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MECHANISMS OF INFLAMMATION IN MENIERE'S DISEASE

MECANISMOS DE INFLAMACIÓN EN LA ENFERMEDAD DE MENIERE

Universidad de Granada

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TESIS DOCTORAL CON MENCIÓN EUROPEA

LIDIA FREJO NAVARRO

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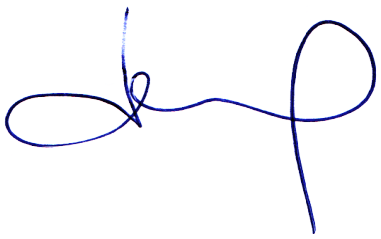

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Abstract

Meniere's disease [MD; MIM 156000] is a chronic disorder with a prevalence of ~ 0.5-1 / 1000 which is characterized by attacks of vertigo associated with sensorineural hearing loss involving low to medium frequencies. Although its etiology remains unknown, MD is often found in families with incomplete phenotypic forms, leading to a high clinical heterogeneity. Furthermore, several evidences have associated MD with autoimmunity where allelic variation in the innate immune response and inflammation genes seem to contribute the hearing outcome of the disease. Otherwise, multiple studies have demonstrated an epidemiologic association between allergy status and MD.

With this in mind, the goals of this PhD thesis were: first, to define a clinical group of patients with autoimmune MD within a large cohort of MD patients using two-step cluster analysis. Second, to identify allelic variants associated with autoimmune MD that could be used as genetic markers and, to investigate their role in MD inflammatory response performing gene expression analysis in the SNV carriers and using proliferation and confocal assays. Finally, to define the effect of allergenic extracts from *Penicillium* and *Aspergillus* in proinflammatory cytokines and gene expression profile in MD patients PBMCs.

Our findings support that: 1) There are 5 clinical subgroups in uni- and bilateral MD; a subgroup of patients was associated with another autoimmune disease (MD type 5, 11%). 2) A locus at 6p21.33, suggests an association with bilateral MD, which could regulate pathways involving TNF family members and through NFκB-mediated inflammation. 3) There are two different subgroups of MD patients according to their basal levels of cytokines and, mold extracts are able to trigger a significant release of IL-6 and TNF-α in MD patients when compared to healthy controls.

We have found evidences that support several mechanisms of inflammation involving proinflammatory cytokines, regulation of TWEAK/Fn14 pathway and an aberrant response to mold extracts in MD patients.

Resumen

La enfermedad de Meniere [EM; MIM 156000] es una enfermedad crónica con una prevalencia de ~ 0.5-1 / 1000, que se caracteriza por ataques de vértigo asociados a pérdida de audición neurosensorial de bajas y medias frecuencias. Aunque su etiología se desconoce, la EM suele encontrarse en familias con fenotipo incompleto, conduciendo a una alta heterogeneidad clínica. Además, existen evidencias que asocian la EM con autoinmunidad, donde variaciones alélicas en la respuesta inmune innata así como genes asociados a inflamación parecen contribuir en sobre la audición en la enfermedad. Por otra parte, múltiples estudios han demostrado una asociación epidemiológica entre la alergia y la EM.

Teniendo esto en cuenta, los objetivos de esta tesis son: primero, definir un grupo clínico de pacientes con EM autoinmune dentro de una gran cohorte de pacientes con EM utilizando análisis de clúster en dos pasos. Segundo, identificar variantes alélicas asociadas con EM autoinmune que puedan ser utilizadas como marcadores genéticos mediante un array de genotipado en la respuesta inflamatoria en EM realizando análisis de expresión génica en los portadores de la variante así como ensayos de proliferación y microscopia confocal. Finalmente, definir el efecto de extractos alergénicos *Penicillium* y *Aspergillus* sobre citoquinas proinflamatorias y realizar perfiles de expresión génica en células mononucleares de pacientes con EM.

