ANÁLISIS AGREGADO DE VARIANTES EN EL EXOMA DE PACIENTES CON ENFERMEDAD DE MENIERE FAMILIAR E INICIO PRECOZ

Tesis doctoral con mención internacional

Aggregated variant analysis in exomes from familial and early onset Meniere disease patients

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Resumen

Introducción: La enfermedad de Meniere (EM) es una enfermedad rara del oído interno caracterizada por hipoacusia neurosensorial, vértigo episódico, y tinnitus. Aunque la mayoría de los casos de esta enfermedad son esporádicos, en el 6 al 9% de estos pacientes presentan agregación familiar, lo que sugiere una contribución genética en la EM. La EM es una enfermedad compleja, con una gran variabilidad en el fenotipo que se ve acompañada de heterogeneidad genética. Hasta la fecha, solo se han descrito variantes en familias individuales, no encontrándose replicación entre familias no relacionadas.

Objetivos: Identificar los principales genes implicados en la EM familiar mediante secuenciación de exoma completo, así como demostrar un efecto agregado de variantes en determinados genes. Así mismo, se identificarán las principales rutas metabólicas implicadas y los resultados serán comparados con una serie de pacientes con EM esporádica.

Métodos: Un total de 138 casos con EM (94 pacientes con EM familiar y 44 con EM esporádica) diagnosticados de acuerdo con los criterios definido por la Barany Society fueron seleccionados y secuenciados con el objetivo de buscar variantes raras. Las frecuencias alélicas de las variantes identificadas fueron anotadas para llevar a cabo análisis uni y multivariante. Las frecuencias alélicas en nuestro grupo de pacientes fueron comparadas con las frecuencias encontradas en bases de datos de referencia europeas y españolas. Se llevaron a cabo análisis de sobrerrepresentación para obtener las principales rutas y procesos biológicos afectados.

Resultados: En un primer abordaje del trasfondo genético de la EM, se pudo observar que el 40% de los casos familiares y el 68% de los casos esporádicos portaban una variante nueva o ultrarara en un gen ya relacionado con hipoacusia neurosensorial. Analizando estos genes, identificamos un enriquecimiento de variantes en algunos de ellos, destacando sobre los demás el gen que codifica la proteína otogelina, *OTOG.* Se encontraron un total de 10 variantes en 15 familias no relacionadas. Estudiando la clínica de estos pacientes, se observó que podrían conformar un endofenotipo, caracterizado por hipoacusia pantonal con poca progresión. Finalmente, se llevó a cabo un análisis más general, incluyendo todos los genes codificantes. Los resultados de este análisis sugieren que existe una contribución poligénica y/o polialélica en la EM, siendo diferentes los genes involucrados en la forma familiar y esporádica. Basados en los resultados de estos análisis, se pudo determinar mediante un análisis de sobrerrepresentación que rutas como la guía de señalización axonal podría ser importantes en el desarrollo de la enfermedad.

Conclusiones: En esta tesis doctoral se han definido los posibles principales genes y rutas candidatas para la EM familiar, encontrando a su vez una estructura genética diferente a la EM esporádica. Estos resultados podrían ser la base para futuros estudios genéticos y funcionales en la EM.

Abstract

Introduction: Meniere's disease (MD) is a rare inner ear disorder characterized by sensorineural hearing loss, episodic vertigo and tinnitus. Although most of MD patients are sporadic, familial aggregation is observed in 6-9% of these patients, suggesting a genetic contribution in MD. MD is a complex disease, showing phenotypic heterogeneity as well as genetic heterogeneity. To date, only variants in single families have been associated to familial MD, finding no replication in non-related families.

Objectives: To identify the main genes involved in familial MD by whole exome sequencing, and to demonstrate an aggregate effect of variants in certain genes. Furthermore, the main biological processes and pathways will be analyzed, and the results will be compared with sporadic MD patients.

Methods: A total of 138 MD patients (94 familial MD patients and 44 sporadic MD patients) diagnosed according to the criteria defined by the Barany Society were recruited and sequenced to look for rare variants. Minor allelic frequencies of identified variants were annotated to undertake a single rare variant and a gene burden analyses. Allelic frequencies were compared with the frequencies from European and Spanish reference datasets. Over-representation analyses were done to identify the main biological processes and pathways.

Results: In a first approach of the genetic MD background, we identified that 40% of familial MD patients and 68% of sporadic MD patients carried, at least, a novel or ultrarare variant in a gene linked to sensorineural hearing loss. Analyzing these genes, enrichment of rare variants were identified in some of them, standing out the gene which encodes otogelin, *OTOG*. Ten variants were found in 15 nonrelated families. Studying the clinical information of these patients, an endophenotype characterized by flat hearing loss with no progression was observed. Finally, we analyzed all the genes within the human genome. The results obtained from this analysis suggest a polygenic and/or a polyallelic contributions in MD, being different the genes involved in familial and sporadic MD. Based on these results, pathways such as axon guidance were identified throughout an over-representation analysis as key pathways in MD.

Conclusions: In this doctorate thesis, the main potential genes and pathways for familial MD have been defined, finding a differential genetic background between sporadic and familial MD. These results could be the basis for future genetic and functional studies on MD.

Abbreviations

AAO-HNS	American Academy of Otolaryngology-Head and Neck Surgery
ACMG	American College of Medical Genetics
AD	Autosomal dominant
AIED	Autoimmune Inner Ear Disease
AMP	Association of Molecular Pathology
CADD	Combined Annotation Dependent Depletion
cHCs	cochlear hair cells
CI	Confidence Interval
CNV	Copy Number Variant
CSVS	Collaborative Spanish Variant Server
DFNB18B	Autosomal recessive deafness 18B
ECM	Extracellular matrix
EH	Endolymphatic Hydrops
ENU	N-ethyl-N-nitrosourea
ExAC	Exome Aggregation Consortium
FATHMM	Functional Analysis Through Hidden Markov Models
FDR	False discovery rate
FLAGS	Frequently Mutated Genes)
FMD	Familial Meniere Disease
GATK	Genome Analysis ToolKit
GBA	Gene Burden Analysis
gnomAD	Genome Aggregation Database
HCs	Hair cells
HGP	Human Genome Project
HL	Hearing Loss
HPO	Human Phenotype Ontology
IGV	Integrative Genomics Viewer
IHC	Inner Hair Cells
Indel	INsertions and DELetions

LRT	Likelihood Ratio Test
MAF	Minor Allele Frequency
MD	Meniere's Disease
MGI	Mouse Genome Informatic
MP	Mammalian phenotype
mRNA	messenger RNA
NFE	Non-Finnish European
NGS	Next-Generation Sequencing
oHC	Outer Hair Cells
OMIM	Online Mendelian Inheritance in Man
OR	Odds Ratio
ORA	Over-representation analysis
PAMPs	Pathogen-associated molecular pattern
PROVEAN	Protein Variation Effect Analyzer
QC	Quality Control
RefSeq	Reference Sequence
SCs	Supporting cells
SEMA	Semaphorin
SG	Spiral ganglion
SIFT	Sorting Intolerant From Tolerant
SNHL	Sensorineural Hearing Loss
SNV	Single Nucleotide Variant
SRVA	Single Rare Variant Analysis
uCHs	utricular hair cells
UTRs	Untranslated regions
VCF	Variant Call Format
VEST	Variant Effect Scoring Tool
VG	Vestibular ganglion
VM	Vestibular Migraine
VQSLOD	Variant Quality Score Log-Odds

VQSR	Variant Quality Score Recalibration
VUS	Variant of Uncertain Significance
vWD	Von Willebrand Factor D-type domain
WES	Whole Exome Sequencing

1. Introduction

1.1. The inner ear

The sensorineural epithelia located in the organ of Corti and the vestibular organs (utricle, saccule and semicircular canals) are the sensorial receptors located in the inner ear responsible for processing auditory and vestibular information. Prior to this, the sound arrives at the external ear, which includes the auricle and the external auditory canal, and it is transmitted by the ossicular chain (malleus, incus and stapes) of the middle ear, which transfers the sound vibrations from the external ear to the inner ear (LeMasurier & Gillespie 2005). These three parts compose the human ear (Figure 1).

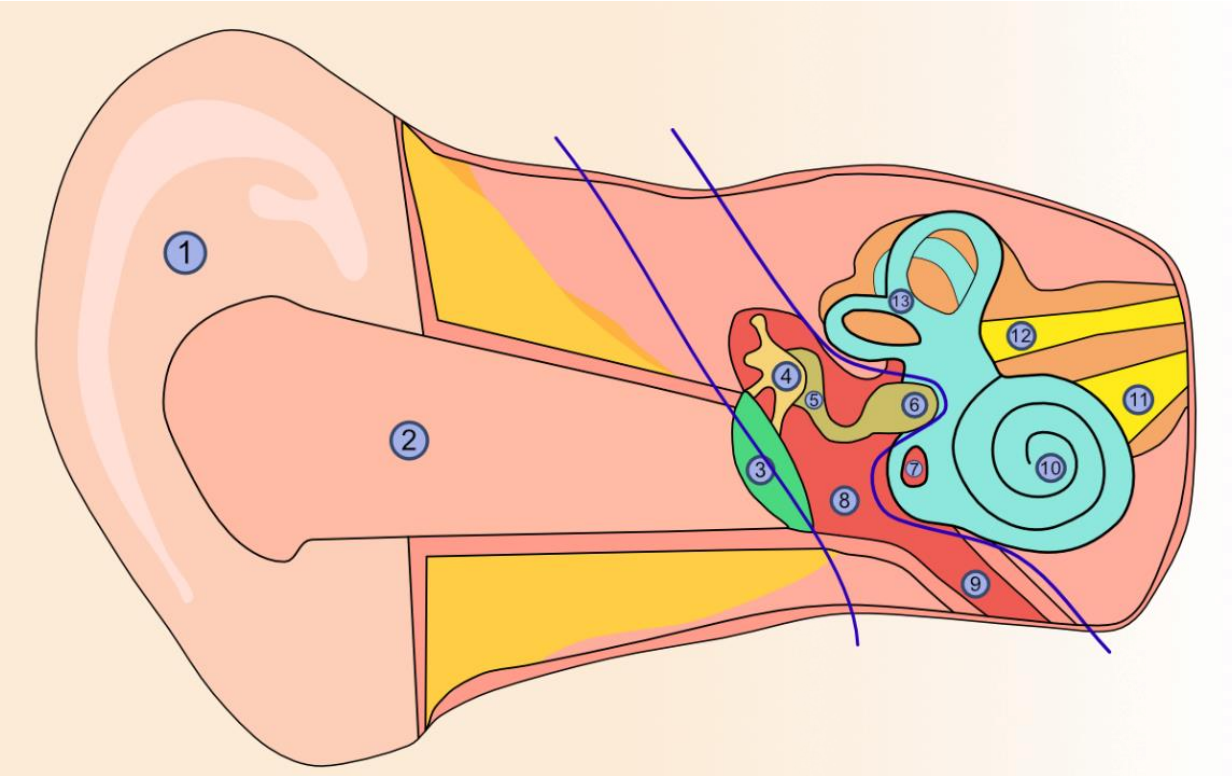

Figure 1: *Anatomy of the inner ear. Blue lines define, from left to right, the outer, middle and inner ear. (1) Auricle (2) Auditory canal (3) Eardrum (4) Malleus (5) Incus (6) Stapes (7) Round window (8) Tympanic cavity (9) Auditory tube (10) Cochlea (11) Cochlear nerve (12) Vestibular nerve (13) Semicircular ducts.*

Head accelerations are perceived as rotations, tilting or linear displacement, according to the sensorial receptor involved and produce a tilting of the stereocilia in the apical surface of vestibular hair cells with opening of mechanotransduction channels located in the tip links and the entry of K^+ ions in the cells (Ohmori 1985; Ottersen et al. 1998).

The inner ear can be morphologically divided into two parts: bony and membranous labyrinths. The membranous labyrinth contains endolymph whereas the bony labyrinth

surrounds the membranous labyrinth. The perilymph fills the spaces between these two structures. Endolymph and perilymph are essentials for correct hearing and balance functions (Ekdale 2016). From a functional point of view, the inner ear can be divided into two systems, since it includes the sensory organs of hearing and balance, the cochlea and the vestibule respectively. In addition, a third non-sensory structure, the endolymphatic sac, located at the distal end of endolymphatic duct, is thought to fulfill two functions: the secretion and reabsorption of endolymph and the immune response within the inner ear (Rask-Andersen et al. 1981; TOMIYAMA & HARRIS 1986). The composition of the endolymph varies throughout these structures, containing a high concentration of K^+ and a low concentration of Na^+ in the cochlea and the vestibular system, while the concentrations of these two ions are completely the opposite in the endolymphatic sac (Marcus & Shipley 1994; Wangemann et al. 1996).

1.1.1. Anatomy and function of the cochlea

The main function of the cochlea is to translate sound vibrations into neural impulses. The cochlea, located in the bony labyrinth, is a spiral-shaped structure divided in three fluid-filled cavities: the scala media, the scala vestibuli and the scala tympani (Rask-Andersen et al. 2012) (Figure 2).

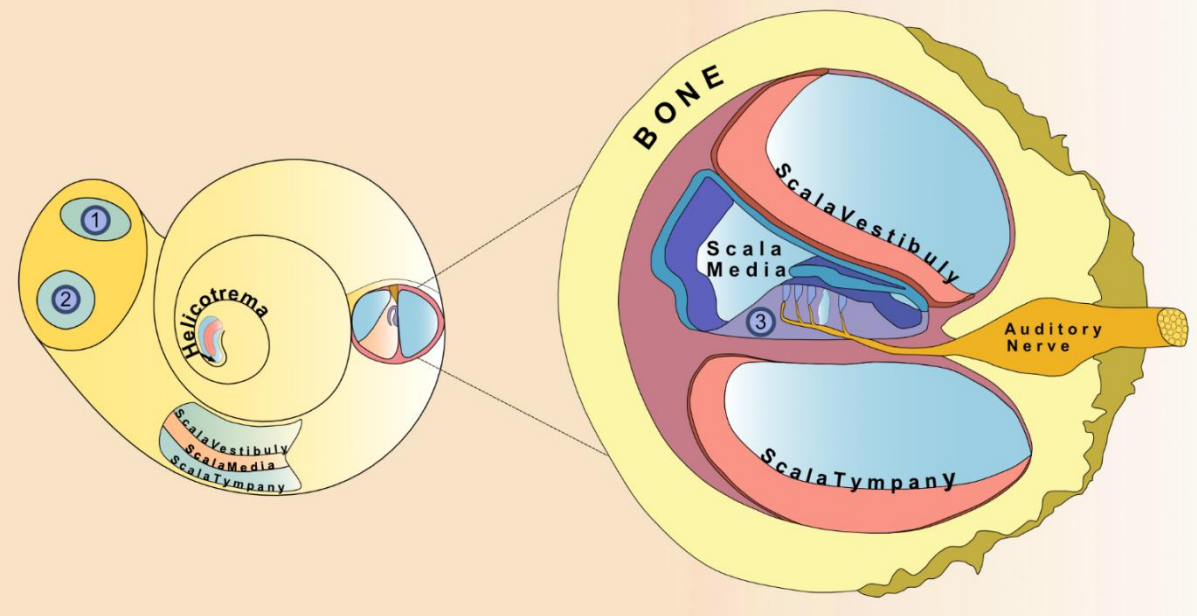

Figure 2: *Anatomy of the cochlea. (1) Oval window (2) Round window (3) Organ of Corti.*

Scala vestibuli and scala timpani contain perilymph whereas scala media is filled with endolymph and includes the Organ of Corti. The basilar membrane, which separates the scala media and scala timpani, supports the organ of Corti, and its thickness varies throughout its length. These changes in thickness make the basilar membrane tuned to different frequencies

at the basal (high frequencies) and the apical area of the cochlea (low frequencies). This allows the tonotopic organization of the cochlea (Ruben 2020) (Figure 3).

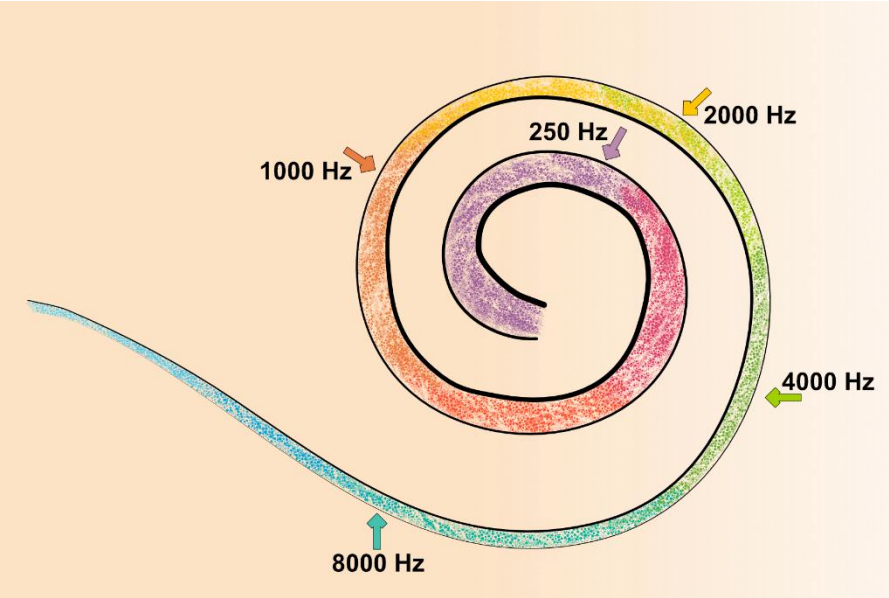

Figure 3: *Distribution of frequencies (Hz) along the basilar membrane. Tonotopy.*

The organ of Corti contains the hair cells, specialized cells responsible for transducing the sound waves into neural signals. Distinguished for having precise patterns of stereocilia, the hair cells are organized in four rows: one row of inner hair cells (iHC) and three rows of outer hair cells (oHC). The oHC are the most numerous hair cells in the cochlea and their stereocilia are attached to the tectorial membrane. The main function of these cells is to improve the hearing sensitivity by amplifying the basilar membrane motion. On the other hand, the iHC are the sound receptors, being activated when their stereocilia are displaced by the traveling wave. iHC stereocilia are not attached to the tectorial membrane, allowing this movement which implies the depolarization of the attached afferent nerve fiber and the stimulation of the auditory cortex as a last step (Schwander et al. 2010) (Figure 4).

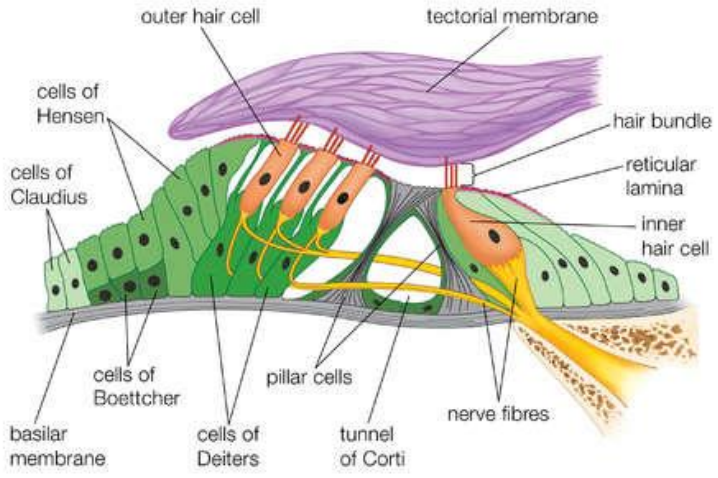

Figure 4: *Schematic representation of the organ of Corti. From: Encyclopædia Britannica (https://www.britannica.com/science/ear)*

1.1.2. Anatomy and function of the vestibular system

The main role of the vestibular system is to participate in the maintenance of proper balance. The vestibular labyrinth is structurally divided into semicircular canals and the otolith organs.

The semicircular canals are three bony canals (posterior, anterior and lateral semicircular canals) arranged in right angle each other filled by endolymph. Each semicircular canal has a dilation called ampulla containing sensory cells which are sensitive to angular acceleration and are covered with a gelatinous membrane called cupula (Figure 5a).

The otoliths organs, the sacculus and the utricle, respond to linear acceleration and the position of the head. These structures contain the macula, a sensory epithelium with hair cells covered by the otolithic membrane. This membrane contains calcium carbonate crystal called otoconia (Baloh et al. 2010) (Figure 5b).

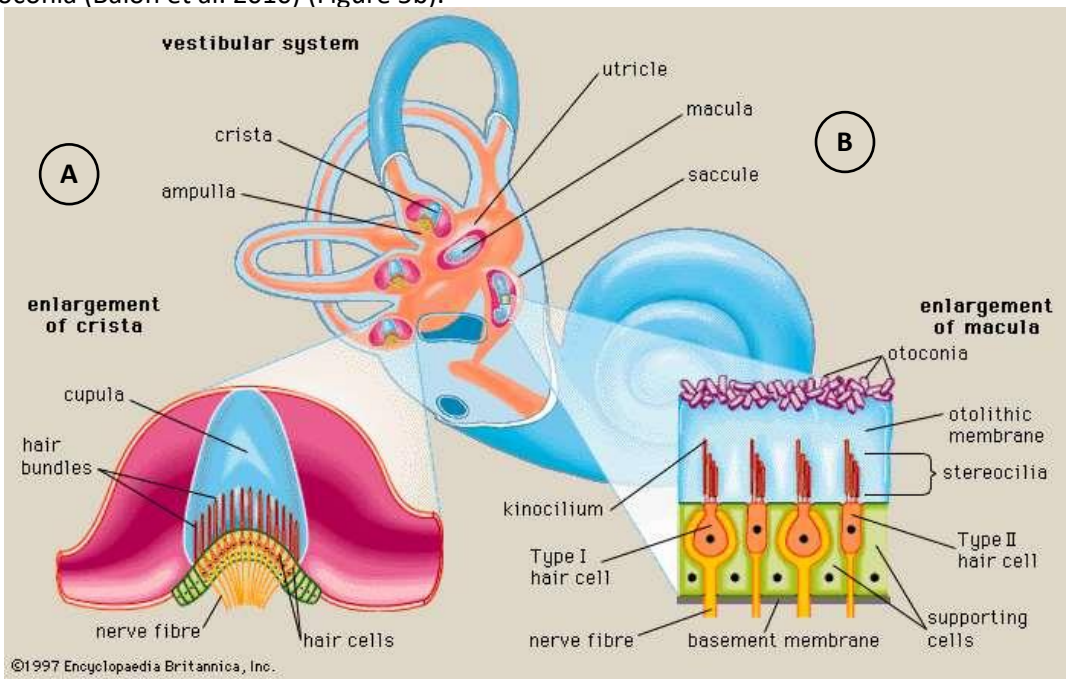

Figure 5: *Vestibular system. A) Cristae of the semicircular canals B) Macula of the utricle and saccule. Figure from Encyclopædia Britannica, inc. Accesed date: May 28, 2020.*

1.2. Meniere's disease

Meniere's disease (MD) is a rare multifactorial disorder probably triggered by a combination of genetic and environmental factors. MD is mainly characterized by episodic vertigo, progressive sensorineural hearing loss (SNHL), tinnitus and aural fulness (Nakashima et al. 2016).

1.2.1. Clinical symptoms

1.2.1.1. Vertigo

Vertigo is a sensation of self-motion when no motion is occurring caused by a disfunction in the vestibular system. In MD, vertigo spells can last from minutes to hours, resulting in a higher risk of falls during these periods. Besides, at long term, patients can report episodic dizziness or unsteadiness, becoming vestibular symptoms more disabling and increasing its impact on quality of life.

Vertigo spells are more common in the first years of the disease. Majority of patients have recurrent episodes of spontaneous vertigo, reporting 35-65% of them acute vertigo spells with sudden appearance. These episodes are usually preceded by tinnitus, decreased hearing in the affected ear and aural fullness (Paparella 1991; Strupp & Brandt 2008).

1.2.1.2. Sensorineural hearing loss

The most characteristic trait of MD is SNHL. Being more fluctuating during the first years, SNHL is usually more stable as the disease progresses. It can affect one ear (unilateral disease) or both ears (bilateral disease) (House et al. 2006; Lopez-Escamez et al. 2009). Hearing loss (HL) is diagnosed by serial pure tone audiograms, being critical to assess the progression of MD and to differentiate it from other inner ear diseases (Figure 6).

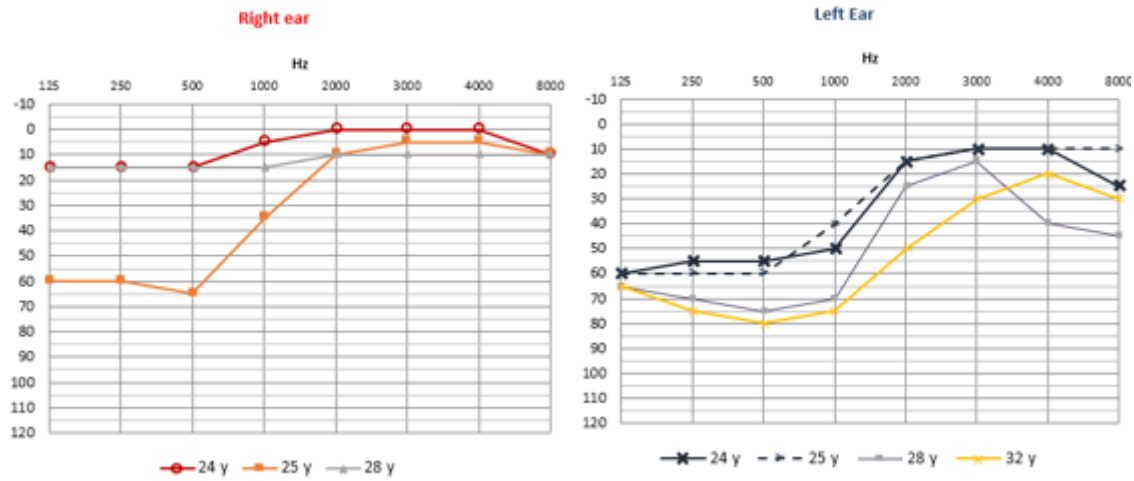

Figure 6: *Audiogram in a MD patient at different ages. In this example, the patient has low frequency bilateral hearing loss.*

1.2.1.3. Tinnitus

Tinnitus, defined as a condition associated with a continuous auditory percept, is a symptom of many disorders. In MD, tinnitus intensity usually increases during vertigo spells. Nowadays, tinnitus is an unmet trait and, particularly in case of severe tinnitus, it is a very disabling symptom (McCormack et al. 2016; Henry et al. 2020).

1.2.1.4. Aural fulness

Aural fullness is a sensation of pressure, also reported as a "clogging sensation", in the affected ear. This symptom usually fluctuates, although it can be constant, and its intensity may increase during vertigo episodes.

1.2.2. Diagnosis and classification of patients

MD diagnosis has been challenging due to the heterogeneity of the disorder. This led to the publication of the first diagnostic criteria for MD by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) in 1995 (Anon 1995), which was jointly reviewed in 2015 by the Classification Committee of the Bárány Society, the Japan Society for Equilibrium Research, the European Academy of Otology and Neurotology, the AAO-HNS and the Korean Balance Society (Lopez-Escamez et al. 2015). Unlike other diseases, the diagnostic criteria for MD is based only on the symptoms during the attacks, not considering any biological marker or findings on magnetic resonance imaging helpful for diagnosing (Table 1).

Symptoms	Definite MD	Probable MD
Vertigo	2 or more episodes of vertigo during 12min to 12h	2 or more episodes of vertigo during 20min to 24h
Hearing loss	Audiometrically documented low-to-medium frequency SNHL on an affected ear during/after one vertigo episode	-
Tinnitus/Aural fullness	Fluctuating aural symptoms	Fluctuating aural symptoms
Other	Not better explained by another vestibular disease	Not better explained by another vestibular disease

Table 1: *Diagnostic criteria defined by the Barany Society.*

MD may show symptom overlap with other disorders, complicating even more the diagnosis of this disease. The most common are vestibular migraine (VM) and autoimmune inner ear disease (AIED) (Liu & Xu 2016; Gazquez et al. 2011). VM is a common cause of episodic vertigo where vestibular symptoms overlap with migraine. Recently, Flook *et al* (2019) described

a method based on cytokines capable of differentiating VM patients from two subgroups of MD patients according to their proinflammatory signature (Flook et al. 2019). AIED is characterized by episodes of sudden to progressive bilateral SNHL. Initially, the HL of AIED patients fluctuates, and it can be confused with MD. To distinguish them from MD, pure tone audiogram is required for a correct differential diagnosis.

By using clustering analysis, the Meniere's disease Consortium defined five subgroups of patients with unilateral and bilateral MD according to few clinical variables: Group 1 includes those patients with metachronic SNHL (HL starts in one ear and involves the second ear in the next months or years); Group 2 clusters MD patients with synchronic SNHL (simultaneous SNHL); Group 3 characterized by familial MD (FMD); Group 4 associated with migraine and Group 5 associated with an autoimmune disease in addition to MD (Frejo et al. 2016; Frejo et al. 2017) (Table 2).

Subgroup	Frequency	Definition
1	46%	Metachronic hearing loss without migraine and without AD
2	17%	Synchronic hearing loss without migraine or AD
3	13%	Familial MD
4	12%	Comorbid migraine
5	11%	Presence of a comorbid AD

Table 2: *Clinical subgroups in MD.*

1.2.3. Epidemiology

There is not an agreement about the incidence (new cases per year) and prevalence (cases in population) of MD in general population. This situation is partly due to the fact that MD diagnosis is based on the reported symptoms of the patients. Besides, epidemiological studies in populations from United States, England, Africa, Japan or Finland have yielded different results, varying the prevalence of MD considerably between them. Thus, the reported prevalence in United States is 195/100,000 people (Alexander & Harris 2010), whereas in Japanese and Finish populations the prevalence was reported as low as 36/100,000 and 43/100,000 people, respectively (Shojaku & Watanabe 1997; Kotimäki et al. 1999).

The differences in these results could be explained in two ways: absence of standardized diagnosis and/or ethnic (or geographical) differences between the studied populations. As it has been explained above, it not was until 1995 that clinicians had a first guideline for diagnosing MD. This fact could influence the diagnosis of MD, varying its prevalence up or down. On the

other hand, it is already well-known the impact of ethnicity in complex diseases, and the results of the previous studies lead us to think that it can be modifying the MD prevalence.

MD is usually diagnosed at age 40, with a slight female predominance. Characterized by a variable course, the appearance of vestibular and cochlear symptoms may not coincide in time, taking years to fulfill MD diagnostic criteria. The vast majority of cases are sporadic, although 8% of these patients show familial aggregation. Most families follow an autosomal dominant (AD) inheritance pattern, and some of them exhibit genetic anticipation, a phenomenon in which symptoms tend to appear at an earlier age in consecutive generations (Requena et al. 2014).

1.2.4. Treatments

Since MD often occurs along with other diseases, such as migraine, allergy or an autoimmune condition, a personalized approach for MD patients is highly recommended. The first strategy to treat MD is through a proper diet, with a low content of sodium and a high-water intake (Naganuma et al. 2006). The rationale underlying this diet is the prevention of the vasopressin release and maintaining of the inner ear homeostasis (Degerman et al. 2015). As a supplement, betahistine is usually used since it shows a positive effect in MD patients, reducing the vertigo episodes (Murdin et al. 2016). If this first approach does not control MD episodes, intratympanic treatment can be applied, being dexamethasone the most used drug (Lavigne et al. 2016; Beyea et al. 2017). As a previous alternative to surgery, gentamicin can be also applied in an intratympanic way to reduce vertigo attacks, however this treatment poses the risk to worsen hearing loss (Patel et al. 2016). Finally, the surgery techniques most used are labyrinthectomy and vestibular neurectomy (De La Cruz et al. 2007; Nevoux et al. 2018).

1.2.5. Pathophysiology

According to human histopathological findings in temporal bones from patients with MD, the disorder has been related to the accumulation of endolymph in the cochlear duct (endolymphatic hydrops). On this basis, MD is considered a complex disease where multiple factors, such as genetics, autoinflammation, autoimmunity and allergy, could be interacting to result in endolymphatic hydrops (Semaan et al. 2005).

1.2.5.1. Endolymphatic hydrops

In normal conditions, endolymph is mainly produced in the stria vascularis and, in a slow way, it is absorbed in the endolymphatic duct and sac. In MD, this absorption is defective, finally causing endolymphatic hydrops (EH) and dilation of the Reissner's membrane. The rupture of this membrane could explain all of the symptoms of MD, however, according to a review of

human temporal bones with MD, in two thirds of patient there is no evidence of rupture (Paparella & Djalilian 2002).

EH involves molecular and cellular changes throughout the inner ear. It has been found that in MD patients the mastoid is smaller in size and the vestibular aqueduct, a bony canal running from the vestibule to the temporal bone, is shorter. Some cell populations are also affected: loss of hair cells, defective supporting cells (SCs) and atrophy of the tectorial membrane (Yoda et al. 2011). In addition, the neurons which innervate the hair cells of the Organ of Corti, the spiral ganglion neurons, are also affected in number before a damage in the inner hair cells (Momin et al. 2009). This loss of neurons could be leading the subsequent decrease and loss of hair cells.

There is no evidence that a larger loss of hair cells could bring a more severe EH (Momin et al. 2009). Therefore, it should be something other than EH involved in the origin of changes mentioned above. This opens the way to new hypotheses where other factors, such as genetic predisposition, could be playing an important role in the development of MD.

1.3. Basis and state of the art of human genetics

Since the DNA was identified as the hereditary material by Avery, MacLeod and McCarty (Avery et al. 1944); and its structure was deduced by Watson and Crick as a double helix, human genetics have shown a rapid progress (Watson & Crick 1953). DNA consists of two complementary strands made up of the combination of four nucleotides: Adenine (A) which bonds to thymine (T), and cytosine (C) which bonds to guanine (G). This molecule is extremely condensed inside the nucleus of the cells, forming a total of 46 chromosomes (23 pairs of chromosomes; diploid genome) as it was concluded in 1956 (TJIO & LEVAN 1956). From them, 22 pairs of chromosomes are autosomes and one pair of sex chromosomes.

A milestone in genetics was the development of DNA sequencing methods in 1977. It was due to this progress that it was possible to identify the first genetic variants at the DNA level (Sanger et al. 1977). However, the analysis of the DNA also needed a reference sequence, a normal DNA sequence to compare with another given DNA sequence. With this aim, to sequence the entire 3 billion of nucleotides in the human genome, the Human Genome Project (HGP) was born in 1990 funded mainly by the National Institutes of Health from United State (Watson 1990). In April 2003 was published the result of this project, with about 3.2 billion base pairs sequenced encoding 30,000-35,000 genes (Collins et al. 2003). Genes are the regions of DNA

that contain the information for coding proteins (exons). However, genes also contain regions that do not code for proteins (introns), but these non-coding regions have regulatory effects and contribute to make cell-specific proteins. DNA can be divided in coding and non-coding regions. The latter represent almost 99% of the human genome and they contain regulatory elements that modify the gene products such as gene expression.

