants remain with the same classification: *PHF8* (Pathogenic) and *SYN1* and *MED12* as VUS.

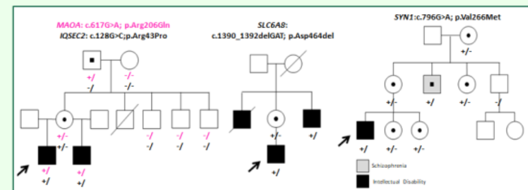

Figure 1. Pedigrees and variant segregation of 3 of the studied families.

Conclusions

Targeted next generation sequencing has proven to be useful in unravelling the genetic origin on individuals suspected to have non-syndromic XLID. Indeed we have identified candidate variants in 16 out 61 of the patients studied.

Variant segregation together with additional studies also helped in re-evaluating and further classifying the genetic variants found.

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8. Poster presented at “II. Congreso Interdisciplinar en Genética Humana” in Madrid (Spain) (in Spanish).

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ESTUDIOS GENÉTICOS MOLECULARES EN HERMANOS VARONES CON DISCAPACIDAD INTELECTUAL EN EL PAÍS VASCO

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Introducción

La Discapacidad Intelectual (DI) supone un serio problema tanto médico como social en los países desarrollados. A pesar de su alta prevalencia (1-3%),cada caso puede ser único debido a la gran heterogeneidad de las causas que producen DI. Se estima que en casi un 60% de los pacientes las causas que producen la DI son genéticas.

Objetivos:

Valorar la contribución de las causas genéticas en parejas de hermanos varones del País Vasco con DI no sindrómica.

n Total=53

Síndrome X Frágil (n=9)

Anomalías de número de copia (n=6)

Secuenciación masiva (n=10)

n sin diagnóstico= 28

Figura 1. Representación del número de parejas de hermanos estudiados y diagnósticos obtenidos. 53 parejas de hermanos han sido estudiados, de los cuales 9 han sido positivos para el Síndrome X-frágil. Además se han detectado anomalías de número de copia en 6 casos bien por array-CGH o MLPA. Por último, estudios de secuenciación masiva han detectado variantes que puedan ser causa de la patología en 10 casos, quedando así 28 parejas de hermanos sin diagnóstico molecular.

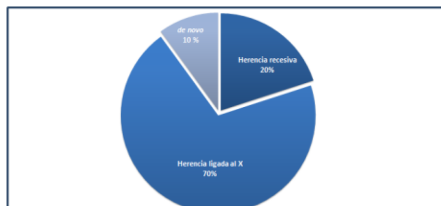

Figura 2. Proporción de cada tipo de herencia en los casos en los que se han encontrado variantes por secuenciación masiva (n total=10).

Material y Métodos:

Revisión de todas las parejas de hermanos varones que llegaron a nuestro laboratorio inicialmente para el estudio del gen *FMR1* desde el año 1991. Se realizaron en ellos los siguientes estudios: el test *FMR1*, los array-CGH; MLPAs (subteloméricas, del X y autismo) y estudios de secuenciación masiva, bien paneles dirigidos o exomas completos.

Resultados:

Se han estudiado 53 parejas de hermanos varones con DI, llegando al diagnóstico en el 47% de los casos (25/53). Ver Figuras 1 y 2 y Tabla 1.
Hay discrepancias entre hermanos para las variantes puntuales (parejas 1 y 4); dos hermanos resultaron ser monocigóticos (parejas 3 y 6) y dos tenían diferente padre (pareja 5).

Tabla 1. Anomalías de número de copia y variantes puntuales halladas mediante las diversas metodologías aplicadas.

		Variante	Herencia	Clasificación	1er hermano	2º hermano	Notas
Anomalías de número de copia	1	*IL1RAPL1* c.178-?_249+?del	materna-XL	Patogénica	+	+	
	2	*OPHN1* c.169-?_239+?del	materna-XL	Patogénica	+	+	
	3	dup(X)(p22.12)	materna-XL	Patogénica	+	+	
	4	dup(X)(p11.22)	materna-XL	Patogénica	+	+	
	5	del(3p26.3-1); dup(5q35.1-3)	materna-t(3;5)	Patogénica	+	+	
	6	dup(15)(q11.2-q13.1)	materna-met	Patogénica	+	+	
Variantes puntuales	1	*ZMYMND11*: c.1798T>C; p.Arg600Trp	*de novo*	Patogénica	+	-	Cada hermano una variante diferente
		USP9X: c.4841G>A; p.Arg1614Lys	materna-XL	VUS	-	+	
	2	*AGA*: c. 631G>A; p.Trp168*	recesiva	Patogénica	+	+	
	3	*NLGN1*: c.74 T>A; p.Leu25*	recesiva	Patogénica	+	+	Los dos hermanos son gemelos monocigóticos
	4	*PHF8*: c.252C>A; p.Tyr84*	materna-XL	Patogénica	-	+	El hno no habló hasta los cinco años. Hoy día ha evolucionado bien.
	5	*IQSEC2*: c.128G>C; p.Arg43Pro	materna-XL	Patogénica	+	+	2 hnos diferente padre
	6	*HUWE1*: c.1125G>T; p.Met375Ile	materna-XL	Probable patogénica	+	+	Los dos hermanos son gemelos monocigóticos
	7	*PDZD4*: c.1442C>T; p.Pro481Leu	materna-XL	Probable patogénica	+	+	
	8	*UPF3B*: c.398-1G>C	materna-XL	Probable patogénica	+	NE	
	9	*SLC9A6*: c.316A>G; p.Met106Val	materna-XL	Probable patogénica	+	+	
	10	*HUWE1*: c.12209C>G; p.Ser4070Cys	materna-XL	VUS	+	NE	

Conclusiones

Nuestros resultados:

- Corroboran que el SXF es el mas prevalente.
- Subrayan la necesidad de realizar estudios de array-CGH
- Confirman la importancia de la DI ligada al X en parejas de 2 hermanos varones

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