DIAGNOSTIC YIELD OF EXOME SEQUENCING IN A GENETICS PRACTICE

RENDIMIENTO DIAGNOSTICO DEL EXOMA EN UNA CONSULTA DE GENETICA

Master's Degree Project

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1.- ABSTRACT

1.1 Purpose: To evaluate the diagnostic yield of exome sequencing in paediatric patients with clinical features consistent with a potential genetic disease.

1.2 Material: We studied a cohort of 30 consecutive paediatric patients of a Genetics Practice between 2014 and 2016. Patients had one or more of the following clinical features: intellectual disability, neurodevelopmental disorder, congenital malformations and dysmorphic features.

1.3 Methods: Exome sequencing was requested based on the clinical features and the geneticist's judgment. We describe gender, age, ethnicity, consanguinity, family history, clinical features, cost, laboratory results and turnaround time.

1.3 Results: In our cohort of study, the exome sequencing tests showed an average diagnostic rate of 26,7%.

1.4 Conclusion: In our study, exome sequencing resulted in a relatively low yield of final specific diagnosis, although the diagnostic rate obtained was similar to some of the previously published studies in the same time frame. Further research is needed to accurately estimate the efficiency of exome sequencing in pediatric patients with suspected genetic diseases.

2.- KEYWORDS: Next-Generation Sequencing (NGS). Whole Genome Sequencing (WGS). Whole Exome Sequencing (WES). Diagnostic yield. Genetic diseases.

3.- INTRODUCTION

3.1 Next Generation Sequencing: Exome sequencing.

The **Human Genome Project** carried out at the beginning of the 21st century is considered to be one of the most remarkable advances in science and, particularly in Medicine, in human history. This international project not only provided the whole sequence of the human DNA but also the capacity to fully understand human genetics: gene structure and regulation, genetic variation and expression, among many other biological processes. It is after this great advance that **genomic medicine** became a new branch in the field of Human Genetics. From then on, we have been able to sequence the genetic code of any individual, understand underlying processes related to gene modulation and its phenotype consequences and recognise new genetic patterns of illnesses.

A major goal of Medical Genetics and Genomics is the identification of specific genes and elucidating their roles in health and disease. Virtually, any disease is the result of the combined action of genes and environment. The quickening pace of discovery in the last decade has helped to explain the contribution of genes to disease generation.

Classic **cytogenetic techniques** (karyotype or FISH) were widely performed to uncover gross structural genetic anomalies. They have been recently replaced by newer molecular cytogenetics tools (MLPA and Array-CGH) that are able to identify smaller genetic variations by using molecular-based techniques.

Next-Generation Sequencing (NGS), as a further step from 'Sanger sequencing', are those technologies that allow a rapid nucleotide mapping of large DNA fragments efficiently in a short period of time. According to the DNA region coded, there are three different NGS techniques:

- Whole Genome Sequencing (WGS): describes the DNA molecule, that is, the complete sequence of the genome, including exons (20-22.000 in humans) and introns.

- Whole Exome Sequencing (WES): studies the coding fraction of the DNA molecule (exons).

- 'Target' (panel) Sequencing: Includes only a limited number of genes (panel) related to a specific disease or group of diseases.

Recent advances in NGS techniques have improved the accuracy of sequencing and increased the generation of sequencing data at a decreasing cost.

The most universally accepted indications for NGS studies are undiagnosed cases and syndromes resulting from pathogenic variants in various genes. Next-Generation technology has allowed a more rapid diagnosis and has led to the discovery of new disease-causing genes.

Exome Sequencing (ES) is among NGS techniques of diagnostic purpose. This recent diagnostic tool provides a virtually complete sequence of the coding DNA. It analyses around 20.000 genes. Exome sequencing is the ideal test to perform when other first-tier tests fail to identify a pathogenic variant that explains the clinical phenotype of the patient.

Exons are the coding segments of the DNA molecule. The coding regions as a whole are known as the exome. Exons are the lead to protein synthesis and, thus, phenotype expression. A deep description of the exome results ultimately in a profound knowledge of human genetic information. Nevertheless, the exome sequencing coverage is not 100%. SNPs -Single Nucleotide Polymorphisms-, CNVs -Copy Number Variants-, deletions and insertions are detected through this test. However, its interpretation is complex and needs of comprehensive information of the proband to fully accomplish the task. Clinical exome sequencing is not intended to identify uniparental disomy, somatic heterozygous variants, pathogenic repeat expansions, some copy number variants or variants in the mitochondrial genome.

Nonetheless, this test has limitations: since it is new, there are constant database updates. Its diagnostic yield ranges from 20 to 60 percent, so those cases that remain undiagnosed are subject to further genomic testing or periodic re-evaluation.

Exome testing can be performed alone or simultaneously with the proband's relatives' genetic material. That is, analysing and correlating the information provided by the proband (single exome) and the proband's parents (exome-duo or exome-trio) significantly increases the chance of understanding a newly identified variant.

Exome-trio sequencing offers a great advance: it readily detects "*de novo*" pathogenic variants, that is, variations identified in the proband that are absent in his or her parents. This way, if the variant explains the patient's phenotype, its pathogenicity is corroborated and the variant is not present in neither the father nor the mother, we can firmly establish the variant to be the cause of the proband's disease. Because single '*de novo'* pathogenic variants are observed in exome-trio sequencing, these '*de novo'* pathogenic variants have the potential to highlight novel disease-causing genes.

The maximum benefit obtained from NGS testing requires a multidisciplinary approach and a precise and exhaustive study protocol.

Data collection starts with a thorough study of the proband: detailed clinical history (in Paediatrics, pregnancy, birth-related and neonatal period data are not to be overlooked) and a systematic and detailed physical examination from head to toe. Previous diagnostic clinical workup is needed: complementary tests and medical assessment, somatometric measurements and phenotype

findings' universal codification -Human Phenotype Ontology (HPO) nomenclature-. NGS techniques are particularly useful in complex and atypical phenotypes.

Genetic counseling is a crucial step for a successful genetic assessment. All information related to the proband's pathological medical record is relevant as well as any information from his or her close family members. Thus, a standardised detailed family pedigree, including at least three generations is mandatory. Inheritance patterns, consanguinity, candidate genes, expression, variability and epigenetic changes may become apparent after this initial step.

A strict selection of study candidates is essential. Not all individuals are eligible to undergo (NGS) genetic testing. The process has to be properly explained to the to-be examined probands for many reasons. In the case of paediatric probands, the process will be explained to their parents, who will consent to undergo the genetic testing process or decline it.

All the information collected from the abovementioned interventions helps the clinical geneticist to build an initial diagnostic approach and a rational interpretation of the study start line.

Results obtained from the test show several options. Evaluation of the potential pathogenicity of variants with clinically relevant characteristics is recommended. Results are classified as follows:

- Benign: the variant does not relate to the illness or condition.

- Likely benign: the variant is not likely to produce the disease.

- Likely pathogenic: the variant is considered the probable cause of the disease.

- Pathogenic variant (formerly known as 'mutation'): the variant is considered to be the cause of the disease, then it establishes the molecular diagnosis.

- Variant of unknown significance (VUS): the variant has characteristics of being an independent disease-causing variant, but insufficient or conflicting evidence exists. Variants that are present in control subjects but have not yet been linked to a certain pathology.

- Incidental findings: pathogenic variants not related to the initial suspected clinical diagnosis. Incidental findings result from an already-set mutation screening for deleterious variants recommended by scientific societies. These variants are not primarily looked for and, although they don't respond to a primary indication, they demand careful handling. In the case of children, as well as in adults, incidental findings should be reported if they entail actionable medical measures.

- Secondary findings: variants of clinical significance that are not linked to the initial suspected clinical diagnosis.

In a clinical setting, the main goal of genetic diagnosis is to reveal whether the patient carries a pathogenic or likely pathogenic variant. In this last case, a certain degree of uncertainty should be considered.

With our expanding knowledge of genes involved in hereditary disease pathogenesis and the rapidly falling DNA sequencing costs, molecular diagnosis will soon become the standard of care for many conditions. In this sense, the scope of Medicine is switching towards a personalised approach by direct detection of disease-causing variations. For this reason, rare diseases genetic assessment represents the paradigm of personalized health.

3.2 Rare diseases

A rare disease is defined as a condition with a prevalence threshold of not more than 5 affected people per 10.000 individuals of the general population. **Rare genetic diseases** affect 1 in 50 individuals and add up to 6000-7000. [1, 2] It is estimated that 8% of the world's population is thought to be affected by a rare disease. [3] Around 80% of rare diseases have a monogenic origin. [4] More than half of the individuals affected with a rare disease never receive a diagnose, despite extensive diagnostic workup (i.e. diagnostic odyssey).