Since the results of the HGP were published, and the next development of parallel massive sequencing, the interest in genomics is constantly growing. As the demand for genomics services has increased, the prices for sequencing DNA have decreased repeatedly, making DNA sequencing an attractive approach to study complex diseases (Figure 7)

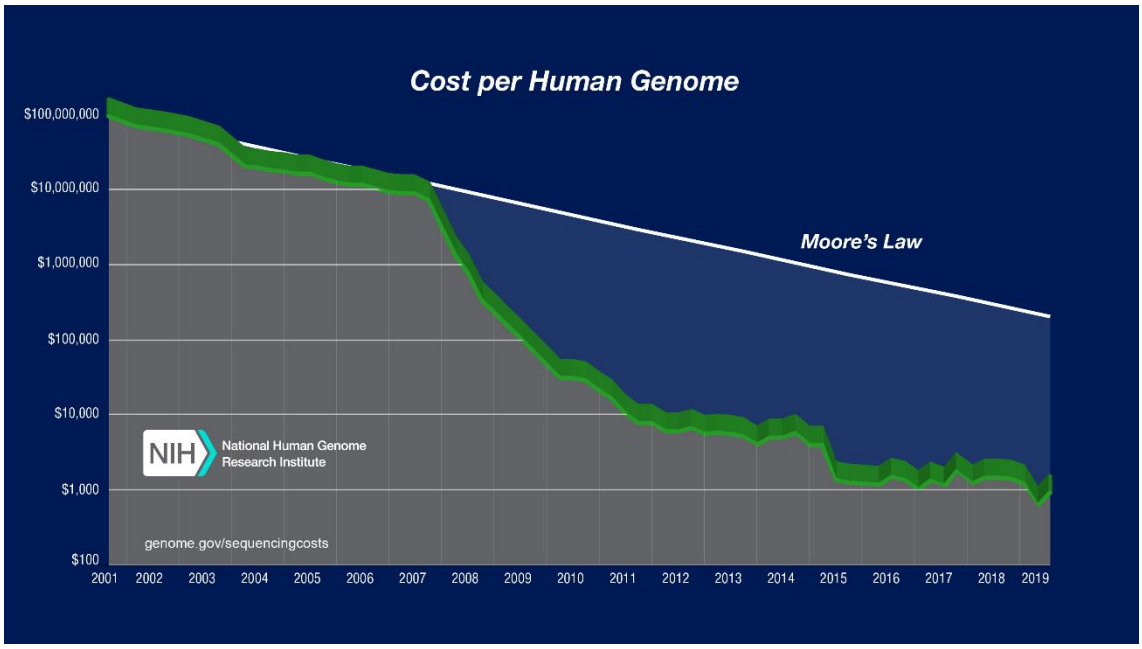

Figure 7: *Cost per genome data. Figure from National Human Genome Research Institute. Accessed data: 29 May, 2020.*

1.3.1. From genes to proteins

Most genes contain the information to produce protein chains. Nevertheless, there is a required intermediate state between DNA and protein: the messenger RNA (mRNA). The production of mRNA from a gene sequence is called transcription, process where DNA serves as a template for complementary base-pairing. Genes consist of coding regions (exons) separated by non-coding regions (introns), regions which are ruled out from the resulting functional mRNA transcript. Thus, the mRNA is a single-stranded copy of the coding regions of the gene. After the transcription, the mRNA should be translated. During the translation, the mRNA is read following a set of rules called genetic code, where RNA sequence is linked to the amino acid sequence. The combination of three bases in mRNA makes a codon, and each codon codes for a specific

amino acid. Since there are 4 bases in mRNA, there are 64 possible codons for a total of 20 different amino acids. It must be considered that there is a unique start codon, the AUG codon, and three codons for ending the translation of the mRNA, known as STOP codons.

1.3.2. Genetic variants

A variant is an alteration in a given DNA sequence. These changes in the DNA happen in a natural way during the cell division and subsequent DNA replication, being the principal source of genetic variation. The replication machinery, the proteins responsible for DNA replication, sometimes inserts a wrong nucleotide, or even too many/few nucleotides into the DNA sequence (McCulloch & Kunkel 2008) (Figure 8). Most of these changes are repaired through DNA repair mechanisms by recognizing the unpaired nucleotides, however some of these errors skip these processes, passing down to the next generation cells, becoming permanent mutations. If the affected cells are gametes, variants will be transmitted from parents to children.

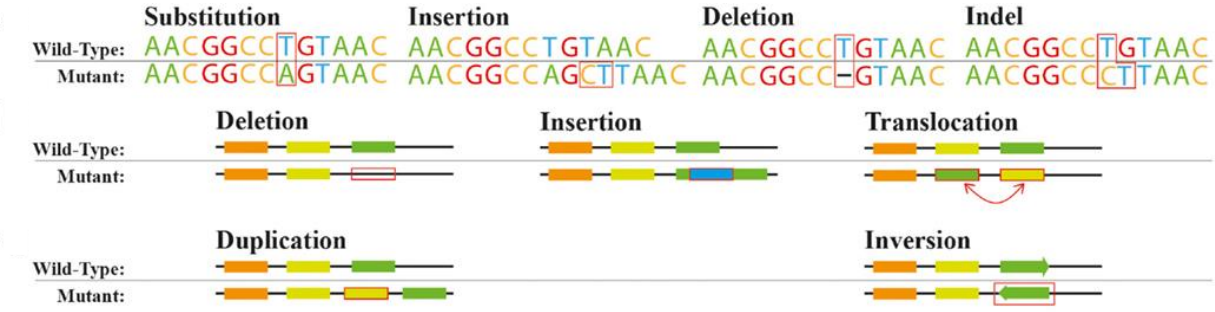

Figure 8: *Types of variants found in human genomes. From: Cardoso J et al (2015). Analysis of Genetic Variation and Potential Applications in Genome-Scale Metabolic Modeling. Front Bioeng Biotechnol.*

When a variant is compared with a reference population, we can discern its frequency. Mainly, variants are divided in two groups according to its frequency: a) common variants, when its frequency is higher than 5% of the population and b) rare variants, when its frequency is lower than 5% of the population.

Functionally, **single nucleotide variants (SNV)** are substitutions in one nucleotide (i.e., A is replaced by T), and can be divided in two different categories: coding and non-coding variants. As mentioned above, DNA has coding regions and non-coding regions. Thus, coding variants are those variants localized in coding regions and non-coding variants are those affecting non-coding regions. Within the coding variants, we can find the nonsynonymous and synonymous variants, depending on whether the involved variant results in a codon change in the translated amino acid or not, respectively. The genetic code is redundant, meaning that the same amino acid can be coded by multiple codons. Therefore, a SNV can provoke a change in the DNA sequence but the resulting new codon may continue coding the same amino acid (i.e.

synonymous variants). If that does not happen the coded amino acid will change, existing the possibility that this change leads to the loss of the original STOP codon (stop loss) or the appearance of a premature one (stop gain, both types named nonsense variants).

Genetic variants can involve more than a single nucleotide. So, **insertion and deletions (indels)** affect a region from 2 to hundreds base pairs, and they can be divided in frameshift or non-frameshift indels depending if they change the reading frame during the translation or not, respectively. When the involved region is larger, variants are known as **structural variants**. Within them, we can find copy number variants (CNV), inversions and translocations. CNV affect regions of DNA that have a variable number of repeats when it is compared with a reference genome. CNV can imply a gain or a loss of copies. Inversions are inverted regions in the same position than the reference sequence. Finally, translocations are the result of transferring a sequence of DNA from one region to another. They do not involve a loss or gain of nucleotides.

1.3.3. Assessing the pathogenicity of genetic variants

The ability to distinguish between benign and pathogenic variants is a key to obtaining relevant results from genetics studies. Consequently, multiple predictive algorithms have been implemented in the last decade to assess the pathogenicity of genetic variants. So, the most used algorithms are the Combined Annotation Dependent Depletion (CADD) algorithm (Rentzsch et al. 2019), the Sorting Intolerant From Tolerant (SIFT) algorithm (Sim et al. 2012) and the Polymorphism Phenotyping v2 (PolyPhen-2) algorithm (Adzhubei et al. 2013). Since the result from these tools sometimes are inconsistent between them, in 2015 was published the guidelines for the interpretation of sequence variants by the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP), being used nowadays as a gold standard for variant interpretation (Richards et al. 2015). These criteria take into account different evidences for classifying variants into five categories: pathogenic, likely pathogenic, uncertain significance, likely benign and benign (Table 3).

	Benign		Pathogenic			
	Strong	Supporting	Supporting	Moderate	Strong	Very Strong
Population data	MAF is too high for disorder OR observation in controls inconsistent with disease penetrance			Absent in population databases	Prevalence in affected statistically increased over controls	
Computational and predictive data		Computational evidences suggest no impact on gene /gene product	Computational evidences support a deleterious effect on the gene /gene product	Novel missense change at an aa residue where a different pathogenic missense change has been seen before. Protein length changing variant	Same aa change as an established pathogenic variant	Predicted null variant in a gene where LOF is a known mechanism of disease
Functional data	Well-established functional studies show no deleterious effect		Missense in gene with low rate of benign missense variants and path. Missenses common	Mutational hot spot or well-studied functional domain without benign variation	Well-established functional studies show a deleterious effect	
Segregation data	Nonsegregation with disease		Cosegregation with disease in multiple affected family members			
De novo data				De novo (without paternity & maternity confirmed)	De novo (paternity & maternity confirmed)	
Allelic data		In trans with a dominant variant OR in cis with a pathogenic variant		For recessive disorders, in trans with a pathogenic variant		
Other database		Reputable source w/out shared data = benign	Reputable source = pathogenic			
Other data		Found in case with an alternate cause	Patient's phenotype or FH highly specific for gene			

Table 3: *Evidences and guideline for the interpretation of variants by the ACMG and AMP.*

1.4. Genetics of hearing loss and Meniere's disease

To date, 119 genes have been related to non-syndromic HL (i.e. hearing impairment without abnormalities of the external ear or medical problems), indicating a high genetic heterogeneity (Van Camp G. 2018). Although HL etiology is very heterogenous, it has been estimated that in half of the cases its origin is genetic (Koffler et al. 2015). Different inheritance patterns have been also described for HL, usually being more severe when it is inherited in a recessive manner. Pathogenic variants in the gene coding the connexin 26 (*GJB2*) are the most common cause of severe autosomal recessive HL (Sloan-Heggen et al. 2016). AD inheritance pattern can be also observed in non-syndromic HL, existing 47 genes related to this condition, and it is mainly characterized as post-lingual (i.e. with an onset between the age of 20 and upwards) and progressive (Petersen 2002). Genes related to non-syndromic HL are shown in supplementary table 1.

One of the major evidences that support a genetic contribution in MD is the occurrence of families with more than 1 case fulfilling its diagnostic criteria (multiplex families). This is known as familial aggregation. So, in a cross-sectional study with 500 MD patients, Paparella found that a 20% of cases had a familial history (Paparella 1984). Also, in a large cohort of Spanish and Italian MD patients, Requena *et al.* reported a familial history in 34% of studied cases (Requena et al. 2014). Most of families follow an AD inheritance pattern with incomplete penetrance, existing also evidences of anticipation and more severe phenotypes as the age of onset is lower (Morrison et al. 2009).

The first approaches to study families with MD considered the disease as a monogenic syndrome. By using whole exome sequencing (WES), Requena *et al*. found the first genes related to MD in a single family from south Spain. These genes, *DTNA* and *FAM136A*, encode a-dystrobrevin and a mitochondrial protein with unknown function respectively, and it was demonstrated that they are expressed in the neurosensorial epithelium of the crista ampullaris of the rat (Requena et al. 2015a). However, variants in these genes have not been reported in additional MD families. Later, Martin-Sierra *et al.* described a variant in *PRKCB* gene in a second Spanish MD family. In this study, a high expression of this gene was observed in tectal and inner border cells, showing a tonotopic expression from the base to the apex of the cochlea (Martín-Sierra et al. 2016). Additionally, missense variants in *SEMA3D* and *DPT* were found in two different Spanish families (Martín-Sierra et al. 2017). More recently, rare variants in *HMX2* and *TMEM55B* genes were pointed out in a Finish family. In this study, a grandchild with early onset MD and his grandfather, with definite MD, shared two variants in these genes (Skarp et al. 2019). Putting it all together, these studies reflect the genetic heterogeneity in FMD, even in a same

population (Spanish population), and the need to address the study of MD with a different approach. Genes related to FMD are shown in table 4.

Gene	Variants	Function	Ref.
DTNA	chr18:32462094G>T	Formation and stability of synapses	Requena et al.
FAM136A	chr2:70527974C>T	Unknown	Requena et al.
PRKCB	chr16:23999898G>T	Neutrophil chemotaxis, melanoma cell growth and proliferation	Martin-Sierra et al.
DPT	chr7:84642128C>T	Extracellular matrix protein	Martin-Sierra et al.
SEMA3D	chr1:168665849C>T	Involved in axon guidance	Martin-Sierra et al.

***Table 4**: Genes described to this day for familial Meniere disease.*

A more complex approach has been used to study the genetic of sporadic MD cases, who represent the majority of MD cases (92%). Using polygenic inheritance as a model, Gallego *et al*. described an excess of rare variants in HL-related genes in a large Spanish sporadic MD cohort when they were compared to the Spanish and the non-Finish European reference populations. Among these genes, it can be highlighted genes related with the regulation of the ionic concentration of the endolymph, such as *SLC26A4* or *CLDN14*, genes associated to connexons in GAP junctions, causing recessive deafness such as *GJB2,* and even genes related to syndromic HL such as Usher syndrome type IG (USH1G) (Gallego-Martinez et al. 2019b). These results remark the importance of genes related to deafness in MD. In a later study focused on the axonal guidance signaling pathway, a burden of rare variants was also found in genes such as *NTN4* and *NOX3* in a large cohort of Spanish sporadic MD cases (Gallego-Martinez et al. 2019a). Genes related to sporadic MD are shown in table 5.

Gene	Odds ratio		Corrected P values	
	Spanish population	NFE population	Spanish population	NFE population
GJB2	2.06 (1.33-3.19)	3.2 (2.12-4.83)	$6.85x10^{-03}$	$1.65 x10^{-06}$
*SEMA3D**	2.67 (1.94-3.68)	0.8 (0.53-1.21)	$4.06x10^{-09}$	0.5
CLDN14	4.64 (2.65-8.11)	23.18 (13.81-38.9)	$1.49x10^{-07}$	$<1.00x10^{-15}$
SLC26A4	2.33 (1.513.59)	2.88 (1.89-4.38)	$7.37x10^{-04}$	$4.88x10^{-06}$
*NFKB1**	2.73 (2.03-3.66)	1.43 (1.03-1.98)	$6.62x10^{-11}$	0.1
ESRRB	1.84 (1.33-2.54)	3.39 (2.52-4.55)	$6.12x10^{-04}$	$<1.00x10^{-15}$
USH1G	4.67 (2.68-8.17)	20.27 (12.06-34.06)	$3.05x10^{-07}$	$<1.00x10^{-15}$
*NTN4**	7.22 (3.07-18.30)	3.05 (2.40-4.49)	$4.58x10^{-02}$	0.4
*NOX3**	6.96 (3.87-12.91)	1.59 (0.98-2.43)	$2.33x10^{-03}$	0.5

Table 5: *Genes found to be related to sporadic Meniere disease. * indicates that this gene only showed an enrichment of rare variants in the Spanish population.*

2. Hypothesis

Meniere disease has a hereditary background which causes the disease to appear early or late in sporadic and FMD cases.

The working hypothesis is that in FMD cases and early-onset sporadic MD patients showing anticipation are more likely to have a genetic origin of the disease and, in these cases, the accumulation of rare variants in different genes would have a larger effect size to MD phenotype.

3. Objectives

The main objective of this Thesis is to identify the main genes associated with familial Meniere's disease by WES. Likewise, we address the following specific objectives:

1. To identify potential *de novo* or singletons variants in early-onset MD patients.
2. To demonstrate the aggregated effect of rare variants through a selective variant enrichment in certain genes in FMD.
3. To define the major pathways implied in FMD and sporadic MD.

4. Methods

4.1. Diagnosis of cases

All patients were diagnosed according to the diagnostic criteria described by the International Classification Committee for Vestibular Disorders of the Barany Society (Lopez-Escamez *et al*. 2015). A complete hearing and vestibular assessment were carried out in all cases, including brain magnetic resonance imaging to exclude other causes of neurological symptoms. Serial pure tone audiograms were retrieved from clinical records to assess hearing loss since the initial diagnosis.

A total of 138 patients with MD were selected for exome sequencing for this Thesis. Ninety-four MD patients over 18 years old from 62 different families with one or more affected first-degree relatives, and 44 sporadic MD cases with an age of onset under 35 years were recruited.

4.2. DNA extraction and WES

Blood and saliva samples were taken from patients to perform WES, a method which targets only coding regions of the genome. DNA samples were extracted from saliva with prepIT-L2P (DNA Genotek, Ottawa, Canada) and from blood with QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands) using manufacturer's protocols, and they passed the quality controls required for WES (i.e. DNA concentration higher than 50 ng/µl, a ratio A260/A280 higher than 1.7 and a total DNA amount higher than 1 µg). DNA quality and concentrations were checked by three different methods: Qubit dsDNA BR Assay kit (ThermoFisher Scientific, NY, USA), Nanodrop 200C and Quant-iT Picogreen (Invitrogen, CA, USA). The condition of the DNA was assessed by gel electrophoresis method.

WES data were generated for the 138 recruited MD patients. DNA samples from familial and sporadic MD cases were captured using SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) and were paired-end sequenced on the Illumina NovaSeq 6000 system at 100X coverage. Raw reads were stored in two FASTQ files for each individual.

4.3. Bioinformatics

4.3.1. Data generation and pre-processing

The FASTQ files are text files storing the sequence data. Since our samples were sequenced in a paired-end way, two FASTQ files representing the forward and reverse sequenced reads were generated for each of the samples. These files have, in addition to the DNA sequence, information about the quality for every base. The quality scores are PHRED-encoded using ASCII characters, representing the estimated probability of an error (Cock et al. 2009). Thus, each entry of a FASTQ file is divided into four lines:

@SEQ_ID	**[Sequence ID]**
GATTTGGGGTTCAAAGCAGTATCGATCAAATAGTAAATCCATTTGTTCAA	**[DNA sequence]**
+	**[Separator]**
!''*((((***+))%%%++)(%%%%).1***-+*''))**55CCF>>>>>	**[Phred Score]**

If P is the probability of error of a base call, the PHRED quality score or Q score is defined as:

$$Q_{PHRED} = -10\ x\ log_{10}(P)$$

PHRED SCORE (Q)	Probability of base call error	Base call accuracy	ASCII characters
10 (Q10)	1 in 10	90%	!"#$%&'()*+
20 (Q20)	1 in 100	99%	,-./012345
30 (Q30)	1 in 1000	99.9%	6789:;h=i?
40 (Q40)	1 in 10000	99.99%	@ABCDEFGHIJ

Table 6: *Summary of PHRED quality scores*

Higher Q values mean higher accuracy, giving on the whole an image of how well the sequencing process was (Table 6 & Figure 9).

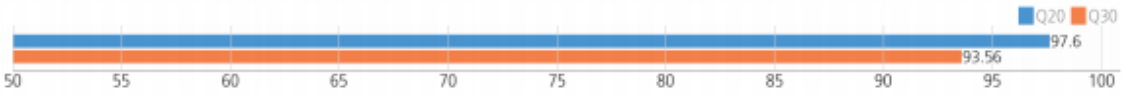

Figure 9*: Example of Q20/Q30 scores of raw fastq data from a sample included in this thesis*

4.3.1.1. Generation of analysis-ready BAM files

FASTQ files should be processed before having files ready for analysis. To this end, the BWA-MEM algorithm and the SAMtools view command were used (Li et al. 2009; Li & Durbin 2009). Taking as input the FASTQ files and a reference genome, the BWA-MEM algorithm generates a SAM file for each sample, where the paired-end reads are aligned to a reference genome (GRCh37/hg19 was used). Due to the size of SAM files, they should be converted to BAM files, a binary version of SAM files, using SAMtools view command. Most of the next-generation sequencing (NGS) tools work with BAM files, becoming the starting point for the generation of genetic variation data. This first BAM file generated by SAMtools must be refined through a post-alignment processing, using Picard tools and the Genome Analysis Tool Kit (GATK) (McKenna et al. 2010; Wysoker A, Tibbetts K 2011). Picard tools were used for locating and removing duplicated reads, which arise from artifacts during the sequencing amplification cycles. GATK was used for assessing the quality of the generated BAM file.

4.3.2. Generation of callsets

Using the analysis-ready BAM files as input, the Haplotypecaller function from GATK was applied for identifying genetic variants. SNVs and Indels were retrieved for each of the patients, generating 138 Variant Call Format (VCF) files.

Tabix (Li 2011) and the vcf-merge function from the VCFtools package (Danecek et al. 2011) were used for indexing and merging the resulting individual VCF files. Four different merged VCF files were generated:

A. *FMD cases*: Variants called in each FMD patient (N=94) were recorded in this VCF file.
B. *One case per family*: Only the variants called in one patient for each family (N=62) were selected for this file. Whenever possible, the selected patient was in the last generation.
C. *SMD cases*: Variants called in each early-onset SMD patient (N=44) were recorded in this VCF file.
D. *All cases*: Variants called in each MD patient (N=138) were recorded in this VCF file.

Indels were retrieved in individual VCF files as well. The same strategy was followed, generating four groups to undertake a differential analysis according to their familial or sporadic condition.

4.3.3. Variants quality assessment

Variants were tagged according to its base-calling quality. The most used tool for this purpose is the VariantRecalibrator function from GATK, which is the first stage of a two-step process called Variant Quality Score Recalibration (VQSR). Based on "good quality sites" given as input (hapmap, omni2.5, 1000Ghigh_confidence sites and dbsnp), this first step assesses the probability that a variant is a genuine variant and it is not a sequencing artifact. The second step is done with the ApplyRecalibration function from GATK, where each variant is annotated with a VQSLOD score. This score is the log-odds ratio of being a genuine variant with regards to being a false positive according to the results obtained with the VariantRecalibrator function.

4.3.4. Variant annotation

Since VCF files contain only information about reference and alternative alleles, chromosomal positions of the variants, base calling quality scores and genotypes should be annotated.

4.3.4.1. Annovar

Annovar is a tool to functionally annotate genetic variants (Wang et al. 2010). During this process, four tab-delimited text (.txt) files were generated taking as input each of the four merged VCF files. Two types of annotations were made with Annovar:

A. *Gene-based annotations*: They classify variants according to its functional consequences on genes (coding or non-coding variants) and the amino acid affected. To this end, NCBI's Reference Sequence (RefSeq) was used (Pruitt et al. 2007).

B. *Filter-based annotations*: Annovar takes information from previously recorded variants in different databases. Using the '-downdb' argument from Annovar, the Exome Aggregation Consortium (ExAC) database, the Genome Aggregation Database (gnomAD), the Combined Annotation Dependent Depletion (CADD) scores and the dbNSFP database (v3.0) were downloaded and used.

4.3.4.2. Collaborative Spanish Variant Server (CSVS)

Since all patients included in this study were mainly from Spanish ascendants, the allelic frequencies of the variants were annotated with the Collaborative Spanish Variant Server (CSVS) database (Dopazo et al. 2016). This database contains allelic information from a total of 1942 samples from Spanish origin. Three of them (classified as 'Group VIII: Diseases of the ear and mastoid process') were discarded from our analysis. This database is freely available on its website (http://csvs.babelomics.org/; accessed: 19/01/2020).

4.3.5. Variant filtering

A two-step variant filtering process was applied for selecting proper candidate variants. Before the annotation, a soft-filtering process was used, adding information to the 'FILTER' column of VCF files. No hard-filtering processes (i.e. remove the variant entry) were applied in order not to lose information.

4.3.5.1. VQSR

By using the VariantRecalibrator and ApplyRecalibration functions from GATK, the probability of each variant to be true was calculated. This probability is also known as variant quality score log-odds (VQSLOD) and its main objectives are to increase the sensitivity (i.e. to detect real variants) and the specificity (i.e. to diminish the false positive variants). Using the VQSLOD score the variants were ranked and divided into quality tranches (Figure 9).

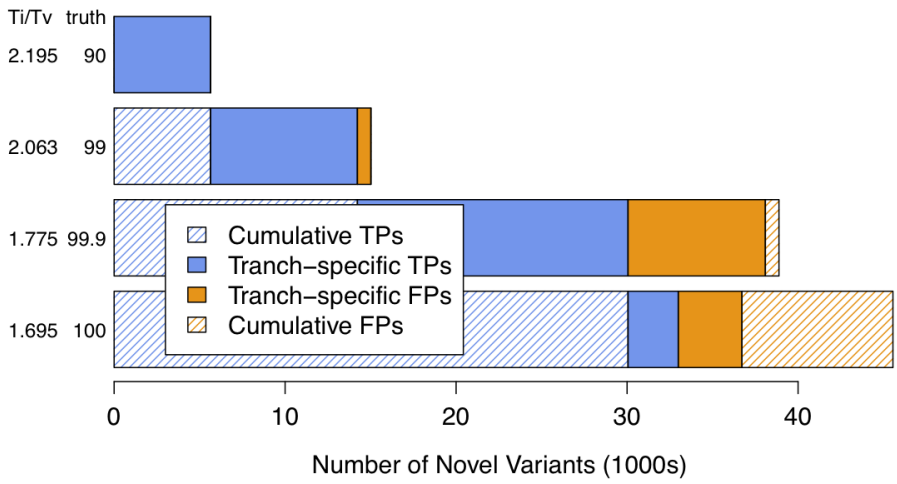

Figure 10: *Example of tranches plot generated by the VariantRecalibrator walker*

In the first filtering step, these quality tranches were used. Variants with a low VQSLOD value were tagged using a tranche sensitivity threshold. A sensitivity of 99% was selected for this step.

4.3.5.2. Allelic frequency

A second post-annotation filtering step based on minor allelic frequencies (MAF) was applied. The MAF values from three public databases were considered: ExAC, gnomAD and CSVS. Different MAF cutoffs were applied according to the prevalence of MD and the different analysis strategies explained below ('Callsets analysis strategy' section).

4.3.5.3. Frequently mutated genes in public databases

It is known that some genes are frequently affected by rare variants. These genes, listed by Shyr et al. (2014), are known as FLAGS (FrequenLy mutated GeneS). They showed that these genes possess characteristics, such as gene length, that make them less likely to be critical for disease development. Thus, the top 100 FLAGS list was also used to prioritize genes in our datasets.

4.3.6. Callsets analysis strategy

Two pipelines were conducted to search for rare variants. The first was a single rare variant analysis (SRVA) for studying individual families and sporadic cases; the second approach was a gene burden analysis (GBA) to obtain a gene-level mutational profile. These analyses were applied in the four generated callsets. Since the estimated prevalence of MD is 7.5/10,000 individuals, variants with a MAF<0.001 (1‰) were selected for SRVA. On the other hand, the MAF cutoff was increased up to 0.005 (5‰) and 0.05 (5%) for the GBA to analyze the combined effects of rare variants.

4.3.7. Variant prioritization

4.3.7.1. Phenix

A genetic analysis considering the different clinical subtypes or endophenotypes of MD was conducted using Human Phenotype Ontology (HPO) terms. Candidate genes were prioritized based on predicted variant pathogenicity and phenotypic similarity of diseases associated with the genes harboring these variants using PhenIX pipeline (available at http://compbio.charite.de)

Variants with a MAF > 0.001 in ExAC-non-Finnish European (ExAC-NFE) or gnomAD-NFE populations were discarded from this analysis. Variant frequencies were also checked in CSVS database. Three HPO terms were used to define the MD phenotype since MD disease has no a specific HPO term. This way, sensorineural hearing impairment (HP:0000407), vertigo (HP:0002321) and tinnitus (HP:0000360) terms were used in every patient. Furthermore, different HPO terms, such as migraine (HP:0002076), type 1 diabetes mellitus (HP:0100651) or hypothyroidism (HP:0000821), were used to represent specific phenotypes of each patient. Whole exome sequencing data from 82 MD cases (62 familial MD cases and 20 sporadic MD cases) were used for this analysis.

4.3.7.2. Association with a phenotype or pathway

As a method of variant prioritization, candidate genes were associated to phenotypes, biological processes or pathways. Genes were associated to mammalian phenotypes (MP) using the batch query from the Mouse Genome Informatic (MGI) database (http://www.informatics.jax.org/batch). Similarly, the Human Phenotype Ontology (HPO) project was used to associate genes to human phenotypes (https://hpo.jax.org/app/), and the Online Mendelian Inheritance in Man (OMIM) database was used to determine associations between genes and diseases (https://omim.org/). With regard to the association of candidate genes to biological processes and pathways, PantherDB (http://www.pantherdb.org/) and WebGestalt (http://www.webgestalt.org/) with Reactome database as reference (https://reactome.org/) were used.

4.3.7.3. Sensorineural hearing loss genes

Genes previously associated with SNHL were prioritized to identify rare variants with likely strong effects on MD phenotype. A SNHL gene set was generated by using three different databases: the Hereditary Hearing Loss Homepage (Van Camp G. 2018), the Deafness Variation Database (Azaiez et al. 2018) and Harmonizome (Rouillard et al. 2016), containing a total of 116 genes related with SNHL (Supplementary Table 1).

4.3.7.4. Gene expression analysis

The expression of candidate genes in the inner ear and vestibular/spiral ganglion neurons were assessed according to the results of three different gene expression studies in mice:

- Vestibular and cochlear HC gene expression data were retrieved from an RNA-Seq study during mice inner ear development (E16 to P7) (Scheffer et al. 2015).
- Supporting cells (Deiter's and pillar cells) and cochlear hair cells (outer and inner) gene expression data were obtained from an RNA-Seq study of the mouse organ of Corti (Liu et al. 2018).
- A microarray analysis studying the gene expression in vestibular and spiral ganglion neurons at six development stages (E12, E13, E16, P0, P6 and P15) (Lu et al. 2011) was used in conjunction with the Auditory and Vestibular Gene Expression Database from Goodrich Lab (http://goodrich.med.harvard.edu/resources.html)

In general, genes were considered specific in given cell population if its expression was >2-fold when it was compared with a second cell population from the same study.

4.3.7.5. Pathogenicity scoring and prediction

Pathogenicity predictions were obtained from dbNSFP database (version 3.0) (Liu et al. 2016) using ANNOVAR. Thus, each variant was annotated with CADD (Kircher et al. 2014), Functional analysis through Hidden Markov Models (FATHMM) (Shihab et al. 2013), MetaLR and MetaSVM (Dong et al. 2015), MutationAssessor (Reva et al. 2007), Likelihood Ratio Test (LRT) (Chun & Fay 2009), MutationTaster2 (Schwarz et al. 2014), Polymorphism Phenotyping v2 (PolyPhen-2) (Adzhubei et al. 2013), Sorting Intolerant From Tolerant (SIFT) (Ng & Henikoff 2003), Protein Variation Effect Analyzer (PROVEAN) (Adzhubei et al. 2013) and Variant Effect Scoring Tool (VEST) (Carter et al. 2013). Likewise, all candidate variants were assessed according to the standards and guidelines described by the ACMG and the AMP (Richards et al. 2015). These annotations were used for prioritizing and classifying each variant in pathogenic, likely pathogenic, unknown significance, likely benign and benign.

4.3.8. Variant validation

4.3.8.1. Integrative Genome Viewer

Candidate variants were visually verified in BAM files using the Integrative Genomics Viewer (IGV) (Thorvaldsdóttir et al. 2013). IGV was used to assess the mapped reads in each genomic region where a candidate variant is located.

4.3.8.2. Sanger sequencing

Rare and novel candidate variants were validated using Sanger sequencing with a 3130 Genetic Analyzer (Applied Biosystems) and they were visualized with Sequence Scanner Software 2.0 (Applied Biosystems). Primers were a) designed in the regions flanking the variants by Primer3 v4.1 (http://bioinfo.ut.ee/primer3/), b) checked if they were specific for the region of interest by Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and c) checked for primer heterodimers between forward and reverse primers by Oligoanalyzer (https://eu.idtdna.com/calc/analyzer/).

4.3.9. Statistics

For each selected variant in the SRVA, odds ratio (OR) with 95% confidence interval (CI) were calculated using the MAF values from the CSVS database (N=1,942), the NFE population (N=33,365) from ExAC, and the NFE population from gnomAD v3 (N=32,299). A 2 x 2 contingency table was applied counting alternate allele for each variant in cases and controls.

For GBA, we counted the total exonic alternate alleles per gene in our cohort against the three reference datasets. The GBA assumes that all selected variants within a given gene have

an additive effect and we considered that all variants have the same weight. The OR were calculated using the combined allele frequency of the selected variants for each gene.

Allele	A	a
Cases	m11	m12
Controls	m21	m22

A: Alternate allele

$$OR = \frac{m11 * m22}{\mathrm{m12} * \mathrm{m21}}$$

After calculating OR with 95% CI, we obtained one-sided p-values that were corrected for multiple testing by the total number of genes encoded by human genome (20,000) (Abdellah et al. 2004) following the Bonferroni approach.

Standard audiometric evaluations for air and bone conduction elicited by pure tones from 125 to 8000 Hz were retrieved from the clinical records to analyze the time course of the hearing profile in FMD cases with candidate variants. Regression analysis was performed to estimate the outcome of hearing loss for each frequency.

5. Results

5.1. Phenix

A subset of 82 patients was analyzed using the Phenix pipeline as a preliminary study of the genetic background of our MD cohort. The results from this analysis showed that **40% of familial MD cases and 68% of sporadic MD cases carried, at least, a novel or ultrarare variant in genes related with SNHL** (Table 7).

GENE	Position	REF	ALT	Variant Function	CADD	MAF (ExAC-NFE)	MAF (CSVS)
ESPN	chr1:6488328	C	T	Nonsyn SNV	33	4.1x10-4	1x10-3
	chr1:6509058	C	T	Nonsyn SNV	22.8	0	0
DIAPH1	chr5:140953193	G	C	Nonsyn SNV	23.1	4.0x10-4	1.0x10-3
TJP2	chr9:71845053	G	T	Nonsyn SNV	27.9	4.5x10-5	0
TECTA	chr11:120998763	G	A	Nonsyn SNV	25.1	7.5x10-5	0
	chr11:121008594	G	C	Nonsyn SNV	23.5	7.5x10-5	2x10-3
	chr11:121023689	G	C	Nonsyn SNV	27.2	0	1x10-3
	chr11:121028725	T	C	Nonsyn SNV	23.5	1.2x10-4	1x10-3
	chr11:121036077	C	T	Nonsyn SNV	23.1	0	0
MYO7A	chr11:76922875	G	A	Nonsyn SNV	21.7	4.0x10-4	0
	chr11:76925719	G	A	Nonsyn SNV	23.0	0	0
	chr11:76890920	G	A	Nonsyn SNV	24.4	4.8x10-4	0
	chr11:76910646	G	A	STOP gain	44	0	0
	chr11:76885923	G	A	Nonsyn SNV	32	1.1x10-4	3x10-3
USH1C	chr11:17531093	G	C	Nonsyn SNV	23.6	8.4x10-4	1x10-3
	chr11:17552836	C	G	Nonsyn SNV	24.8	0	0
DIABLO	chr12:122701379	G	C	Nonsyn SNV	26.7	0	0
GJB2	chr13:20763437	A	G	Nonsyn SNV	29.6	0	0
MYH14	chr19:50785088	A	G	Nonsyn SNV	28.9	0	0
	chr19:50810310	C	T	Nonsyn SNV	29.9	0	1x10-3
MYH9	chr22:36691716	C	T	Nonsyn SNV	26.4	6.7x10-5	0

Table 7: *Candidate variants in SNHL genes found using Phenix pipeline. REF: Reference allele; ALT: Alternative allele; MAF: Minor allele frequency.*

Among these results, we highlight a novel nonsynonymous heterozygous variant in ***DIABLO* gene** (chr12:122701379G>C) which was validated by Sanger sequencing in three affected relatives; a rare nonsynonymous heterozygous variant in ***TECTA* gene** (chr11:121028725T>C) validated in four relatives; and a novel nonsynonymous variant **in *MYH14* gene** (chr19:50785088A>G) validated in three relatives (Figure 11).