Nuestros hallazgos sustentan que: 1) Hay 5 subgrupos clínicos en EM uni- y bilateral; un subgrupo de pacientes se asoció a otra enfermedad autoinmune (EM tipo 5, 11%). 2) Un locus en 6p21.33, sugiere una asociación con la EM bilateral, que podría regular vías que implican miembros de la familia de TNF y a través de inflamación mediada por NFκB. 3) Hay dos grupos distintos de EM según sus niveles basales de citoquinas y además la exposición a extractos fúngicos induce una liberación exacerbada de IL-6 TNF-α en pacientes con EM cuando los comparamos con controles sanos.

Hemos encontrado evidencias que apoyan distintos mecanismos de inflamación que se relacionan con citoquinas proinflamatorias, la regulación de la vía TWEKFn14 y una respuesta anormal a extractos fúngicos en pacientes con EM.

Abbreviations

AAO-HNS	American Academy of Otolaryngology-Head and Neck Surgery
AD	Autoimmune Diseases/comorbidities
AIED	Autoimmune Inner Ear Disease
BBB	Blood-Brain Barrier
BMD	Bilateral Meniere Disease
DH	Delayed Hydrops / Delayed MD
EAONO	European Academy of Otology and Neurotology
EH	Endolymphatic Hydrops
ES	Endolymphatic Sac
FMD	Familial Meniere Disease
HC	Hair cells
HL	Hearing loss
IHC	Inner Hair Cells
IL-1	Interleukin-1
IL-1RA	Interleukin-1 Receptor Antagonist
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
MD	Meniere Disease
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
OHC	Outer Hair Cells
PBMC	Peripheral Blood Mononuclear Cells
RA	Rheumatoid Arthritis
RL	Reticular Lamina
SC	Supporting Cells
SD	Standard Deviation

SGN	Spiral Ganglion Neurons
SL	Spiral Ligament
SLE	Systemic Lupus Erythematosus
SMD	Sporadic Meniere Disease
SNHL	Sensorineural Hearing Loss
SNV	Single Nucleotid Variant
TNF-α	Tumor Necrosis Factor Alpha
UMD	Unilateral Meniere Disease
VM	Vestibular Migraine

1. Introduction

1.1. Inner ear anatomy

The inner ear is a sensory organ totally enclosed in the temporal bone. It consists of three main structures: the cochlea, the vestibule and the endolymphatic sac (ES) (**Figure 1**). The cochlea and vestibule are peripheral sensory organs that detect sound and angular/linear acceleration, respectively; conversely, the ES is a cystic structure and does not contribute to detection of these stimuli.