Congenital malformations, neurodevelopmental delay and intellectual disability are among the most common indications for genetic referral in the paediatric population.

Congenital malformations or anomalies are structural or functional defects that occur during intrauterine life and can be identified prenatally, at birth or shortly after birth. Congenital defects have consequences to the individual's quality of life and life expectancy and normally require medical surveillance. The European Surveillance of Congenital Anomalies (EUROCAT) estimates a prevalence of major congenital anomalies of 23,9 per 1.000 births (from 2003 to 2007). [5] Half of all congenital anomalies cannot be linked to a specific cause.

Neurodevelopmental disabilities are a group of conditions due to mental and/or physical impairments that arise before adulthood. This causes difficulties in communication, learning, language acquisition, behavior, self-care or mobility. When many of these areas are affected, we might refer to such conditions as global developmental delay. Intellectual disability is probably the most frequent neurodevelopmental disability and the most frequent indication for exome sequencing overall.

Intellectual disability represents a remarkable subgroup of neurodevelopmental disorders and is defined by an intellectual coefficient under 70. It involves a substantial delay in the acquisition of functioning and adapting abilities of environmental, genetic or multifactorial causes.

The extensive genetic and phenotypic heterogeneity of intellectual disability is the major hindrance to obtaining a precise molecular diagnosis. High-throughput NGS-based strategies are increasingly replacing direct sequencing strategies (sequentially screening candidate genes). [6]

Diagnostic odyssey is a newly coined term used to describe the situation of vulnerability that many rare disease-affected individuals experience throughout their diagnostic process. It refers to patients that have not yet received a molecular diagnosis despite numerous genetic testing and specialists visits in years. They undergo a time-consuming, costly and uninformative journey that lasts decades.

By providing a timely diagnosis, the course of some human conditions can be modified or, in cases in which no substantial interventions can be implemented, at least the diagnostic odyssey finally stops.

Genetically uncharacterized diseases find in rapid scanning of the most informative fraction of the DNA molecule -exons-, detailed genetic information for identifying causal variants in Mendelian disease genes. Hence, exome sequencing emerges as an effective approach for diagnostic purposes in routine clinical set-ups.

The increasing use of NGS techniques has uncovered a broader spectrum of diseases related to genetic variants in a way that now, rare disorders are not the only target in genetics studies, but also common and complex diseases.

3.3 Clinical usefulness

Establishing the underlying molecular diagnosis for diseases generates different possible **outcomes** for patients:

- Modification of morbidity and mortality.

- Change in clinical management: medication and diet changes, surgical procedures, etc.

- Referral to specialists to follow up care. 25% of patients change their subspecialty consult or have the first visit, after obtaining an exome or genome sequencing result.

- Halt of unproductive and unnecessary treatment and invasive procedures.

- Inclusion in surveillance strategies.

- Further diagnostic tests: imaging testing, biochemistry testing, additional molecular testing.

- Obviate the need for further testing.

- Transition to palliative care or withdrawal of care in cases of expected negative outcomes in severe illnesses.

- Enrollment in experimental clinical trials or other research studies.

- Predict the risk of recurrence.

- Reproductive decision-making and planning: sperm or egg donation, assisted reproductive technologies, preimplantation diagnosis, termination of pregnancies, invasive fetal diagnosis techniques. The decision to terminate a pregnancy or to avoid future pregnancies is also an expected outcome. This points out the importance of exome testing: the usefulness expands beyond the proband to further family members.

- Cascade familial genetic testing: identification of at-risk family members.

- Joining support groups and rare diseases organisations.

- Distress, depression or anxiety as a psychological and social negative outcome. Psychosocial negative impact on the patient and their family is a downside to genetic testing.

The literature shows that 5% of patients experienced some kind of change in their clinical management as a result of exome sequencing or genome sequencing.[7]

There are many articles that describe findings to these techniques as ‘medically actionable’ since they somehow imply the discontinuation or the implementation of some medical procedures.

3.4 Paediatric population

The **paediatric population** is peculiar for many reasons. The diagnosis process needs to be shortened, as time at this age could have a great impact on the morbidity and mortality of the individual. Establishing a diagnosis at an early age could be life-changing, since some genetic-based diseases are life-threatening or, in other cases, the course of the disease can be altered if the patient is a candidate for specific target treatment.

Therefore, when a genetic condition is identified through genetic testing, we are able to predict future clinical manifestations or even prevent them from affecting the individual’s quality of life by early intervention. That is, the prognosis of an individual depends largely on an established genetic diagnosis because it allows the implementation of medical care, medication changes, clinical management and follow-up, psychological benefit, educational resources, reproductive and prenatal choices, at-risk relatives identification and family counseling.

Phenotyping or, in other words, firmly describing a proband's clinical features and acknowledgeable clinical disorders is imperative for more successful and more profitable genetic testing, particularly, exome sequencing.

During early infancy, the assessment of syndromes may be tedious, for many phenotypical features might be absent, not fully expressed or hardly recognisable at a young age. That is, hallmark features are yet to be expressed. Since an early diagnosis in rare diseases with a progressive nature and missing hallmarks at a young age is a challenge for medical geneticists, clinical and phenotypical re-evaluation is compelled.

The wide phenotypical spectrum of expression of rare diseases plus clinical variations and uncommon presentations in infancy makes WES a reasonable approach to fulfil our knowledge gap in paediatric rare diseases' clinical and genetic heterogeneity.

Congenital anomalies, intellectual disability and neurodevelopmental disorders are the most common conditions that lead patients to the genetics practice, that is, the most frequent medical study requests. These clinical findings can be part of a syndrome or an isolated feature in an individual. Due to heterogeneity in phenotypical expression and genetic ground of these entities, establishing an accurate diagnosis based on signs and symptoms is not an easy task, especially in the newborn period and infancy.

3.5 Whole exome sequencing

The inclusion of **Next-Generation Sequencing** in clinical practice is a relatively recent progress for genetics: clinical laboratories started offering new DNA techniques to maximize genetic information. Whole exome and clinical exome studies are among NGS techniques of newly implementation in the clinical setting.

The evidence of the use of **whole exome sequencing** as part of the routine clinical care is limited and, though it appears to be a comprehensive genomic test, reports of its diagnostic utility are yet sparse.

4.- MATERIAL AND METHODS

The aim of this study is to assess the diagnostic yield of exome sequencing analysis in patients referred from 2014 to 2016 to our Genetics Practice at the third-level Hospital Clínico Universitario 'Lozano Blesa' in Zaragoza. That is, to analyse the proportion of patients that have undergone exome testing and have obtained a pathogenic variant or likely-pathogenic variant as a result in a gene presumably related to the individual's condition. Variants that explain only partially the phenotype have not been considered as part of diagnostic yield (not causative).

For that purpose, an observational descriptive study was designed. A total number of 30 patients is the cohort of our study. All the probands had previously been evaluated at the genetics practice by a clinical genetics specialist. Needless to mention that only those with high suspicion of a genetic disease were included in the cohort.

There were two selection criteria: 1) the patient must have undergone exome sequencing (either clinical exome test or exome-trio test) and 2) the patient must be of paediatric age (that is usually under 18 years old) at the time of referral.

The study cohort included consecutive patients that, either had a suspected or clinically recognizable genetic diagnosis or disease or, if the diagnosis was unknown or unsuspected, had an exhaustive and detailed description of his or her clinical features.

All selected patients lacked a specific genetic diagnosis and had, in all cases, already taken other genetic tests. Most probands had already undergone at least one of the following first-tier genetic tests: Karyotype, FISH, Array-CGH or gene-panel sequencing. All patients had a normal, non-diagnostic or inconclusive Array-CGH. These tests didn't shed any light on diagnostic findings. They had also been tested for metabolic inborn errors of metabolism included in the newborn screening test performed in Aragon prior to arriving at the Genetics Practice.

The patients ended up at our genetics practice either by a referral order from a different paediatric department (Neurology, Psychiatry, Gastroenterology, etc), the hospitalisation floor, primary care centres, the neonatal intensive care unit, etc.

The age of patients included in our cohort ranged from birth to 17 years. Regarding ethnicity and ancestry, patients and families were mainly from Spain, but there were also patients and families from other European countries and from South America.

Indications for exome sequencing were one or more of the following: intellectual disability, neurodevelopmental disorders, congenital malformations and/or dysmorphic features.

The clinical geneticist was first to select candidates for the test, but the choice was later corroborated by the Hospital's Genetics Committee.

The cost of the tests was assumed by Aragon's public health service (SALUD). Laboratories chosen to carry out the exome sequencing tests were private. The decision was based on both economic and experience criteria.

The present study has the Hospital's approval and has initiated the validation process for final approval at CEICA (Aragon's Investigation Ethics Committee).

4.1 Genetics committee

The eligibility of patients was evaluated at the genetics practice by a medical geneticist.