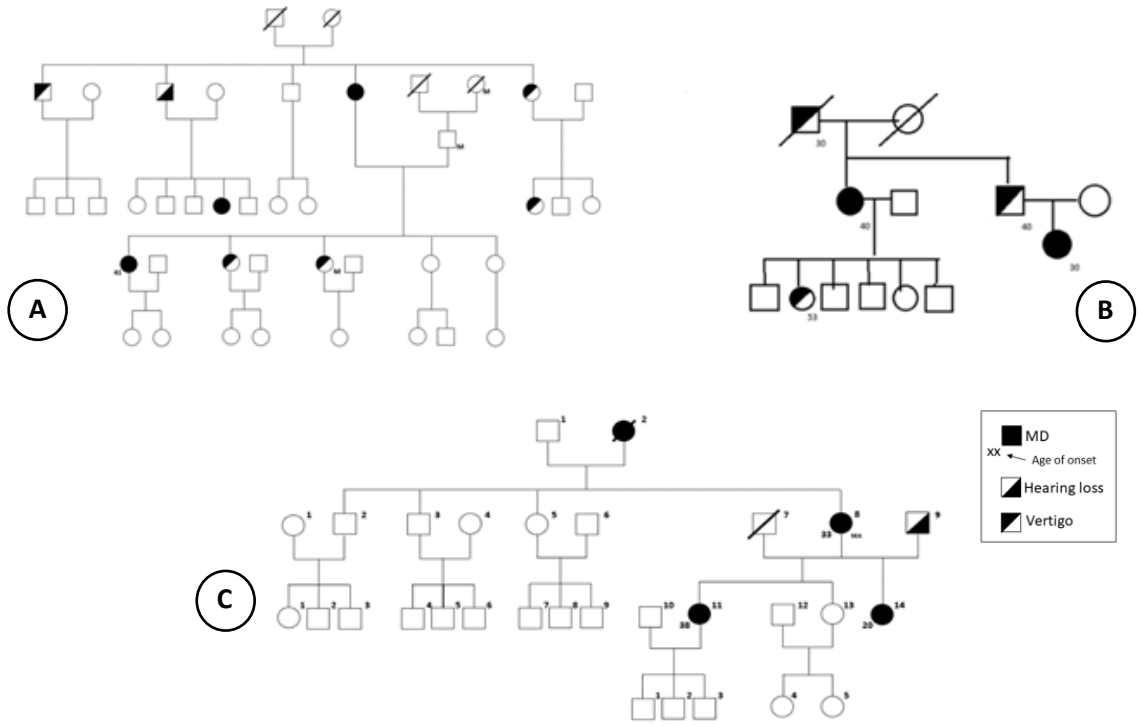

***Figure 11**: Pedigrees of families carrying variants in A) TECTA gene,* B) *MYH14* gene and C) *DIABLO* gene.

These variants were classified as likely pathogenic according to its CADD value (>15). Table 8 shows the phenotypes of the cases considered for this analysis.

Variables (%)		HPO term	More information
Gender			
Female	58 (70.7)	-	-
Male	24 (29.3)	-	-
Age (yo)			
≤20	7 (8.5)	-	-
(20-30]	23 (28.0)	-	-
(30-40]	24 (29.3)	-	-
>40	28 (34.2)	-	-
Subtype			
Familial MD	58 (70.7)	-	-
Sporadic MD	24 (29.3)	-	-
Laterality			
Unilateral	43 (52.4)	HP:0009900	https://hpo.jax.org/app/browse/term/HP:0009900
Bilateral	39 (47.6)	HP:0008619	https://hpo.jax.org/app/browse/term/HP:0008619

Other conditions			
Headache	33 (40.2)	HP:0002315	https://hpo.jax.org/app/browse/term/HP:0002315
Migraine	16 (19.5)	HP:0002076	https://hpo.jax.org/app/browse/term/HP:0002076
Type 1 diabetes mellitus	1 (1.2)	HP:0100651	https://hpo.jax.org/app/browse/term/HP:0100651
Type 2 diabetes mellitus	4 (4.9)	HP:0005978	https://hpo.jax.org/app/browse/term/HP:0005978
Hypertension	6 (7.3)	HP:0000822	https://hpo.jax.org/app/browse/term/HP:0000822
Hypercholesterolemia	4 (4.9)	HP:0003124	https://hpo.jax.org/app/browse/term/HP:0003124
Inflammatory bowel	1 (1.2)	HP:0002037	https://hpo.jax.org/app/browse/term/HP:0002037
Hypothyroidism	1 (1.2)	HP:0000821	https://hpo.jax.org/app/browse/term/HP:0000821
Asthma	4 (4.9)	HP:0002099	https://hpo.jax.org/app/browse/term/HP:0002099

Table 8: *Clinical features and HPO terms given as input to Phenix pipeline for MD patients included in this study.*

5.2. SNHL gene set analysis

Motivated by the results obtained by Phenix pipeline, a single rare variant analysis and a gene burden analysis focused on SNHL genes were performed in a larger cohort of MD patients to identify rare variants in these genes. Seventy-three MD patients from 46 different families and 36 early onset sporadic MD patients were included in this analysis.

5.2.1. Single rare variant analysis on SNHL genes

In the SRVA, a total of 5136 variants were found in FMD patients (N=46) on SNHL genes. After applying quality controls (QC), 4247 SNV remained. Only 114 nonsynonymous or splice site SNV fulfilled the MAF (<0.001) filtering criteria in at least one reference population dataset (Figure 12). From them, 80 SNV were already described in the NFE population (ExAC/gnomAD databases) or Spanish population (CSVS database) (Supplementary Table 2)

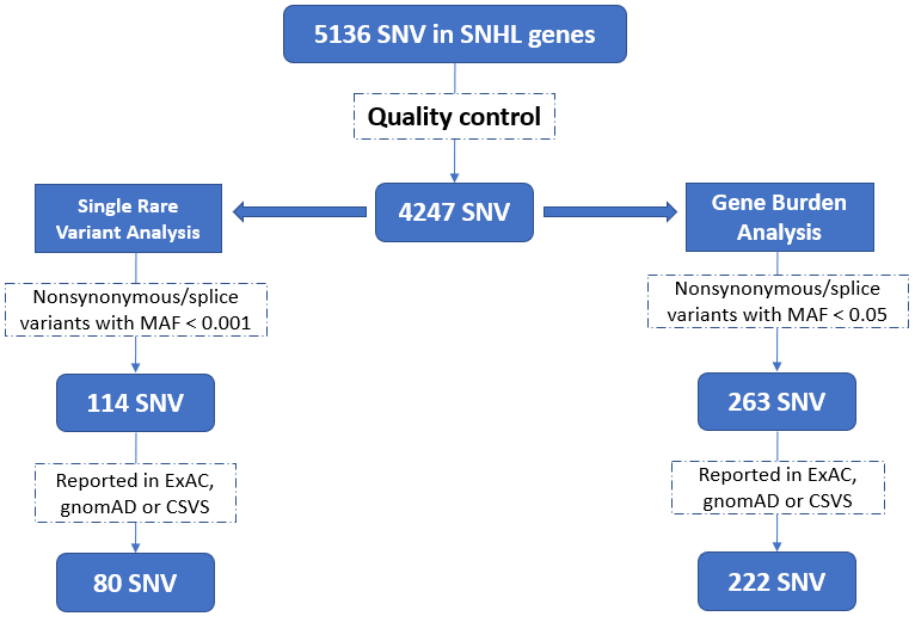

Figure 12: *Flowchart summarizing the bioinformatic analysis on familial MD cases.*

Some of these rare variants could be highlighted. A heterozygous variant located in ***OTOG* gene** was observed in cases from two unrelated families (F1 and F14). The variant located in chr11:17574758G>A (rs552304627; p.V141M), is in the last nucleotide of the fourth exon in the *OTOG* canonical transcript (ENST00000399391), and it was classified as likely pathogenic according to the ACMG and AMP guidelines. This multiplex variant is in a Von Willebrand Factor D-type domain (vWD) with a MAF=0.0008 in NFE population from ExAC and a MAF=0.0011 in NFE population from gnomAD. Multiple in silico tools supported a likely pathogenic effect of this variant (SIFT score=0.001; M-CAP=0.153; CADD=28.2; GERP++=5.36). In addition, a heterozygous missense variant located in ***DMXL2* gene** (chr15:51828804C>A) was found in two cases from two unrelated families (F17 and F31). This variant was classified as a variant of uncertain significance (VUS) according to the ACMG and AMP guidelines. As far as its frequency in reference populations is concerned, it has been only described in Latino population from gnomAD v3 with a frequency of 0.00007. These variants were validated by Sanger sequencing (Supplementary Figure 1).

The rest of the rare SNV were considered private familial variants because none of them were found in other FMD cases.

5.2.2. Gene burden analysis on SNHL genes

Seventy-four genes with 222 SNV fulfilling the criteria of this analysis (MAF≤0.05 and reported in ExAC, gnomAD or CSVS) were retained after QC and filtering steps in FMD cases (Figure 12). Most of the genes (72%) carried less than 3 variants, thus they were discarded for

further analysis. An **enrichment of rare missense variants in *OTOG* gene in FMD cases** against either NFE population from ExAC (OR= 3.7 (2.4-5.7), p=3.3x10^{-8}) and gnomAD (OR= 4.0 (2.6-6.1), p= 4.6x10^{-9}) or Spanish population from CSVS (OR= 3.0 (1.9-4.8), p= 1.2x10^{-5}) was the most significant finding. **Nine different rare missense variants in *OTOG* gene were found in 14/46 non-related families** (Supplementary figure 2), existing 6 families with 2 or more shared variants (Table 9 and supplementary table 3). The variants rs61978648 and rs61736002 were shared by individuals from 4 unrelated families. Likewise, the variants rs552304627 and rs117315845 were found in patients from other 2 unrelated families.

Moreover, a novel missense variant in *OTOG* gene (chr11:17594747C>A) was found in two cases from a 15th family. This variant, not included in the GBA and located in exon 18, was found in heterozygous state affecting the sequence of the C8 domain. The distribution of the variants found in *OTOG* is scattered across the gene sequence (Figure 13).

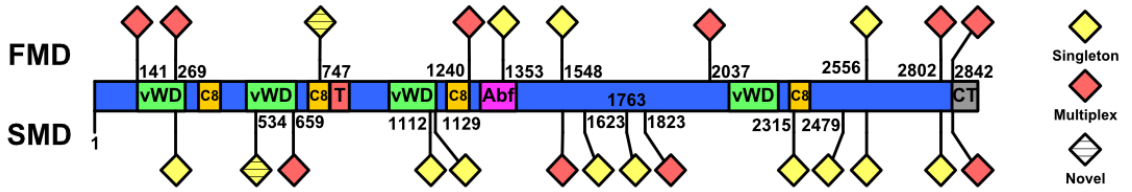

Figure 13: *Variant distribution across OTOG gene domains. On the upper part, variants which were found in familial MD (FMD) patients. On the bottom, variants which were found in sporadic MD (SMD) patients. Variants p.V269I, p.L1548F, p.R2802H and p.K2842N were observed in both FMD and SMD cases. Yellow-colored variants indicate variants found in only one patient, whereas red-colored variants represent variants found in 2 or more cases in a cohort. vWD, von Willebrand factor type D domain; T, Trypsin inhibitor-like domain; Abf, Alpha-L-arabinofuranosidase B domain; CT, Cysteine knot domain.*

5.2.2.1. Assessment of the *OTOG* familial endophenotype

We studied the hearing profile for the 14 patients (3 males, 11 females) with rare variants in *OTOG* gene (Supplementary figure 3). Ten of them showed bilateral hearing loss, 3 had left-sided hearing loss and only 1 patient showed right-sided SNHL (Supplementary table 4). From these 14 patients, 16 ears from 12 patients showed a flat shaped audiogram (57.1%), 5 ears from 5 patients showed a ski-slope shaped audiogram (17.8%), 3 ears from 3 patients showed a reverse-slope shaped (10.7%) and 4 ears had a normal pure-tone audiogram (14.2%).

The hearing loss at onset and the outcome for each frequency was estimated by regression analysis. Significant negative correlations were found at 1000 Hz (R2=0.143; p=0.033) and 2000 Hz (R2=0.246; p=0.004). There was no statistical correlation at 125 Hz, 250 Hz, 500 Hz, 4000 Hz nor 8000 Hz, suggesting no progression at these frequencies (Figure 14). The age of onset of the symptoms was 41.93±8.66 and the estimated hearing loss at onset was 62.14±12.83 for low frequencies (125-250-500 Hz) and 58.75±14.1 for high frequencies (1000-2000-4000 Hz).

Variant position	Exon	Families	Sporadic cases	MAF FMD	MAF ALL MD	MAF NFE	
						ExAC	GnomAD
11:17574758G>A	4	F1; F14	—	0.041 (3/73)	0.028 (3/109)	0.00080	0.0011
11:17578774G>A	7	F2; F3; F4; F5	S24	0.068 (5/73)	0.055 (6/109)	0.0090	0.0041
11:17594747C>A*	18	F34	—	0.027 (2/73)	0.018 (2/109)	—	—
11:17621218C>T	30	F6; F7	—	0.027 (2/73)	0.018 (2/109)	0.0026	0.0058
11:17627548G>A	32	F14	—	0.012 (1/73)	0.009 (1/109)	0.0056	0.0045
11:17631453C>T	35	F8	S11; S24	0.012 (1/73)	0.028 (3/109)	0.017	0.011
11:17632921C>T	35	F2; F3; F4; F5	—	0.068 (5/73)	0.046 (5/109)	0.0015	0.0011
11:17656672G>A	45	F10	S9	0.013 (1/73)	0.018 (2/109)	0.0034	0.0052
11:17663747G>A	52	F1; F13; F14	S7	0.055 (4/73)	0.046 (5/109)	0.0058	0.0024
11:17667139G>C	54	F9; F11; F12	S12; S20	0.082 (6/73)	0.073 (8/109)	0.019	0.023

Table 9: *Rare variants found in the GBA on SNHL in OTOG gene for familial MD patients*

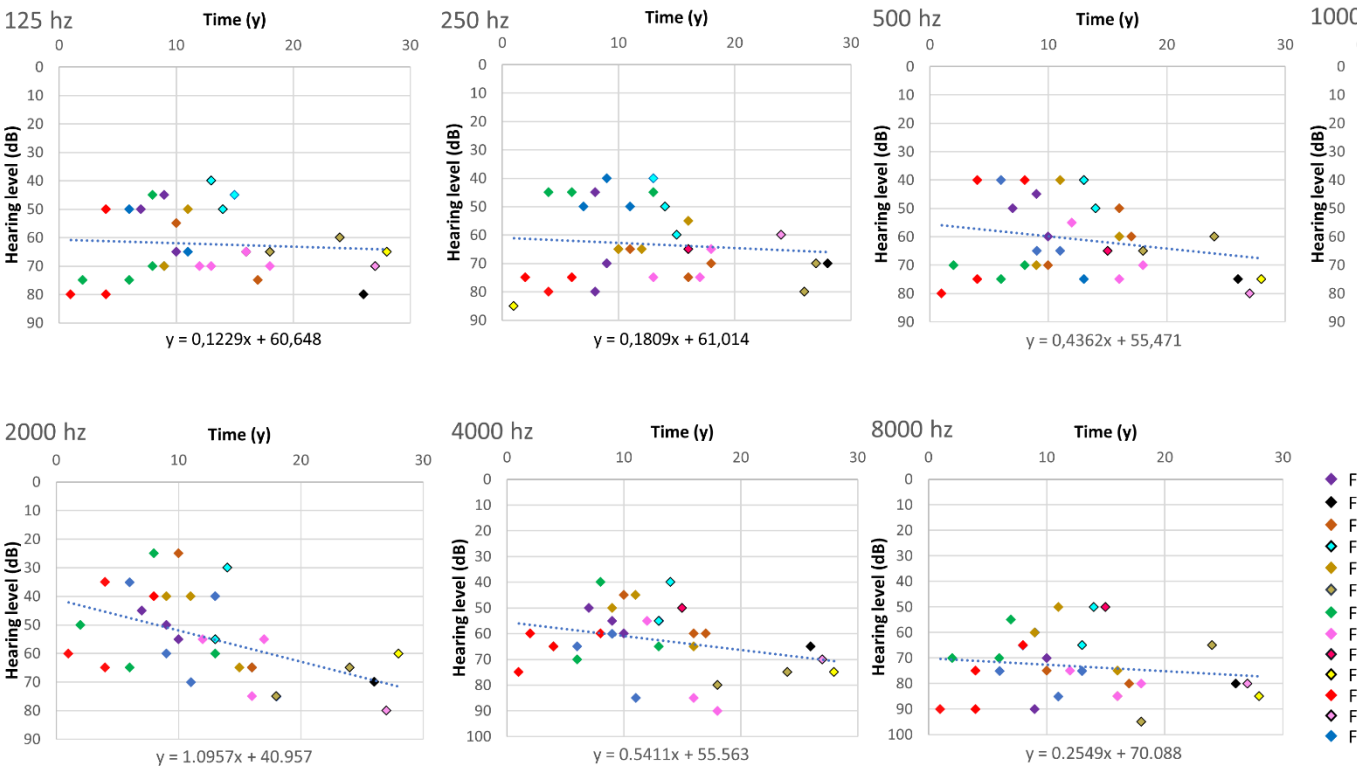

Figure 14: *Scattered plot showing air conduction hearing thresholds obtained and the duration of the disease for each fre*

5.2.3. SNHL gene set analysis in early onset sporadic MD

The same bioinformatic analyses were performed in a series of patients with sporadic MD with an age of onset younger than 35 (Figure 15). Sixty-six nonsynonymous or splice site SNV with MAF < 0.001 were found in the SRVA in 48 SNHL genes. Among them, three variants were found in two sporadic cases and another variant was also found in a familial case. The rest of the SNV were considered simplex variants found in singletons and none of them were found in homozygous state (Supplementary table 5).

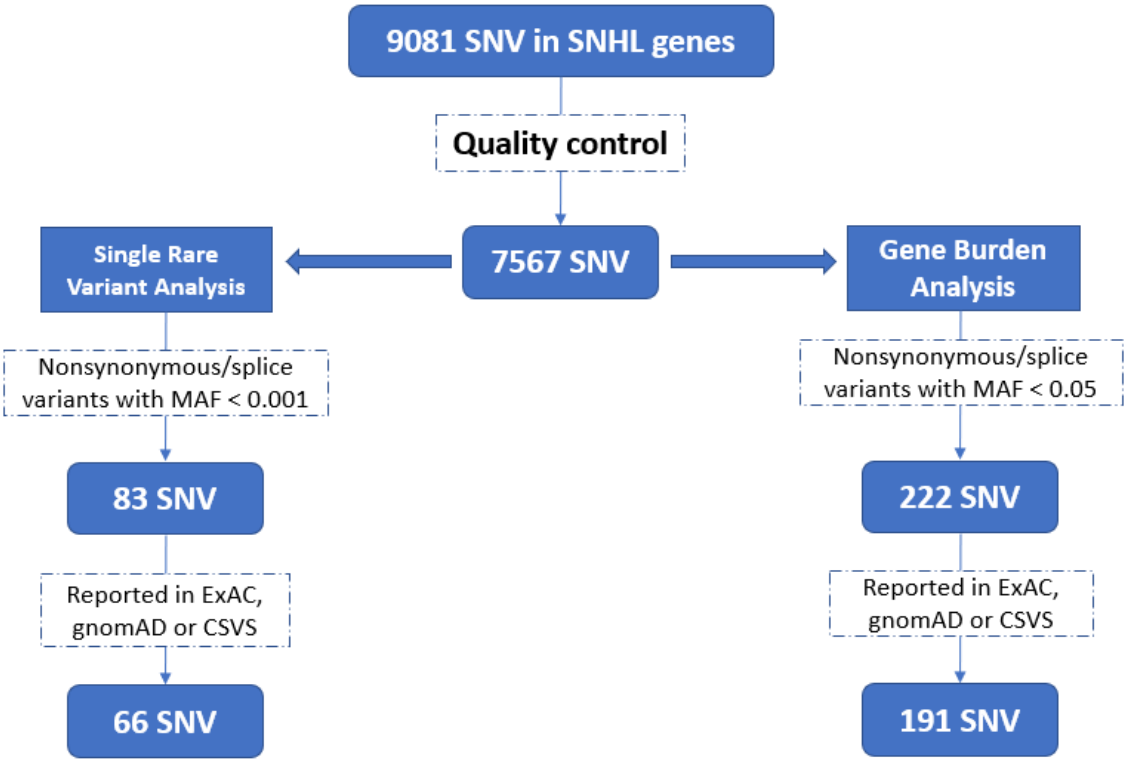

Figure 15: *Flowchart summarizing the bioinformatic analysis on familial SD cases. SRVA: Single rare variant analysis; GBA: Gene burden analysis*

A heterozygous nonsynonymous SNV was found in ***OTOG* gene** carried by two unrelated sporadic MD cases. The variant chr11:17632279C>T (rs779658224; p.A1823V), located in exon 35 of the canonical transcript of *OTOG* gene, was classified as a VUS according to the ACMG and AMP guidelines. This variant has a MAF=0.00054 in the NFE population from gnomAD v3 and it is not described in the Spanish reference population from the CSVS. In addition, two heterozygous nonsynonymous variants were found in two sporadic MD cases in ***DMXL2* gene**. These two SNVs (rs762424714; p.H2287N & rs117017152; p.I699T) are not located in any known domain of this protein and both are classified as likely benign according to their CADD scores (9.64 and 9.74, respectively). Finally, a heterozygous nonsynonymous SNV in ***OTOGL* gene** was

found in one sporadic case and in one familial case. The variant chr12:80752642T>G (rs145929269; p.C2068G) is in exon 51 of the canonical transcript of *OTOGL* gene (ENST00000458043), a region which encodes a cysteine-rich region. This variant was also classified as a VUS according to the ACMG and AMP guidelines.

Thirteen rare SNV were found in *OTOG* gene fulfilling the GBA criteria in patients with early onset MD (Table 10). However, in contrast with the results obtained in FMD cases, **there was not an excess of rare variants in this gene** against neither the NFE population from ExAC (OR=2.0 (1.2-3.2), p=0.067) or gnomAD (OR=2.0 (1.2-3.2), p=0.075) nor Spanish population (OR=1.9 (1.1-3), p=0.16).

Position	Exon	Sporadic cases	MAF NFE		MAF CSVS	CADD	Domain
			ExAC	gnomAD			
chr11:17578774G>A	7	S24	0.0090	0.0041	0.018	15.95	vWD
chr11:17591922C>T	16	S1; S12	0.032	0.035	0.030	25.4	vWD
chr11:17615604C>T	27	S12	0.00090	0.00079	0.0070	23.1	vWD
chr11:17615655C>T	27	S12	0.00090	0.00076	0.0070	33	vWD
chr11:17631453C>T	35	S11; S24	0.016	0.011	0.016	12.89	—
chr11:17631679C>G	35	S2	—	0.000062	—	23.1	—
chr11:17632099G>A	35	S5	0.00060	0.0019	0.0030	0.073	—
chr11:17632279C>T	35	S1; S23	0	0.00054	—	26.4	—
chr11:17653443C>T	40	S13	0.00060	0.0013	0.0030	34	C8
chr11:17655748G>A	43	S1	0.031	0.034	0.022	14.23	—
chr11:17656672G>A	45	S9	0.0034	0.0052	0.0040	31	—
chr11:17663747G>A	52	S7	0.0058	0.0024	0.0060	19.41	—
chr11:17667139G>C	54	S12; S20	0.018	0.023	0.019	27.2	CT

Table 10: Rare variants found in the GBA on SNHL in OTOG gene for sporadic MD patients

5.3. Hypothesis-free data-driven analysis

After analyzing the SNHL gene set, we were able to recruit more familial and sporadic patients, obtaining new DNA samples for WES. Next, we analyzed all the genes within the human genome. Thus, we increased the total number of FMD cases to 94 cases from 62 unrelated families and the number of early-onset SMD cases to 44.

5.3.1. Familial Meniere Disease

5.3.1.1. Single rare variant analysis

After applying QC, 14855 SNV remained with a MAF≤0.001 in at least one reference population dataset. From them, 10466 SNV were already described in the NFE population or

Spanish population. Only 627 nonsynonymous or splice site SNV in 570 genes were found in 2 or more cases, and 65 variants in 65 genes were found in 3 or more cases.

Among these variants, 3 of them could be highlighted since they were found in cases from three unrelated families: **a)** A heterozygous variant, with a MAF=0.00018 in NFE population from gnomAD and MAF=0.001 in the Spanish population from CSVS, located in ***ALPK1* gene** was observed in cases from families F1, F13 and F25. This variant chr4:113350387C>A (rs116427224) was classified as likely pathogenic according to multiple in silico tools (SIFT score=0; CADD=34; GERP++ =5.4); **b)** A heterozygous variant, with a MAF=0.00034 in NFE population from gnomAD and MAF=0.002 in the Spanish population from CSVS, located in ***NEK5* gene** was observed in cases from families F29, F44 and F61. This variant chr13:52650273C>T (rs139136964) was classified as likely pathogenic according to multiple in silico tools (SIFT score=0.028; CADD=23; GERP++ =4.65); **c)** A heterozygous variant, with a MAF= 0.00014 in NFE population from gnomAD and MAF=0.001 in the Spanish population from CSVS, located in ***GNAS* gene** was observed in cases from families F41, F45 and F61. This variant chr20:57428474G>A (rs527488103) was classified as likely pathogenic according to several in silico tools (SIFT score=0.002; CADD=18.17), however, this variant had a low conservation score (GERP++=-9.12). Furthermore, a variant in ***PIEZO2* gene** was identified in two patients from unrelated families (PT60 and EX125). The variant chr18:10671666C>T, with a MAF=0.0001 in NFE population from gnomAD and MAF=0.006 in the Spanish population from CSVS, was found in homozygous state in one of these two patients. It was classified as likely pathogenic according to multiple in silico tools (SIFT=0, Polyphen=0.992, GERP=4.620, CADD=34, M-CAP=0.449).

5.3.1.2. Gene burden analysis

Following the same strategy explained before for the GBA, 26757 variants with a MAF≤0.05 and located in 11093 genes were retained after QC and filtering steps. As it happened in the GBA with SNHL genes, most of the genes (82%) carried less than 3 rare variants, being discarded for further analysis. Of the genes remaining, only **100 genes had a significant enrichment of rare variants** (corrected p-value < 0.05) in our FMD cases against the Spanish population from CSVS (Table 11). The gene with variants in more cases (17/62) and most significant when it was compared to the 3 control populations was ***DCP1B* gene**. On the other hand, the gene with more variants in our FMD cohort was ***ACAN* gene**, with 16 different rare missense variants in 13/62 cases.

Gene	# Variants	# Cases	OR (CI)			Corrected p-value		
			CSVS	ExAC	gnomAD	CSVS	ExAC	gnomAD
ACAN	16	13	3.2(2-5.1)	4(2.5-6.3)	3.3(2.1-5.2)	0.040	$3.8x10^{-5}$	0.0052
MYO7A	12	12	3.9(2.3-6.8)	3.5(2.1-5.8)	4.5(2.7-7.4)	0.012	0.017	0.00019
ALDH16A1	11	10	5.4(2.9-10.1)	3.6(2.1-6.5)	4.3(2.4-7.6)	0.0014	-	0.011
RADIL	11	15	3.2(2.2-4.5)	1.9(1.3-2.6)	2.7(1.9-3.7)	$1.1x10^{-5}$	-	0.00065
CFAP65	10	8	10.4(5.1-21.4)	20.8(11-39.2)	29.6(15.7-55.7)	$3.8x10^{-6}$	$<10^{-15}$	$<10^{-15}$
KIF17	10	10	4.3(2.4-7.7)	4.1(2.4-7.2)	5.4(3.1-9.4)	0.019	0.0046	$2.7x10^{-5}$
KIF26A	10	12	5.5(3-10.1)	2.2(1.3-3.9)	3.9(2.2-6.8)	0.0013	-	-
UNC5B	10	21	2.9(1.9-4.4)	2.3(1.5-3.4)	2.3(1.5-3.4)	0.016	-	-
CCDC116	9	16	3.5(2.4-5.1)	2(1.4-2.9)	2(1.4-2.9)	$1.6x10^{-6}$	-	-
OPRM1	9	7	7.8(4.1-14.8)	7.2(4.1-12.8)	8.1(4.6-14.4)	$4.4x10^{-6}$	$1.6x10^{-7}$	$1.4x10^{-08}$
PCDHAC1	9	9	4.5(2.7-7.2)	3.2(2-5)	3.4(2.2-5.4)	$3.1x10^{-5}$	0.012	0.0021
ADAMTSL4	8	21	2.6(1.8-3.9)	2(1.4-2.9)	2(1.4-2.9)	0.023	-	-
ANO7	8	6	8.1(3.7-17.6)	6(3-12)	9.7(4.8-19.6)	0.0030	0.0069	$3.5x10^{-6}$
DCP1B	8	17	2.7(2.1-3.4)	2.1(1.6-2.7)	2.2(1.7-2.8)	$1.9x10^{-10}$	$2.8x10^{-5}$	$1.5x10^{-6}$
MTCL1	8	10	4.7(2.6-8.5)	2.4(1.4-4.2)	2.5(1.5-4.4)	0.0043	-	-
ATG2B	7	7	9.7(4.1-22.8)	4.8(2.3-10.2)	4.9(2.3-10.3)	0.0034	-	-
ATP8B4	7	12	5.2(2.9-9.3)	3.8(2.2-6.5)	3.9(2.2-6.7)	0.0011	0.047	0.030
CFAP43	7	8	5.3(2.7-10.4)	7.8(4.2-14.7)	8.7(4.6-16.3)	0.026	$2.5x10^{-6}$	$2.7x10^{-7}$
CSPG4	7	7	21.2(9-49.7)	5.9(3-11.4)	19.4(10-37.6)	$4.5x10^{-8}$	0.0018	$<10^{-15}$
KIF14	7	13	5.8(3.2-10.5)	16.8(9.6-29.3)	15.3(8.8-26.6)	0.00016	$<10^{-15}$	$<10^{-15}$
NLRP6	7	7	8.8(4-19.3)	7(3.5-14.2)	12.9(6.4-26)	0.0014	0.00099	$1.7x10^{-8}$
PIK3C2B	7	10	6.1(3.2-11.8)	3.2(1.8-5.8)	4.8(2.6-8.6)	0.00092	-	0.0060
PPL	7	6	9.3(4-21.8)	15.8(7.4-33.6)	16.3(7.7-34.6)	0.0051	$9.0x10^{-9}$	$6.1x10^{-9}$
THADA	7	8	5.2(2.7-10.2)	4.2(2.2-7.8)	4.6(2.4-8.5)	0.031	-	0.038
VWA5B1	7	8	5.7(2.8-11.7)	5.8(3-11.1)	8.5(4.4-16.5)	0.034	0.0040	$3.9x10^{-6}$
WDR49	7	8	5.2(2.8-9.7)	8.4(4.7-14.9)	7.7(4.4-13.7)	0.0031	$8.0x10^{-9}$	$4.7x10^{-8}$
WDR90	7	8	11.7(5.2-26.5)	16.5(8.1-33.5)	20(9.9-40.3)	$7.4x10^{-5}$	8.9E-11	$<10^{-15}$
ANP32C	6	4	18.3(6.6-50.5)	232.5(89.1-606.8)	150(63-357.1)	0.00039	$<10^{-15}$	$<10^{-15}$
BRWD1	6	5	9.3(3.7-23.3)	6.7(3-15.1)	7.6(3.4-17)	0.035	0.046	0.017
CEP170B	6	5	27.3(10.5-71)	4.7(2.3-9.4)	7.5(3.7-15)	$2.3x10^{-7}$	-	0.00035

Table 11: *Top 30 genes with an enrichment of rare variants (MAF≤0.05) in FMD patients against Spanish control population. Genes are ordered by number of rare variants. The symbol '-' indicates not significance (corrected p-value>0.05).*

In contrast to the GBA carried out with the SNHL genes dataset, where genes were selected according to a phenotype (SNHL), the related phenotypes of the genes in this analysis were not known in advance. Thus, through searches in databases and relevant literature, each gene was associated to a phenotype for prioritizing purposes (Table 12). Interestingly, ***ACAN* and *MYO7A* genes have been related before to deafness in mice** (MP:0001967), being ***MYO7A* gene also associated to autosomal dominant deafness 11 (DFNA11) in humans**.

Gene	Mammalian Phenotype (MP)		OMIM	
	ID	Term	ID	Disease
ACAN	MP:0001967	Deafness	OMIM:612813	Spondyloepimetaphyseal Dysplasia
	MP:0004403	Absent cochlear outer hair cells		
KIF14	MP:0001525	Impaired balance	OMIM:616258	Meckel Syndrome 12
	MP:0000433	Microcephaly	OMIM:617914	Microcephaly 20
MYO7A	MP:0001967	Deafness	OMIM:601317	Deafness, autosomal dominant 11
RADIL	MP:0000689	Abnormal spleen morphology	-	-
CFAP65	MP:0002674	Abnormal sperm motility	-	-
KIF17	MP:0004768	Abnormal axonal transport	-	-
KIF26A	MP:0003651	Abnormal axon extension	-	-
ANP32C	-	-	-	-
UNC5B	MP:0001648	Abnormal apoptosis	-	-
CCDC116	-	-	-	-
PCDHAC1	-	-	-	-
ADAMTSL4	MP:0002092	Abnormal eye morphology	OMIM:225100	Ectopia Lentis
ANO7	MP:0010053	Decreased grip strength	-	-
MTCL1	MP:0001516	Abnormal motor coordination/balance	-	-
WDR90	-	-	-	-
ATG2B	-	-	-	-
ATP8B4	-	-	-	-
CSPG4	MP:0005331	Insulin resistance	-	-
WDR49	-	-	-	-
PPL	MP:0002060	Abnormal skin morphology	OMIM:169610	Paraneoplastic Pemphigus
PIK3C2B	-	-	-	-
THADA	-	-	-	-
VWA5B1	-	-	-	-
BRWD1	MP:0000242	Impaired fertilization	-	-
OPRM1	MP:0002912	Abnormal excitatory postsynaptic potential	-	-
CEP170B				
CFAP43	MP:0001925	Male infertility	OMIM:236690	Hydrocephalus, Normal pressure
DCP1B	-	-	-	-
NLRP6	MP:0008537	Increased susceptibility to induced colitis	-	-
ALDH16A1	MP:0001765	Abnormal ion homeostasis	-	-

Table 12: *Associated phenotype and disease for TOP30 genes with an enrichment of rare variants in FMD patients (MAF≤0.05))*

Since the total number of variants in the callset was larger in the WES dataset than in the SNHL dataset, a **GBA taking as a cutoff a MAF≤0.005 was also done**. A total of 15706 variants located in 8544 genes were retained after QC and filtering steps. From these genes, 7773 (90.98%) carried less than 3 rare variants, and **188 genes with 3 or more variants showed a significant enrichment** (corrected p-value < 0.05) in FMD cohort against the Spanish control population. The gene with more variants in our FMD cohort continued to be ***ACAN* gene**, with 15 different rare missense variants (MAF≤0.005) in 11/62 cases. On the other hand, different genes which did not show a significant accumulation of variants using a cutoff MAF≤0.05,

showed it using a cutoff MAF≤0.005. Table 13 shows the results from the GBA for the top 30 genes with a significant accumulation of variants.