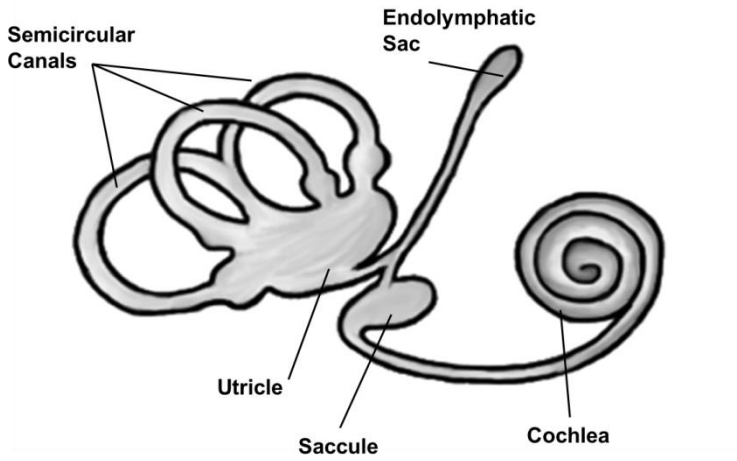


Figure 1. General diagram of the Inner ear

These structures are divided in bony and membranous constructions; each of them is filled with fluids of a unique ion composition called Endolymph and Perilymph, which are essential for maintaining hearing and balance. Endolymph in the cochlea and vestibular system has high [K^+] and low [Na^+], while the ES has high [Na^+] and low [K^+][1]. High [K^+] in the cochlea and vestibule allows depolarization of the sensory epithelium by providing K^+ ions to the cytoplasm through mechanosensitive cation channels, propagating sound and acceleration stimulation transmission to the central nervous system over the vestibulo-cochlear nerve[2, 3]. The ES is considered to be involved in ion homeostasis and Endolymph volume regulation[4]; however the regulatory mechanisms for ion homeostasis and Endolymph volume have not been conclusively evidenced.

1.1.1. The Cochlea

The cochlea is the auditory portion of the inner ear. It is a spiral-shaped cavity in the bony labyrinth. Its main function is to separate sounds according to their frequencies before they are translated by the hair cells into a neural code in the fibers of the auditory nerve.

A core component of the cochlea is the Organ of Corti, the sensory organ of hearing, which is distributed along the partition separating fluid chambers in the coiled tapered tube of the cochlea. The sensory epithelium within the Organ of Corti is a cellular layer on the basilar membrane, in which sensory hair cells are powered by the potential difference between the perilymph and the endolymph (**Figure 2**). Hair cells transform the vibration of the basilar membrane into neural stimuli, which are conducted by spiral ganglion. Cochlear hair cells in humans are organized in one row of inner hair cells and three rows of outer hair cells[5].

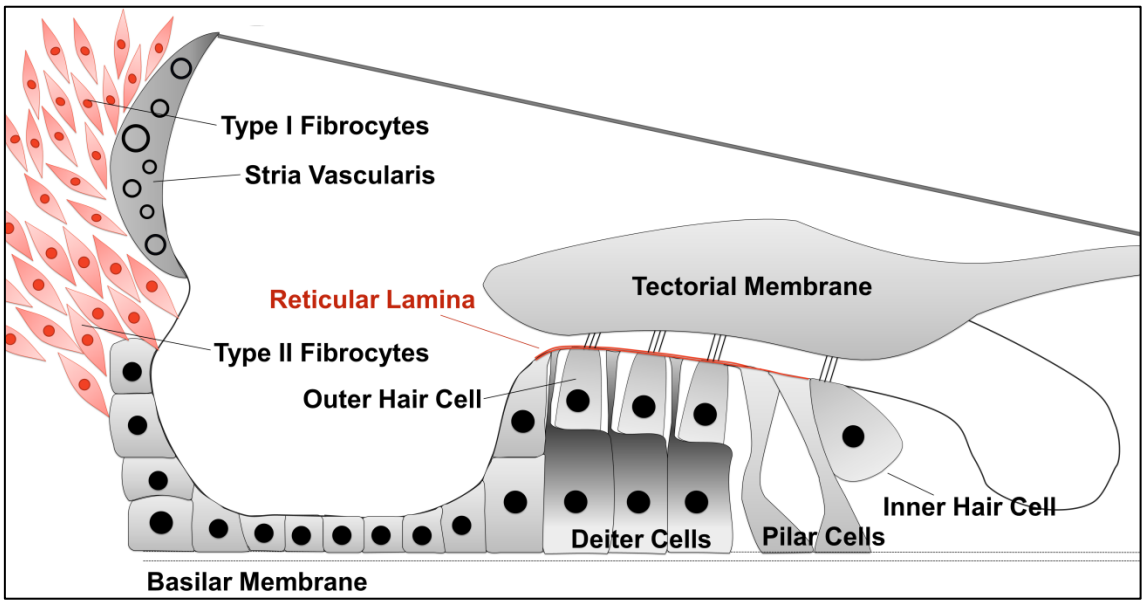


Figure 2. Diagram of the Organ of Corti epithelia.

- **Inner Hair cells (IHC):** These are the actual sensory receptors. IHC transform the sound vibrations in the fluids of the cochlea into electrical signals that are then relayed via the auditory nerve to the auditory brainstem and to the auditory cortex.