An interdisciplinary Genetics Committee made out of clinicians and personnel from our Hospital had periodic meetings to discuss the feasibility (cost) and relevance of all the genetic tests requested to external laboratories.

4.2 Informed consent

After the proband was considered entitled to undergo exome testing, the clinical geneticist thoroughly explained the risks and benefits of the procedure to the patient's parents or legal guardians. That was prior to obtaining the written informed consent. Since most of the study population are children, parents act as representatives for their sons and daughters and are responsible for the decision-making process.

The indication of the test was outlined for the patient's family, but they were also advised about the fact that the disclosure of further results was to be expected. These findings would then be classified as variants of unknown significance, pathogenic, benign or unwanted (incidental and secondary) findings. They had to state whether they wanted to receive such information or not. Some of the results could later unleash additional medical actions.

4.3 Blood samples

Peripheral blood samples were provided in all cases to extract DNA. Samples from parents or siblings of the proband were required in the case of exome-trio and exome-duo sampling. No tissue or organ probes were used as sources for DNA.

Each sample was carefully handled. Laboratories participating in the study collected samples together with a brief description of the patient's phenotype or suspected syndrome and previous diagnostic workup, including laboratory tests (clinical report). Laboratory coordinators ensured an appropriate dealing of samples and monitored their submission and preservation.

Testing and analysis were carried out according to each laboratory standard. The overall strategy of the clinical exome sequencing workflow requires a very intensive collaboration with clinicians before, during, and after WES analysis to provide relevant and accurate laboratory reports.

Data collection was made through the electronic and physical clinical records of patients. Data were dealt with under strict confidentiality and anonymity.

Variables analysed included: age, gender, ethnicity, indication for exome testing, exome sequencing results, turnaround time and costs.

The **time** when exome sequencing was performed was, in all cases, only after minimum first-tier testing had already been completed and showed no positive results. The time gap between the initial patient assessment at the practice and the exome request is highly variable. We must consider that request dates range from 2014 to 2016, and the availability and profitability of WES were yet to be proved.

The **clinical interpretation** was carried out by the same genetic specialist leading the Genetics Practice (who had previously evaluated the patients) and the latest literature evidence available at the time of the genetic counseling visit.

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5.- RESULTS

The study cohort is made up of a total of 30 patients.14 patients were male and 16 patients were female. All patients (probands) were children (younger than 18 years old). The majority of patients had Spanish origins, although other nationalities and ethnicities were part of the study:

- Case 7's mother was from Romania.

- Case 20's family belonged to the gypsy ethnic community.

- Case 23's father came from Romania and the mother was from Bulgaria.

- Case 28's family was from Bulgaria.

- Case 29's father was from Italy.

- Case 30's family was from Colombia.

Consanguinity was found in 5 families: cases 17, 20, 21, 23, and 25.

Pedigrees showed that 5 families had more than one affected individual and/or another family member with similar clinical features:

- Case 1's mother had similar facial dysmorphic features.

- Case 3 had a deceased sister with the same phenotype.

- Case 7 had a family history of neonatal deaths on the mother's side due to undisclosed cardiac and cerebral anomalies.

- Cases 8 and 9 were brothers with identical clinical features.

- Case 13 had an uncle on the mother's side of the family with psychomotor delay.

The referral physicians belong to various settings:

- Paediatric Neurology Hospital Practice (7): cases 7, 8, 15, 17, 21, 22 and 28.

- Paediatric Gastroenterology Hospital Practice: (1): case 18.

- ENT (2): cases 19 and 30.

- Paediatric Psychiatry Hospital Practice (3): cases 16, 23 and 24.

- Centres of Child Development and Early Attention (CCDEA) (1): case 13.

- Neonatal Intensive Care Unit (6): cases 4, 12, 14, 20, 26 and 27.
- Hospitalisation floor (2): cases 6 and 25.
- Primary care centres (1): case 19.
- Different hospital (1): case 1.
- On request of the patient's family (5): cases 2, 3, 9,10 and 11.
- Unknown (1): case 5.

The main indications for referral to the Genetics Practice included:

- Intellectual disability (n=8): cases 3, 5, 6, 8, 9, 10, 17 and 24.
- Malformations and/or dysmorphic features (n=10): cases 2, 4, 11, 12, 14, 18, 19, 20, 26 and 29.
- Psychomotor delay (n=6): cases 7, 13, 21, 22, 28 and 30.
- Absence or delay of speech (n=1): case 23.
- Clinical suspicion of a specific syndrome (n=1): case 1.
- Microcephaly (n=1): case 25.
- Hypotonia (n=1): case 27.
- Autism spectrum disorder (n=1): case 16.
- Unknown (n=1): case 15.

Samples submitted included only the proband in 15 cases, the proband and both parents in 12 cases and the proband and another family member in 3 cases. According to this:

- Single (proband) exome test was performed on cases: 1, 5, 6, 8, 12-17, 20, 22, 26, 28 and 30.
- Single exome of the proband and of one family member was performed on cases: 9, 10 and 21.
- Exome-trio test was performed on cases: 2, 3, 4, 7, 11, 18, 19, 23, 24, 25, 27 and 29.

One patient (case 15) is deceased.

All patients had undergone prior genetic testing that included one or more of the following: karyotype, comprehensive gene panel tests, Sanger sequencing of a specific gene, metabolic screening and chromosomal microarray (Array-CGH) analysis. Results obtained from these tests didn't help to establish a solid molecular diagnosis for the probands.

The cost of the tests ranges from 1.200 € to 1.600 € at the time of the request. In our cohort, most exome tests had a cost of 1.200 €.

Bioinformatics and data analysis pipeline was carried out by each laboratory. Variants with suboptimal quality scores were not considered. Other variants removed from the final report included common variants with a frequency of >1% in the general population, synonymous variants and variants that were not included in the Human Genome Mutation Database at the time of the study.

Variants were later classified, according to the American College of Medical Genetics and Genomics (ACMGG) as:

- Benign.

- Likely benign.

- Variant of unknown significance (VUS).

- Likely pathogenic.

- Pathogenic (deleterious).

Some patients' studies had an additional chart (annex) with VUS as an extended report, as shown below in Fig.1:

Cr.	Pos	Ref.	Obs.	Estado	N.Sec.	Gen	Tipo	Cambio	Frec.Controles	OMIM	Modelo
2	179436580	G	A	het	160	TTN	*missense*	NM_003319:exon154:c.C47084T:p.T15695I	0 %	#188840	Rec/Dom
3	31659457	-	GTTT	het	77	STT3B	*frameshift*	NM_178862:exon8:c.1149_1150insGTTT:p.R383fs	0 %	#608605	Recesivo
3	170198485	T	C	het	199	SLC7A14	*missense*	NM_020949:exon7:c.A1586G:p.Y529C	0 %	#615720	Recesivo
5	6625719	G	C	het	33	NSUN2	*missense*	NM_001193455:exon3:c.C318G:p.H106Q	0 %	#610916	Recesivo
9	77418730	C	T	het	63	TRPM6	*missense*	NM_001177310:exon15:c.G1696A:p.A566T	0 %	#607009	Recesivo
9	103035151	C	G	het	41	INVS	*missense*	NM_014425:exon12:c.C1577G:p.T526R	0 %	#243305	Recesivo
10	23481722	G	A	het	19	PTF1A	*missense*	NM_178161:exon1:c.G263A:p.G88D	0 % (rs569569636)	#607194	Recesivo
10	97471736	C	G	het	14	ENTPD1	*missense*	NM_001098175:exon1:c.C23G:p.T8R	0 %	#601752	Recesivo
11	66335808	G	T	het	10	CTSF	*missense*	NM_003793:exon1:c.C150A:p.F50L	0 %	#603539	Recesivo
12	21796952	C	T	het	167	LDHB	*missense*	NM_001174097:exon4:c.G338A:p.R113K	0 %	#150100	Rec/Dom
12	54339012	C	T	het	27	HOXC13	*missense*	NM_017410:exon2:c.C965T:p.S322L	0 %	#142976	Recesivo
12	106799679	G	T	het	35	POLR3B	*missense*	NM_001160708:exon11:c.G717T:p.W239C	0 %	#614366	Recesivo
14	64518340	A	G	het	80	SYNE2	*missense*	NM_015180:exon48:c.A7709G:p.E2570G	0 %	#608442	Dominante
15	45361217	G	-	het	40	SORD	*frameshift*	NM_003104:exon7:c.753delG:p.T251fs	0 % (rs397787697)	#182500	-
17	5487025	G	T	het	14	NLRP1	*missense*	NM_001033053:exon1:c.C253A:p.Q85K	0 %	#606636	Dominante
19	3978074	T	G	het	156	EEF2	*missense*	NM_001961:exon12:c.A1810C:p.M604L	0 %	#130610	Dominante

Figure 1. Annex with additional findings likely not related to the patient's phenotype.