Gene	# Variants	# Cases	OR (CI)			Corrected p-value		
			CSVS	ExAC	gnomAD	CSVS	ExAC	gnomAD
ACAN	15	11	9.2(5.2-16)	15.2(9.2-25)	13.4(8.1-22)	$1.5x10^{-10}$	$<10^{-15}$	$<10^{-15}$
SPTA1	14	8	6.2(3.6-10.8)	22.5(13.4-37.8)	18.5(11-30.9)	$2.3x10^{-6}$	$<10^{-15}$	$<10^{-15}$
ALDH16A1	10	9	11.2(5.6-22.3)	7.1(3.9-12.9)	9.1(5-16.6)	$1.4x10^{-7}$	$2.5x10^{-6}$	$8.7x10^{-9}$
ZNF142	10	10	12.8(6.4-26)	22(12-40.2)	30.6(16.6-56.3)	$2.3x10^{-8}$	$<10^{-15}$	$<10^{-15}$
CFAP65	10	8	10.9(5.3-22.3)	20.8(11-39.2)	37.2(19.6-70.9)	$1.8x10^{-6}$	$<10^{-15}$	$<10^{-15}$
ARHGAP8	9	12	7.7(4.2-14.3)	19.7(11.3-34.4)	24.3(13.9-42.5)	$9.2x10^{-7}$	$<10^{-15}$	$<10^{-15}$
CACNA1S	9	10	17.5(8.1-38)	8.2(4.4-15.3)	7.4(4-13.9)	$9.1x10^{-9}$	$1.1x10^{-6}$	$7.0x10^{-6}$
MYO7A	9	9	11.3(5.3-24.4)	7.9(4.1-15.4)	15.8(8.1-30.7)	$9.4x10^{-6}$	$1.6x10^{-5}$	$8.9x10^{-12}$
TICRR	8	7	15.3(6.5-35.9)	20.8(10.2-42.2)	20.6(10.2-41.6)	$6.5x10^{-6}$	$<10^{-15}$	$<10^{-15}$
CCDC40	8	9	6.2(3.3-11.6)	11.9(6.7-21.1)	13.2(7.4-23.5)	0.00017	$<10^{-15}$	$<10^{-15}$
LAMC3	8	9	7.7(4-15)	10.4(5.7-18.9)	11.6(6.3-21.1)	$2.9x10^{-5}$	$4.3x10^{-10}$	$2.7x10^{-11}$
KIF17	8	7	6.4(3.3-12.7)	16.3(8.6-30.6)	27.5(14.5-52.1)	0.0018	$<10^{-15}$	$<10^{-15}$
OPRM1	8	6	8.1(3.9-16.9)	12.4(6.4-24)	15.8(8.1-30.7)	0.00049	$2.2x10^{-9}$	$8.9x10^{-12}$
KIF26A	8	8	50.4(16.5-154.5)	14.4(7.1-29.1)	37.8(18.4-77.7)	$1.3x10^{-7}$	$2.5x10^{-9}$	$<10^{-15}$
KIF14	7	13	6(3.3-10.8)	16.8(9.6-29.3)	15.2(8.7-26.5)	$8.8x10^{-5}$	$<10^{-15}$	$<10^{-15}$
OTOG	7	12	6.2(3.3-11.6)	9.2(5.2-16.4)	6.3(3.5-11.1)	0.00017	$6.4x10^{-10}$	$6.9x10^{-6}$
NOS1	7	7	6.6(3.1-14.2)	15.5(7.6-31.3)	15.4(7.6-31)	0.028	$5.8x10^{-10}$	$4.8x10^{-10}$
KIF26B	7	9	6.5(3.1-13.3)	11.9(6.1-23.2)	20.5(10.5-40)	0.0078	$5.0x10^{-9}$	$<10^{-15}$
SPTB	7	9	8.4(4-17.5)	15.7(8.1-30.5)	14.8(7.6-28.7)	0.00034	$8.9x10^{-12}$	$4.4x10^{-11}$
UNC5B	7	7	8.1(3.9-16.9)	55(27.6-109.5)	25.7(13.1-50.5)	0.00049	$<10^{-15}$	$<10^{-15}$
GPR179	7	7	11(4.9-24.6)	7.1(3.5-14.2)	6.3(3.1-12.6)	0.00012	0.00090	0.0056
LY75	7	7	9.7(4.4-21.5)	8.6(4.2-17.3)	8.6(4.3-17.4)	0.00043	$4.0x10^{-5}$	$3.5x10^{-5}$
MYBPC2	7	5	9(4.1-19.8)	13.6(6.7-27.5)	9.7(4.8-19.6)	0.00094	$8.1x10^{-9}$	$4.4x10^{-6}$
MYH7B	7	8	6.5(3-13.9)	11(5.5-22.3)	7.9(3.9-15.9)	0.033	$4.5x10^{-7}$	0.00015
LRRN4	6	7	8.1(3.7-17.8)	19.7(9.7-40)	21.9(10.8-44.6)	0.0027	$4.4x10^{-12}$	$<10^{-15}$
ANKAR	6	6	9.4(4.2-20.7)	19.9(9.8-40.4)	26.7(13.1-54.4)	0.00064	$4.4x10^{-12}$	$<10^{-15}$
ATR	6	5	6.3(2.9-13.5)	9.2(4.5-18.5)	8.3(4.1-16.7)	0.044	$1.3x10^{-5}$	$7.4x10^{-5}$
CFH	6	7	7.4(3.8-14.4)	13(7.1-23.8)	123.6(63.9-239.3)	$5.6x10^{-5}$	$<10^{-15}$	$<10^{-15}$
DNAH14	6	8	7.9(3.6-17.2)	10(4.9-20.2)	10(4.9-20.2)	0.0039	$2.8x10^{-6}$	$2.8x10^{-6}$
GEMIN4	6	8	9.7(4.4-21.5)	9.1(4.5-18.3)	7.3(3.6-14.7)	0.00043	$1.6x10^{-5}$	0.00058

Table 13: *Top 30 genes with an enrichment of rare variants (MAF≤0.005) in FMD patients*

Table 14 shows the genes with an enrichment of rare variants in the table above which were not associated to a phenotype in table 12.

Gene	Mammalian Phenotype (MP)		OMIM	
	ID	Term	ID	Disease
SPTA1	MP:0000245	Abnormal erythropoiesis	OMIM:270970	Spherocytosis
ZNF142	MP:0001399	Hyperactivity	OMIM:618425	ND with impaired speech and hyperkinetic movements
ARHGAP8	MP:0001304	Cataract		
CACNA1S	MP:0002106	Abnormal muscle physiology	OMIM:170400	Hypokalemic periodic paralysis type 1
TICRR	-	-	-	-
CCDC40	MP:0004131	Abnormal motile primary cilium morphology	OMIM:613808	Ciliary dyskinesia type 15
LAMC3	MP:0008039	Increased NK T cell number	OMIM:614115	Cortical Malformations
NOS1	MP:0001525	Impaired balance	-	-
OTOG	MP:0006328	Nonsyndromic hearing impairment	OMIM:614945	DFNB18B
KIF26B	MP:0000527	Abnormal kidney development	-	-
SPTB	MP:0002424	Abnormal reticulocyte morphology	OMIM:616649	Spherocytosis type 2
GPR179	MP:0004021	Abnormal rod electrophysiology	OMIM:614565	Stationary night blindness
LY75	MP:0005078	Abnormal cytotoxic T cell physiology	-	-
MYBPC2	MP:0011100	Preweaning lethality	-	-
MYH7B	-	-	-	-
ANKAR	-	-	-	-
ATR	MP:0000854	Abnormal cerebellum development	OMIM:210600	Seckel syndrome 1
DNAH14	-	-	-	-
GEMIN4	MP:0009850	Embryonic lethality	OMIM:617913	ND with microcephaly, cataracts, and renal abnormalities

Table 14: *Associated phenotype and disease for genes with an enrichment of rare variants in FMD patients (MAF≤0.005).*

5.3.1.3. Gene expression of candidate genes in the inner ear

Although the evident candidate genes for FMD cases are those that are already associated to any hearing or vestibular phenotypes in human, such as *OTOG* or *MYO7A* genes, inner ear gene expression studies were reviewed in order to identify additional suitable candidate genes.

Since **hair cells (HCs) are essential for hearing and balance**, an RNA-Seq study of gene expression during mouse inner ear development (from E16 to P7) was used (Scheffer et al. 2015). So, **155 genes** from the 188 candidate genes were found in this study. These genes were classified in **three groups**: HCs-enriched genes, SCs-enriched genes and nonspecific genes. Fifty-two genes were classified as HCs-enriched genes, 31 genes as SCs-enriched genes, whereas 72 genes had nonspecific expression (Figure 16).

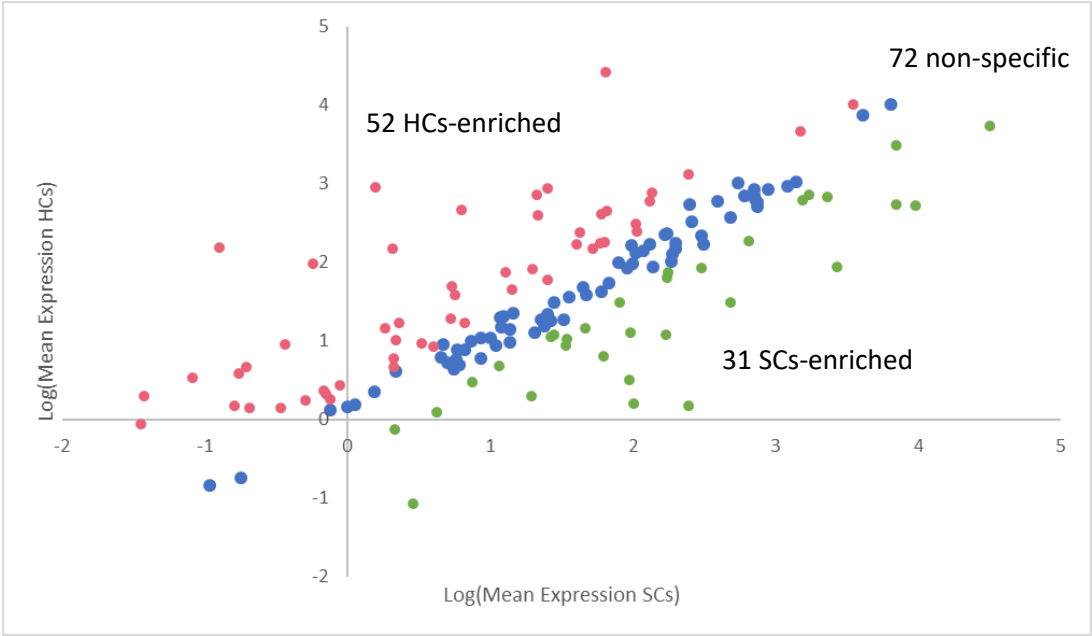

Figure 16: *Dispersion plot showing the expression of candidate genes for FMD patients in hair cells (HCs) and supporting cells (SCs). Red dots represent HCs-enriched genes. Green dots represent SCs-enriched genes. Blue dots represent genes with nonspecific expression for these two cell populations.*

Hair cells can be divided in two subpopulations: **cochlear HCs (cHCs) and utricular HCs (uHCs)**. From the 52 genes HCs-enriched, 31 genes were at least four-fold enriched in HCs compared with SCs. **Fourteen genes were classified as uHCs-enriched, 5 as cHCs-enriched** and 12 genes had nonspecific expression between HCs at postnatal stages (Figure 17). Genes such as *NEK5* (24.5-fold), *UNC5B* (16.4-fold) and *CCDC40* (5.7-fold) can be highlighted as uHCs-enriched genes, while *SEZL6* (8.2-fold), SCN11A (3.5-fold) and INSC (2.2-fold) genes are the most remarkable cHCs-enriched genes.

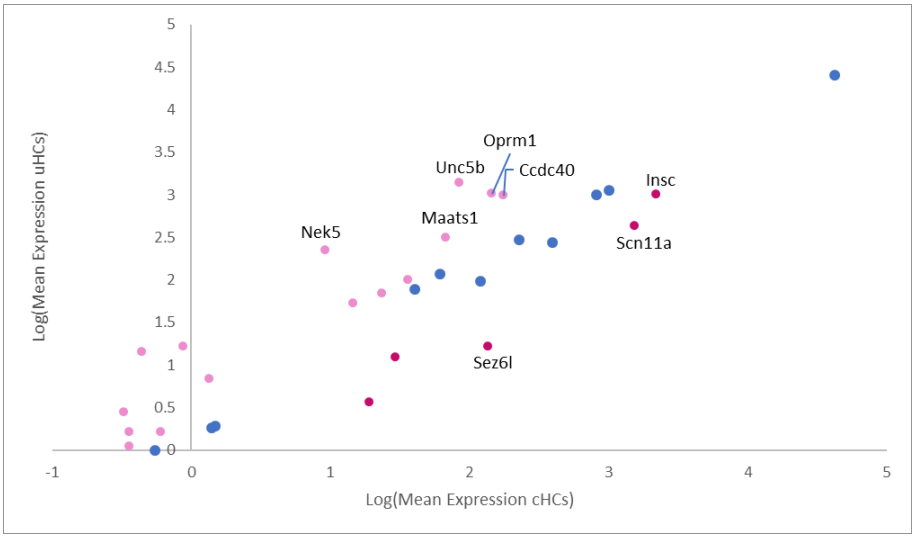

Figure 17: *Dispersion plot showing the expression of candidate genes in utricular hair cells (uHCs) and cochlear hair cells (cHCs). Light pink dots represent uHCs-enriched genes. Dark pink dots represent cHCs-enriched genes. Blue dots represent genes with nonspecific expression for these two cell populations.*

To analyze the **expression of candidate genes in SC**, data were retrieved from another RNA-Seq study in the organ of Corti examining gene expression in pillar and Deiters' cells (Liu et al. 2018). Expression data from inner and outer cochlear hair cells was retrieved as well from this study. **One hundred fifty-two genes out of 188 FMD candidate genes were found**. From them, 94 genes showed, in at least one of the four cell populations, expression specificity. **Forty and 30 genes were enriched (>2-fold) in Deiters' and pillar cells**, respectively, when their expressions in these two cells populations were compared to each other. We performed the same analysis in HCs populations, and **14 and 39 genes were differentially expressed in iHCs and oHCs**, respectively (Figure 18).

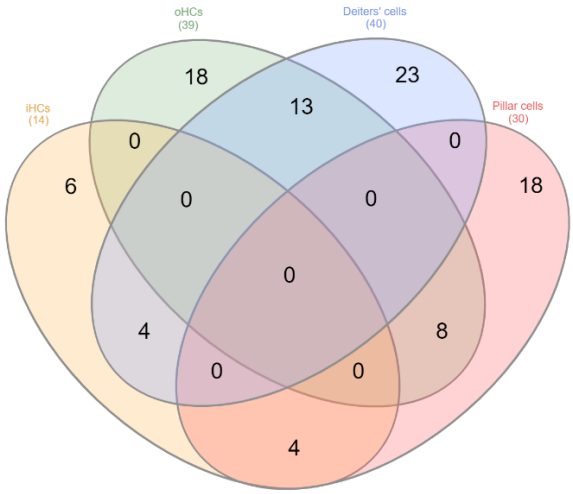

Figure 18: *Venn diagram showing the distribution of candidate genes for FMD patients according to their expression in inner hair cells (iHCs - yellow), outer hair cells (oHCs - green), Deiter's cells (blue) and pillar cells (red).*

Comparing the four populations of cochlear cells together, only *MADD* gene was specific (>2-fold against any of the other three populations) for iHCs, 8 genes for oHCs, 16 genes for Deiters' cells and 13 for pillar cells (Figure 19).

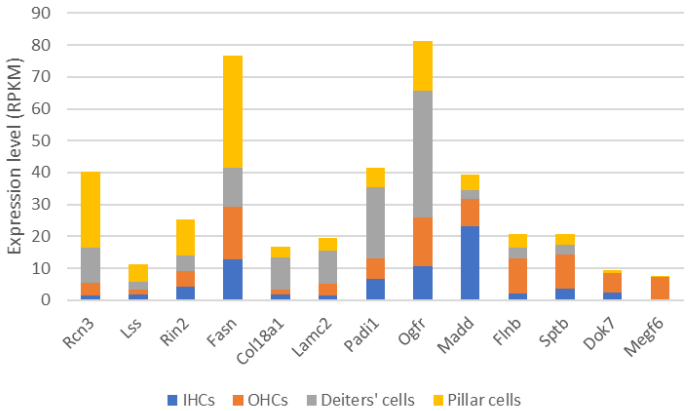

Figure 19: *Stacked bar plot showing the expression of certain candidate genes for FMD patients in inner hair cells (iHCs), outer hair cells (oHCs), Deiter's cells and pillar cells.*

5.3.1.4. Gene expression of candidate genes in spiral and vestibular ganglion neurons

The cochlear afferent nerve fibers generate nerve impulses that are transmitted from the cochlear iHC to the central nervous system throughout the **spiral ganglion (SG) neurons**. Likewise, **vestibular ganglion (VG) neurons** carry acceleration information to the brain from the vestibular hair cells in otolithic and semicircular canal organs. Thus, **genes expressed in these neurons are good candidates for auditory system development and function**.

To analyze the gene expression in SG neurons, mice expression data at six development stages (E12, E13, E16, P0, P6 and P15) from a microarray analysis was retrieved (Lu et al. 2011). So, **130 genes** out of 188 candidate genes were found in this study. Interestingly, 9 of these genes were included in a "signature gene list" consisting of genes whose expression levels differed significantly between the SG and VG neurons or/and changed significantly over time (Figure 20).

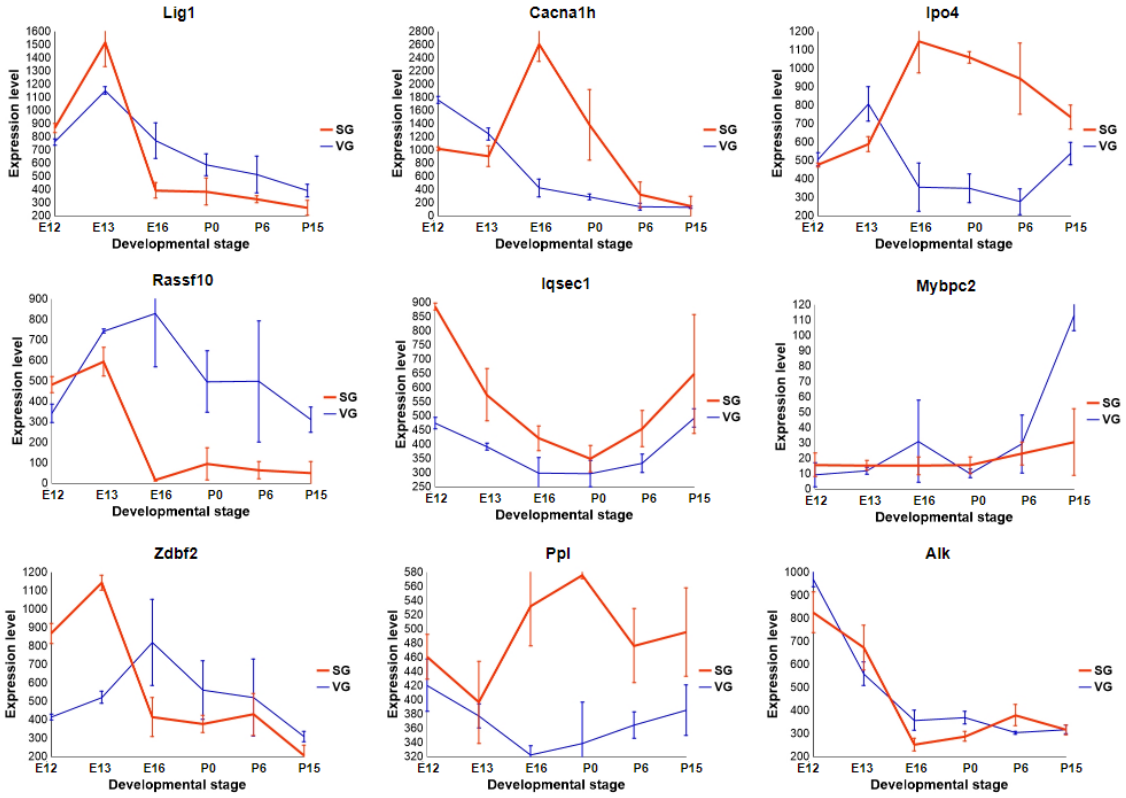

***Figure 20**: Line graph showing the expression of candidate genes included in the 'signature gene list' across different age stages (E12, E13, E16, P0, P6 and P15) in spiral ganglion neurons (SG) and vestibular ganglion neurons (VG) for FMD patients.*

Finally, average expressions for each gene were determined at each stage in both cells populations: early stage (E12-E13), mid stage (E16-P0) and late stage (P6-P15). Table 15 shows the top 10 expressed genes from each stage and cell population.

Spiral Ganglion Neurons			Vestibular Ganglion Neurons		
Early	**Mid**	**Late**	**Early**	**Mid**	**Late**
ACLY	*ACLY*	*ACLY*	*ACLY*	*ACLY*	*ACLY*
AGRN	*AGRN*	*AGRN*	*AGRN*	*AGRN*	*AGRN*
BRWD1	*CIC*	*ANK1*	*BRWD1*	*DIDO1*	*COL27A1*
DIDO1	*PCM1*	*CCDC80*	*CIC*	*LAMC1*	*DIDO1*
NOTCH1	*PRR12*	*LAMC1*	*DIDO1*	*NRP1*	*LAMC1*
PCM1	*PTCH1*	*NRP1*	*NOTCH1*	*PCM1*	*NRP1*
PTCH1	*PXDN*	*PTCH1*	*PTCH1*	*PTCH1*	*PCM1*
PXDN	*REV3L*	*PXDN*	*PXDN*	*PXDN*	*PTCH1*
REV3L	*SHROOM2*	*REV3L*	*REV3L*	*REV3L*	*REV3L*
UNC5B	*UNC5B*	*UNC5B*	*UNC5B*	*SHROOM2*	*SHROOM2*

Table 15: *Top10 expressed genes from each stage and neuron population*

5.3.1.5. Gene ontology and pathway over-representation analyses

To obtain a better molecular insight from the list of FMD candidate genes, a gene ontology (biological process) and pathway **over-representation analyses (ORA)** were undertaken (Figure 21).

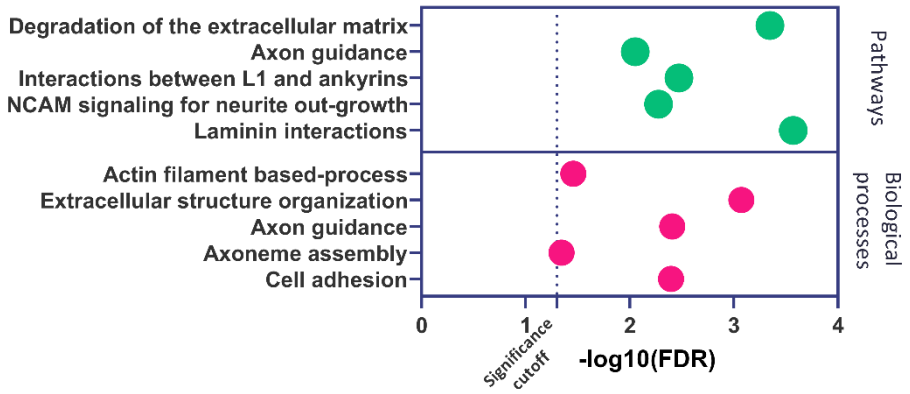

Figure 21: *Dot plot showing a summary of the results obtained by the pathway and biological processes over-representation analyses. More detailed information in Table 16 and 17.*

Regarding the gene ontology ORA, each of the 188 candidate genes were mapped and annotated to, at least, a biological process. **A significant over-representation (FDR<0.05) was found in five main biological processes**: a) Cell adhesion; b) Axoneme assembly; c) Axon guidance; d) Extracellular structure organization, and e) Actin filament based-process. Table 16 shows more information about the results of this analysis.

Biological Process	# Total genes	# Candidate genes	Fold enrichment	P-value	FDR
Cell adhesion	924	25	2.89	2.78x10-6	4.03x10-3
Axoneme assembly	70	6	9.12	7.69x10-5	4.54x10-2
Axon guidance	265	13	5.22	2.21x10-6	3.92x10-3
Extracellular structure organization	341	16	4.99	2.56x10-7	1.02x10-3
Actin filament based-process	590	17	2.95	5.68x10-5	3.49x10-2

***Table 16**: Detailed information about the results obtained by the biological processes over-representation analysis in FMD patients.*

One hundred candidate genes were found associated to a pathway in Reactome. Using them, a pathway ORA analysis was performed. The **five main pathways with a significant over-representation** were: a) Degradation of extracellular matrix; b) Axon guidance; c) Interaction between L1 and ankyrins; d) NCAM signaling for neurite out-growth, and e) Laminin interactions. Table 17 summarizes the results of this analysis. Variants in genes belonging to these pathways are shown in supplementary table 6.

Pathway	# Total genes	# Candidate genes	Fold enrichment	P-value	FDR
Laminin interactions	30	6	21.06	3.11x10-7	2.27x10-4
NCAM signaling for neurite out-growth	63	6	10.03	2.77x10-5	5.30x10-3
Interaction between L1 and ankyrins	31	5	16.982	9.76x10-6	3.37x10-3
Axon guidance	549	16	3.07	5.47x10-5	8.87x10-3
Degradation of extracellular matrix	140	10	7.52	7.82x10-7	4.50x10-4

***Table 17**: Detailed information about the results obtained by the pathway over-representation analysis in FMD patients.*

5.3.2. Sporadic Meniere Disease

5.3.2.1. Single rare variant analysis

After applying QC and following the steps taken in FMD variant analysis, 10047 SNV remained with a MAF≤0.001 in at least one reference population. From them, 7198 SNV were already described in the NFE population from ExAC or the Spanish population from CSVS, and 848 (11.8%) were also identified in the FMD cohort. Only 255 (3.6%) nonsynonymous or splice site SNV were found in 240 genes in 2 or more sporadic cases, 62 (24.2%) of these SNV were also found in at least a FMD patient and only 5 SNVs in 2 or more FMD patients (Table 18).

Position	Gene	# FMD Cases	# SMD Cases	CADD	MAF CSVS	MAF ExAC	MAF gnomAD
chr3:112357653C>T	CCDC80	3	2	16.23	0.003	0.0006	0.0005
chr17:42451817C>T	ITGA2B	3	3	14.09	0	0.0007	0.0007
chr1:7897189A>G	PER3	2	2	13.19	0.003	0.0008	0.0008
chr16:52107718C>T	C16orf97	2	3	13.83	0.002	0.0004	0.003
chr8:145692936A>G	KIFC2	2	2	2.78	0.003	0.0006	0.0005

Table 18: *The five variants which were found in at least 2 familial MD patients and 2 sporadic MD patients*

Among the variants affecting 2 or more SMD cases, three nonsynonymous SNV variants affecting three genes linked to deafness in mice (MP:0001967) could be highlighted. A heterozygous nonsynonymous SNV in ***ADGRV1* gene** was found in two unrelated SMD cases (S21 and S17). This variant chr5:89989802A>G (rs111033430; p.Y2410C), located in exon 33/90, has a MAF=0.0002 in the NFE population from GnomAD v3 and MAF=0.001 in the Spanish population from CSVS. According to multiple tools, this variant was classified as likely pathogenic (SIFT score=0; CADD=23.3; Polyphen=0.926). Besides, two additional variants in *ADGRV1* gene (p.I2526V and p.F3347L) fulfilling the MAF criteria in NFE population from gnomAD were found in the same two cases. Another variant, chr17:72916507C>T (rs111033466; p.E142K) affecting ***USH1G* gene***,* was identified in two SMD cases (S15 and S26). Although its MAF for both NFE population and Spanish population fulfilled the threshold, it was classified as likely benign since it occurs at a poorly conserved nucleotide (GERP++=3.8). In addition, a second rare variant affecting *USH1G* gene (p.K130E) was found in the same two patients. The third variant in a gene related to deafness was chr11:17632279C>T (***OTOG* gene**, rs779658224; p.A1823V), affecting two SMD patients and discussed before in '*SNHL gene set analysis in early onset sporadic MD'* section. None of these three variants were found in FMD cases.

5.3.2.2. Gene burden analysis

To compare the results obtained in FMD cohort with the series of SMD patients, GBA using two MAF threshold (MAF≤0.05 and MAF≤0.005) were done. A total of 19975 variants with a **MAF≤0.05** in 9578 genes were retained after QC and filtering steps. From them, 7261 genes (75.8%) carried 1 or 2 variants, being discarded for further analysis. After comparing with the Spanish population from CSVS, **70 genes had a significant enrichment of rare variants** (corrected p-value<0.05) in our series of SMD patients. **Only two genes** (*BCL9L* and *RASSF10*) **matched between FMD-enriched and SMD-enriched genes sets** (Figure 22).

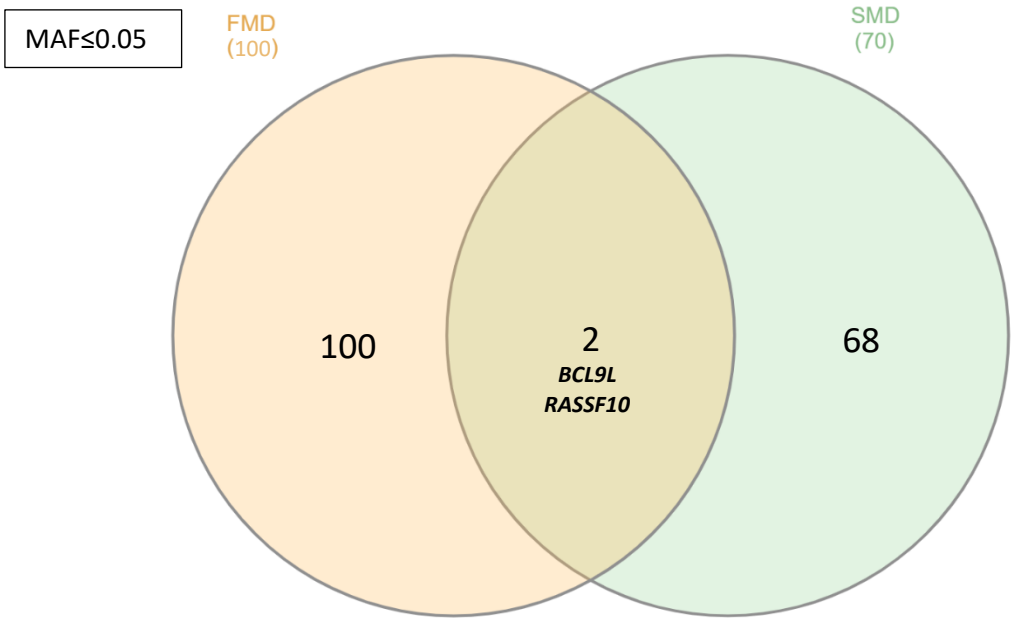

Figure 22: *Venn diagram showing the total number of candidate genes for familial and sporadic MD for MAF≤0.05, and the genes in common between them.*

As regard to the GBA taking as cutoff a **MAF≤0.005**, 10937 variants located in 6711 genes were retained after QC and filtering steps. From them, 5815 genes (86.6%) carried less than 3 variants, whereas 1**21 genes with 3 or more variants showed a significant enrichment** in the series SMD patients when they were compared to the Spanish control population from CSVS. A comparison between enriched genes in FMD and SMD patients was done, resulting in **13 enriched genes in common** (Figure 23).

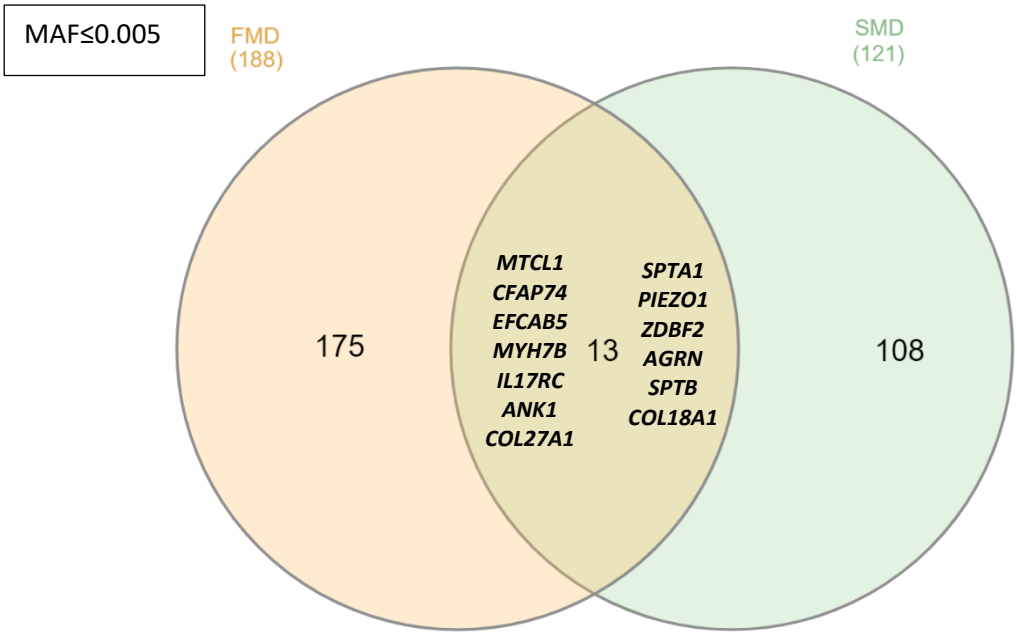

Figure 23: *Venn diagram showing the total number of candidate genes for familial and sporadic MD for MAF≤0.005, and the genes in common between them.*

Table 19 shows the results from the GBA for the top 30 genes with a significant accumulation of rare variants in SMD patients.