The deflection of the hair-cell stereocilia opens mechanically gated ion channels that allow any small, positively charged ions (primarily Ca^{2+} and K^{+}) to go in the cell[6]. The influx of positive ions from the endolymph in the scala media depolarizes the cell, resulting in a receptor potential. This receptor potential opens voltage gated calcium channels; calcium ions then enter the cell and trigger the release of neurotransmitters at the basal end of the cell. The neurotransmitters diffuse across the narrow space between the hair cell and a nerve terminal, where they then bind to receptors and thus trigger action potentials in the nerve. In this way, the mechanical sound signal is converted into an electrical nerve signal. Repolarization of hair cells is done in a special manner. The perilymph in the scala tympani has a very low concentration of positive ions. The electrochemical gradient makes the positive ions flow through channels to the perilymph.

Hair cells chronically leak Ca^{2+}. This leakage causes a tonic release of neurotransmitter to the synapses. It is thought that this tonic release is what allows the hair cells to respond so quickly in response to mechanical stimuli. The velocity of the hair cell response may also be due to the fact that it can increase the amount of neurotransmitter release in response to a change as little as 100μV in membrane potential[7].

▪ **Outer Hair Cells (OHC):** These cells mechanically amplify low-level sound that enters the cochlea. The amplification may be driven by the movement of their hair bundles, or by an electrically driven motility of their cell bodies. The terminations on the outer hair cells are almost all from efferent axons that arise from cells in the brain.

In mammalian OHC, the receptor potential triggers active vibrations of the cell body. This mechanical response to electrical signals is called somatic electromotility[8], and drives oscillations in the cell's length, which occur at the frequency of the incoming sound and provide mechanical feedback amplification. The effect of this system is to non-linearly amplify quiet sounds more than large ones so that a wide range of sound pressures can be reduced to a much smaller range of hair displacements[9]. This property of OHC is called the cochlear amplifier.

The development, function, and maintenance of inner ear sensory epithelia are heavily dependent upon supporting cells (SC), which are non-sensory cells that reside

between hair cells[10]. Unlike hair cells, which contact only the luminal surface of the epithelium, supporting cells extent the entire depth of the epithelium, from the basal lamina to the lumen. At the apical surface of the sensory epithelium, the membrane of hair cells and supporting cells are interconnected by tight and adherens junctions to form the reticular lamina[11]. SCs are linked to each other and to hair cells by tight and adherens junctions; and they communicate directly with other supporting cells by gap junctions. SCs serve a diverse set of functions in the sensory epithelia like maintaining the structural integrity of the sensory organs during sound stimulation and head movements, which is particularly provided by Pillar and Deiter's cells[12]. They also help to maintain an environment in the epithelium that enables hair cells to function.

1.1.2. The vestibule

The cochlea is opened to a central cavity or vestibule, which collects three bony canals, the semicircular canals. These structures are also known as Pars superior. Perilymph fluid runs inside them and they are connected to the ES. The membranous labyrinth goes inside of this bony labyrinth and is comprised into the otolithic organ. The vestibular system consists of three different parts:

- The **saccule** function is associated to the equilibrium and gravity perception, since sensory cells detect linear accelerations and head tilts in the vertical plane. When the head moves vertically, the sensory cells of the saccule are disturbed and the neurons connected to them begin transmitting impulses to the brain.
- The **utricle** macula lies almost in the horizontal plane. Hair cells can distinguish different degrees of tilting of the head, thanks to their apical stereocilia and kinocilium. Depending on whether the tilt is towards the direction of the kinocilium or not, the resulting hair cell polarization is excitatory (depolarizing) or inhibitory (hyperpolarization), respectively. So, the afferent nerve fibers response can be interpreted by the vestibular nuclei
- The **semicircular canals** derive from the utricle, each with an ampulla containing sensory cells at one end in a structure called crista ampullaris, which function is to sense angular acceleration and deceleration of the head.