For exome-trio cases, variants were filtered into 4 categories: "*de novo*" (new variant, usually heterozygous in the proband, not observed in either parent and likely causing an autosomal dominant condition), homozygous (both parents are heterozygous for the same variant and the proband inherited it from both parents, likely causing an autosomal recessive condition), compound heterozygous (the proband has one pathogenic variant from the mother and a different pathogenic variant inherited from the father, potentially causing an autosomal recessive condition); and 'inherited' variants (this is the largest group of variants: these variants are inherited from one of the parents and are usually considered as no disease-causing variants).[8]

Pathogenic and likely pathogenic variants were, afterward, analysed and compared to assess the potential causative relation to the proband's phenotype. A literature review was then performed upon each VUS, to evaluate its potential pathogenic effect. No functional studies were carried out in our cohort.

Only variants identified in genes included in OMIM at the time of the report and for which other pathogenic variants had been previously described were studied. Moreover, their pathogenicity should result in clinical features consistent with the proband's phenotype.

Our results show a total overall diagnostic rate of 26,7% for exome sequencing. The rate of inconclusive molecular diagnosis was 53,3%. The remaining 20% of exome studies were reported as normal.

Exome result	Frequency (n)	Percentage
Normal	6	20%
Inconclusive	16	53,3%
Conclusive (diagnostic)	8	26,7%

Table I. Overall results of exome studies in our cohort.

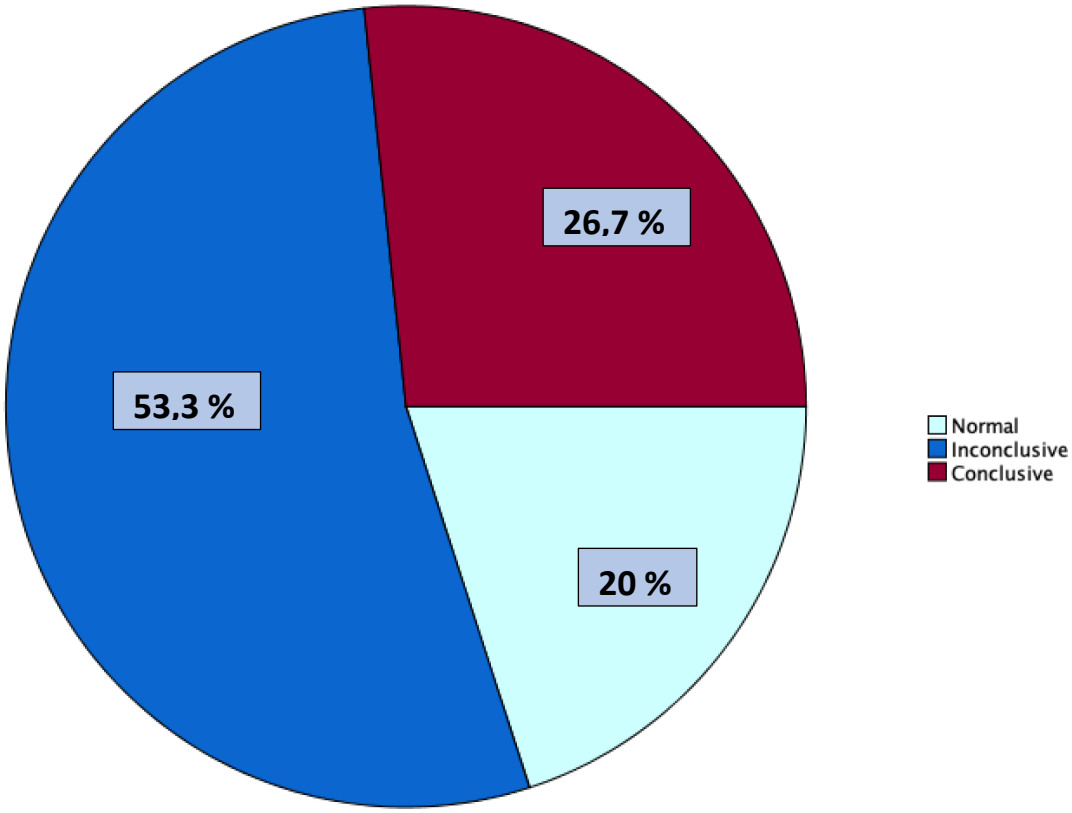
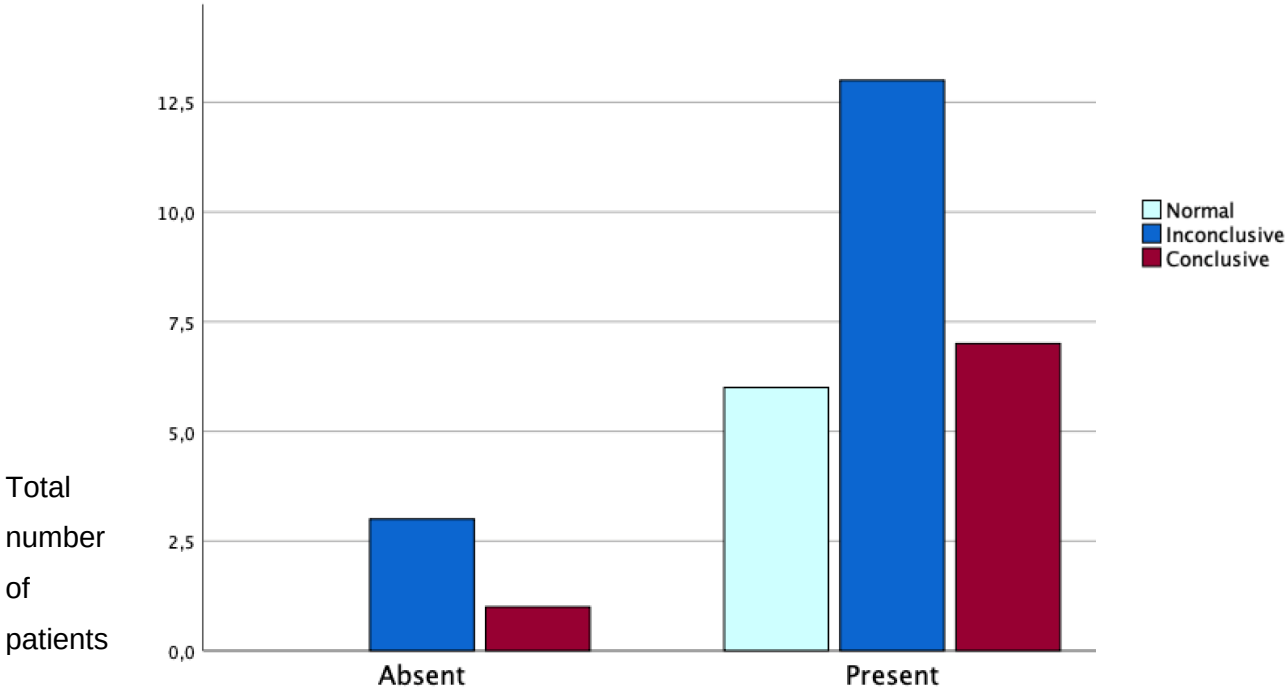


Figure 2. Graphic representation of overall results of exome testing.

There were differences in the diagnostic rate according to the patients' phenotype: the rate among intellectual disability, neurodevelopmental disorders, congenital abnormalities and dysmorphic features is shown in the following graphics.