Gene	# Variants	# Cases	OR (CI)			Corrected p-value		
			CSVS	ExAC	gnomAD	CSVS	ExAC	gnomAD
ADGRV1	12	9	7.1(4.1-12.3)	9.8(5.9-16.4)	10.7(6.4-17.9)	$4.52x10^{-8}$	$<10^{-15}$	$<10^{-15}$
SEC16A	10	10	6.5(3.4-12.3)	17.6(9.6-32.1)	18.8(10.3-34.3)	$1.63x10^{-4}$	$<10^{-15}$	$<10^{-15}$
STARD9	10	5	7(3.6-13.8)	10.2(5.4-19)	9.7(5.2-18.2)	$2.48x10^{-4}$	$4.38x10^{-13}$	$2.12x10^{-08}$
PIEZO1	9	9	16.8(8.1-35)	20.6(10.9-38.7)	16.4(8.8-30.8)	$8.93x10^{-10}$	$<10^{-15}$	$<10^{-15}$
SPTA1	9	6	11.2(5.6-22.4)	16.3(8.7-30.5)	18.3(9.8-34.3)	$2.29x10^{-7}$	$<10^{-15}$	$<10^{-15}$
TRIOBP	9	5	12.1(5.8-25.4)	23.3(12-45.4)	23.9(12.3-46.5)	$8.01x10^{-7}$	$<10^{-15}$	$<10^{-15}$
ZNF469	9	8	6.2(3.1-12.5)	9.2(4.8-17.9)	6.7(3.5-13)	$7.03x10^{-3}$	$4.27x10^{-11}$	$2.71x10^{-04}$
COL18A1	8	7	13.4(6.3-28.3)	18.7(9.6-36.4)	21.5(11.1-41.8)	$2.18x10^{-7}$	$<10^{-15}$	$<10^{-15}$
DLEC1	8	5	14.6(6.5-32.6)	9.2(4.6-18.6)	9.3(4.6-18.7)	$1.24x10^{-6}$	$5.46x10^{-10}$	$8.36x10^{-06}$
MYH7B	8	8	7.2(3.5-14.6)	8.1(4.2-15.7)	8.4(4.3-16.2)	$1.02x10^{-3}$	$5.76x10^{-10}$	$5.40x10^{-06}$
NBEAL1	8	7	10.6(4.9-23)	11.4(5.7-23)	10.2(5.1-20.5)	$5.13x10^{-5}$	$1.06x10^{-11}$	$1.64x10^{-06}$
PNPLA7	8	5	6.1(3.3-11.6)	15.9(8.7-29)	19.9(10.9-36.3)	$4.36x10^{-4}$	$<10^{-15}$	$<10^{-15}$
PRUNE2	8	8	17.4(7.7-39.7)	14(6.9-28.2)	15.4(7.6-31.1)	$2.03x10^{-7}$	$1.99x10^{-13}$	$4.13x10^{-10}$
COL20A1	7	8	8.3(3.9-17.8)	10.7(5.3-21.6)	13.2(6.6-26.7)	$9.42x10^{-4}$	$3.62x10^{-11}$	$1.04x10^{-08}$
SCNN1D	7	5	7.3(3.2-16.2)	6(2.9-12.8)	7.1(3.4-15.1)	$2.72x10^{-2}$	$2.47x10^{-6}$	$5.11x10^{-03}$
ABCC12	6	2	66.6(18.7-236.8)	36.8(16.2-83.9)	37.1(16.4-83.8)	$1.72x10^{-6}$	$<10^{-15}$	$<10^{-15}$
ADAMTSL4	6	5	8.9(3.7-21.4)	36.5(16-83.2)	43.4(19.1-98.3)	$2.37x10^{-2}$	$<10^{-15}$	$<10^{-15}$
AFF1	6	6	9.7(4-23.5)	6.4(2.9-14.5)	6.8(3-15.3)	$1.14x10^{-2}$	$6.21x10^{-6}$	$6.11x10^{-02}$
ALKBH8	6	5	8.5(4.1-17.4)	13(6.7-25.2)	11.7(6-22.6)	$1.14x10^{-4}$	$4.09x10^{-14}$	$6.83x10^{-09}$
BDP1	6	4	10.9(4.4-26.7)	20.7(9.2-46.8)	17.5(7.8-39.3)	$3.96x10^{-3}$	$3.20x10^{-13}$	$8.78x10^{-08}$
CAPN15	6	6	133.3(26.8-661.9)	177(72.6-431.9)	113.9(49.2-263.8)	$4.38x10^{-5}$	$<10^{-15}$	$<10^{-15}$
CCDC171	6	6	17.5(6.8-45.3)	29.7(13.1-67.4)	25.8(11.4-58.1)	$7.23x10^{-5}$	$<10^{-15}$	$9.33x10^{-11}$
CPAMD8	6	5	13.7(5.4-34.4)	18(8-40.7)	29.5(13.1-66.5)	$5.28x10^{-4}$	$3.30x10^{-12}$	$8.88x10^{-12}$
MYCBPAP	6	6	8.8(3.9-19.8)	31.8(14.9-68.1)	39.7(18.6-84.5)	$3.74x10^{-3}$	$<10^{-15}$	$<10^{-15}$
NID1	6	6	8.9(3.7-21.5)	11.4(5-25.5)	14.5(6.5-32.6)	$2.25x10^{-2}$	$4.25x10^{-9}$	$1.87x10^{-06}$
OTOGL	6	5	9.3(3.8-22.6)	10.2(4.6-23)	12.2(5.4-27.4)	$1.58X10^{-2}$	$1.82x10^{-8}$	$2.71x10^{-05}$
PTPN21	6	6	17.3(7.2-41.6)	14.8(7-31.4)	14.3(6.8-30.3)	$3.81x10^{-6}$	$2.27x10^{-12}$	$6.83x10^{-08}$
TNRC18	6	6	12.1(4.8-30.7)	86(37.3-198)	102(44.3-234.4)	$2.65x10^{-3}$	$<10^{-15}$	$<10^{-15}$
TRPV1	6	6	7.3(3.4-15.5)	16.2(8-32.7)	23.8(11.7-48)	$4.47x10^{-3}$	$1.09x10^{-14}$	$<10^{-15}$
ABCA5	5	5	10.5(4.3-25.7)	17.3(7.6-39)	16.8(7.5-37.9)	$5.54x10^{-3}$	$7.16x10^{-12}$	$1.75x10^{-07}$

Table 19: Top 30 genes with an enrichment of rare variants (MAF≤0.005) in SMD patients

5.3.2.3. Gene expression of candidate genes in the inner ear

As it was done with genes enriched in FMD cohort, the expression of enriched genes in SMD patients was studied in a cochlear gene dataset. So, **101 genes** among 121 candidate genes were found in a RNA-seq study of gene expression during mouse inner ear development (Scheffer et al. 2015). The resulting genes were classified in HCs-enriched genes (35), SCs-enriched genes (26) and nonspecific genes (40). The distribution of these genes is summarized in Figure 24.

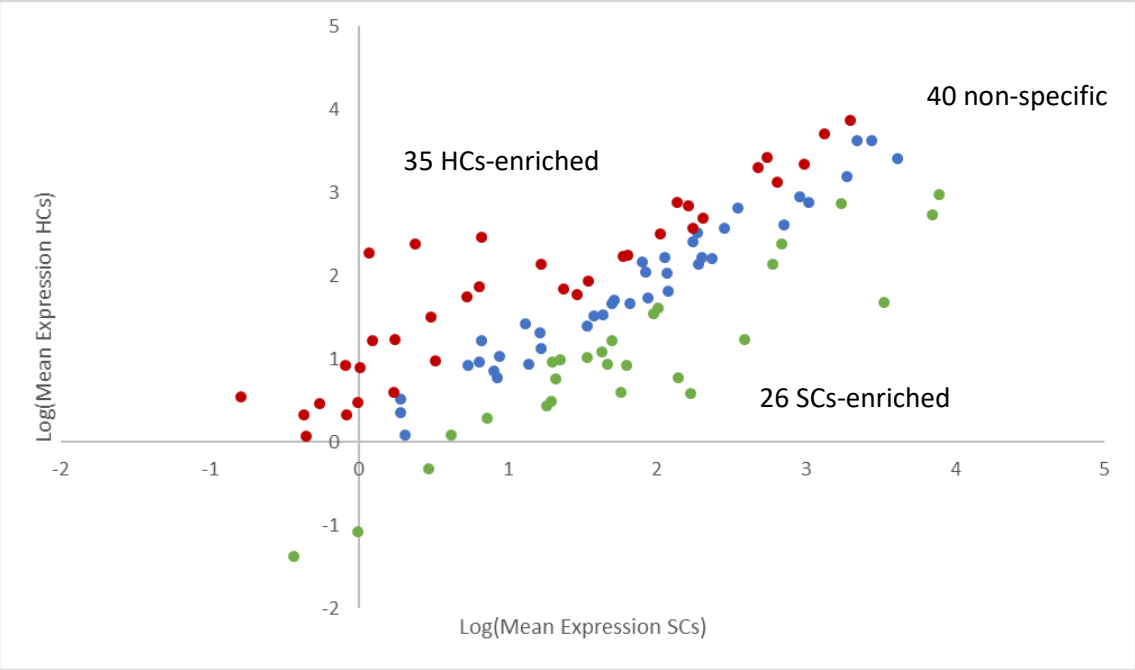

Figure 24: *Dispersion plot showing the expression of candidate genes for SMD patients in hair cells (HCs) and supporting cells (SCs). Red dots represent HCs-enriched genes. Green dots represent SCs-enriched genes. Blue dots represent genes with nonspecific expression for these two cell populations.*

Only 18 genes were at least four-fold enriched in HC compared with SCs, and when they were divided in hair cells subpopulations, we found that **6 of them were classified as uHCs-enriched, 2 as cHCs-enriched** and 10 had a nonspecific expression between HCs at postnatal stages (Figure 25). ***SEMA5B*** **and** ***MYCBPAP*** **genes** could be highlighted since showed a 11.28- and 9.48-fold enrichment, respectively, in cHCs when their expressions were compared with uHCs.

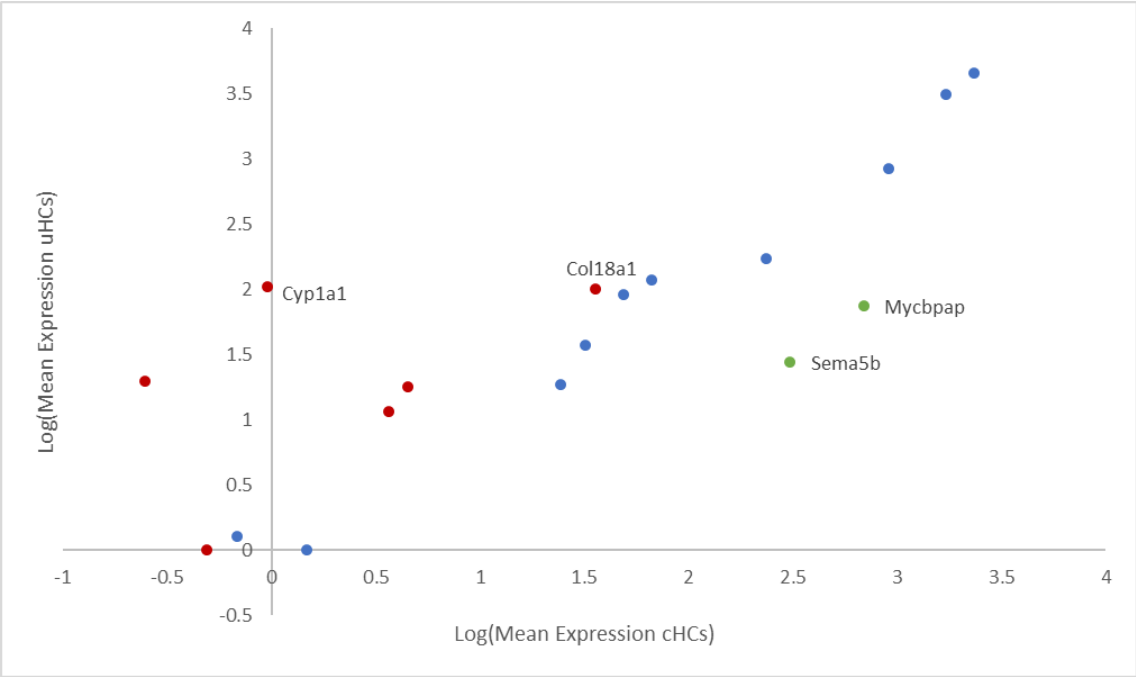

Figure 25: *Dispersion plot showing the expression of candidate genes in utricular hair cells (uHCs) and cochlear hair cells (cHCs). Red dots represent uHCs-enriched genes. Green dots represent cHCs-enriched genes. Blue dots represent genes with nonspecific expression for these two cell populations.*

The expression of candidate genes was also analyzed in **SCs (Deiters' and pillar cells) and cochlear HCs (inner and outer HCs)** using data from an RNA-Seq study in mice (Liu et al. 2018). A total of **103 genes** from the SMD candidate gene set were found in this study. Eighteen and 27 genes showed an enrichment >2-fold in Deiters' and pillar cells, respectively, when their expressions in these two cells populations were compared to each other. Regarding HCs populations, 11 and 20 genes were specific for iHCs and oHCs, respectively (Figure 26).

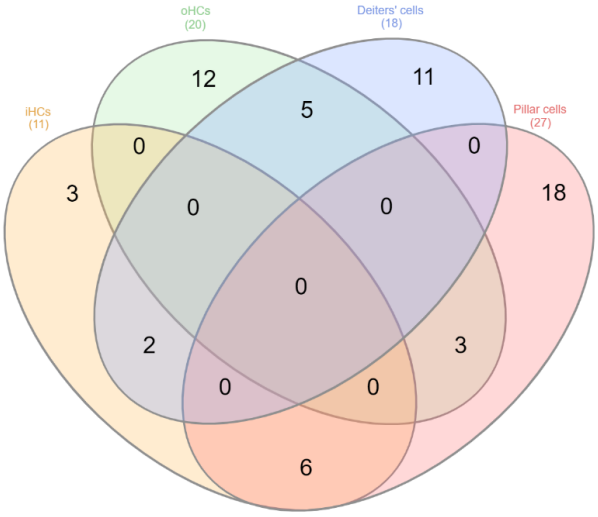

Figure 26: *Venn diagram showing the distribution of candidate genes for SMD patients according to their expression in inner hair cells (iHCs - yellow), outer hair cells (oHCs – green), Deiter's cells (blue) and pillar cells (red).*

When the four populations of cochlear cells from the organ of Corti were compared to each other, it was found that the expression of 3 genes were specific (>2-fold against any of the other three populations) for iHCs, 6 genes for oHCs, 8 genes for Deiters' cells and 8 for pillar cells. (Figure 27).

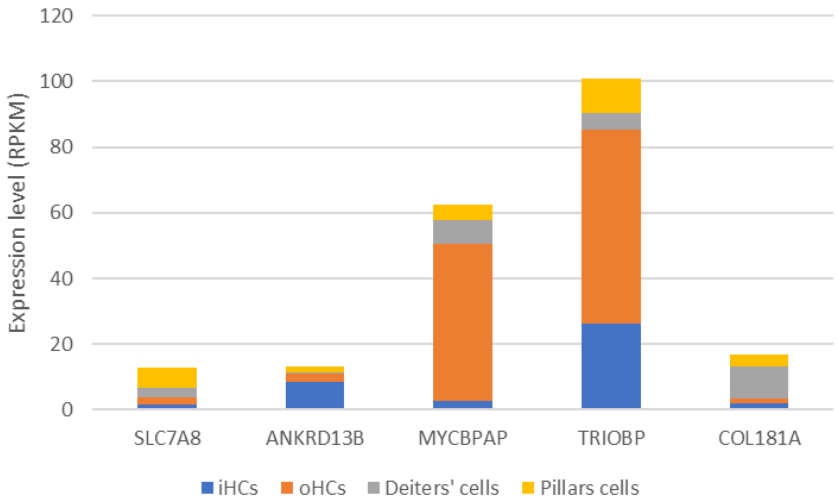

***Figure 27**: Stacked bar plot showing the expression of certain candidate genes for SMD patients in inner hair cells (iHCs), outer hair cells (oHCs), Deiter's cells and pillar cells.*

5.3.2.4. Gene expression of candidate genes in spiral and vestibular ganglion neurons

In order to compare FMD candidate gene expression results with the SMD cases, the expressions of candidate genes for this series of patients in **SG and VG mice neurons** were retrieved (Lu et al. 2011). So, **89 (73.5%) genes were found in this study**. From them, 7 genes were included in the "signature gene list, indicating in these genes a differential expression over time or/and population-dependent. (Figure 28).

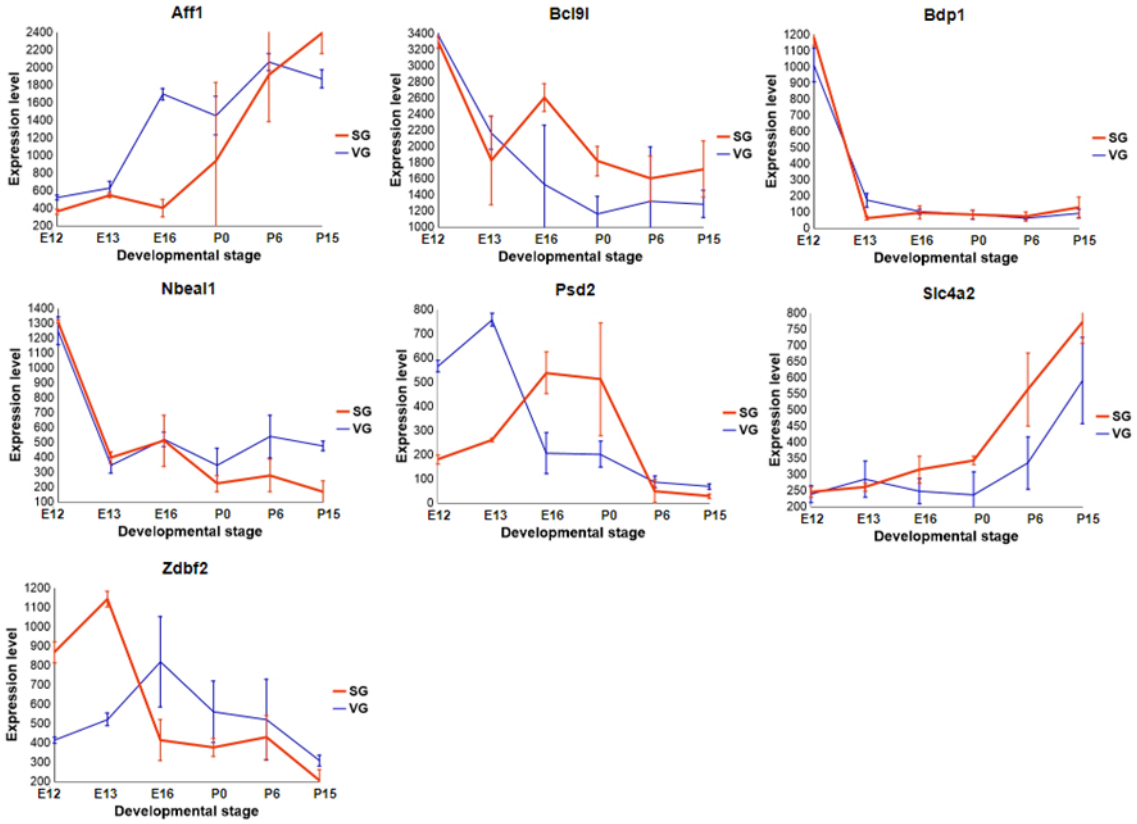

Figure 28: *Line graph showing the expression of candidate genes included in the 'signature gene list' across different age stages (E12, E13, E16, P0, P6 and P15) in spiral ganglion neurons (SG) and vestibular ganglion neurons (VG) for SMD patients.*

Similarly, average expressions for each gene were determined at early, mid and late stages in both neuron populations. Table 20 shows the top 10 expressed genes from each stage and cell population.

Spiral Ganglion Neurons			Vestibular Ganglion Neurons		
Early	**Mid**	**Late**	**Early**	**Mid**	**Late**
AGRN	*AGRN*	*AFF1*	*AGRN*	*AFF1*	*AFF1*
BAZ2B	*ANK1*	*AGRN*	*BAZ2B*	*AGRN*	*AGRN*
BCL9L	*ANKRD13B*	*ANK1*	*BCL9L*	*BAZ2B*	*BAZ2B*
CWF19L2	*BAZ2B*	*COL27A1*	*COL27A1*	*COL27A1*	*COL27A1*
DMXL1	*BCL9L*	*CWF19L2*	*CWF19L2*	*CWF19L2*	*CWF19L2*
KIF13B	*COL27A1*	*DMXL1*	*DMXL1*	*DMXL1*	*DMXL1*
LYSMD3	*CWF19L2*	*FNDC1*	*LYSMD3*	*NID1*	*KIF13B*
NID1	*PFKP*	*NID1*	*NID1*	*PFKP*	*NID1*
TNRC6C	*SPEG*	*SPEG*	*PFKP*	*SPEG*	*PFKP*
URB2	*TNRC6C*	*TNRC6C*	*TNRC6C*	*TNRC6C*	*SPEG*

Table 20: Top10 expressed genes from each stage and neuron population

5.3.2.5. Gene ontology and pathway over-representation analyses

Finally, a **gene ontology and pathway ORA** were undertaken to obtain a list of candidate biological processes and pathways (Table 21). Contrary to ORA in FMD cohort, where several biological processes and pathways were found over-represented, **only a biological process and a pathway were found significantly enriched in the series of sporadic cases**.

Biological Process	**# Total genes**	**# Candidate genes**	**Fold enrichment**	**P-value**	**FDR**
Cytoskeleton organization	1187	24	3.46	1.09x10-7	1.73x10-3
Pathway	**# Total genes**	**# Candidate genes**	**Fold enrichment**	**P-value**	**FDR**
Interaction between L1 and ankyrins	31	5	27.456	8.97x10-7	1.54x10-3

Table 21: *Detailed information about the results obtained by the biological processes and pathway over-representation analyses in SMD patients.*

6. Discussion

The underlying hypothesis leading this research is that **MD has a significant genetic contribution that influences the onset and the clinical course of the condition**. The genetic structure of familial MD is complex and includes few monogenic families, but the majority show a **polygenic involvement**. In this doctorate thesis we have analyzed the genetic background of FMD cases and SMD with early onset (≤ 35 years old) by WES to find the **main genes and pathways involved in this disease**. We considered that individuals with onset <35 years old could be carriers of recessive inheritance or de novo mutations.

Familial MD has an AD inheritance with **incomplete penetrance** (Morrison et al. 2009; Requena et al. 2014), and few genes have been involved in singular families (Requena et al. 2015b; Martín-Sierra et al. 2016; Martín-Sierra et al. 2017). However, these findings have not been replicated neither in other MD families nor SMD cases. Thus, the starting point of this thesis should be to **find the shared genes among most cases**.

Hearing loss genes have a contribution to FMD

In the first part of this thesis, we obtained an initial approach of the genetic background in MD patients using the Phenix pipeline. We found in a subset of 82 MD patients that **40% of FMD cases and 68% of SMD cases carried, at least, a novel or ultrarare variant in genes related with SNHL**. Although more than 150 genes have been associated to deafness (Azaiez et al. 2018), and 116 of them are related with nonsyndromic SNHL (Van Camp G. 2018), none of them have been previously associated to MD.

Several likely pathogenic variants in SNHL genes were found and validated as part of this pipeline. A novel variant (chr12:122701379G>C; p.T21R) in ***DIABLO* gene** segregating the MD phenotype was found in a Spanish family including three affected women (F22), suggestive of an AD pattern of inheritance. This gene has been previously associated to dominant progressive nonsyndromic hearing loss (DFNA64) (Cheng et al. 2011). This nonsynonymous variant is localized 8 amino acids downstream of the causative variant described by Cheng *et al* (p.S126L). A variant in ***TECTA* gene** (chr11:121028725T>C; p.V1494A) was found segregating the phenotype in other Spanish family (F8). *TECTA* gene encodes alpha-tectorin, one of the major non-collagenous components of the tectorial membrane, and it is associated to DFNA8/12 (Hildebrand et al. 2011). The variant found in this family has not been reported before in literature, however, it has been associated to non-syndromic hearing loss in ClinVar

(SCV000368188). Finally, a novel nonsynonymous variant in ***MYH14* gene** was found in three MD cases of another Spanish family (F4). *MYH14* encodes one of the heavy chains of the class II non-muscle myosins, and it has been demonstrated its expression in the cochlea and association to DFNA4A (Donaudy et al. 2004).

Although the Phenix pipeline showed a foreseeable implication of certain rare or novel variants in SNHL genes, we could not get more than private variants for singular families, suggesting that **the monogenic hypothesis (i.e. classic Mendelian inheritance) in FMD should be reconsidered**. Thus, more complex inheritance models are needed to explain the incomplete penetrance or variable expressivity observed in MD.

OTOG gene is a relevant gene in FMD

Thus, in the second part of this thesis, by applying a GBA, we found an **enrichment of rare missense variants in several unrelated patients with FMD in the *OTOG* gene**. These variants were identified in **15 of 46 non-related families**, representing the 33% of them. Incomplete peentrance was observed in 7 of the 15 families with rare variants in *OTOG* (47%), whereas partial syndromes, such as episodic vertigo or hearing loss, were found in relatives from 5 of 15 families. Most of these rare variants were found in 2, 3 or 4 unrelated individuals from different families with MD and they were considered multiplex variants. On the other hand, the majority of the variants in *OTOG* found in non-familial patients with early onset were not observed in other sporadic cases (singletons variants).

***OTOG* encodes the otogelin protein,** which was first described by Cohen-Salmon et al (Cohen-Salmon et al. 1997). Otogelin is a 2925 amino acid protein (ENST00000399391) constituted by vWD and C8 domains, and a cysteine knot-like domain in its C-terminal. **It is mainly expressed in** acellular structures which cover the sensory inner ear epithelia: the **tectorial membrane, the otoconial membranes and the cupula** over the cristae ampullaris of the semicircular canals. This structural protein plays an **important role in both auditory and vestibular functions** due to its localization in the extracellular structures overlying the stereocilia of the hair cells involved in the mechanotransduction of sound and acceleration (Schrauwen et al. 2016).

The effects of variants in otogelin were first demonstrated in the orthologous gene in a mouse model. Three **mice models** have been generated for evaluating the phenotypic changes resulting from *OTOG* variants. In the **Otog^{tm1Prs} model**, authors inactivated Otog by deleting the

first three exons. At P4 in Otog$^{-/-}$ mice, vestibular dysfunction was detected, observing anomalies in the saccule and utricule. The auditory function was evaluated by Pleyer reflex, showing profound hearing impairment. The Otog$^{+/-}$ mice did not present any anomalies (Simmler et al. 2000a). The second model was the **twister (twt) mice**, mice characterized by a spontaneous recessive mutation entailing absence of Otog expression. **Similarly to Otog^{tm1Prs}, in Otog^{twt} the vestibular dysfunction was detected at P4, while the hearing loss was progressive and moderate to severe/profound** (Simmler et al. 2000b). The last mouse model published is the otogelin N-ethyl-N-nitrosourea **(ENU) induced mouse model**. In this model, a homozygous variant at the splice donor site of intron 29, Otog$^{vbd/vbd}$, cause a frameshift and a premature codon. Otog$^{vbd/vbd}$ mice showed abnormal hearing and vestibular functions (El Hakam Kamareddin et al. 2015).

Four variants have been described in *OTOG* gene causing autosomal recessive deafness 18B (DFNB18B). Schraders et al. were the first to describe causative variants in *OTOG* gene. A homozygous 1bp deletion, c.5508delC (p.Ala1838Profs*31) in four related patients, and two compound-heterozygous variants, c.6347C>T (p.Pro2116Leu) and c.6559C>T (p.Arg2187*) in other two related patients, were described to cause hearing loss and vestibular dysfunction (Schraders et al. 2012). More recently, a homozygous nonsense variant c.330C>G (p.Tyr110*) in a Korean patient was identified, showing early-onset mild hearing loss without vestibular dysfunction (Yu et al. 2019). Imaging studies in families with DFNB18B with homozygous mutations in OTOG gene did not find abnormalities in computed tomography scans of the temporal bone (Oonk et al. 2014; Ganaha et al. 2019).

In contrast to studies mentioned above, ***OTOG* variants found in this thesis were all in heterozygous state** and, despite 6 FMD cases and 3 SMD cases studied had two or more variants, compound heterozygous variants could not be demonstrated because samples from the parents were not available and *OTOG* variant segregation could not be fully assessed in each family. Nevertheless, the variants chr11:17574758G>A and chr11:17663747G>A found in F14 were also identified in his mother, the variants chr11:17578774G>A and chr11:17632921C>T found in F5 were also identified in her sister (II-7), and a novel variant chr11:17594747C>A not considered for the GBA were found in F34 and her brother. Furthermore, variants located in untranslated regions (UTRs) and promoter regions, which modulate gene expression and different protein features (Chatterjee & Pal 2009), could not be evaluated because of the study design. Altogether, **the results obtained by GBA on SNHL genes suggested a different genetic architecture in FMD cases and SMD cases**, since the enrichment of rare variants in *OTOG* gene

was only found in FMD cases and most of the variants found in sporadic cases with early onset were singletons (not observed in multiple individuals).

Each region of the cochlea is specifically stimulated by a specific frequency, a property of the basilar membrane termed **tonotopy**. Thus, the base of the cochlea mainly responds to high-frequency sounds, whereas **the apex responds to low-frequency sounds**, frequencies commonly affected in MD (Robles & Ruggero 2017; Nakashima et al. 2016). Of note, **otogelin shows a tonotopic gene expression in mice** (Yoshimura et al. 2014). *OTOG* gene showed a **2.43-fold change in expression for apex vs base**, making this gene a possible candidate for SNHL in MD. In addition, an RNA-seq study of the inner ear from patients with normal hearing showed a **high expression of *OTOG* gene in the vestibule** (Schrauwen et al. 2016). This could explain the vestibular dysfunction in patients with pathogenic variants in this gene.

Otogelin is an extracellular protein located in the **tectorial and otolithic membranes of the saccule and utricle**. In a study performed in zebrafish ear, otogelin and tectorin alpha are required for otolith tethering in the otolithic membrane. It seems that there are two stages in this process: seeding and maintenance of the otoliths. The initial seeding step, in which otolith precursor particles tether directly to the tips of hair cell kinocilia, fails to occur in the einstein (*eis*) zebrafish mutant, an *OTOG* knock-out (Stooke-Vaughan et al. 2015). Although there is a large difference between *eis* zebrafish and the human phenotype in MD, the *eis* mutation disrupts otolith seeding and **we speculate the carriers of *OTOG* variants may have a fragile tectorial and otolithic membranes with lower tethering of otoconia that will lead to a severe perturbation of the endolymphatic fluid**.

Carriers of rare variants in *OTOG* have an endophenotype in MD

The audiograms of FMD patients carrying rare variants in *OTOG* gene showed a **moderate-to-severe flat hearing loss ≈60 dB since the first years of onset involving all frequencies**. Low-frequency hearing had slight variations throughout the years, while a negative correlation was found at mid (1000Hz) and high-frequency (2000Hz) hearing. Data from F14 were considered as an outlier and discarded because his hearing profile was not comparable to the rest of FMD patients (Supplementary Figure 3). Since all frequencies are involved since the onset of the disease, we can speculate that the damage of the tectorial membrane mediated by mutations in otogelin will involve the entire cochlea from base to apex.

According to our results, the **clinical picture** of patients with mutations in *OTOG* would be a female of 43 years old with sudden or rapidly progressive flat SNHL around 60 dB and vertigo attacks with a family history of MD, vertigo or early onset SNHL.

Gene burden analyses have been previously used to investigate the genetic background of sporadic MD. Gallego-Martinez et al. have published 2 studies selecting sporadic (non-familial) MD patients using two custom gene panels. The first study included 45 autosomal genes related with SNHL, however *OTOG* was not selected for this panel (Gallego-Martinez et al. 2019b). The second study included genes from 2 main pathways showing differentially expressed genes in SCs of the cochlea and vestibular organs: axonal guidance signaling and leukocyte extravasation pathway (Gallego-Martinez et al. 2019a). These studies found an enrichment of multiplex rare variants in several SNHL genes such as *GJB2*, *USH1G*, *SLC26A4*, *ESRRB*, and *CLDN14* and axonal guidance signaling genes such as *NTN4* and *NOX3* in non-familial patients with MD.

In conclusion, in the second part of this thesis **we have found an enrichment of rare missense variants in the *OTOG* gene in FMD cases**. These findings support a **multiallelic contribution in MD, where *OTOG* gene seems to be playing a relevant role** in the pathophysiology of hearing and vestibular functions in MD.

Beyond SNHL genes

In the third and last part of this thesis, we increased the sample size and analyzed rare variations in the coding regions of the entire human genome to run a hypothesis-free data-driven analysis. Through single rare variant and gene burden analyses, new candidate genes for MD not related before with SNHL in humans were suggested. Likewise, candidate biological processes and pathways for MD were pointed out by over-representation analyses.

Single rare variant analysis suggests polygenic contribution to MD

As it was seen in previously performed analyses, **SRVA failed to identify a gene that explains the onset of the disease as a whole**. On the contrary, when this analysis was done taking as input the variants found in both FMD and SMD patients, a total of 865 SNVs affecting 2 or more MD patients in 785 different genes were found. From them, only 5 variants fulfilling the criteria of MAF<0.001 were identified in both FMD and SMD patients. **Since no clear**

candidate gene for MD was found, we suggest a polygenic inheritance pattern for MD. This type of inheritance could explain the variable expressivity or the incomplete penetrance seen in some families (Martín-Sierra et al. 2017). Despite this, some single variants could be pointed out given their predicted pathogenicity, the function of the gene harboring the variant or their expression in the inner ear or related cells.

Familial MD

Concerning FMD patients, a rare variant (rs139136964) was found in ***NEK5* gene** in three unrelated patients (F29, F44 and F61). This gene, which encodes a kinase, was identified as a marker for vestibular HCs in a study where other 182 genes were classified as HC or epithelial non-HCs type-specific markers at P1 mice (Elkon et al. 2015). In addition, in a mouse model ($Nek5^{mpc234H}$; MGI:5792410) where a single point mutation was induced through ENU mutagenesis (p.A554T), progressive hearing loss was observed (Potter et al. 2016). A second mutation in ***ALPK1* gene** (rs116427224) was found in other 3 unrelated FMD patients (F1, F13 and F25). This gene encodes a kinase whose main function is to detect bacterial pathogen-associated molecular pattern (PAMPs) metabolites and initiate an innate immune response, triggering proinflammatory NF-kappa-B signaling (Zhou et al. 2018). However, we did not find any direct link of *ALPK1* gene to hearing loss. Finally, a variant in ***PIEZO2* gene** was identified in 2 unrelated patients (PT60 and EX125). *PIEZO2* gene encodes a mechanosensitive channel which converts mechanical stimuli in biological signals. An immunolocalization study in the organ of Corti of mice identified *Piezo2* gene expression at the apical surface of cochlear and vestibular hair cells, being controlled by the intracellular Ca^{2+} concentration. *Piezo2* seems to be required for the reverse-polarity currents that are observed during hair cell development, after tip-link breakage and in hair cells lacking TMC1/2. *Piezo2* knockout mice showed hearing impairment with no vestibular defects (Wu et al. 2017).

Sporadic MD

Similar results, in terms of number of variants and genes affected, were seen in SMD patients, not being able to define an obvious candidate for the disease. In addition, we found only a **small overlap between rare variants in FMD and SMD patients**, a 11.8%. This is a first glimpse of a **differential genetic background between sporadic and familial MD** patients. Variants in genes already related to hearing loss, such as *ADGRV1*, *USH1G* and *OTOG* genes, were highlighted. ***ADGRV1* gene**, which encodes adhesion G protein-coupled receptor V1, has been related to Usher syndrome type 2C (USH2C), a disorder where sensory HCs of the inner ear

and the photoreceptors in the eye are affected (Weston et al. 2004). Hearing loss in USH2C patients is present at birth, showing a slope-shaped audiogram (high frequencies more affected). Usher syndrome is usually cause by compound heterozygous variants (Zhang et al. 2018). This kind of inheritance pattern could be a candidate in the two patients with three rare variants in *ADGRV1* described in this thesis, although no visual phenotype was reflected in our clinical database. In close connection to *ADGRV1*, since variants in this gene have been observed in Usher syndrome type 1G, are the variants found in ***USH1G* gene** (p.K130E and p.E142K). These two variants were found in two unrelated SMD patients and could be good candidates for a recessive inheritance pattern. However, both of them were classified as polymorphisms in a *USHG1* gene screening in Spanish patients with Usher syndrome (Aller et al. 2007).