1.2. Meniere's Disease

Meniere's disease (MD) is an inner ear syndrome characterized by episodes of spontaneous vertigo lasting from 20 minutes to hours, usually associated with low to medium frequencies fluctuating sensorineural hearing loss (SNHL), tinnitus and aural fullness [13] (**Table 1**).

Table 1: Meniere's disease natural history: symptoms and phases

Symptom	Initial Phases	Middle phase	Final phase
Vertigo	Balance disorder may occur abruptly as a persistent episode of vertigo	This is the phase between vertigo crises	Vertigo disappears
Hearing loss	Hardly perceived	Perceived after vertigo attack	Raise to total deafness
Tinnitus	At the same time that vertigo attack	Previously to vertigo spell	Patients could present constant tinnitus in the affected ear

1.2.1. Clinical features

1.2.1.1. Vertigo

Vertigo is experienced as an episode of sensation of rotation in which the individual perceives how his environment or himself turns and moves. As a result, the individual could develop chronic imbalance, and an increased risk of falls. Nevertheless, these attacks are not associated with decreased level of consciousness. Attacks of vertigo vary in duration, with a minimum of 20 minutes to several hours.

Between 35-65% of the patients have acute vertigo spells with sudden appearance[14]. The episode may be preceded by an ipsilateral low-frequency tinnitus. Nystagmus is of peripheral features and if it is too intense could cause blurred vision. However, it is difficult to predict a crisis because they show a variable irregular pattern.

The etiology of vertigo spells is unknown, but the hypothesis more extended is that crises occur by changes in endolymph pressure, rupture of the membranous labyrinth or sudden changes in the composition of the endolymph that irritate the sensory epithelia of the saccule, utricle and semicircular canals. A progressive destruction of vestibular receptors and loss of vestibular neurons could explain the decrease and disappearance of the vertigo spells during the progression of the disease [14, 15].

1.2.1.2. Sensorineural hearing loss (SNHL)

SNHL is an important symptom in MD because is more stable over time than vertigo. SNHL can arise in one or both ears (bilateral disease)[16, 17] and may be rapidly progressive (weeks or months) or slowly progressive (years), which is the most common form of presentation. Initially, hearing loss and vertigo may occur at the same time, with a fluctuation of tonal ranges in the early years. However, some patients can show only episodic vertigo or hearing loss (Delayed MD)[18] with tinnitus and later all the symptoms can follow simultaneously. Later, the disease progresses and hearing loss worseness, reaching in some cases profound deafness.

Hearing loss is diagnosed by pure-tone audiometry. Subsequent audiograms are required to differentiate MD from other inner ear diseases[19] and, to study the time course and disease progression[17] **(Figure 3).**