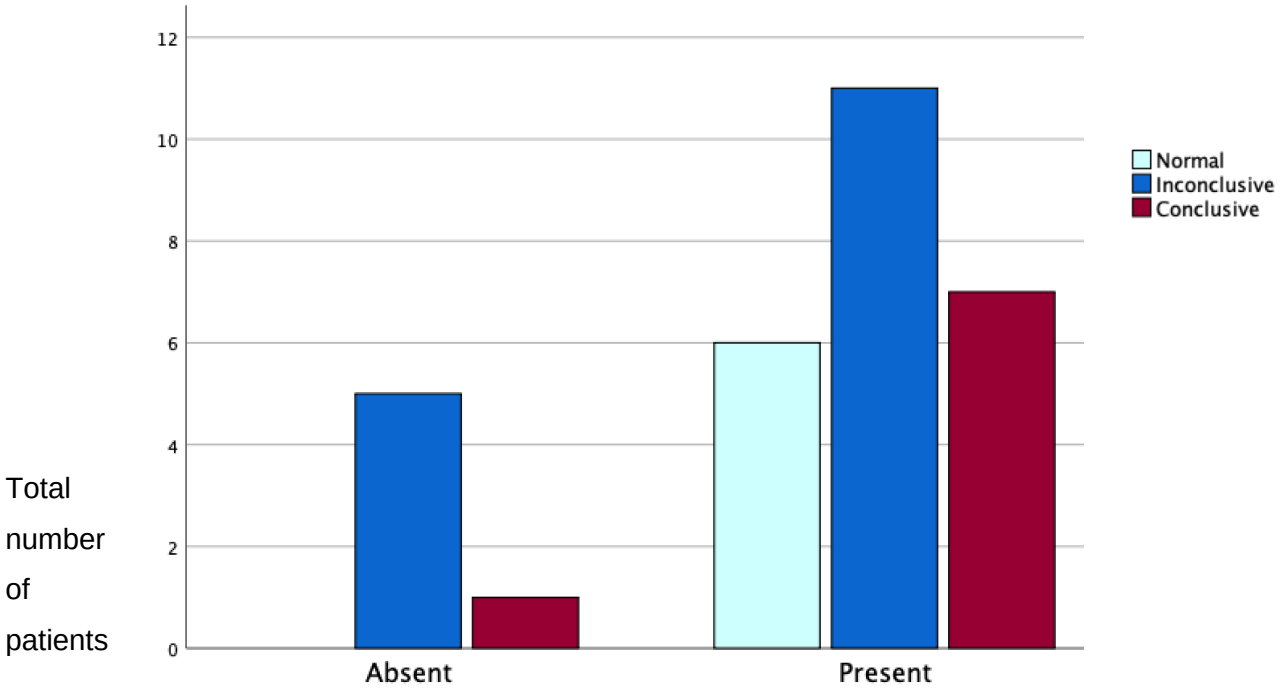


Intellectual disability

Figure 3. Overall diagnostic rate of exome testing in probands with intellectual disability.

Intellectual disability	Normal		Inconclusive		Conclusive		Total
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Absent	0	0%	3	75%	1	25%	4
Present	6	23,1%	13	50%	7	26,9%	26

Table II. Overall results of exome testing in probands with intellectual disability.

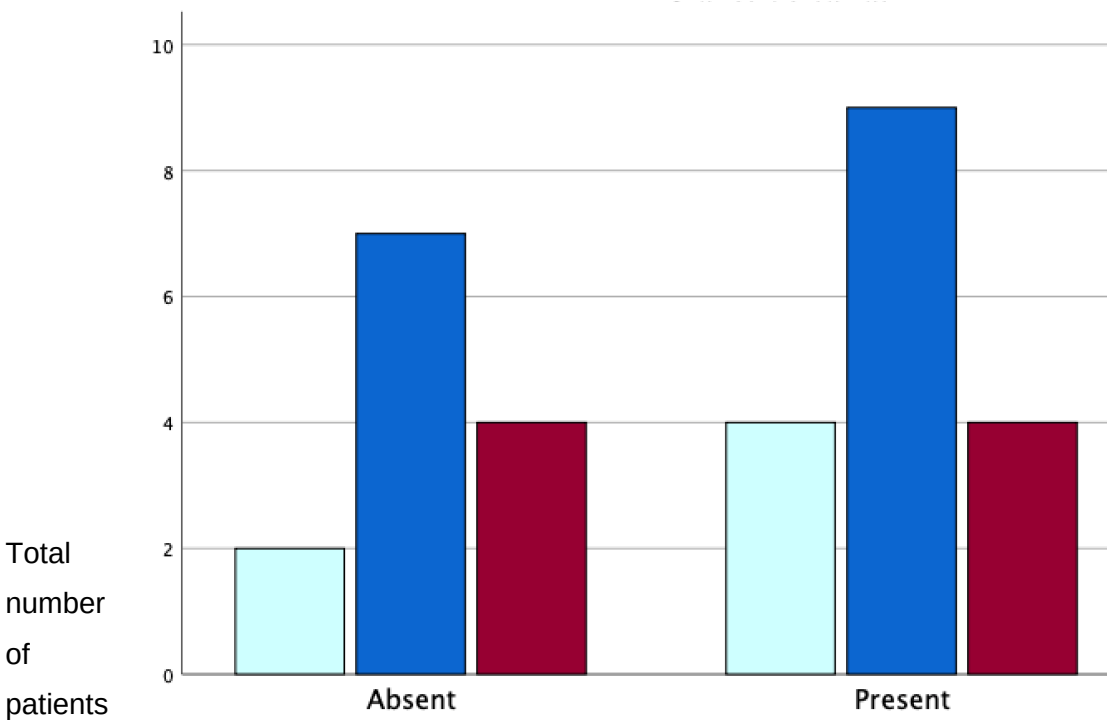


Neurodevelopmental disorders

Figure 4. Overall diagnostic rate of exome testing in probands with neurodevelopmental disorders.

Neuro-developmental disorders	Normal		Inconclusive		Conclusive		Total
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Absent	0	0%	5	83,3%	1	16,7%	6
Present	6	25%	11	45,8%	7	29,2%	24

Table III. Overall results of exome testing in probands with neurodevelopmental disorders.

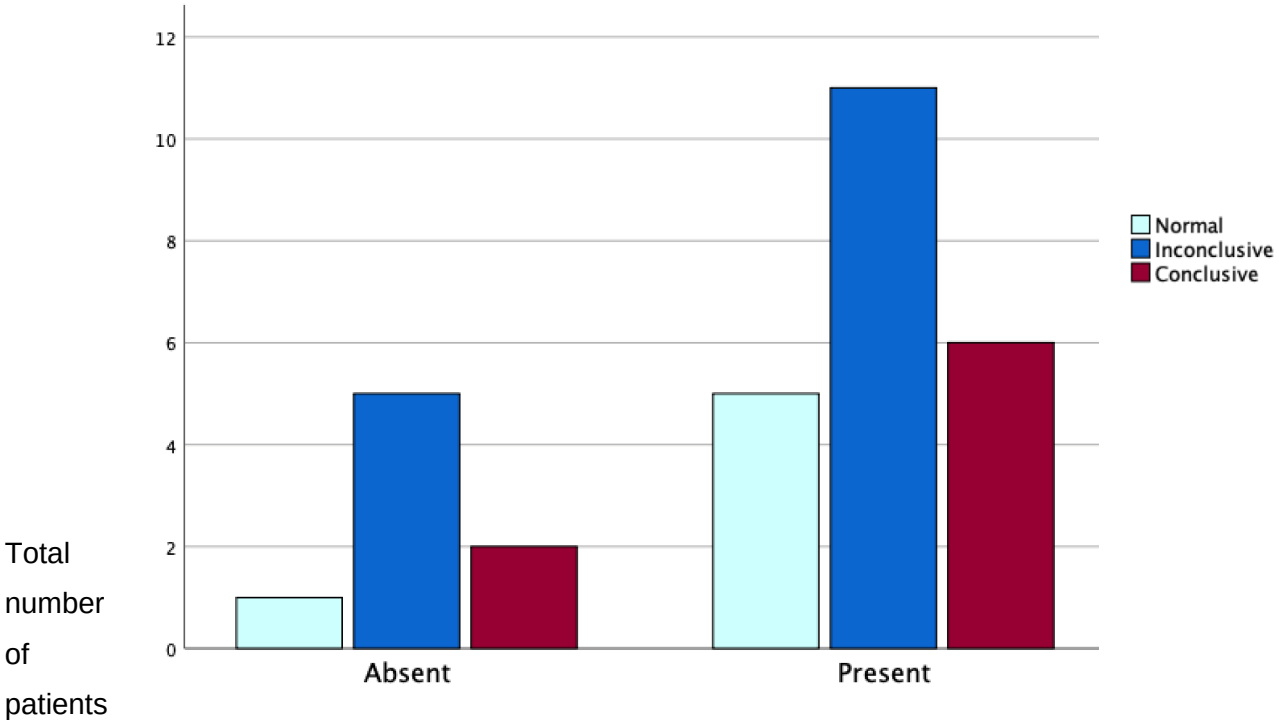


Congenital malformations

Figure 5. Overall diagnostic rate of exome testing in probands with congenital malformations.

Congenital malformations	Normal		Inconclusive		Conclusive		Total
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Absent	2	15,4%	7	53,8%	4	30,8%	13
Present	4	23,5%	9	52,9%	4	23,5%	17

Table IV. Overall results of exome testing in probands with congenital malformations.



Dysmorphic features

Figure 6. Overall diagnostic rate of exome testing in probands with dysmorphic features.

Dysmorphic features	Normal		Inconclusive		Conclusive		Total
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	
Absent	1	12,5%	5	62,5%	2	25%	8
Present	5	22,7%	11	50%	6	27,3%	22

Table V. Overall results of exome testing in probands with dysmorphic features.