Gene burden analysis suggests polyallelic inheritance involving several genes

Gene burden analysis of rare variations yielded good results when it was applied to SNHL genes. Thus, we decided to apply it to the entire genome and obtain a general insight into the genetic background of MD. Contrary to the GBA done in SNHL genes, two MAF cutoff were applied, obtaining different results and candidate genes.

On the one hand, applying **MAF≤0.05** as cutoff, **100 and 70 genes with a significant enrichment of rare variants were found in FMD and SMD patients**, respectively. Surprisingly, **only 2 genes were found in common** between them: ***RASSF10* and *BCL9L* genes**. Little is known about these genes, however *Rassf10* gene is expressed by embryonic and postnatal HCs in mice (Scheffer et al. 2015), while *Bcl9l* gene was classified as a signature gene for both SG and VG neurons, since its expression changed considerably over time, showing higher expressions at E12 and E13 stages (Lu et al. 2011).

Specific enriched genes for FMD patients, as well as for SMD patients, were identified using the **MAF≤0.05** threshold. **For FMD patients**, genes such as *ACAN*, *MYO7A*, *DCP1B* and *KIF14* genes could be highlighted. ***ACAN* gene** encodes aggrecan, which is a non-collagenous component of the extracellular matrix (ECM). It was found that aggrecan, along with other ECM proteins (i.e. brevican, neurocan, hyaluran and tenascin-R), formed perineural nets surrounding the base of inner HCs and their synaptic contacts. These structures are supposed to control the synaptic transmissions. Besides, a high expression of aggrecan in the spiral limbus and in the lower part of the spiral ligament was revealed by immunohistochemistry in mice (Sonntag et al. 2018). In the same study was also suggested that, because of its strong anionic charge (Aspberg 2012), aggrecan could contribute to maintain the ion compositions of the endolymph and

perilymph (Sonntag et al. 2018). Furthermore, aggrecan showed a tonotopic gene expression within the cochlea in a microarray study, being more expressed in the basal turn compared with the apical turn (6.26-fold) (Yoshimura et al. 2014). Altogether, these findings could make *ACAN* gene a good candidate for our FMD cohort, where 15 variants affecting 11 unrelated patients were found. ***MYO7A* gene** is linked to autosomal dominant deafness 11 (DFNA11), autosomal recessive deafness 2 (DFNB2) and Usher syndrome type 1B. These syndromes have in common hearing and vestibular dysfunctions (Di Leva et al. 2006; Weil et al. 1997; Jaijo et al. 2007). Twelve rare variants in 12 unrelated patients (F14, F28, F51, F54 and F59) were found in our FMD cohort. In ***KIF14*** and ***DCP1B*** genes were also found a significant enrichment of rare variants, however we did not find any link of these genes with hearing or vestibular function. **For SMD patients**, *ADGRV1*, *PIEZO1*, *COL18A1*, *ATM* genes could be highlighted since they were the most mutated genes in the series of sporadic patients. As discussed in "Single rare variant analysis suggests polygenic contribution to MD" discussion subsection, ***ADGRV1*** is related to USH2C, a disorder characterized by hearing loss and visual defects. Nineteen rare variants (MAF≤0.05) were identified in 16 SMD patients out of 44. ***PIEZO1* gene** encodes a mechanosensitive channel, channels which can mediate touch, hearing or blood pressure regulation among other functions (Ranade et al. 2015). Although the expression of genes from its same family, such as *PIEZO2* gene, have been reported in the inner ear, significant levels of *PIEZO1* mRNA was not detected (Wu et al. 2017). No hearing or vestibular phenotypes have been described for any of the mice with variants in *Piezo1* gene (MGI:3603204). ***COL18A1*** gene encodes the alpha chain of type XVIII collagen, expressed in multiple organs playing an important role in the function and development of the kidney, eye and nervous system. Null variants in *COL18A1* leads to a rare heterogenous disorder called Knobloch syndrome, characterized by myopia, vitreoretinal degeneration, macular abnormalities and occipital encephalocele (A L Sertié et al. 2000). Although *COL18A1* gene expression has been detected in non-sensory cells and SCs in chicken utricle (Herget et al. 2013), neither hearing or vestibular dysfunction have been described for this gene. Ten out of 46 SMD patients carried variants in *COL18A1* gene. Variants in the ***ATM*** gene cause ataxia telangiectasia, a disorder characterized by cerebellar degeneration, telangiectasia, immunodeficiency and cancer susceptibility (Rothblum-Oviatt et al. 2016). Vestibular dysfunction caused by cerebellar degeneration is characteristic of this disease. No hearing phenotype has been observed for this condition (Shaikh et al. 2011). Ten different variants were found in 13 SMD patients.

On the other hand, 188 and 121 genes with a significant excess of variants applying MAF≤0.005 as cutoff were found in FMD and SMD patients, respectively. Of note, more and

different genes showed an enrichment of variants using a more restrictive MAF cutoff. This could suggest a **greater contribution in MD of ultrarare variants** than rare variants (MAF≤0.05). In this analysis, 13 genes showed an enrichment of variants in both FMD and SMD. From them, we could underline *ANK1*, *SPTB*, *SPTA1*, *MYH7B* and *MTCL1*. Encoding ankyrin 1, ***ANK1*** gene showed an excess of rare variants (8 variants) affecting 4 SMD and 5 FMD unrelated patients. This gene is expressed across the cochlea, presenting a tonotopic gradient expression, being higher in the apex turn in comparison with the basal turn (2.94-fold) (Yoshimura et al. 2014). *ANK1* variants were observed to account 23/85 patients with hereditary spherocytosis from Netherlands (van Vuren et al. 2019). Interestingly, from the 13 genes shared between FMD and SMD patients, there were other 2 genes related to this condition: ***SPTB*** and ***SPTA1*** genes. The proteins encoded by these genes are expressed in erythrocytes membrane. Thus, variants affecting them can cause a change in erythrocytes shape called spherocytosis. ***MYH7B*** gene encodes one of the two heavy chains of myosin II, and it was related to SNHL in a family of five individuals where 3 affected children were compound heterozygous for two rare variants (p.D557N and p.R1693) (Haraksingh et al. 2014). Although none of these two variants were observed in our cohort, 8 SMD and 8 FMD patients carried rare variants (12) in this gene, representing an enrichment of rare variants. One of the variants observed in a SMD patient (S9) was an amino acid far from one of the variants reported in the study cited before (p.T1692R; $CSVS_{MAF}$:Novel; $gnomAD_{MAF}$: 0.00001). This patient also carried another variant in MYH7B gene ($CSVS_{MAF}$:0.01). ***MTCL1*** gene encodes microtubule-associated protein highly expressed in cerebellum Purkinje neurons (Satake et al. 2017). Furthermore, *MTCL1* was classified as a signature gene in spiral and vestibular ganglion neuron, with an increased expression at mid stage in mice (E16-P0) (Lu et al. 2011). Diseases such as cerebellar ataxia have been linked to *MTCL1* gene dysfunction (Krygier et al. 2019). Four FMD and 4 SMD patients carried 8 variants, representing an enrichment of rare variants in this gene.

Some genes could be highlighted specifically for FMD and SMD patients. Specific enrichment in several genes already discussed, such as *ACAN*, *COL4A6* or *OTOG* genes, but also new enriched genes using a MAF≤0.005 were **identified for FMD**. Among other genes, *ESPNL*, *COL4A4*, *NOTCH1* and *NRP1* showed enrichment of rare variants. ***ESPNL*** encodes espin-like, a protein that was identified in developing stereocilia and essential for normal hearing. It was found that *ESPNL* is transported by MYO3A and MYO3B, proteins that regulates stereocilia length by transporting at stereocilia tips cargos that control actin polymerization. Thus, *ESPNL* seems to be implied in controlling the spacing of the stereocilia staircase. Besides, *Espnl* null mice showed high frequency hearing loss (Ebrahim et al. 2016). Six rare variants were found in

6 FMD patients. ***COL4A4*** gene encodes one of the 6 subunits of collagen type IV, which is only found in basement membranes, and being its major structural component (Momota et al. 1998). Variants in this gene have been linked to Alport Syndrome, characterized by progressive glomerulonephritis and hematuria, which is often associated with SNHL (high-frequencies initially affected) and damage of the eye (Vega et al. 2003). Seven rare variants in 6 FMD patients were found. ***NOTCH1*** is a member of the NOTCH family, a group of receptors involved in differentiation, proliferation and survival processes. NOTCH signaling pathway seems to play multiple roles during inner ear development. NOTCH1 is expressed throughout the developing cochlear duct from E12 to, at least, P3 stages (Lanford et al. 1999), being involved in HCs development (Murata et al. 2006; Murata et al. 2013). Five rare variants were found in 5 FMD patients. ***NRP1*** gene encodes neuropilin-1, a transmembrane receptor associated to neural and cardiovascular development. This receptor is known to bind to semaphorin (SEMA) classes 3A, 3B, 3C and 3D and the vascular endothelial growth factor beta. It was shown that the NRP1/SEMA3A signaling has a key role in cochlear innervation (Salehi et al. 2017). More interestingly, NRP1 interacts with SEMA3D. a gene already related to FMD (Martín-Sierra et al. 2017) and whose expression in the non-sensory epithelium flanking the sensory cristae have been demonstrated in the developing chicken inner ear (Scott et al. 2019). Five variants in 6 FMD patients were found. Specifically **for SMD patients**, some of the most noteworthy genes are *TRIOBP*, *TRPV1*, *SPTBN4*, and *BDP1*. ***TRIOBP*** gene is known to cause autosomal recessive deafness 28 (DFNB28) (Shahin et al. 2006; Riazuddin et al. 2006). This gene encodes a protein that seems to participate in cytoskeletal organization. Several families segregating variants in this gene reported prelingual severe hearing loss without vestibular abnormalities (Riazuddin et al. 2006). Nine variants in 5 SMD cases were found. Encoding a vanilloid receptor, ***TRPV1*** gene is expressed in the organ of Corti of rat and guinea pigs and spiral ganglion neurons. In this study it was also hypothesized that TRPV1 could participate in the cochlear homeostasis (Zheng et al. 2003). Six rare variants in 6 SMD patients were found in this gene. ***SPTBN4*** gene encodes beta-IV-spectrin and it is associated to congenital myopathy, neuropathy and central deafness (i.e. as a result of defects in central nervous system) in mice (Parkinson et al. 2001) and human (Knierim et al. 2017). Five SMD patients carrying 5 rare variants were found in our dataset. Variants in ***BDP1*** gene are associated to autosomal recessive deafness 112 (DFNB112), characterized by postlingual progressive hearing loss. An homozygous stop-loss variant was identified in a Qatari family where affected patients had bilateral progressive hearing loss, affecting first mid-high frequencies, but progressing to include low frequencies (Girotto et al. 2013). Five variants were found in 4 SMD patients.

Over-representation analysis pointed out certain candidate pathways

Based on the results obtained in the GBA with MAF≤0.005 for FMD and SMD patients, a pathway and a gene ontology ORA were undertaken to obtain a better molecular interpretation. The results from **FMD** for both analyses were robust since the biological processes and pathways were very related. Biological processes and pathways linked to the nervous system, such as axon guidance (GO:0007411; Reactome:R-HSA-422475), NCAM signaling (Reactome:R-HSA-375165) and interaction between L1 and ankyrins (Reactome:R-HSA-445095) were found overrepresented. Axon guidance pathway has been related to SMD in a study based on a targeted-sequencing panel including genes related with the main pathways in SCs (Gallego-Martinez et al. 2019a); NCAM is expressed during the development in the cochlea suggesting roles in axon guidance, synaptogenesis and nerve-target recognition due to its temporo-spatial distribution (Whitlon & Rutishauser 1990); and a role in guiding the direction of type 1 spiral ganglion afferent dendrites towards their target zone on the iHCs were suggested for L1 (Brand et al. 2013). This could suggest that the onset of hearing loss in some patients with FMD is caused by a damage on the vestibulocochlear nerve. The second subgroup of enriched pathways and biological processes could be defined as 'cell-cell and cell-matrix interaction', encompassing biological processes such as cell adhesion (GO:0007155) or extracellular structure organization (GO:0043062) and pathways such as laminin interactions (Reactome:R-HSA-3000157) and degradation of extracellular matrix (Reactome:R-HSA-1474228). Although it is a broad topic, it is known that extracellular matrix, cell-cell adhesion and cell-matrix interactions play a role in inner ear development (Legan & Richardson 1997). Furthermore, it is somehow related to the first topic (nervous system-axon guidance), since proteins such as laminins have been shown to enhance neurite outgrowth (Webber & Raz 2006). Little could be discussed about the pathways and biological processes enriched in **SMD**. Only a pathway and a biological process were found with a significant over-representation. This may be due to the sample size of SMD group.

Conclusiones

1. Las variantes raras en genes asociados a hipoacusia juegan un papel importante en la estructura genética de la EM, contribuyendo varios de ellos en a la EM familiar y esporádica.
2. El gen *OTOG* es un buen candidato para un subgrupo de pacientes con EM familiar. Estos pacientes tienen un endofenotipo caracterizado por hipoacusia pantonal moderada a severa desde el principio de la enfermedad.
3. El análisis agregado de variantes muestra un mejor resultado que el análisis de variante única a la hora de encontrar genes candidatos en la EM familiar y esporádica, apoyando la hipótesis de heterogeneidad genética.
4. Los pacientes con EM familiar y esporádica tienen una arquitectura genética diferente. Cuando se compararon los resultados obtenidos del análisis agregado de variantes, solo se pudo encontrar un pequeño solapamiento, obteniendo genes candidatos para cada condición. Genes como *ACAN* o *OTOG* parecen buenos candidatos para la EM familiar, mientras que el gen *ADGRV1* podría ser destacado para la EM esporádica.
5. Se encontraron determinadas rutas y procesos biológicos importantes, especialmente para la EM familiar, que pueden jugar un papel importante en el inicio de la EM, como la guía axonal o las interacciones de la laminina.

Conclusions

1. Rare variation in hearing loss-related genes play an important role in the genetic structure of Meniere disease, and several genes contribute to familial MD and sporadic MD.
2. *OTOG* gene is a strong candidate gene for a subgroup of FMD patients. These patients have an endophenotype characterized by moderate-to-severe flat hearing loss since the first years of onset.
3. Gene burden analysis showed a better performance than single rare variant analysis to target candidate genes in familial and sporadic MD, supporting genetic heterogeneity.
4. Familial and sporadic MD patients have a different genetic architecture. When gene burden analysis results were compared, only a small overlap was observed, obtaining different candidate genes for each condition. Genes such as *ACAN* or *OTOG* seem good candidates for FMD, whereas *ADGRV1* gene could be highlighted for SMD.
5. Certain candidate pathways and biological processes were pointed out, especially for FMD, where pathways such as axon guidance or laminin interactions could play an important role in the onset of the disease.

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Supplementary Material

Supplementary Table 1: List of sensorineural hearing loss genes selected for analysis, chromosome position, phenotypes and references.

Gene	Position	Phenotype	Reference
ESPN	chr1:6,484,848-6,521,430	DFNB36	https://www.ncbi.nlm.nih.gov/pubmed/15930085
IFNLR1	chr1:24,480,647-24,514,449	DFNA2C	https://www.ncbi.nlm.nih.gov/pubmed/29453195
GJB3	chr1:35,246,790-35,251,970	DFNA2B	https://www.ncbi.nlm.nih.gov/pubmed/9843210
KCNQ4	chr1:41,249,684-41,306,124	DFNA2A	https://www.ncbi.nlm.nih.gov/pubmed/10369879
BSND	chr1:55,464,606-55,476,556	DFNB73	https://www.ncbi.nlm.nih.gov/pubmed/19646679
ROR1	chr1:64,239,690-64,647,181	DFNB108	https://www.ncbi.nlm.nih.gov/pubmed/27162350
CDC14A	chr1:100,810,584-100,985,833	DFNB32	https://www.ncbi.nlm.nih.gov/pubmed/27259055
COL11A1	chr1:103,342,023-103,574,052	DFNA37	https://www.ncbi.nlm.nih.gov/pubmed/30245514
GPSM2	chr1:109,417,972-109,477,167	DFNB82	https://www.ncbi.nlm.nih.gov/pubmed/20602914
LMX1A	chr1:165,171,104-165,325,952	DFNA7	https://www.ncbi.nlm.nih.gov/pubmed/29754270
NLRP3	chr1:247,579,458-247,612,410	DFNA34	https://www.ncbi.nlm.nih.gov/pubmed/28847925
OTOF	chr2:26,680,071-26,781,566	DFNB9	https://www.ncbi.nlm.nih.gov/pubmed/10903124
PNPT1	chr2:55,861,198-55,921,045	DFNB70	https://www.ncbi.nlm.nih.gov/pubmed/23084290
ELMOD3	chr2:85,581,517-85,618,875	DFNB88	https://www.ncbi.nlm.nih.gov/pubmed/24039609
PJVK	chr2:179,316,163-179,326,117	DFNB59	https://www.ncbi.nlm.nih.gov/pubmed/16804542
TMIE	chr3:46,742,823-46,752,413	DFNB6	https://www.ncbi.nlm.nih.gov/pubmed/12145746
ILDR1	chr3:121,706,170-121,741,127	DFNB42	https://www.ncbi.nlm.nih.gov/pubmed/21255762
MCM2	chr3:127,317,066-127,341,279	DFNA70	https://www.ncbi.nlm.nih.gov/pubmed/26196677
CCDC50	chr3:191,046,866-191,116,459	DFNA344	https://www.ncbi.nlm.nih.gov/pubmed/17503326
WFS1	chr4:6,271,576-6,304,992	DFNA6	https://www.ncbi.nlm.nih.gov/pubmed/10424813
GRXCR1	chr4:42,895,283-43,032,675	DFNB25	https://www.ncbi.nlm.nih.gov/pubmed/20137778
REST	chr4:57,774,042-57,802,010	DFNA27	https://www.ncbi.nlm.nih.gov/pubmed/29961578
GAB1	chr4:144,257,915-144,395,721	DFNB26	https://www.ncbi.nlm.nih.gov/pubmed/29408807
MARVELD2	chr5:68,710,939-68,740,157	DFNB49	https://www.ncbi.nlm.nih.gov/pubmed/15538632
BDP1	chr5:70,751,442-70,863,649	DFNB112	https://www.ncbi.nlm.nih.gov/pubmed/24312468
PPIP5K2	chr5:102,455,853-102,548,500	DFNB100	https://www.ncbi.nlm.nih.gov/pubmed/15538632
SLC22A4	chr5:131,630,136-131,679,899	DFNB60	https://www.ncbi.nlm.nih.gov/pubmed/27023905

Gene	Position	Phenotype	Reference
DIAPH1	chr5:140,894,583-140,998,622	DFNA1	https://www.ncbi.nlm.nih.gov/pubmed/9360932
GRXCR2	chr5:145,239,296-145,252,531	DFNB101	https://www.ncbi.nlm.nih.gov/pubmed/24619944
POU4F3	chr5:145,718,587-145,720,083	DFNA15	https://www.ncbi.nlm.nih.gov/pubmed/18228599
SERPINB6	chr6:2,948,393-2,972,399	DFNB91	https://www.ncbi.nlm.nih.gov/pubmed/20451170
DCDC2	chr6:24,171,983-24,383,520	DFNB66	https://www.ncbi.nlm.nih.gov/pubmed/25601850
RIPOR2	chr6:24,797,601-25,042,238	DFNB104	https://www.ncbi.nlm.nih.gov/pubmed/24958875
COL11A2	chr6:33,130,458-33,160,276	DFNA13	https://www.ncbi.nlm.nih.gov/pubmed/10581026
LHFPL5	chr6:35,773,070-35,801,651	DFNB67	https://www.ncbi.nlm.nih.gov/pubmed/16752389
CLIC5	chr6:45,866,188-46,048,132	DFNB103	https://www.ncbi.nlm.nih.gov/pubmed/17021174
MYO6	chr6:76,458,909-76,629,254	DFNA22	https://www.ncbi.nlm.nih.gov/pubmed/11468689
CD164	chr6:109,687,717-109,703,762	DFNA66	https://www.ncbi.nlm.nih.gov/pubmed/26197441
EYA4	chr6:133,561,736-133,853,258	DFNA10	https://www.ncbi.nlm.nih.gov/pubmed/17567890
GSDME	chr7:24,737,972-24,809,244	DFNA5	https://www.ncbi.nlm.nih.gov/pubmed/9771715
PDE1C	chr7:31,790,793-32,338,941	DFNA74	https://www.ncbi.nlm.nih.gov/pubmed/29860631
ADCY1	chr7:45,613,739-45,762,715	DFNB44	https://www.ncbi.nlm.nih.gov/pubmed/15583425
HGF	chr7:81,328,322-81,399,754	DFNB39	https://www.ncbi.nlm.nih.gov/pubmed/19576567
TRRAP	chr7:98,475,556-98,610,866	DFNA	https://www.ncbi.nlm.nih.gov/pubmed/31231791
SLC26A5	chr7:102,993,177-103,086,624	DFNB61	https://www.ncbi.nlm.nih.gov/pubmed/12719379
SLC26A4	chr7:107,301,080-107,358,254	DFNB4	https://www.ncbi.nlm.nih.gov/pubmed/16570074
MET	chr7:116,312,444-116,438,440	DFNB97	https://www.ncbi.nlm.nih.gov/pubmed/25941349
MIRN96	chr7:129,414,532-129,414,609	DFNA50	https://www.ncbi.nlm.nih.gov/pubmed/19363479
ESRP1	chr8:95,653,302-95,719,694	DFNB109	https://www.ncbi.nlm.nih.gov/pubmed/29107558
GRHL2	chr8:102,504,660-102,681,954	DFNA28	https://www.ncbi.nlm.nih.gov/pubmed/12393799
TJP2	chr9:71,736,209-71,870,124	DFNA51	https://www.ncbi.nlm.nih.gov/pubmed/20602916
TMC1	chr9:75,136,717-75,451,267	DFNB7	https://www.ncbi.nlm.nih.gov/pubmed/18616530
WHRN	chr9:117,164,360-117,267,736	DFNB31	https://www.ncbi.nlm.nih.gov/pubmed/12833159
TNC	chr9:117,782,805-117,880,536	DFNA56	https://www.ncbi.nlm.nih.gov/pubmed/23936043
TPRN	chr9:140,086,069-140,098,645	DFNB79	https://www.ncbi.nlm.nih.gov/pubmed/20170898
MYO3A	chr10:26,223,002-26,501,465	DFNB30	https://www.ncbi.nlm.nih.gov/pubmed/12032315

Gene	Position	Phenotype	Reference
PCDH15	chr10:55,562,531-57,387,702	DFNB23	https://www.ncbi.nlm.nih.gov/pubmed/14570705
CDH23	chr10:73,156,691-73,575,704	DFNB12	https://www.ncbi.nlm.nih.gov/pubmed/11090341
C10orf105	chr10:73,471,458-73,497,581	DFNB12	https://www.ncbi.nlm.nih.gov/pubmed/11090341
PDZD7	chr10:102,767,440-102,790,914	DFNB57	https://www.ncbi.nlm.nih.gov/pubmed/26849169
EPS8L2	chr11:694,438-727,727	DFNB106	https://www.ncbi.nlm.nih.gov/pubmed/26282398
USH1C	chr11:17,515,442-17,565,963	DFNB18A	https://www.ncbi.nlm.nih.gov/pubmed/12107438
OTOG	chr11:17,568,920-17,668,697	DFNB18B	https://www.ncbi.nlm.nih.gov/pubmed/23122587
CABP2	chr11:67,286,383-67,290,899	DFNB93	https://www.ncbi.nlm.nih.gov/pubmed/22981119
LRTOMT	chr11:71,791,377-71,821,828	DFNB63	https://www.ncbi.nlm.nih.gov/pubmed/18953341
ANAPC15	chr11:71,817,424-71,823,826	DFNB63	https://www.ncbi.nlm.nih.gov/pubmed/18953341
MYO7A	chr11:76,839,310-76,926,286	DFNA11A	https://www.ncbi.nlm.nih.gov/pubmed/8776602
NARS2	chr11:78,147,007-78,285,919	DFNB94	https://www.ncbi.nlm.nih.gov/pubmed/25807530
RDX	chr11:110,045,605-110,167,447	DFNB24	https://www.ncbi.nlm.nih.gov/pubmed/17226784
MPZL2	chr11:118,124,118-118,135,251	DFNB111	https://www.ncbi.nlm.nih.gov/pubmed/29961571
TECTA	chr11:120,971,882-121,062,202	DFNA8	https://www.ncbi.nlm.nih.gov/pubmed/16718611
EPS8	chr12:15,773,075-16,035,263	DFNB102	https://www.ncbi.nlm.nih.gov/pubmed/24741995
MSRB3	chr12:65,672,423-65,882,024	DFNB74	https://www.ncbi.nlm.nih.gov/pubmed/21185009
OTOGL	chr12:80,603,233-80,772,870	DFNB84B	https://www.ncbi.nlm.nih.gov/pubmed/23122586
PTPRQ	chr12:80,799,774-81,073,968	DFNB84	https://www.ncbi.nlm.nih.gov/pubmed/20346435
KITLG	chr12:88,886,570-88,974,628	DFNA69	https://www.ncbi.nlm.nih.gov/pubmed/26522471
SLC17A8	chr12:100,750,857-100,815,837	DFNA25	https://www.ncbi.nlm.nih.gov/pubmed/11115382
DIABLO	chr12:122,692,209-122,712,081	DFNA64	https://www.ncbi.nlm.nih.gov/pubmed/21722859
P2RX2	chr12:133,195,366-133,198,972	DFNA41	https://www.ncbi.nlm.nih.gov/pubmed/24211385
GJB2	chr13:20,761,602-20,767,114	DFNA3A	https://www.ncbi.nlm.nih.gov/pubmed/9620796
GJB6	chr13:20,796,101-20,806,534	DFNA3B	https://www.ncbi.nlm.nih.gov/pubmed/10471490
COCH	chr14:31,343,720-31,364,271	DFNA9	https://www.ncbi.nlm.nih.gov/pubmed/9806553
SIX1	chr14:61,110,133-61,124,977	DFNA23	https://www.ncbi.nlm.nih.gov/pubmed/15141091
ESRRB	chr14:76,776,957-76,968,180	DFNB35	https://www.ncbi.nlm.nih.gov/pubmed/18179891
STRC	chr15:43,891,596-44,010,458	DFNB16	https://www.ncbi.nlm.nih.gov/pubmed/11687802

Gene	Position	Phenotype	Reference
DMXL2	chr15:51,739,908-51,915,030	DFNA71	https://www.ncbi.nlm.nih.gov/pubmed/27657680
CIB2	chr15:78,396,948-78,423,886	DFNB48	https://www.ncbi.nlm.nih.gov/pubmed/23023331
HOMER2	chr15:83,509,838-83,654,661	DFNA68	https://www.ncbi.nlm.nih.gov/pubmed/25816005
TBC1D24	chr16:2,525,147-2,555,735	DFNB86	https://www.ncbi.nlm.nih.gov/pubmed/24387994
CLDN9	chr16:3,062,457-3,064,506	DFNB	https://www.ncbi.nlm.nih.gov/pubmed/31175426
CRYM	chr16:21,250,195-21,314,404	DFNA40	https://www.ncbi.nlm.nih.gov/pubmed/12471561
OTOA	chr16:21,689,835-21,772,050	DFNB22	https://www.ncbi.nlm.nih.gov/pubmed/11972037
KARS	chr16:75,661,622-75,682,541	DFNB89	https://www.ncbi.nlm.nih.gov/pubmed/23768514
SPNS2	chr17:4,402,129-4,443,228	DFNB115	https://www.ncbi.nlm.nih.gov/pubmed/30973865
MYO15A	chr17:18,012,020-18,083,116	DFNB3	https://www.ncbi.nlm.nih.gov/pubmed/9603736
GRAP	chr17:18,923,986-18,950,950	DFNB114	https://www.ncbi.nlm.nih.gov/pubmed/30610177
TMEM132E	chr17:32,907,768-32,966,337	DFNB99	https://www.ncbi.nlm.nih.gov/pubmed/25331638
WBP2	chr17:73,841,780-73,852,588	DFNB107	https://www.ncbi.nlm.nih.gov/pubmed/26881968
ACTG1	chr17:79,476,997-79,490,873	DFNA20	https://www.ncbi.nlm.nih.gov/pubmed/14684684
LOXHD1	chr18:44,056,935-44,236,996	DFNB77	https://www.ncbi.nlm.nih.gov/pubmed/21465660
GIPC3	chr19:3,585,551-3,593,539	DFNB15	https://www.ncbi.nlm.nih.gov/pubmed/21660509
S1PR2	chr19:10,332,109-10,341,948	DFNB68	https://www.ncbi.nlm.nih.gov/pubmed/26805784
SYNE4	chr19:36,494,002-36,499,695	DFNB76	https://www.ncbi.nlm.nih.gov/pubmed/23348741
CEACAM16	chr19:45,202,421-45,213,986	DFNA4B	https://www.ncbi.nlm.nih.gov/pubmed/25589040
MYH14	chr19:50,691,443-50,813,802	DFNA4A	https://www.ncbi.nlm.nih.gov/pubmed/15015131
OSBPL2	chr20:60,813,580-60,871,269	DNFA67	https://www.ncbi.nlm.nih.gov/pubmed/25759012
CLDN14	chr21:37,832,919-37,948,867	DFNB29	https://www.ncbi.nlm.nih.gov/pubmed/11163249
TMPRSS3	chr21:43,791,996-43,816,955	DFNB8	https://www.ncbi.nlm.nih.gov/pubmed/11907649
TSPEAR	chr21:45,917,775-46,131,495	DFNB98	https://www.ncbi.nlm.nih.gov/pubmed/22678063
MYH9	chr22:36,677,323-36,784,063	DFNA17	https://www.ncbi.nlm.nih.gov/pubmed/11023810
TRIOBP	chr22:38,092,995-38,172,563	DFNB28	https://www.ncbi.nlm.nih.gov/pubmed/16385458
SMPX	chrX:21,724,090-21,776,281	DFNX4	https://www.ncbi.nlm.nih.gov/pubmed/21549342
POU3F4	chrX:82,763,269-82,764,775	DFNX2	https://www.ncbi.nlm.nih.gov/pubmed/7839145
PRPS1	chrX:106,871,654-106,894,256	DFNX1	https://www.ncbi.nlm.nih.gov/pubmed/20021999

Gene	Position	Phenotype	Reference
COL4A6	chrX:107,386,780-107,682,727	DFNX6	https://www.ncbi.nlm.nih.gov/pubmed/23714752
AIFM1	chrX:129,263,337-129,299,861	DFNX5	https://www.ncbi.nlm.nih.gov/pubmed/25986071

Supplementary Table 2: Rare variants (MAF<0.001) found in SRVA for familial Meniere disease patients.