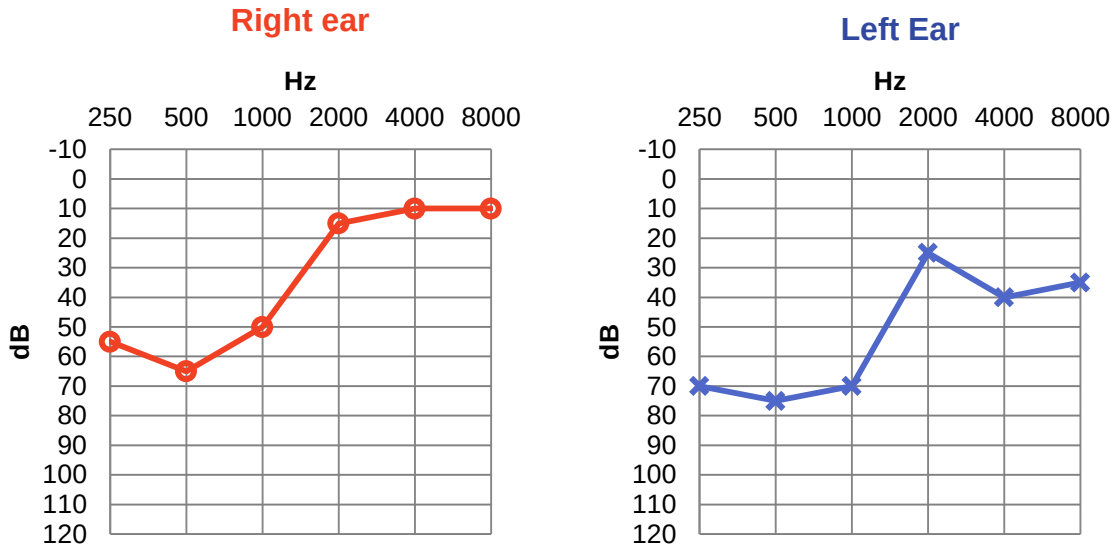


Figure 3. Audiogram of a MD patient with bilateral SNHL.

1.2.1.3. Tinnitus

Tinnitus is defined as the perception of sound within the ear (ringing in the ears) where no actual sound is present[20, 21].

Largely, tinnitus intensity increases during vertigo attacks or if there is a worseness in hearing. In the course of the disease, tinnitus becomes a unceasing and very disabling symptom, which associated with hearing loss, difficult the speech understanding and could become the most annoying and principal long-term disease symptoms.

1.2.1.4. Aural fullness

Aural fullness is perceived as a blocking sensation, fullness and ear pressure. This sensation may be constant and its intensity may increase during vertigo attacks.

1.2.2. Diagnosis and disease classification

MD is a very heterogeneous disease which may overlap with other conditions[22, 23, 24, 25, 26] (**Figure 4**). To define MD the standard criteria usually used are the ones described in the 1995 guidelines established by the American Academy of Otolaryngology-Head and Neck surgery (AAO-HNS)[27] (**Table 2**), but since 2015 a new diagnostic criteria have been jointly formulated by Classification Committee of the Bárány Society, The Japan Society for Equilibrium Research, the European Academy of Otology and Neurotology (EAONO), the Equilibrium Committee of the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) and the Korean Balance Society[13] (**Table 3**).