Conclusive exome test results were defined as diagnostic. Diagnostic exome sequencing results were obtained in a total of 8 patients. Pathogenic variants are included in the following table:

n	Gene	Mutation	Type of mutation	Zygosis	De novo	Inherited	Model of inheritance	OMIM	Associated disease
9	*THOC2*	c.3323C>T (p.Ser108Leu)	missense	hemizygous	NO	YES	X-L	300395	X-L Intellectual developmental disorder 12
10	*THOC2*	c.3323C>T (p.Ser108Leu)	missense	hemizygous	NO	YES	X-L	300395	X-L Intellectual developmental disorder 12
12	*SATB2*	c.C1165T,p.R389C	missense	heterozygous	YES		AD	608148	Glass S. (Rauch)
18	*ARID1B*	c.3223C>T (p.R1075X)	missense	heterozygous	YES		AD	614556	Coffin-Siris S.
22	*TCF4*	c.C1154G:p.T385S	missense	heterozygous	YES		AD	602272	Pitt-Hoppkins S.
25	*CTNNB1*	c.C1543T:p.R515X	Stop codon	heterozygous	YES		AD	116806	Neurodevelopmental disorder with spastic diplegia and visual defects S.
27	*RET*	c.2753T>c (p.Met918Thr)	missense	heterozygous	YES		AD		Central Hypoventilation S.
28	*IRAK1* *MECP2*	Del: chrX:153,261,198-153,325,612	deletion			YES	X- L	300005	Rett S.

Table VI. Detailed exome sequencing results in the 8 probands with diagnostic test results.

The inheritance patterns were autosomal dominant, autosomal recessive or X-linked. Pathogenic mutations met the criteria of adequate inheritance patterns and disease-phenotype concordance. Most variants were missense.

The decision to request exome-duo or exome-trio tests instead of single exome test did not provide a greater diagnostic yield, as represented in the table below:

Type of exome test	Normal		Inconclusive		Conclusive	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Single	5	33,33%	7	46,66%	3	20%
Duo	0	0%	1	33,33%	2	66,66%
Trio	1	8,33%	8	66,66%	3	25%

Table VII. Results of exome studies based on individuals included in the test.

No new candidate disease genes were found among cases with positive exome results.

Turnaround time ranges from 2 to 8 months, with an average of 5,1 months.

No new candidate disease genes were identified among cases with conclusive exome results.

Six patients with no clear eventual diagnosis despite exome testing were offered to take part in the 'IMPaCT' project. This Project is a nationwide project that aims to develop a national network to perform highly complex genome-wide studies. Patients taking part in the studies are, in most cases, individuals affected with rare diseases that have already undergone exome studies that showed inconclusive results. It is an attempt to set the basis for a 'Personalised Medicine' approach within the public health system in Spain.

6.- DISCUSSION

Between 2014 and 2016, exome sequencing was not included in the routine diagnostic workup in patients in our Genetics Practice at the Hospital Clínico Universitario 'Lozano Blesa' in Zaragoza. On the contrary, it was a last resource diagnostic tool to appeal for when previous routine tests failed to confirm a suspected clinical diagnosis of a genetic disease.

Although the selection of our patients was consecutive, all of them ticked at least one of the boxes of the main analysed variants: intellectual disability, neurodevelopmental disorder, congenital malformations and dysmorphic features. The literature supports the use of Next-Generation techniques (including exome sequencing) for elucidating the molecular aetiology of these conditions.

Exome sequencing is a suitable test to perform when investigating the underlying molecular aetiology of intellectual disability, congenital malformations, dysmorphological features and neurodevelopmental disorders, with clear benefit after this intervention (exome sequencing versus no exome sequencing) obtained by patients suffering these conditions. [9]

Microarrays do not detect SNVs -Single Nucleotide Variations-, indels -small insertions and deletions- and small structural variants. Some of these and other variants contribute to congenital anomalies, developmental disorders and intellectual disability, thus, although traditionally considered as a first-tier genetic test, Microarrays (Array-CGH) might prove insufficient for the diagnosis of these conditions.

The diagnostic yield of exome sequencing is usually high because in most clinical practices it is not a first-tier test, but it is mostly performed after other genetic tests have failed to confirm a specific diagnosis.

It is uncertain which clinical diagnosis is most likely to yield a molecular diagnosis after exome testing, for there are numerous indications to perform this test. Plus, the fact that it is unbiased when it comes to checking all human disease-causing genes, it can render to the identification of more than one genetic condition even in the absence of obvious clinical manifestations.[10]

Interestingly, some authors point to a higher diagnostic yield for neurodevelopmental indications.[11]

6.1 Utility

Whole exome sequencing is largely being used early in the diagnostic workup of patients with a suspected genetic origin of their disorder, especially **genetically heterogeneous disorders** (such as congenital anomalies and neurological disorders).[12,13]

Sawyer highlights significant **genetic heterogeneity** as "the most common contributing factor for patients not receiving a molecular diagnosis prior to WES".[14] Iglesias' findings point in the same direction: "WES is an effective tool to diagnose genetically heterogeneous disorders in a clinical setting, especially in young children in whom all the clinical features may not yet be evident. It is also valuable in providing evidence for possible expansion of the phenotypes associated with recognized human diseases and to identify new candidate genes associated with human disease".[15]

Explanation	Number of families ($N = 105$)
Genetic heterogeneity	49
Atypical presentation	26
Missed by another method	9
Gene identified while in the pipeline	9
Extremely rare condition	5
Conflation	4
Limited access to testing	3

This article shows a table with the most common contributing factors to the absence of molecular diagnosis.[14]

Figure 7. Most common contributing factors to the lack of a definitive molecular diagnosis. [14]

In a nutshell, exome sequencing analyses a large number of genes (exons) at a single process, so, despite old conceptions, it is an efficient diagnostic tool, especially in cases of high genetic heterogenicity.

Alternatively, patients with clinical presentations highly suggestive of a specific genetic diagnosis should undergo targeted testing first. That includes patients with a known history of the disorder, suspicion of chromosomal disorder or patients with a firm clinical suspicion of conditions in which sequencing might not result in a diagnosis, such as Fragile X Syndrome, Prader-Willi Syndrome or Angelman Syndrome. [9]

The most medically significant feature abstracted from the proband's medical record can in many cases help us decide whether exome sequencing is an appropriate diagnostic tool to find answers.

6.2 Outcomes

In our descriptive study of a cohort of 30 patients, the diagnostic yield of exome sequencing was 26,7%.

Outcomes from large studies that include 300 patients approximately show success rates for WES to provide a molecular diagnosis ranging from 22% to 46%.[8,10,14] Sawyer's study of the FORGE population showed a diagnostic rate from 12% to 44%, with an average of 29%. [14]

Of the 115 patients of the Iglesias cohort of study, in 32,1% of the cases, a definitive genetic aetiology was identified through WES.[15]

Shickh finds a diagnostic yield of exome and genome sequencing ranging from 3 to 70%, with a higher yield in neurological and acute diseases, whereas standard testing ranges between 4 to 26%. This article additionally points out the increase of the diagnostic yield in recent years 2017-2020, contrary to the decreasing rate of VUS between 2018-2020.

Other studies evidence a diagnostic yield between 28-68%.[16-21]

The systematic review carried out by Manickam shows a diagnostic yield of 34% for exome sequencing.[9] Yang's findings are parallel to our findings: "Whole exome sequencing identified the underlying genetic defect in 25% of consecutive patients referred for evaluation of a possible genetic condition".[10]

The next table sums up a series of studies where exome sequencing was performed under similar indications as to our study (intellectual disability, neurodevelopmental disorders and congenital malformations) with their respective diagnostic yields.

Author	Publication year	Cohort (N)	Diagnostic yield
Scocchia	2019	60	68.3%
Cordoba et al	2018	40	40%
Nair et al	2018	167	34,1%
Powis et al	2018	66	37,9%
Bourchany et al	2017	29	45%
Evers et al	2017	72	35%

Kuperberg et al	2016	57	49,1%
Tarailo-Graovac et al	2016	41	68%
Nolan and Carlson	2016	53	48%
Thevenon et al	2016	43	32,5%
Tammimies et al	2015	95	8,4%
Valencia et al	2015	40	30%
Zhu et al	2015	119	24,3%
Soden et al	2014	119	45%
Iglesias et al	2014	115	32,2%
Dixon-Salazar et al	2012	118	18,6%

Table VIII. Summary of published overall diagnostic yields of exome sequencing. Studies published in the same period of time (2014-2016) as our study are highlighted in green.[15, 20, 22-35]

As proved above, our diagnostic yield is among the expected figures attending to the available scientific evidence up to date.

Literature supports a higher diagnostic yield for exome-trio sequencing compared to single exome sequencing. “The average of the diagnostic yields reported in the studies identified by our review was 34,4% for trio ES and 26,6% for singleton”.[36]

Our diagnostic rate according to single, duo or trio exome sequencing results in 20%, 66,66% and 25% respectively. However, the limited number of probands participating in our study makes it premature for us to draw conclusions on the diagnostic yield of each exome test.