Variant	Gene	Exon	Fam MD Code	MAF NFE		MAF CSVS	CADD	ACMG
				ExAC	gnomAD			
chr1:6488328C>T	*ESPN*	2	F31	0.00040	0.00020	0.0024	35	Uncertain Significance
chr1:24484269G>A	*IFNLR1*	7	F21	0.00006	0	0.00063	3.8	Likely benign
chr1:35250892T>G	*GJB3*	2	F32	0.00010	0	0.0003	24.5	Likely benign
chr1:35250961G>A	*GJB3*	2	F36	0.0016	0.00057	0.0034	11.2	Benign
chr1:109472327C>T	*GPSM2*	15	F19	0.000015	0.000046	0.00030	34	Benign
chr2:26696027G>C	*OTOF*	29	F19	0.00090	0.0007	0.0012	22.7	Uncertain Significance
chr2:26741960C>T	*OTOF*	4	F3	0.000088	0.00015	0.00091	26.2	Likely benign
chr3:46747377C>T	*TMIE*	2	F45	0.00040	0.00038	0.00030	24.2	Uncertain Significance
chr3:127325493G>C	*MCM2*	6	F26	0.00070	0.0008	0.00030	27.8	Uncertain Significance
chr3:127340040T>C	*MCM2*	15	F22	0.000015	0	—	23.4	Uncertain Significance
chr4:6304085T>C	*WFS1*	8	F8	0.00037	0.0004	0	3.78	Likely benign
chr5:70793116A>C	*BDP1*	13	F42	0.000030	0.00001	0.00030	23.9	Uncertain Significance
chr5:70797395G>A	*BDP1*	14	F10	0.00027	0.0002	0.0034	0.002	Likely benign
chr5:70819777C>T	*BDP1*	25	F15	0	0.00003	—	0.038	Likely benign
chr5:102530663C>T	*PPIP5K2*	30	F27	0.00050	0.0006	0.00061	23.7	Uncertain Significance
chr5:140953193G>C	*DIAPH1*	16	F39	0.00040	0.0006	0.00061	18.90	Uncertain Significance
chr5:140953259G>A	*DIAPH1*	15	F22	0.00002	0.00001	—	21.7	Uncertain Significance
chr5:140953280T>C	*DIAPH1*	15	F24	0.000022	0	0	6.04	Uncertain Significance
chr6:2953312T>A	*SERPINB6*	5	F24	0.00024	0.0002	0.00091	11.55	Uncertain Significance
chr6:2954942G>T	*SERPINB6*	5	F1	0.00080	0.00082	0.0024	24.7	Uncertain Significance
chr7:107329557T>C	*SLC26A4*	9	F27	0.00060	0.00044	0.0043	31	Likely benign
chr7:107350627G>A	*SLC26A4*	19	F39	0.00020	0.0003	0.0015	23.2	Benign
chr7:116339248T>C	*MET*	2	F17	0.00010	0.0001	—	8.54	Benign
chr7:116339605C>T	*MET*	2	F40	0.000075	0.00013	0.0091	11.84	Uncertain Significance
chr7:116411646C>T	*MET*	13	F41	0.000060	0.00009	0.00030	21.0	Uncertain Significance
chr9:71845053G>T	*TJP2*	12	F19	0.000045	0.00006	0.00030	32	Uncertain Significance
chr9:71852004T>C	*TJP2*	14	F35	0.00030	0.0004	0.0027	5.54	Benign
chr10:26462782G>A	*MYO3A*	30	F30	0.000030	0.0006	—	9.24	Benign
chr10:26462923T>C	*MYO3A*	30	F3	0.000030	0	0	10.31	Likely benign
chr10:26463125C>G	*MYO3A*	30	F10	0.000090	0.00004	—	16.78	Uncertain Significance
chr10:55581760C>T	*PCDH15*	35	F28	0.00010	0.00009	0.0012	15.06	Uncertain Significance
chr10:55912942C>T	*PCDH15*	15	F39	0.00020	0.0001	0.0015	24.6	Benign

Variant	Gene	Exon	Fam MD Code	MAF NFE		MAF CSVS	CADD	ACMG
				ExAC	gnomAD			
chr10:56106198T>C	*PCDH15*	7	F24	0.000029	0.00001	0.0018	22.7	Uncertain Significance
chr10:73464804G>A	*CDH23*	24	F30	0.000047	0.00001	—	24.9	Uncertain Significance
chr10:73553197G>A	*CDH23*	46	F35	0	0.00004	0.00030	17.72	Uncertain Significance
chr10:102769030C>T	*PDZD7*	16	F45	–	0.00001	–	21.5	Uncertain Significance
chr10:102780378G>C	*PDZD7*	7	F25	–	0.00003	–	22.5	Uncertain Significance
chr11:720123G>A	*EPS8L2*	5	F43	0.000061	0.00003	0.00030	32	Uncertain Significance
chr11:17518327G>A	*USH1C*	19	F6	–	0.00001	0.00061	25.8	Uncertain Significance
chr11:17531093G>C	*USH1C*	18	F27	0.00080	0.0010	0	24.0	Uncertain Significance
chr11:17574758G>A	*OTOG*	5	F1; F14	0.00080	0.0011	0.004	28.2	Uncertain Significance
chr11:67287311A>G	*CABP2*	6	F44	0.00060	0.0004	0.00030	26.1	Uncertain Significance
chr11:67287381C>T	*CABP2*	6	F29	0.000078	0.00004	–	13.73	Likely benign
chr11:76885923G>A	*MYO7A*	17	F37	0.00010	0.0001	0.0030	34	Uncertain Significance
chr11:76890920G>A	*MYO7A*	21	F3	0.00049	0.00003	–	24.4	Uncertain Significance
chr11:76922875G>A	*MYO7A*	46	F27	0.00040	0.0003	0.00030	22.1	Uncertain Significance
chr11:76925719G>A	*MYO7A*	49	F34	–	0.00009	–	23.0	Uncertain Significance
chr11:117965537G>A	*TMPRSS4*	2	F37	0.00024	0.0002	0.0015	23.3	Uncertain Significance
chr11:121023689G>C	*TECTA*	12	F24	0	0.00001	–	27.2	Uncertain Significance
chr11:121028725T>C	*TECTA*	13	F8	0.000091	0.00009	0.00091	22.3	Uncertain Significance
chr12:15819384C>T	*EPS8*	7	F27	0.00012	0.00009	–	23.1	Uncertain Significance
chr12:15823797C>T	*EPS8*	4	F28	0.00080	0.00079	0.0024	18.82	Uncertain Significance
chr12:80735904T>A	*OTOGL*	43	F22	0.00028	0.00009	0	20.8	Benign
chr12:80752642T>G	*OTOGL*	51	F31	0	0.00004	0.00030	27.6	Uncertain Significance
chr12:80770980C>T	*OTOGL*	57	F10	0.00064	0.0006	0.0038	14.19	Benign
chr12:80936029T>C	*PTPRQ*	19	F22	0	0.0001	0.00091	18.56	Likely benign
chr12:100751192C>T	*SLC17A8*	1	F33	0.00030	0.0002	0.0012	19.98	Benign
chr12:122710552G>T	*DIABLO*	1	F39	–	0.00001	–	23.2	Uncertain Significance
chr12:133196619A>G	*P2RX2*	3	F9	0.0013	0.00095	0.0032	21.4	Likely benign
chr13:20763437A>G	*GJB2*	2	F34	–	0.00001	–	29.6	Uncertain Significance
chr14:31355389A>G	*COCH*	11	F23	0.0010	0.0011	0.0021	22.0	Likely benign
chr14:76905955T>G	*ESRRB*	4	F23	0	0.00001	–	18.81	Uncertain Significance

Variant	Gene	Exon	Fam MD Code	MAF NFE		MAF CSVS	CADD	ACMG
				ExAC	gnomAD			
chr15:51828804C>A	*DMXL2*	12	F17; F31	–	0	0	23.6	Uncertain Significance
chr16:21728238C>T	*OTOA*	5	F29	0	0	–	2.05	Uncertain Significance
chr16:21739665C>T	*OTOA*	19	F25	0.00020	0.0001	0.0040	19.35	Likely benign
chr16:75663379T>C	*KARS*	12	F30	0.00045	0.0006	–	20.2	Uncertain Significance
chr17:18042912G>A	*MYO15A*	18	F7	0	0.00003	0	12.7	Uncertain Significance
chr17:18051861C>T	*MYO15A*	31	F40	0.0016	0.0001	0	23.7	Likely benign
chr17:32957039A>G	*TMEM132E*	6	F33	–	0	0	23.1	Uncertain Significance
chr18:44109147C>T	*LOXHD1*	29	F31	0.00060	0.0004	0.0012	19.82	Likely benign
chr18:44184084C>T	*LOXHD1*	7	F27	0.00030	0.0001	0.0024	26.8	Uncertain Significance
chr19:3586840G>A	*GIPC3*	3	F24	0.00040	0.0006	0.00030	23.4	Likely benign
chr19:50785088A>G	*MYH14*	33	F4	0	–	0.00030	31	Uncertain Significance
chr19:50810310C>T	*MYH14*	41	F8	0	0.00017	0.00061	34	Uncertain Significance
chr20:60835075G>A	*OSBPL2*	3	F44	0.00003	0.00006	0	8.74	Benign
chr21:43796787C>T	*TMPRSS3*	11	F17	0.000030	0.00001	0.00030	18.18	Uncertain Significance
chr21:45945541C>T	*TSPEAR*	8	F16	0.000030	0	0.00030	34	Uncertain Significance
chr21:45953710G>A	*TSPEAR*	3	F25	0.00014	0.00018	0.0013	8.98	Uncertain Significance
chr22:36697620G>A	*MYH9*	21	F45	0.00060	0.0002	0.00030	23.1	Uncertain Significance
chr22:38165350C>T	*TRIOBP*	21	F45	0.000021	0.00003	0.00030	33	Uncertain Significance

Supplementary Table 3: Statistical analysis for rare variants in OTOG gene in FMD cases. MAF FMD, minor allele frequency in fam
population from ExAC and gnomAD; CSVS, Collaborative Spanish Variant Server; CADD, Combined Annotation Dependent Depleti

Variant position	CADD	MAF FMD	MAF NFE		MAF CSVS	OR NFE		C
			ExAC	GnomAD		ExAC	gnomAD	
11:17574758G>A	24.8	0.041 (3/73)	0.00080	0.0011	0.0033	28(6.7-116.5)	19.6(4.7-81.3)	1.2
11:17578774G>A	15.95	0.068 (5/73)	0.0090	0.0041	0.017	5(1.8-13.7)	11.1(4.1-30.5)	
11:17621218C>T	34	0.027 (2/73)	0.0026	0.0058	0.0033	8.5(2.1-34.8)	3.8(0.9-15.5)	
11:17627548G>A	23.6	0.012 (1/73)	0.0056	0.0045	0.0054	1.9(0.3-14)	2.5(0.3-17.7)	
11:17631453C>T	12.89	0.012 (1/73)	0.017	0.011	0.014	0.6(0.1-4.6)	1(0,1-6,9)	
11:17632921C>T	7.71	0.068 (5/73)	0.0015	0.0011	0.0054	30.3(10.9-84.1)	43,1(15,4-120,8)	1.5
11:17656672G>A	31	0.013 (1/73)	0.0034	0.0052	0.0039	3.2(0.4-23.2)	2,1(0,3-15,1)	
11:17663747G>A	19.41	0.055 (4/73)	0.0058	0.0024	0.0054	5.8(1.8-18.3)	14,1(4,4-45,1)	
11:17667139G>C	27.2	0.082 (6/73)	0.019	0.023	0.017	1.8(0.6-5.6)	1,4(0,4-4,4)	

Supplementary Table 4: Clinical information of FMD patient carrying variants in *OTOG* gene. HL, hearing loss; NH, normal h
migraine with aura; MO, migraine without aura.

Families	Code		F1	F2	F3	F4	F5	F6
	MD patients	Studied patient	II-4	II-2	II-2	III-7	II-2	II-1
		Other MD relatives	I-1; II-2	I-2; II-1	II-1	II-1	II-1; II-7; II-11	II-2
	Relatives with incomplete phenotype	Probable MD	II-1	—	—	—	—	—
		Episodic Vertigo	—	—	—	I-1; II-3; III-2	—	—
		Hearing loss	—	—	—	—	—	—
Clinical data of studied patient	Sex		Female	Female	Female	Female	Female	Male
	Laterality		Bilateral	Bilateral	Bilateral	Bilateral	Unilateral	Bilatera
	Left ear	Low frequencies	Mild HL	Severe HL	Severe HL	Mild HL	MtoS HL	Modera HL
		Mid frequencies	NH	Severe HL	Severe HL	Mild HL	MtoS HL	MtoS H
		High frequencies	MtoS HL	Severe HL	Severe HL	Mild HL	MtoS HL	Profour HL
		Shape	Mild ski-slope	Flat	Flat	Flat	Flat	Ski-Slo
	Right ear	Low frequencies	Severe HL	Moderate HL	Mild HL	MtoS HL	NH	Severe
		Mid frequencies	Moderate HL	Mild HL	Moderate HL	Mild HL	NH	Severe
		High frequencies	Severe HL	Severe HL	Severe HL	Moderate HL	NH	Severe
		Shape	Reverse-slope	Ski-Slope	Ski-Slope	Reverse-slope	NH	Flat
	Other data	Age of onset	50	31	52	30	53	31
		Headache	Paroxysmal hemicrania	MA	Tension headache	MO	No	Tensio headac
		Autoimmune disease	No	Hypothyroidism	No	No	No	No

Families	Code		F10	F11	F12	F13	F14
	MD patients	Studied patient	II-2	II-2	III-6	III-1	II-3
		Other MD relatives	II-1	II-1	I-1; II-2	I-1; III-2	I-1; II-2
	Relatives with incomplete phenotype	Possible MD	—	—	—	—	II-1
		Vertigo	—	—	—	—	—
		Hearing loss	—	—	—	II-3	IV-4
Clinical data of studied patient	Sex		Female	Female	Male	Female	Male
	Laterality		Bilateral	Bilateral	Bilateral	Unilateral	Bilateral
	Left ear	Low frequencies	MtoS HL	Severe HL	MtoS HL	NH	Moderate HL
		Mid frequencies	Moderate HL	MtoS HL	MtoS HL	NH	Mild HL
		High frequencies	MtoS HL	Profound HL	MtoS HL	NH	MtoS HL
		Shape	Flat	Flat	Flat	NH	Reverse-slope
	Right ear	Low frequencies	Profound HL	Mild HL	MtoS HL	Moderate HL	Mild HL
		Mid frequencies	Profound HL	NH	MtoS HL	Moderate HL	Mild HL
		High frequencies	Profound HL	Moderate HL	MtoS HL	Moderate HL	MtoS HL
		Shape	Flat	Flat	Flat	Flat	Mild Ski-Slope
	Other data	Age of onset	53	38	42	42	40
		Headache	No	No	No	No	No
		Autoimmune disease	Ulcerative colitis	No	No	No	No

Supplementary Table 5: Rare variants (MAF<0.001) found in the SRVA for sporadic MD cases.

Variant	Gene	Exon	Sporadic cases	MAF NFE		MAF CSVS	CADD
				ExAC	gnomAD		
chr1:24484051C>T	*IFNLR1*	7	S33	0.000045	0.000092	0	20.6
chr1:35251146G>C	*GJB3*	2	S22	0.000045	0.000015	0	4.10
chr1:100963756G>A	*CDC14A*	14	S13	0.000015	0	0	0.031
chr2:55863449C>T	*PNPT1*	28	S2	0.000045	0.000062	–	23.9
chr2:179319145G>A	*DFNB59*	3	S32	0.00040	0.00029	–	25.7
chr4:6279258C>T	*WFS1*	2	S10	0	0	–	35
chr4:6296827G>A	*WFS1*	7	S10	0.000015	0	–	3.27
chr4:6303833G>A	*WFS1*	8	S17	0	0.000031	–	22.8
chr5:70785335G>C	*BDP1*	10	S24	0.00020	0.00029	0.0010	16.52
chr5:70806540A>G	*BDP1*	17	S4	0.000030	0.000015	–	21.7
chr5:70855826C>G	*BDP1*	37	S24	0.00060	0.00048	0.0010	21.9
chr5:140908474T>C	*DIAPH1*	21	S29	–	0	–	10.33
chr5:145719938C>G	*POU4F3*	2	S9	0.00050	0.00084	0	26.4
chr6:133769314A>G	*EYA4*	5	S14	–	0	–	14.41
chr7:45632443C>T	*ADCY1*	3	S11	0.00050	0.00065	–	23.6
chr7:103018088T>G	*SLC26A5*	18	S4	0.00020	0.00025	0.0030	25.3
chr8:102570958C>A	*GRHL2*	4	S3	0.000030	0.000015	–	1.62
chr9:117819627C>T	*TNC*	15	S23	0.000075	0.000062	0	17.16
chr9:117852972C>T	*TNC*	2	S22	0	0	–	18.08
chr9:140093585G>A	*TPRN*	1	S6	0.00040	0.00029	0.0010	24.3
chr10:73406348G>A	*CDH23*	13	S22	0.00010	0.00017	0.0010	24.8
chr10:73466770G>A	*CDH23*	25	S18	0.00010	0.000062	–	24.7
chr10:102775470G>A	*PDZD7*	11	S27	0.00040	0.00015	–	20.6
chr10:102783196G>A	*PDZD7*	4	S3	0.00020	0.00019	0	32
chr11:17522618C>T	*USH1C*	23	S29	0	0	–	26.8
chr11:17538971C>T	*USH1C*	14	S12	0.0012	0.00043	0.0027	11.17
chr11:17615604C>T	*OTOG*	28	S12	0.00090	0.00079	0.0070	23.1
chr11:17615655C>T	*OTOG*	28	S12	0.00090	0.00076	0.0070	33
chr11:17631679C>G	*OTOG*	36	S2	–	0.000062	–	23.1
chr11:17632279C>T	*OTOG*	36	S1; S23	0	0.00054	–	26.4
chr11:67290778C>T	*CABP2*	1	S29	0.00011	0.000046	–	10.62
chr11:76883864G>A	*MYO7A*	16	S26	0.00030	0.00019	0.0010	34
chr11:76922974G>A	*MYO7A*	46	S31	0.000062	0.000031	–	26.9
chr11:78239889C>G	*NARS2*	6	S31	0.00020	0.00015	0	22.7
chr11:120998763G>A	*TECTA*	8	S18	0.000075	0.00011	–	24.9
chr12:80616011C>T	*OTOGL*	6	S26	0.0011	0.00067	0.00095	35
chr12:80752642T>G	*OTOGL*	51	S27	0	0.000046	0	27.6
chr12:80771692G>A	*OTOGL*	58	S26	0.00020	0.00011	0.0010	24.3
chr12:80900414G>A	*PTPRQ*	13	S10	–	0.00024	–	23
chr12:80943396A>G	*PTPRQ*	22	S24	–	0.000046	0.00091	0.004
chr12:81013287A>G	*PTPRQ*	28	S35	0.0017	0.000093	0.00061	23.6
chr12:122710554G>C	*DIABLO*	1	S4	0.0015	0.00091	0.0042	23.5

Variant	Gene	Exon	Sporadic cases	MAF NFE		MAF CSVS	CADD
				ExAC	gnomAD		
chr12:133196619A>G	*P2RX2*	3	S5	0.0013	0.00095	0.0032	15.35
chr13:20763104T>C	*GJB2*	2	S15	0.00012	0.00011	0.0012	14.82
chr15:51742460G>T	*DMXL2*	40	S1; S11	0.00003	0	0.0022	9.64
chr15:51828581A>G	*DMXL2*	12	S1; S11	0.00045	0.00050	0.0025	9.74
chr13:20797249T>C	*GJB6*	5	S36	0	0	–	18.08
chr15:83532948T>C	*HOMER2*	4	S25	0.00040	0.00048	0	23.3
chr16:2546934C>T	*TBC1D24*	2	S27	0.00030	0.00022	0.0010	16.76
chr16:21728262T>C	*OTOA*	5	S6	0.0013	0.00050	0.0018	0.21
chr16:75663344C>T	*KARS*	13	S4	0.000029	0.000015	–	23.6
chr17:18024352G>T	*MYO15A*	2	S16	0.0011	0.00023	0.00092	1.47
chr17:18039039G>T	*MYO15A*	12	S5	0.0010	0.00071	0.0030	22.9
chr17:18049397C>T	*MYO15A*	29	S35	0.000046	0.000015	0	24.3
chr17:32961954A>G	*TMEM132E*	8	S16	0.000015	0.000015	–	9.14
chr17:73845743G>C	*WBP2*	3	S35	0.000014	0.000015	—	25.4
chr18:44057473C>T	*LOXHD1*	9	S15	–	0	—	20.4
chr18:44190775G>T	*LOXHD1*	6	S30	–	0.000015	—	22.7
chr19:50755984G>A	*MYH14*	15	S6	0.0020	0.00060	—	22.7
chr22:36714278C>T	*MYH9*	11	S1	0.000030	0.000015	—	33
Chr22:38130508C>A	*TRIOBP*	9	S18	0.000064	0.00020	0.0022	14.95
chr22:38130521C>T	*TRIOBP*	9	S3	0.000074	0.00012	0.0010	20.6
chr22:38155433G>A	*TRIOBP*	9	S21	0.00027	0.00011	0.00061	12.93
chr22:38164179C>T	*TRIOBP*	19	S18	0.000060	0.00020	0.0020	26.2
chr22:38165339A>G	TRIOBP	21	S18	0.000079	0.00020	0.0015	5.99
chr22:38167706T>C	TRIOBP	22	S3	0.000030	0.000062	0.0010	23.6

Supplementary Table 6: Variants found in genes belonging to the five main pathways with a significant over-representation in familial MD patients. Pathway 1: Axon guidance; 2: Degradation of extracellular matrix; 3: Laminin interactions; 4: NCAM signaling for neurite out-growth; 5: Interactions between L1 and ankyrins. The three last columns represent the minor allele frequency in non-Finnish European populations from GnomAD and ExAC, and the Spanish population from CSVS, respectively.

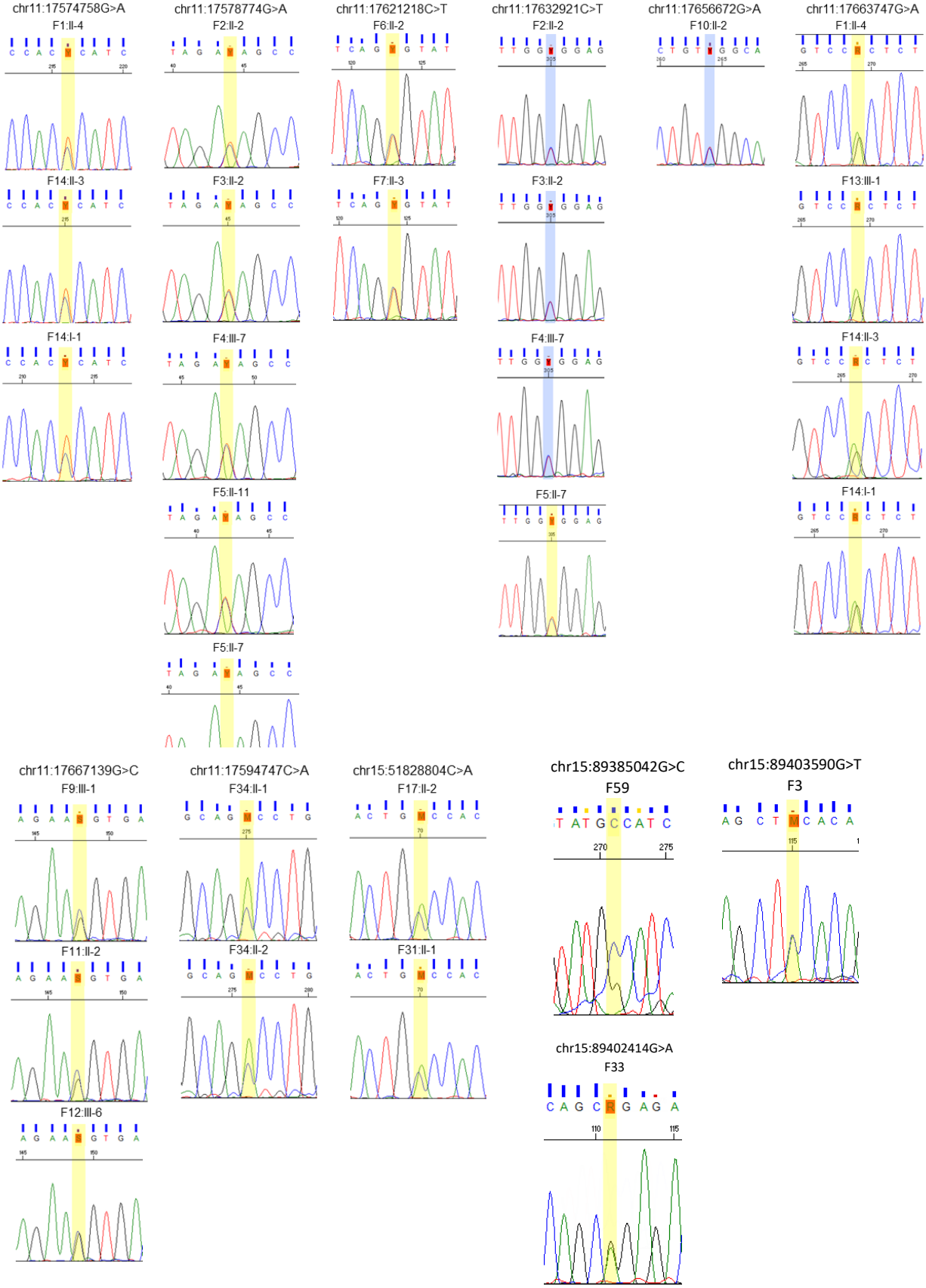
Gene	Pathway	Variant position	Exon	Codes	GnomAD	ExAC	CSVS
ACAN	2	chr15:89382022G>A	3	F48, F33	0.0022	0.0019	0.00466
ACAN	2	chr15:89382129C>A	3	F26	0.0005	0.0009	0.00206
ACAN	2	chr15:89402176C>G	12	F44	0.0005	0.0002	0.00292
ACAN	2	chr15:89403590G>T	13	F3	0	0.00002	0.00051
ACAN	2	chr15:89392786G>A	10	F53	0.0003	0.0003	0.00026
ACAN	2	chr15:89395201G>A	11	F50	0.0001	0.0003	0.00026
ACAN	2	chr15:89400341C>T	12	F52	0.0034	0.0032	0.00159
ACAN	2	chr15:89401134T>C	12	F59	0.0015	0.001	0.0008
ACAN	2	chr15:89401362G>T	12	F53	0.0003	0.0002	0.00053
ACAN	2	chr15:89401814G>A	12	F53	0.0003	0.0002	0.00053
ACAN	2	chr15:89401989C>T	12	F53	0.0003	0.0002	0.00027
ACAN	2	chr15:89400302G>A	12	F17	0	0.0001	0
ACAN	2	chr15:89400561G>A	12	F54	0	0.00003	0
ACAN	2	chr15:89402414G>A	12	F33	0	0	0
ACAN	2	chr15:89385042G>C	5	F59	0	0.00001	0
ADAM15	2	chr1:155026439G>A	4	F13	0	0	0.00027
ADAM15	2	chr1:155030599C>A	14	F52	0.0019	0.0018	0.0008
ADAM15	2	chr1:155028444C>T	8	F28	0	0	0
ADAM15	2	chr1:155026915G>A	6	F56	0.0003	0.0004	0
AGRN	1, 4	chr1:981869G>C	18	F45	0	0.00002	0
AGRN	1, 4	chr1:982302C>A	19	F27	0.0017	0.0035	0.00077
AGRN	1, 4	chr1:985378G>A	27	F46, F36	0.0015	0.0011	0.00182
AGRN	1, 4	chr1:979268C>T	10	F26	0.0001	0.0001	0
AGRN	1, 4	chr1:984711G>A	25	F15	0	0.00003	0
ANK1	1, 3, 5	chr8:41519013G>A	42	F54	0.00007	0.0001	0.00026
ANK1	1, 3, 5	chr8:41548021G>A	32	F54	0.0001	0.0002	0.00077
ANK1	1, 3, 5	chr8:41530362G>A	38	F52	0.0015	0.0011	0.00077
ANK1	1, 3, 5	chr8:41577289C>T	10	F58	0.0015	0.0015	0.00336
ANK1	1, 3, 5	chr8:41561606C>T	20	F5	0	0.00003	0
CACNA1H	1, 4	chr16:1270392T>A	34	F37	0.0001	0.00008	0.00077
CACNA1H	1, 4	chr16:1260082G>T	18	F55	0.0004	0.0012	0.00388
CACNA1H	1, 4	chr16:1250333G>A	7	F5	0	0.00004	0
CACNA1H	1, 4	chr16:1270213C>G	34	F47	0	0	0
CACNA1H	1, 4	chr16:1258141G>A	16	F58	0.00067	0.0004	0
CACNA1H	1, 4	chr16:1258220C>T	16	F50	0.0002	0.0001	0
CACNA1S	1, 4	chr1:201009011C>T	44	F33	0.005	0.0031	0.00129
CACNA1S	1, 4	chr1:201010661C>T	41	F40	0	0.00009	0.00026
CACNA1S	1, 4	chr1:201020165T>A	33	F30, F58	0.0041	0.0038	0.00206
CACNA1S	1, 4	chr1:201038610A>G	18	F39	0.00067	0.00009	0.00052

CACNA1S	1, 4	chr1:201047079G>A	11	F21	0.0015	0.0019	0.00052
CACNA1S	1, 4	chr1:201012485G>A	40	F46	0.0001	0.0007	0
CACNA1S	1, 4	chr1:201021734G>A	32	F26	0.00067	0.0002	0
CACNA1S	1, 4	chr1:201022660A>G	30	F45	0	0.00003	0
CACNA1S	1, 4	chr1:201044667A>T	13	F15	0	0.00004	0
CAPN9	2	chr1:230895279C>T	3	F8	0.0013	0.0006	0.00027
CAPN9	2	chr1:230903451T>C	5	F55	0.0001	0.0002	0.00133
CAPN9	2	chr1:230914750G>A	8	F2	0	0	0.00027
CAPN9	2	chr1:230930967G>T	17	F10	0.0017	0.0026	0.00587
CAPN9	2	chr1:230898397A>G	Splice Site	F8	0.0013	0.0006	0
COL15A1	2	chr9:101817602G>A	34	F44	0.0059	0.0046	0.00106
COL15A1	2	chr9:101824569C>G	38	F16, F60	0.0006	0.0007	0.00106
COL15A1	2	chr9:101830911A>G	41	F14, F38	0.0041	0.0028	0.00213
COL15A1	2	chr9:101748083G>A	3	F46	0	0.00002	0
COL18A1	2, 3	chr21:46895448C>T	4	F45	0	0.00003	0.00052
COL18A1	2, 3	chr21:46924434A>C	Splice Site	F62	0.0001	0	0
COL18A1	2, 3	chr21:46876658C>T	1	F56	0.0014	0	0.00186
COL18A1	2, 3	chr21:46875817G>A	1	F22	0.0009	0.0009	0
COL18A1	2, 3	chr21:46896298C>A	5	F23	0	0	0
COL18A1	2, 3	chr21:46916439C>T	29	F62	0	0.00002	0
COL18A1	2, 3	chr21:46932212C>T	42	F23	0.00067	0.00002	0
COL18A1	2, 3	chr21:46907418C>T	16	F14	0	0.00002	0
COL4A4	1, 2, 3	chr2:227872164G>C	48	F21	0	0	0.00077
COL4A4	1, 2, 3	chr2:227872783G>C	47	F40	0.0033	0.0027	0.00129
COL4A4	1, 2, 3	chr2:227920747C>T	30	F48	0.0019	0.0038	0.00362
COL4A4	1, 2, 3	chr2:227985771C>T	5	F49	0	0	0.00052
COL4A4	1, 2, 3	chr2:227920807G>A	30	F14	0	0.00003	0.00052
COL4A4	1, 2, 3	chr2:227958876C>G	20	F14	0	0.00003	0.00052
COL4A4	1, 2, 3	chr2:227886883G>A	44	F9	0	0.00003	0
COL4A6	2, 3	chrX:107400472C>G	43	F54	0	0	0.00099
COL4A6	2, 3	chrX:107462929G>A	5	F51	0.0033	0.0039	0.00442
COL4A6	2, 3	chrX:107430391C>T	23	F28	0.0013	0.0016	0
COL4A6	2, 3	chrX:107418908G>A	29	F59	0	0.0001	0
COL4A6	2, 3	chrX:107421920T>G	27	F14	0	0.00002	0
EPHB2	1	chr1:23189638A>G	4	F39	0	0.00001	0.00026
EPHB2	1	chr1:23110922C>T	3	F59	0	0.00003	0.00026
EPHB2	1	chr1:23189553G>T	4	F43	0	0.00003	0.00052
EPHB2	1	chr1:23234542C>T	10	F31	0	0	0
EPHB2	1	chr1:23239070G>A	13	F9	0	0.00003	0
FBN2	2	chr5:127713560C>G	13	F35	0	0.00003	0.00026
FBN2	2	chr5:127613643T>C	58	F14	0.00067	0	0.00026
FBN2	2	chr5:127597535C>T	64	F38	0.0005	0.0007	0
FBN2	2	chr5:127729008T>C	10	F35	0	0.00005	0
GAB2	1	chr11:77936215C>T	5	F61	0.0003	0.0002	0.00129

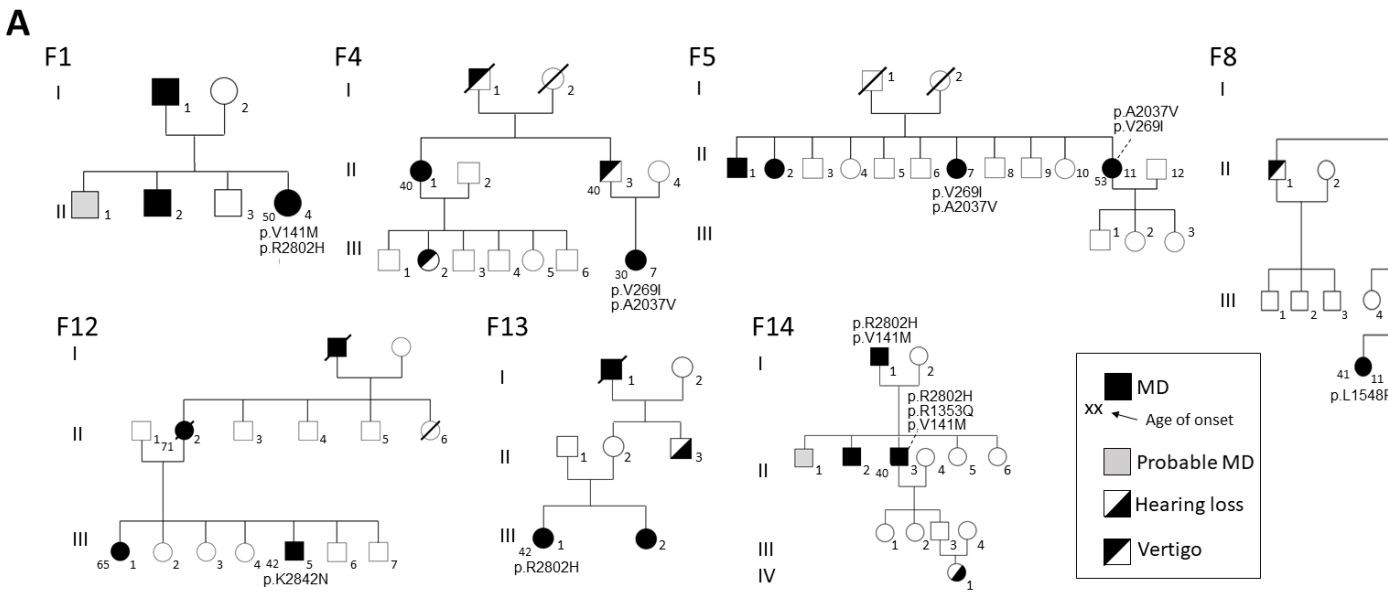
GAB2	1	chr11:77937522C>T	4	F38	0	0	0
GAB2	1	chr11:77937649T>G	4	F16	0.0042	0.00002	0
GAB2	1	chr11:77934541G>A	6	F51	0	0.00008	0
LAMC1	1, 2, 3	chr1:183090920A>G	12	F21	0.001	0.0008	0.00129
LAMC1	1, 2, 3	chr1:183101599G>A	21	F5, F44	0	0.0003	0.00026
LAMC1	1, 2, 3	chr1:183094538A>G	15	F52	0	0.00009	0.00026
LAMC1	1, 2, 3	chr1:183100494G>A	20	F57	0	0.00001	0.00052
LAMC1	1, 2, 3	chr1:183095324G>T	16	F36	0.0009	0.0019	0
LAMC3	3	chr9:133911651G>A	4	F36	0	0	0
LAMC3	3	chr9:133920939C>A	8	F35	0	0.00006	0
LAMC3	3	chr9:133945082G>A	17	F51	0	0.00003	0
LAMC3	3	chr9:133928039G>A	10	F62, F37, F39	0.0015	0.00003	0
LAMC3	3	chr9:133928319C>T	11	F8	0	0.00003	0
LAMC3	3	chr9:133948055G>C	19	F3, F36	0.0049	0.00003	0
LAMC3	3	chr9:133963151G>A	27	F8	0.0003	0.00003	0
LAMC3	3	chr9:133884693G>T	1	F56	0.0003	0.00003	0
MYH14	1	chr19:50770231G>A	23	F7, F61, F8	0.0006	0.00003	0
MYH14	1	chr19:50785088A>G	33	F4	0	0.00003	0
MYH14	1	chr19:50810310C>T	41	F8	0.00067	0.00003	0
MYH14	1	chr19:50755984G>A	16	F50	0.0005	0.00003	0
MYH14	1	chr19:50758572G>C	17	F57	0.0003	0.00003	0
MYO9B	1	chr19:17212814A>G	2	F30	0.0039	0.00003	0
MYO9B	1	chr19:17322565G>A	39	F44	0	0.00003	0
MYO9B	1	chr19:17322742A>C	40	F17, F9	0.0021	0.00003	0
MYO9B	1	chr19:17306138T>C	22	F10	0.00067	0.00003	0
NRP1	1	chr10:33486596C>T	11	F15	0	0.00003	0
NRP1	1	chr10:33502502C>G	9	F3	0	0.00003	0
NRP1	1	chr10:33545258C>T	5	F36, F40	0.0002	0.00003	0
NRP1	1	chr10:33491874A>C	11	F46	0	0.00003	0
NRP1	1	chr10:33496604C>T	10	F21	0	0.00003	0
SCN10A	1, 5	chr3:38743406C>T	25	F34	0	0.00003	0
SCN10A	1, 5	chr3:38760151A>G	19	F3	0.0007	0.00003	0
SCN10A	1, 5	chr3:38781116A>C	13	F6	0	0.00003	0
SCN10A	1, 5	chr3:38739975C>T	26	F5	0.00067	0.00003	0
SCN10A	1, 5	chr3:38755450C>T	20	F50	0.006	0.00003	0
SCN11A	1, 5	chr3:38913189T>C	21	F37, F13	0.0002	0.00003	0
SCN11A	1, 5	chr3:38946687C>T	11	F10	0.00067	0.00003	0
SCN11A	1, 5	chr3:38888494G>C	26	F25	0	0.00003	0
SPTA1	1, 4, 5	chr1:158589121G>A	45	F22	0.0031	0.00003	0
SPTA1	1, 4, 5	chr1:158596683C>T	41	F40	0	0.00003	0
SPTA1	1, 4, 5	chr1:158609685C>T	34	F31	0	0.00003	0
SPTA1	1, 4, 5	chr1:158612732G>A	32	F42	0.0002	0.00003	0
SPTA1	1, 4, 5	chr1:158615292C>A	28	F42	0.0001	0.00003	0
SPTA1	1, 4, 5	chr1:158639300C>A	14	F42	0.0001	0.00003	0

SPTA1	1, 4, 5	chr1:158639328C>T	14	F33	0.0017	0.00003	0
SPTA1	1, 4, 5	chr1:158644397T>C	9	F42	0.00067	0.00003	0
SPTA1	1, 4, 5	chr1:158647584G>T	7	F31	0	0.00003	0
SPTA1	1, 4, 5	chr1:158648255G>A	6	F31	0	0.00003	0
SPTA1	1, 4, 5	chr1:158651394C>T	4	F20	0.0006	0.00003	0
SPTA1	1, 4, 5	chr1:158582673T>G	51	F56, F58	0.0005	0.00003	0
SPTA1	1, 4, 5	chr1:158621162G>A	24	F11	0	0.00003	0
SPTA1	1, 4, 5	chr1:158641928C>T	11	F34	0	0.00003	0
SPTB	1, 4, 5	chr14:65237750G>A	26	F12, F21	0.0038	0.00003	0
SPTB	1, 4, 5	chr14:65261215C>T	12	F31	0.0002	0.00003	0
SPTB	1, 4, 5	chr14:65262093C>T	11	F33	0.0006	0.00003	0
SPTB	1, 4, 5	chr14:65262096G>T	11	F16	0.00007	0.00003	0
SPTB	1, 4, 5	chr14:65262122G>A	11	F36	0	0.00003	0
SPTB	1, 4, 5	chr14:65249092C>A	19	F55	0.0001	0.00003	0
SPTB	1, 4, 5	chr14:65236390A>G	27	F22	0.001	0.00003	0
UNC5B	1	chr10:73045124G>T	4	F2	0	0.00003	0
UNC5B	1	chr10:73050774G>A	8	F31, F3	0.0003	0.00003	0
UNC5B	1	chr10:73056387C>G	14	F31, F3	0.0001	0.00003	0
UNC5B	1	chr10:73056469G>C	14	F21	0.0002	0.00003	0
UNC5B	1	chr10:73057702C>T	15	F37	0	0.00003	0
UNC5B	1	chr10:73057808A>G	15	F24	0.0001	0.00003	0
UNC5B	1	chr10:73056443G>A	14	F40	0.00067	0.00003	0

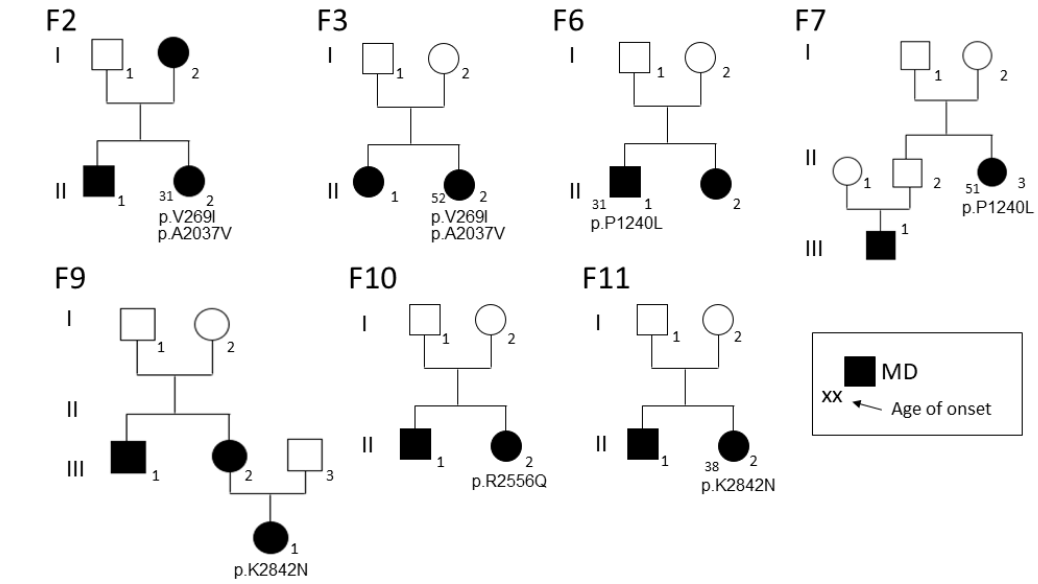
Supplementary Figure 1: Validation of candidate variants by Sanger sequencing.