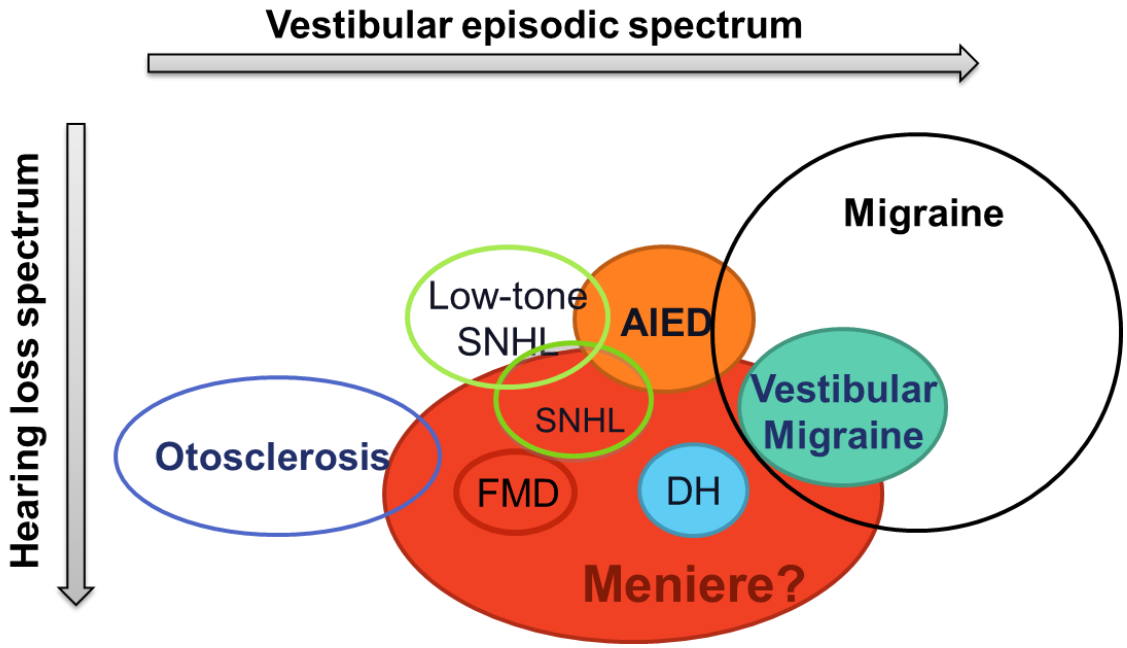


Figure 4. Diagram of diseases overlapping with MD.
Autoimmune inner ear disease (AIED); Vestibular migraine; Migraine; Delayed hydrops (DH); Familial MD (FMD); Sensorineural hearing loss (SNHL); Low-tone SNHL and Otosclerosis.

Since the diagnosis is clinical, pure tone audiometry is required in all patients. Some diseases may have overlapping symptoms and a similar clinical course. In some cases

a differential diagnosis is needed with physical examination and additional tests such as: speech audiometry, auditory evoked potentials, vestibular and imaging tests amongst others, to exclude other causes. Some of these diseases symptoms are as follow:

- **Autoimmune inner ear disease (AIED):** AIED is an inflammatory disease of the inner ear characterized by recurring episodes of sudden or progressive bilateral sensorineural hearing loss[26].
- **Vestibular migraine (VM):** VM is the most common cause of episodic vertigo in adults and children. The diagnostic criteria of the consensus document of the International Bárány Society for Neuro-Otology and the International Headache Society (2012)[22] combine the typical signs and symptoms of migraine with the vestibular symptoms lasting 5 min to 72 h and exclusion criteria of other disorders that also elicit vestibular signs.
- **Delayed Hydrops (DH) or delayed MD:** DH is typically observed in patients who have been suffering from longstanding unilateral profound inner-ear hearing loss. The time that elapses between the occurrence of hearing loss and the onset vertigo symptoms can take several years[18].

Table 2. Diagnostic Criteria from AAO-HNS, 1995.

Symptoms	Certain MD	Probable MD	Possible MD
Spontaneous Vertigo	>2 episodes >20 min	1 episode >20 min	Episodic vertigo without documented HL.
Hearing loss (HL)	Audiometrically documented on at least 1 occasion	Audiometrically documented on at least 1 occasion	Fluctuation or fixed SNHL with disequilibrium, but without definitive episodes
Tinnitus / aural fullness	Present	Present	Present
Other causes	Excluded	Excluded	Excluded

Table 3. Diagnostic criteria for Menière's disease jointly formulated by the Classification Committee of the Bárány Society, The Japan Society for Equilibrium Research, the EAONO, the AAO-HNS and the Korean Balance Society, 2015.

Symptoms	Definite MD	Probable MD
Spontaneous Vertigo	>2 episodes vertigo >20 min to 12h	>2 episodes vertigo or dizziness >20 min to 24h
Hearing loss (HL)	Audiometrically documented low- to medium- frequency SNHL defining the affected ear on at least one occasion before, during or after one of the episodes of vertigo	Fluctuating aural symptoms (hearing, tinnitus or aural fullness) in the affected ear
Tinnitus / aural fullness	Fluctuating aural symptoms	
Other causes	Excluded	Excluded

1.2.3. Epidemiology

1.2.3.1. Incidence and prevalence

The MD prevalence is variable since the diagnosis is based on clinical symptoms. Therefore, some studies carried out in specific populations are considered as biased.
It seem that the prevalence of MD is more common in industrialized countries and in European descent population than in Asian and African populations with a range of 10–225 cases/100000 individuals[28]. In the US, a study described MD prevalence as 190/100000 individuals [29], similar to the prevalence of UK 157/100000[30]. Besides, other study in Caucasian population showed that MD prevalence was higher 1-2/1000[31]. However, in other countries has been reported a lower prevalence, such as Finland with a prevalence of 43/100.000[32] or in Japan, where the prevalence is about 36/100.000[33]. In Spain, a study performed in Cantabria showed an intermediate prevalence of 75/100000[34].