Some of the reviewed articles break down the diagnostic yield into each analysed category: “The individual yield for each category was 53,5% for birth defects and 34,4% for developmental delay/ intellectual disability […]”. [15]

“The highest rate of a positive diagnosis was in the group of patients with multiple congenital abnormalities without ID or with unknown ID status (54% of the conclusive diagnoses). Rates of conclusive diagnoses in groups with multiple congenital abnormalities with ID and with epileptic encephalopathy were respectively 31% and 15%”.[25]

According to Cordoba et al, Fogel et al and Srivastava et al, phenotypes involving the nervous system reported higher diagnostic yields. [22,37-39]

Differences in diagnostic rates of the analysed variants in our study are not significant: of the 26 patients with intellectual disability, 26,9% had a positive exome result; 29,2% of the 24 patients affected with neurodevelopmental disorders; 23,5% of a total of 17 patients with congenital malformations and 27,3% of patients with dysmorphic features that added up to 22 individuals.

Due to the limited number of probands in our study and the fact that most of the patients share more than one analysed condition, a larger cohort is required to draw conclusions. We contemplate assessing such phenotype-associations and diagnostic yield correlations further in our study when probands recruitment allows a greater cohort.

In his study, Iglesias et al state that the more narrowly defined the phenotype, the higher the diagnostic yield. [15] In his study, Trujillano et al reflect how the diagnostic yield rate is influenced by the proband's phenotype description: the more HPO terms given (complex phenotypes) the higher the diagnostic yield.[40]

WES as an effective diagnostic tool, particularly in nonspecific or heterogeneous phenotypes with a better diagnostic yield in consanguineous families, in severe and in syndromic phenotypes and patients with more than just one isolated symptom.[40]

In our study, major outcomes of WES implementation in the diagnostic evaluation were the findings of pathogenic variants among already known genes.

6.3 When to perform exome sequencing

When is the best time to request exome sequencing testing remains unclear. Whether at the beginning of the diagnostic process or towards the end of the diagnostic odyssey, the most convenient time to consider exome sequencing largely depends on the patient's clinical presentation.

When WES is performed is probably a key point to diagnostic yield. It is broadly hypothesized that the diagnostic rate and the economic benefit increase when WES is used early in the diagnostic process, but conflicting literature exists in this respect. Optimal timing to perform WES depends on the patient. A personalised approach and a comprehensive clinical assessment will tell whether it should be performed prior to other first-tier diagnostic procedures enrollment or towards the diagnostic odyssey. Sometimes, WES could even be a first-tier diagnostic strategy when convenient.

Studies like Bourchany's found no significant differences in the diagnostic yield in patients that underwent first-line WES or WES following multiple investigations.[25]

Many authors suggest that performing exome sequencing should not be put off to a dead-end street situation but, instead, be carried out in certain conditions where positive exome results are to be

expected. In his article, Iglesias et al include genetic heterogeneity or involvement of a large number of genes as indications for first-tier exome testing.[15] Namely, exome sequencing widespread availability and decreased costs have led it to be part of first-tier diagnostic tools, providing even higher diagnostic yields.[16,21]

There is strong recommendation to support the use of exome sequencing and as either a first or second-line test, especially, in patients with congenital anomalies, developmental delay and intellectual disability". [9, 40-42]

"Exome sequencing and genome sequencing have a higher diagnostic yield and may be more cost-effective when ordered early in the diagnostic evaluation". [9]

Kandamurugu or Shickh stand up for even suppressing previous first-tier tests to reduce costs.[9,11]

"Exome sequencing as a first or second-tier test yielded more diagnoses at a lower cost than using Exome sequencing only after extensive standard testing or using standard testing alone".[9]

Given the decreasing costs and the potential for improved clinical utility, exome and genome sequencing are anticipated to replace conventional genetic tests such as gene panels and CMA. [8,11]

WES has allowed to cut off the often extensive time gap to diagnose reach. Providing a molecular diagnosis in a timely manner is crucial in the childhood age and has led to more adequate disease management. Most importantly, many families had put an end to the diagnostic odyssey they had for many years experienced.

6.4 Exome sequencing limitations

The DNA coverage of exome sequencing is limited compared to genome sequencing. Subsequently, variations that are not placed in the exome or protein-coding DNA fraction will be omitted. The percentage of not-fully covered exome regions must also be taken into consideration.

Additionally, exome sequencing typically overlooks intronic variants, methylation abnormalities, trinucleotide repeat expansions and some copy number variant changes. Whether it is due to technical restrictions or due to variations resulting from epigenomic actions, a proportion of studied patients remain undiagnosed.

"We know that approximately 70% of those who undergo WES will remain without a molecular diagnosis in a known disease gene. There are a variety of reasons including incomplete coverage of the exome and genetic mutations elusive to the technology itself […] There are a proportion of these cases in which the disease-causing variant is in fact within the WES data but for a variety of

reasons (not enough information on the variant, variant acting on gene function through novel molecular mechanism, nuclear inheritance, etc.), there is insufficient evidence to support a definite diagnosis".

Reassessment of unclear and undefined cases of suspected and presumed genetic origin is basic for an updated genetic approach. Results obtained from our study should now, be re-analysed.

Genetics advances and nonstop growing scientific literature evidence require a constant review and update process. For this reason, the VUS rate tends to decrease as time goes by since new variants evidence is created and novel genes are identified.

There is an urge for the creation of international genetics and genomics databases. The large-scale sharing of data among the scientific community will allow a deeper understanding of genetic variants and their expression. A worldwide collaborative infrastructure of genotypic and phenotypic data could provide valuable information as well as ease the identification and characterisation of variants in novel genes related to potential genetic conditions.

7.- STUDY LIMITATIONS

The main limitation of our study is the number of probands: only 30 patients have been included. Although a greater number of patients had undergone exome sequencing between 2014 and 2016 and their results are registered, many clinical details were missed. Thereupon, many individuals had to be left out of the study. Electronic clinical records were not fully integrated into the patients' reports at that time, hence we found great difficulties assessing and collecting all data from each and every proband. In summary, the total number of analysed patients was cut prematurely due to access difficulties to patients' data.

We intend to expand our analysis from the years 2016 to 2021 and include all exome-tested children. Results from our study will be more robust since a larger cohort will be described. In subsequent years to analyse, thanks to the ubiquity of electronic medical patient records, information will be readily available for further investigation.

The highest level of evidence is usually obtained by clinical trials. However, clinical trials are not an adequate study design for our purpose. Case reports and small case series are the most popular form of evidence for patients with rare diseases, considering the affected population is usually reduced.

Spanish public health system allows theoretical universal and free access to medical care. Back in 2014, when the exome tests were performed, the availability and accessibility to such techniques were limited. A, in the past, not included in the standard-of-care diagnosis methods test meant only very few probands became ideal candidates to undergo exome sequencing. Unlike today, not everyone with an obscure diagnosis even after first-tier testing could aim for exome sequencing. Thereby, we find a high risk of bias and lack of generalizability. Selected patients resulted from an exquisite patients' screening. Test candidates were narrowed down by criteria that differ from criteria used nowadays. The question in 2014 was not when to perform exome sequencing but if to perform it or not. The eligibility of a proband was not a big issue but the profitability of the result.

On the other hand, there are constraints to the clinical application of NGS techniques due to financial, technical, feasibility and logistics issues The financial burden of the costs of exome sequencing included not only the test itself but all the medical effects that appear as a consequence of exome results.

The use of exome-trio sequencing is also limited for many reasons, some of them include a higher cost of sampling three DNA probes instead of one or concerns raised after incidental findings report. Besides, exome-trio sequencing constitutes a further diagnostic step when VUS and other unclear variants raise from single exome sequencing, therefore, not routinely performed.

Unclear diagnosis is the most frequent outcome from exome sequencing up to date. We suggest that patients undergo reanalysis every 5 years or at regular intervals after the preliminary results. Periodic databases and literature reviews will help diagnose additional unresolved cases.

Furthermore, up to now, there is no robust body of evidence to support the use of exome technologies: interpretation of results is not uniform. Thus, it is critical to create a universal framework for international data collection and reporting, outcomes interpretation and metrics to develop. A global effort to build evidence-based care guidelines for rare diseases is imperative, in order to help to assess the clinical judgment of clinical geneticists under homogeneous criteria coverage. The creation of a worldwide support network for undiagnosed patients immersed in a diagnostic odyssey could also be of help.

Limitations to this study will be addressed in our next research project.

8.- CONCLUSIONS

1. Our study shows a diagnostic rate of 26,7%, a similar figure to analogous studies. Our diagnostic rate will probably increase in future studies that include a larger cohort of probands.