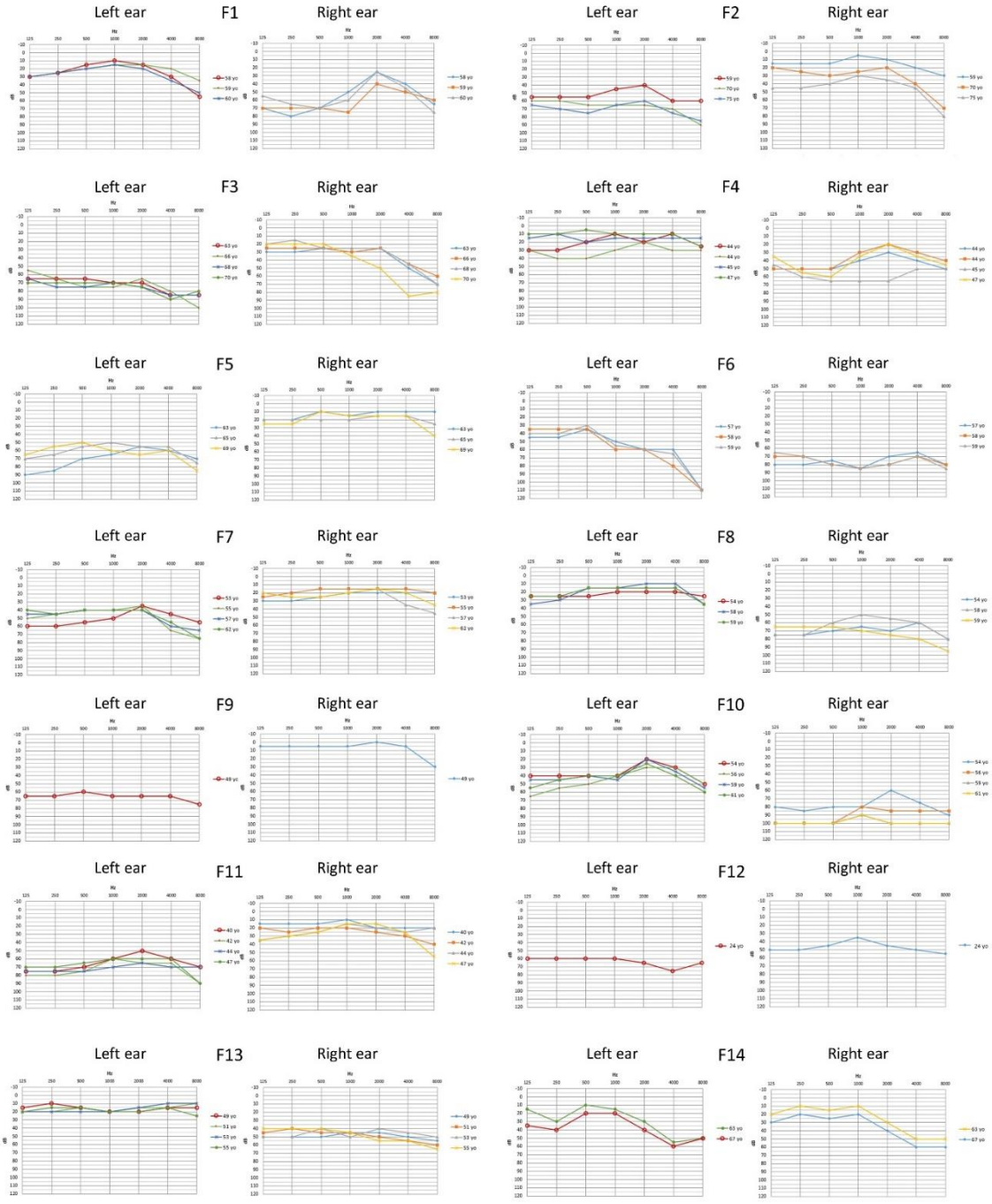
Supplementary Figure 2: Pedigrees of the 14 families carrying variants in the OTOG gene. Seven families showed incomplete pen
complete penetrance (2B). Thirty-eight individuals with MD were diagnosed in these families. Likewise, 5 families (F1, F4, F8, F13
(probable MD, hearing loss or vertigo), suggesting phenotypic heterogeneity. Incomplete penetrance was observed in 7 families (
variants found in gene burden analysis are indicated next to each patient by protein nomenclature.



B


F2
I
1
2
II
1
31
2
p.V269I
p.A2037V
F3
I
1
2
II
1
52
2
p.V269I
p.A2037V
F6
I
1
2
II
31
1
2
p.P1240L
F7
I
1
2
II
1
2
51
3
p.P1240L
III
1
F9
I
1
2
II
III
1
2
3
1
p.K2842N
F10
I
1
2
II
1
2
p.R2556Q
F11
I
1
2
II
1
38
2
p.K2842N
MD
xx
Age of onset

Supplementary Figure 3: Pure tone audiograms for the 14 familial MD patients with variants in *OTOG* gene



Original research article

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2. Morales, Ivan; **Román-Naranjo, Pablo**; Requena, Teresa. Encoding regions with low coverage in massive sequencing of genes of no syndromic autosomal dominant hearing loss. Actualidad Medica: 2017 - 102(802):135-139 – doi: 10.15568/am.2017.802.or01
3. Gallego-Martinez, Alvaro; Requena, Teresa; **Roman-Naranjo, Pablo**; Lopez-Escamez, Jose Antonio. Excess of rare missense variants in hearing loss genes in sporadic Meniere disease. Frontiers in Genetics: February 15, 2019 - 10:76 – doi: 10.3389/fgene.2019.00076
4. Gallego-Martinez, Alvaro; Requena, Teresa; **Roman-Naranjo, Pablo**; May, Patrick; Lopez-Escamez, Jose Antonio. Enrichment of Damaging Missense Variants in Genes Related With Axonal Guidance Signalling in Sporadic Meniere's Disease. Journal of Medical Genetics: February, 2020 - 57(2):82-88 – doi: 10.1136/jmedgenet-2019-106159.

Burden of Rare Variants in the *OTOG* Gene in Familial Meniere's Disease

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Conflicts of Interest and Source of Funding:

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Abstract

Objectives: Meniere's disease (MD) is a rare inner ear disorder characterized by sensorineural hearing loss, episodic vertigo and tinnitus. Familial MD has been reported in 6-9% of sporadic cases, and few genes including *FAM136A, DTNA, PRKCB, SEMA3D* and *DPT* have been involved in single families, suggesting genetic heterogeneity. In this study, the authors recruited 46 families with MD to search for relevant candidate genes for hearing loss in familial MD.

Design: Exome sequencing data from MD patients were analyzed to search for rare variants in hearing loss genes in a case-control study. A total of 109 patients with MD **(**73 familial cases and 36 early-onset sporadic patients) diagnosed according to the diagnostic criteria defined by the Barany Society were recruited in 11 hospitals. The allelic frequencies of rare variants in hearing loss genes were calculated in individuals with familial MD. A single rare variant analysis (SRVA) and a gene burden analysis (GBA) were conducted in the dataset selecting one patient from each family. Allelic frequencies from European and Spanish reference datasets were used as controls.

Results: A total of 5136 single nucleotide variants in hearing loss genes were considered for SRVA in familial MD cases, but only one heterozygous likely pathogenic variant in the *OTOG* gene (rs552304627) was found in two unrelated families. The GBA found an enrichment of rare missense variants in the *OTOG* gene in familial MD. So, 15/46 families (33%) showed at least one rare missense variant in the *OTOG* gene, suggesting a key role in familial MD.

Conclusions: The authors found an enrichment of multiplex rare missense variants in the *OTOG* gene in familial MD. This finding supports *OTOG* as a relevant gene in familial MD and set the groundwork for genetic testing in MD.

INTRODUCTION

Meniere's disease [MD (OMIM 156000)] is a rare inner ear disorder with three major symptoms: sensorineural hearing loss (SNHL), episodic vertigo and tinnitus (Lopez-Escamez et al. 2015; Espinosa-Sanchez & Lopez-Escamez 2016). Hearing loss always involves low and medium frequencies in one or both ears (unilateral or bilateral MD) at the onset of the disease. However, MD also affects high frequencies in early or advanced stages of the disease (Belinchon et al. 2011). Epidemiological studies indicate that MD is most common in European population, suggesting a genetic predisposition (Ohmen et al. 2013). Although the majority of MD patients are considered sporadic (Frejo et al. 2016; Frejo et al. 2017), familial clustering has been reported in 8-9% of sporadic cases in the European descendent (Requena et al. 2014), and in 6% of Korean population (Lee et al. 2015), which also supports a genetic contribution to the disease (Roman-Naranjo et al. 2017). MD shows a wide range of phenotypic variations among patients, even within the same families (Lee et al. 2015b), and it is commonly associated with migraine and systemic autoimmune disorders (Tyrrell et al. 2014; Cha et al. 2008). Familial MD (FMD) shows an autosomal dominant (AD) pattern of inheritance with incomplete penetrance and anticipation, showing an earlier onset compared to sporadic cases (Morrison et al. 2009; Birgerson et al. 1987; Klar et al. 2006). Different whole exome sequencing (WES) based studies have identified several genes related with FMD. Single nucleotide variants (SNV) in *DTNA*, *FAM136A*, *PRKCB*, *DPT* and *SEMA3D* were identified in 4 different families AD inheritance with incomplete penetrance (Requena et al. 2015b; Martín-Sierra et al. 2016; Martín-Sierra et al. 2017). However, these findings have not been replicated neither in other MD families nor sporadic MD (SMD) cases.

WES continues to be an efficient tool to determine disease-causing variants (Williams et al. 2016; Adams & Eng 2018; Suwinski et al. 2019), although the monogenic hypothesis in FMD should be reconsidered to achieve results beyond private rare variants for singular families. Thus, the "one

variant-one disease" hypothesis, described for classic Mendelian inheritance cannot explain the incomplete penetrance or variable expressivity observed in MD (Martín-Sierra et al. 2017) and more complex inheritance models are needed (Cooper et al. 2013; Kousi & Katsanis 2015). Oligogenic and multiallelic models have been already applied in different diseases, such as Parkinson (Lubbe et al. 2016) and Huntington's disease (Lee et al. 2015a), explaining changes in disease progression and phenotypic variability. Furthermore, a digenic inheritance of deafness was reported by variants in *CDH23* and *PCDH15* (Zheng et al. 2005), and recently, an enrichment of rare missense variants in certain SNHL genes, such as *GJB2*, *SLC26A4* or *USH1G*, was found in a large cohort of SMD cases (Gallego-Martinez et al. 2019b), supporting the hypothesis of multiallelic inheritance in MD.

More than 150 genes have been associated to deafness (Azaiez et al. 2018), and 116 of them are related with non-syndromic SNHL (Van Camp G. 2018). We have investigated the genetic background of FMD, focusing on SNHL genes by analyzing 46 families with MD by WES. We have found an enrichment of rare missense variants in the *OTOG* gene compared with non-Finnish European (NFE) and Spanish populations. The *OTOG* gene, which encodes otogelin, has been previously associated with deafness and imbalance and causes autosomal recessive deafness 18B (Simmler et al. 2000a; Schraders et al. 2012). A total of 15 families out of 46 showed, at least, one rare missense variant in this gene, suggesting a key role of otogelin in MD.

MATERIALS AND METHODS

Patient assessment and selection

All patients were diagnosed following the diagnostic criteria described by the International Classification Committee for Vestibular Disorders of the Barany Society (Lopez-Escamez et al. 2015, see Table 1, Supplemental Digital Content 1) . Seventy-three MD patients over 18 years old from 46 different families with one or more affected first-degree relatives, and 36 sporadic MD cases with an age of onset under 35 years were recruited. A complete hearing and vestibular assessment was carried out in all cases, including a brain magnetic resonance imaging to exclude other causes of neurological symptoms. Serial pure tone audiograms were retrieved from clinical records to assess hearing loss since the initial diagnosis.

A summary of the clinical information of these patients is presented in Table 1 and Supplemental Digital Content 1 (see Table 2 and Table 3, Supplemental Digital Content 1).

This study protocol was approved by the Institutional Review Board for Clinical Research (MS/2014/02), and a written informed consent to donate biological samples was obtained from all subjects.

DNA extraction and whole exome sequencing

Blood and saliva samples were taken from patients with MD to perform WES. DNA samples were extracted with prepIT-L2P (DNA Genotek, Ottawa, Canada) and QIAamp DNA Blood Mini Kit (Qiagen, Venlo, The Netherlands) using manufacturer's protocols and quality controls previously described (Szczepek et al. 2019). DNA libraries were prepared by using the SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) and were paired-end sequenced on the Illumina HiSeq 4000 platform at 100X coverage. Raw reads were stored in two FASTQ files for each individual.

Bioinformatic analysis

Dataset generation and processing

Analysis-ready BAM files and VCF files were generated from raw unmapped reads using the GATK Best Practices pipeline. Reads were aligned to the GRCh37/hg19 human reference genome using the BWA-MEM algorithm. For obtaining the final dataset, SNV and small structural variants were filtered according to its Variant Quality Score Recalibration (VQSR) and depth of coverage (DP) values. Thus, variants were excluded if their VQSR value were under the VSQR threshold or their average DP < 10. Variants were functionally annotated using ANNOVAR version 2018Apr16. RefSeq was used for gene-based annotation and the Exome Aggregation Consortium (ExAC) database, the Combined Annotation Dependent Depletion (CADD) scores and the dbNSFP database (v3.0) were used for filter-based annotation.

Sensorineural hearing loss gene set

The SNHL gene set was generated by using three different databases: the Hereditary Hearing Loss Homepage (Van Camp G. 2018), the Deafness Variation Database (Azaiez et al. 2018) and Harmonizome (Rouillard et al. 2016), containing a total of 116 genes related with SNHL (see Table 4, Supplemental Digital Content 1).

Data analysis and prioritization strategy

Two pipelines and filtering/prioritization strategies were conducted to search for rare variants as we have previously described (Gallego-Martinez et al. 2019b). The first was a single rare variant analysis (SRVA) for studying individual families; the second approach was a gene burden analysis (GBA) to obtain a gene-level mutational profile (Figure 1). For these analyses only one patient from each family was selected. Whenever possible, the patient selected was in the last generation. Sporadic cases with an early onset were also investigated to search for singleton variants in candidate genes in both analyses.

All variants were assessed according to the standards and guidelines described by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (Richards et al. 2015). Variants not described in the NFE population from ExAC or gnomAD v3 and the Spanish population from CSVS were discarded to minimize false calls and population-specific variants (Shearer et al. 2014). Selected variants were checked in patients BAM files with IGV and/or sequenced by Sanger sequencing to minimize false calls.

Statistics and databases

Three independent datasets were used as reference to compare the observed MAF in FMD and to calculate odds ratios (OR): NFE population from ExAC, NFE population from gnomAD v3 and the Collaborative Spanish Variant Server (CSVS) database (Lek et al. 2016; Dopazo et al. 2016).

For each selected variant in the SRVA, OR with 95% confidence interval (CI) were calculated using the MAF values from the CSVS database (N=1,579), the NFE population (N=33,365) from ExAC, and the NFE population from gnomAD v3 (N=32,299).

For GBA, we counted the total exonic alternate alleles per gene in our cohort against the three reference datasets. After calculating OR with 95% CI, we obtained one-sided p-values that were corrected for multiple testing by the total number of variants found in each gene following the Bonferroni approach.

Standard audiometric evaluations for air and bone conduction elicited by pure tones from 125 to 8000 Hz were retrieved from the clinical records to analyse the time course of the hearing profile in FMD cases with candidate variants. Regression analysis was performed to estimate the outcome of hearing loss for each frequency.

RESULTS

Main genetic findings in familial MD

Single rare variant analysis

A total of 5136 variants located in SNHL genes were considered in FMD cases. After applying quality controls (QC), 4247 SNV remained. Only 114 nonsynonymous or splice site SNV fulfilled the MAF (<0.001) filtering criteria in at least one reference population dataset (Figure 1). From them, 80 SNV were already described in the NFE population or Spanish population (see Table 5, Supplemental Digital Content 1, which shows the rare variants found in the SRVA for FMD cases). A heterozygous variant located in *OTOG* gene was observed in cases from two unrelated families (F1 & F14). The variant chr11:17574758G>A (rs552304627; p.V141M), which is in the last nucleotide of the fourth exon in the *OTOG* canonical transcript (ENST00000399391), is likely pathogenic according to the ACMG and AMP guidelines. This multiplex variant is located in a Von Willebrand Factor D-type domain (vWD) with a MAF=.0008 in NFE population from ExAC and a MAF=.0011 in NFE population from gnomAD. Multiple *in silico* tools supported a likely pathogenic effect of this variant (SIFT score=.001; M-CAP=.153; CADD=28.2; GERP++ =5.36). In addition, a heterozygous missense variant located in *DMXL2* gene (chr15:51828804C>A) was found in two individuals with FMD from two unrelated families (F17 & F31). This variant was classified as an uncertain significance according to the ACMG and AMP guidelines and it has been only described in Latino population from gnomAD v3 with a frequency of 0.00007. These variants were validated by Sanger sequencing (see Figure 1, Supplemental digital content 2).

The rest of the rare SNV were considered private familial variants because no of them were found in other FMD cases. None small structural variant (insertion or deletion) was found in any SNHL genes.

Gene burden analysis

Seventy-four genes with 222 SNV with a MAF<0.05 were retained after QC and filtering steps. Most of the genes (72%) carried less than 3 variants, thus they were discarded for further analysis. The most significant finding was an enrichment of rare missense variants in *OTOG* gene in our FMD cases against either NFE population from ExAC (OR= 3.7 (2.4-5.7), p=3.3x10^{-8}) and gnomAD (OR= 4.0 (2.6-6.1), p= 4.6x10^{-9}) or Spanish population (OR= 3.0 (1.9-4.8), p= 1.2x10^{-5}). Nine different rare missense variants were found in *OTOG* in 14/46 non-related families, existing 6 families with 2 or more shared variants (Table 2 & see Table 6, Supplemental Digital Content 1 & Figure 2, Supplemental Digital Content 2). The variants rs61978648 and rs61736002 were shared by individuals from 4 unrelated families (F2, F3, F4 & F5). Likewise, the variants rs552304627 and rs117315845 were found in patients from other 2 unrelated families (F1 & F14).

In addition, a novel variant in *OTOG* not included in the GBA was found in two cases from a 15th family (F34). This variant, located in exon 18 (chr11:17594747C>A), was found in heterozygous state affecting the sequence of the C8 domain. The distribution of the variants found in *OTOG* is scattered across the gene sequence (Figure 2).

Hearing profile in familial patients with rare variants in *OTOG*

The hearing profile for the 14 patients (3 males, 11 females) with rare variants in *OTOG* gene was studied (see Figure 3, Supplemental Digital Content 2, which shows the pure tone audiograms for these patients). Ten of them showed bilateral hearing loss, 3 had left-sided hearing loss and only 1 patient showed right-sided SNHL (Table 1). From these 14 patients, 16 ears from 12 patients showed a flat shaped audiogram (57.1%), 5 ears from 5 patients showed a ski-slope shaped audiogram (17.8%), 3 ears from 3 patients showed a reverse-slope shaped (10.7%) and 4 ears had a normal pure-tone audiogram (14.2%).

A regression analysis was done to estimate the hearing loss at onset and the outcome for each frequency. We found a negative correlation at 1000 Hz (R^2=.143; *p*=.033) and 2000 Hz (R^2=.246; *p*=.004). There was no statistical correlation at 125 Hz, 250 Hz, 500 Hz, 4000 Hz nor 8000 Hz, suggesting no progression at these frequencies (see Figure 4, Supplemental Digital Content 2). The age of onset of the symptoms was 41.93±8.66 and the estimated hearing loss at onset was 62.14±12.83 for low frequencies (125-250-500 Hz) and 58.75±14.1 for high frequencies (1000-2000-4000 Hz).

Early onset sporadic MD

The same analytical pipeline was used in a series of patients with sporadic MD with an age of onset younger than 35 (see Figure 5, Supplemental Digital Content 2). For the SRVA, we found 66 nonsynonymous or splice site SNV with MAF < 0.001 in SNHL genes. Among them, three variants were found in two sporadic cases and another variant was also found in a familial case. The rest of the SNV were considered simplex variants found in singletons and none of them were homozygous (see Table 7, Supplemental Digital Content 1, which shows the rare variants found in the SRVA for SMD cases).

A heterozygous nonsynonymous SNV in *OTOG* gene was found in two unrelated sporadic MD cases (S1 and S23). The variant chr11:17632279C>T (rs779658224; p.A1823V) is located in exon 35 of the canonical transcript of *OTOG* gene and it is a variant of uncertain significance (VUS) according to the ACMG and AMP guidelines. This variant has a MAF=.00054 in the NFE population from gnomAD v3 and it is not described in the Spanish population from the CSVS. In addition, two heterozygous nonsynonymous variants were found in two sporadic MD cases (S1 & S11) in *DMXL2* gene. These two SNVs (rs762424714; p.H2287N & rs117017152; p.I699T) are not located in any known domain of this protein and both are classified as likely benign according to their CADD scores (9.64 and 9.74, respectively). Finally, a heterozygous nonsynonymous SNV in *OTOGL* gene was found in one sporadic case and in one familial case (S27 and F31). The variant

chr12:80752642T>G (rs145929269; p.C2068G) is located in exon 51 of the canonical transcript of *OTOGL* gene (ENST00000458043). This region encodes a cysteine-rich region and this variant was also classified as a VUS according to the ACMG and AMP guidelines.

For the GBA, we found 13 rare SNV in *OTOG* gene in patients with early onset MD (see Table 8, Supplemental Digital Content 1). However, in contrast with the results obtained in FMD cases, there was not an excess of rare variants in this gene against neither the NFE population from ExAC (OR=2.0 (1.2-3.2), *p*=.067) or gnomAD (OR=2.0 (1.2-3.2), *p*=.075) nor Spanish population (OR=1.9 (1.1-3), *p*=.16) (Figure 2).

DISCUSSION

Familial MD has an AD inheritance with incomplete penetrance (Morrison et al. 2009; Requena et al. 2014), and few genes have been involved in singular families (Requena et al. 2015b; Martín-Sierra et al. 2016; Martín-Sierra et al. 2017). In this study, we have found an enrichment of rare missense variants in several unrelated patients with FMD in the *OTOG* gene. These variants were observed in 15 of 46 non-related families (33% familial cases). Seven of the 15 families with rare variants in *OTOG* showed incomplete penetrance (47%) and partial syndromes (episodic vertigo or hearing loss) were found in relatives from 5 of 15 families (Morrison et al. 2009; Requena et al. 2015b; Martín-Sierra et al. 2016; Martín-Sierra et al. 2017). Most of these rare variants were found in 2, 3 or 4 unrelated individuals from different families with MD and they were considered multiplex variants. However, the majority of the variants in *OTOG* found in non-familial patients with early onset were not observed in other sporadic cases (singletons variants).

OTOG, which encodes otogelin, was described for the first time by Cohen-Salmon *et al* (Cohen-Salmon et al. 1997). Otogelin is a 2925 amino acid protein (ENST00000399391) constituted by several vWD and C8 domains, and a cysteine knot-like domain in its C-terminal. It is mainly expressed in acellular structures which cover the sensory inner ear epithelia: the tectorial membrane, the otoconial membranes and the cupula over the cristae ampullaris of the semicircular canals. Because of its localization in the extracellular structures overlying the stereocilia of the hair cells involved in the mechanotransduction of sound and acceleration, this structural protein plays an important role in both auditory and vestibular functions (Schrauwen et al. 2016).

The effects of variants in otogelin were first demonstrated in the orthologous gene in a mouse model. Three mouse models have been generated to evaluate the phenotypic changes resulting from *OTOG* variants. In the $Otog^{tm1Prs}$ model, authors inactivated Otog by deleting the first three

exons. Vestibular dysfunction was detected at P4 in Otog$^{-/-}$, observing anomalies in the saccule and utricule. The auditory function was evaluated by Pleyer reflex, showing profound hearing impairment. The Otog$^{+/-}$ mice did not present any anomalies (Simmler et al. 2000a). The second model is the twister (twt) mice, mice with a spontaneous recessive mutation entailing absence of Otog expression. Similarly to Otog^{tm1Prs}, in Otog^{twt} the vestibular dysfunction was detected at P4, and the hearing loss was progressive and moderate to severe/profound (Simmler et al. 2000b). The last mouse model published is the otogelin ENU-induced mouse model. In this model, a homozygous variant at the splice donor site of intron 29, Otog$^{vbd/vbd}$, cause a frame-shift and a premature codon. Otog$^{vbd/vbd}$ mice showed abnormal hearing and vestibular functions (El Hakam Kamareddin et al. 2015).

Four variants have been described in *OTOG* gene causing DFNB18B. Schraders *et al*. were the first to describe causative variants in *OTOG*. A homozygous 1bp deletion, c.5508delC (p.Ala1838Profs*31) in four related patients, and two compound-heterozygous variants, c.6347C>T (p.Pro2116Leu) and c.6559C>T (p.Arg2187*) in other two related patients, were described to cause hearing loss and vestibular dysfunction (Schraders et al. 2012). More recently, a homozygous nonsense variant c.330C>G (p.Tyr110*) in a Korean patient has been identified, showing early-onset mild hearing loss without vestibular dysfunction (Yu et al. 2019). Imaging studies in families with DFNB18B with homozygous mutations in *OTOG* gene did not found abnormalities in CT scans of the temporal bone (Oonk et al. 2014; Ganaha et al. 2019).

In contrast to studies mentioned above, *OTOG* variants found in this study were all in heterozygous state and, despite 6 FMD cases and 3 SMD cases studied had two or more variants, compound heterozygous variants could not be demonstrated because samples from the parents were not available and *OTOG* variant segregation could not be fully assessed in each family. However, the variants chr11:17574758G>A and chr11:17663747G>A found in F14 were also identified in his mother, the variants chr11:17578774G>A and chr11:17632921C>T found in F5

were also identified in her sister (II-7), and a novel variant chr11:17594747C>A not considered for the GBA were found in F34 and her brother. Furthermore, variants located in untranslated regions (UTRs) and promoter regions, which modulate gene expression and different protein features (Chatterjee & Pal 2009; Buckland 2006), could not be evaluated because of the study design. Altogether, the results obtained by GBA suggested a different genetic architecture in FMD cases and SMD cases, since the enrichment of rare variants in *OTOG* gene was only found in FMD cases and most of the variants found in sporadic cases with early onset were singletons (not observed in multiple individuals).

Each region of the cochlea is specifically stimulated by a specific frequency. Thus, the base of the cochlea mainly responds to high-frequency sounds, whereas the apex responds to low-frequency sounds, frequencies mostly affected in MD (Robles & Ruggero 2017; Nakashima et al. 2016). Of note, otogelin shows a tonotopic gene expression in mice (Yoshimura et al. 2014). *OTOG* gene showed a 2.43-fold change in expression for apex vs base, making this gene a possible candidate for SNHL in MD. In addition, an RNA-seq study of the inner ear from patients with normal hearing showed a high expression of *OTOG* gene in the vestibule (Schrauwen et al. 2016), which could explain the vestibular dysfunction in patients with pathogenic variants in this gene.

Otogelin is an extracellular protein located in the tectorial and otolithic membranes of the saccule and utricle. In a study performed in zebrafish ear, otogelin and tectorin alpha are required for otolith tethering in the otolithic membrane. It seems that there are two stages in this process: seeding and maintenance of the otoliths. The initial seeding step, in which otolith precursor particles tether directly to the tips of hair cell kinocilia, fails to occur in the einstein (*eis*) zebrafish mutant, an *OTOG* knock-out (Stooke-Vaughan et al. 2015). Although there is a large difference between *eis* zebrafish and the human phenotype in MD, the *eis* mutation disrupts otolith seeding and we speculate the carriers of *OTOG* variants may have a fragile

tectorial and otolithic membranes with lower tethering of otoconia that will lead to a severe perturbation of the endolymphatic fluid.

Moreover, the lack of otogelin in mice results in outer hair cells dysfunction due to a loss of a) horizontal top connectors between stereocilia and b) the tectorial membrane attachment crowns which couple the tallest stereocilia to the tectorial membrane. Besides, otogelin forms homodimers and, through its interaction with otogelin-like and stereocilin, they are part of the horizontal top connectors and the tectorial membrane attachment crowns. The otogelin-deficient mice *Otog*$^{tm1Prs/tm1Prs}$ showed moderate-to-severe hearing impairment as well as a balance disorder. Interestingly, mice carrying heterozygous variants showed a small progressive hearing loss, whereas mice carrying homozygous variants showed hearing loss from early stages (Avan et al. 2019). This could support a dominant negative pathogenic mechanism in MD patients with variants in *OTOG* gene, since rare variants in otogelin could impair the formation of dimers with the wild-type protein.

The audiograms of FMD patients who carried rare variants in *OTOG* gene showed a moderate-to-severe flat hearing loss ≈60 dB since the first years of onset involving all frequencies. Low-frequency hearing had slight variations throughout the years, while a negative correlation was found at mid (1000Hz) and high-frequency (2000Hz) hearing. Data from F14 were considered as an outlier and discarded because his hearing profile was not comparable to the rest of FMD patients (see Figure 3, Supplemental Digital Content 2). Since all frequencies are involved since the onset of the disease, we can speculate that the damage of the tectorial membrane mediated by mutations in otogelin will involve the entire cochlea from base to apex.

According to our results, the clinical picture of patients with mutations in *OTOG* would be a 43 years old female with sudden or rapidly progressive flat SNHL around 60 dB and vertigo attacks with a family history of MD, vertigo or early onset SNHL.

Gene burden analysis have been previously used to investigate the genetic background of sporadic MD. Gallego-Martinez *et al.* have published 2 studies selecting sporadic (non-familial) MD patients using two custom gene panels. The first study included 45 autosomal genes related with SNHL, however *OTOG* was not selected for this panel (Gallego-Martinez et al. 2019b). The second study included genes from 2 main pathways showing differentially expressed genes in supporting cells of the cochlea and vestibular organs: axonal guidance signaling and leukocyte extravasation pathway (Gallego-Martinez et al. 2019a). These studies found an enrichment of multiplex rare variants in several SNHL genes such as *GJB2, USH1G, SLC26A4, ESRRB,* and *CLDN14* and axonal-guidance signalling genes such as *NTN4* and *NOX3* in non-familial patients with MD.

In conclusion, we have found an enrichment of rare missense variants in the *OTOG* gene in FMD cases. These findings support a multiallelic contribution in MD, where *OTOG* gene seems to be playing a relevant role in the pathophysiology of hearing and vestibular functions in MD.

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Authors declare no conflict of interest.

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Figure legends

Figure 1: Flowchart summarizing the bioinformatic analysis on familial MD cases. On the left, single rare variant analysis (SRVA). On the right, the gene burden analysis (GBA) pipeline. SNV, single nucleotide variants.

Figure 2: Variants distribution across OTOG gene domains. On the upper part, variants which were found in familial Meniere disease (FMD) cases. On the bottom part, variants which were found in sporadic Meniere disease (SMD) cases. Variants p.V269I, p.L1548F, p.R2802H and p.K2842N were observed in both FMD and SMD cases. Yellow-colored variants indicate variants found in only one case, whereas red-colored variants represent variants found in 2 or more cases in a cohort. vWD, von Willebrand factor type D domain; T, Trypsin inhibitor-like domain; Abf, Alpha-L-arabinofuranosidase B domain; CT, Cysteine knot domain.