2. No significant differences were found in the diagnostic rate according to the major clinical features analysed: intellectual disability, neurodevelopmental disorders, congenital malformations and dysmorphic features.

3. Consequently, we strongly support the use of exome sequencing, as part of the routine clinical management and diagnostic process of patients in a genetics practice and, particularly, individuals that suffer from rare diseases.

4. Conclusive molecular results from exome sequencing will, thus, limit time-consuming and expensive cascade diagnostic testing.

5. Despite the increasing rates of conclusive exome sequencing, many patients remain undiagnosed. Technical limitations may contribute largely to undiagnosed cases.

8.1 Final comments

Improvements in the accuracy of Next-Generation Sequencing techniques, exome sequence coverage and alignment, detection of CNV -copy number variants-, pathogenicity prediction algorithms and application of genome sequencing will undoubtedly lead to higher diagnostic yields to genomic tests. In addition, parallel use and pairing of exome sequencing with other approaches such as proteome/transcriptome and methylome will help us better understand genetic pathology and, therefore, improve diagnostic yield overall.

Many questions remain still unsolved. Which are the requirements to order exome sequencing when physicians have not reached a diagnosis? which clinical indication renders a higher diagnostic rate? which indications shouldn't be considered to demand exome sequencing? how do we deal with VUS results? how do we interpret new results? when should incidental and secondary findings be communicated to the patient? We firmly believe that these questions will find evidence-based answers as time goes by and more scientific evidence is generated.

In the meantime, we should not forget the true aim of all our efforts: to improve our patients' quality of life. Patients, in general, value the support received in the practice throughout the diagnosis process, by the information and accompaniment provided and, ultimately, by learning the final diagnosis outcomes. Patients appreciate receiving reliable and updated information from

professionals to help them achieve a better understanding and management of their condition, even in absence of conclusive results.

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Case number	Age (moths)	Age when WES (months)	Origin	Referall	Clinical Features (ID, ND, CA, DF)	Exome test	Result	Gene: Variant	Turnaround time (months)	Cost	Year
1	53	128	Spain	Different Hospital	ID, ND, CA, DF	Single	VUS	***AURKC*** : c.1+1G>A	8	-	2014
2	2	3	Spain	Self request	CA, DF	Trio	VUS	***SOS*** *T* : c.C566T:p.P189L (Father) ***GFAP*** **:** c.C1190T:p.S397L (Father) ***IFT140*** **:** c.A2048G:p.D683G.	6	1.200 €	2014
3	2	65	Spain	Self request	ID, ND	Trio	VUS	***HCN1*** **:** c.G119A:pG67S (Father). ***NDUFA13*** : c.380_382delGC (Father) ***NDUFV1*** : c.G1222A (Father) ***AGK*** : c.G655A (Mother)	6	1.200 €	2014
4	99	181	Spain	NICU	CA, DF	Trio	VUS	***TMEM43*** : c.758_759 ins: p.P253fs (Mother) ***NOTCH1*** : c. G5273A: p. R1758H (Father) & c.C1682T: p.T561M (Father).	3	1.200 €	2014
5	9	24	Spain	?	ID, ND CA, DF	Single	Normal		4	1.200 €	2015
6	12	86	Mother: Romania. Father: Spain	Hospitalisation floor	ID, ND, DF	Single	Normal		4	1.200 €	2015
7	40		Spain	Neurology	ID, ND	Trio	VUS	***RBM8A*** : c.48C>T p.(Ala16Ala). ***WARS 2*** : c.37T>G p.(Trp13Gly) (Father) & c.939G>A p.(Lys313Lys) (Mother)	4	1.350 €	2015
8	17	92	España	Neurology	ID, ND, CA, DF	Single	Normal		4	1.200 €	2015
9	2a 9 m	10a 8 m	Spain	Self request	ID, ND, CA, DF	Duo	PAT	***THOC2*** **:** c.3323C>T (p.Ser108Leu)		0 €	2015
10	79	169	Spain	Self request	ID, ND, CA, DF	Duo	PAT+ VUS	***THOC2*** : c.3323C>T (p.Ser108Leu) ***MANIB1*** :c.1471G>A (p.Glu492Lys) ***CC2DIA*** *:* c.1723C>T (p.Arg575Cys)		0 €	2015
11	29	136	Spain	Self request	ID, ND, CA, DF	Trio	VUS	8p22 (13,445,907-13,771,045) x1 (Father) Xp22.33/Yp11.32(543,192-1,362,098)x3 (Father)	2	1.600 €	2015
12	4	168	Spain	NICU	ID, ND, CA	Single	PAT	***SATB2:*** c.C1165T:p.R389C	4	1.600 €	2015

13	24	71	Spain	CCDEA	ID, ND	Single	Normal		5	1.600 €	2015
14	72	230	Spain	NICU	ID, ND, CA, DF	Single	VUS	***ERCC6L2*** :c.C412G:p.Q138E & c.G2779A:p.D927N ***FTCD*** : c.T1607A:p.L536X & c.G625A:p.G209R	5	1.600 €	2015
15		142	Spain	Neurology	ID, ND, CA, DF	Single	VUS	***COL4A4*** **:** c.C2570T:p.P857L & c.G1334C:p.G445A. ***FBN2*** **:** c.C1658A:p.P553H ***COL10A1*** : c.C619T:p.P207S	5	1.200 €	2015
16	174	192	Spain	Psychiatry	ID, DF	Single	VUS	***COL11A2*** **:** c.G509A (p.R170Q) ***ARID1B*** :c.T6233C (p.I2078T) ***EHMT1*** **:** c.C1525G:p.L509V	5	1.200 €	2015
17	103	137	Spain	Neurology	ID, ND, DF	Single	VUS	***SHOX*** **:** c.G508C:p.A170P (Father). ***MEIS2*** **:** c.934_937del:p.L312fs	7	1.200 €	2015
18	26	?	Spain	Gatroenterology	ID, ND, CA, DF	Trio	PAT	***ARID1B*** : c.3223C>T (p.R1075X)		1.200 €	2015
19	26	142	Spain	PCC	CA, DF	Trio	VUS	***ASXL3*** **:** c.C2273G:p.S758C,c.C2724G:p.I908M, & c.T2758C:p.S920P (Mother). ***KMT2B*** : c.G3032A:p.R1011Q. **ABCA4**: c.C4918T:p.R1640W & c.T4222C:p.W1408R ***ELP4*** :c.G300T:p.K100N	4	1.200 €	2016
20	0,5	59	Spain	NICU	ID, ND, CA, DF	Single	VUS	***TBX1*** : c.C257T:p.P86L	8	1.200 €	2016
21	47	72	Spain	Neurology	ID, CA, ND, DF	Duo	VUS	***KMDSC:*** c.G3156A:p.M1052I (Mother) ***GNPTAB:*** c.T1429G:p.Y477D &c.T500A:p.I167N (Mother)	6	1.200 €	2016
22	22	31	Spain	Neurology	ID, ND, DF	Single	PAT + VUS	***TCF4*** : c.C1154G:p.T385S. ***MAGEL2*** : c.C3131T:p.S1044L. **COL1A1:** c.G3842A(p.G1281D).	7	1.200 €	2016
23	69	83	Mother: Bulgaria. Father: Romania.	Psychiatry	ID, ND	Trio	VUS	***KIAA2022*** : c.C1418T:p.S473F (Mother) ***ASXL1*** : c.T3826A:p.S1276T	9	1.200 €	2016
24	14a 6m	17a 2m	Spain	Psychiatry	ID, ND	Trio	No concluyente	***KIRREL3*** : c.G1567A:p.A523T (Mother) ***TSPAN7*** :c.C515A:p.P172H (Mother)	8	1.200 €	2016

25	8	168	España	Hospitalisation floor	ID, ND, DF	Trio	PAT	***CTNNB1:*** c.C1543T:p.R515X ***CDH15*** : c.G520A:p.A174T.	7	1.200 €	2016
26	1	177	Spain	NICU	ID, ND, CA, DF	Single	Normal		7	1.200 €	2016
27	0,5	0,5	Spain	NICU	.	Trio	PAT	***MTM1*** :c:141_144del (p.Glu48Leufs*24) (Mother). ***RET*** : c.2753T>c (p.Met918Thr).	1	1.200 €	2016
28	19	48	Bulgaria	Neurology	ID, ND, DF	Exoma	PAT	***IRAK1*** & ***MECP2*** : delchrX:153,261,198-153,325,612	5	1.200 €	2016
29	6	157	Mother: Spain. Father: Italy.	ENT	ID, ND, CA, DF	Exoma trio	Normal		4	1.200 €	2016
30	71	192	Colombia	ENT	ID, ND	Single	VUS	***CPA6*** **:**c.107G>T; NP_065094.3:p.R36L.		1.200 €	2016