These differences in the prevalence rate of MD could be as result of both, geographical and ethnic differences, as it has been found in other diseases. Other causes could be methodological errors in the estimation, but the most probable cause would be the lack of standardization of the diagnostic criteria and the diagnostic difficulties, mainly in early stages, when all the symptoms are not always present.

1.2.4. Treatment

Several medical and surgical therapies have been offered to patients with MD over the past 150 years. The excess of medical and surgical therapies indicates that no effective treatment is available for these patients. However, most will be helped by a combination of lifestyle and dietary changes, medical therapy, psychological advice and patients organization support [35].

1.2.4.1. Lifestyle changes

All patients with Meniere's disease are encouraged to reduce their salt intake to a maximum of 1.5 - 2 g/day. They are also asked to avoid all sources of caffeinated products, to reduce their intake of chocolate, and to avoid all tobacco and alcoholic products as much as possible. In 1934, Furstenberg showed the relation between sodium retention and MD, and recommended a substantial reduction in intake of sodium[36].

A strong association with seasonal allergies and circulating immune-complexes exists in patients with known diagnoses of MD[37]. Allergy-avoidance could relieve some of the allergy symptoms associated with the disease and allow an improved quality of life for patients. Some studies have reported a significant reduction (up to 62%) in both frequency and severity of attacks of vertigo in patients with Meniere's disease after starting immunotherapy for allergies[38].

1.2.4.2. Surgery

Currently, surgery in MD patients is not very common through its controversial effectiveness[39]. However, different surgical techniques could be used, when previous treatments fail, such as ES surgery, labyrinthectomy or vestibular neurectomy.

1.2.4.3. Pharmacological treatment

- **Diuretics** regulate the homeostasis of the inner ear, avoiding fluid overload. So, electrolyte levels should be checked frequently to avoid other consequences. Nonetheless, recent studies have suggested no relation between use of diuretics and MD [40]; however, some clinicians believe that most of the time diuretics are a fairly safe option and are offered to all patients.

▪ **Steroid therapy** has been used in treatment of acute and chronic symptoms of MD, both oral steroids and intratympanic steroid injections have been tried. Trials of steroids could be of substantial value to most patients since a large number with Meniere's disease have allergies and/or immune-mediated events[41, 42]. If patients do not respond to the oral steroids and their hearing continues to deteriorate, intratympanic injections can be given. Patients who have a pronounced response to oral or intratympanic steroids might have other immune-mediated diseases and should have a complete work-up for autoimmune problems, including psoriasis, rheaumatoid arthritis, SLE, ankilosis spondilytis or autoimmune hypothyroidism.

▪ **Gentamicin trans-tympanic perfusion**: Destructive treatments can be used in patients with intractable vertigo. The present drug of choice is gentamicin, which causes direct damage to both the sensorineural epithelium and the dark cells of the labyrinth, thus affecting vestibular function and cochlear function. So the treatment is based on the dose-dependent ototoxic effect produced in the vestibule, sensory and secretory cells[43]. There is a low effect on hearing and, long-term hearing impairment occurs in 16% of patients[44].

1.3. Pathophysiology

Meniere's disease etiology remains unknown, but most authors believe that MD is multifactorial[45] (**Figure5**). Despite, three main theories regarding the cause of MD exist.

Figure 5. Diagram of MD etiology and its symptomatology.
Figure adapted from Merchant et al[46].

1.3.1. Endolymphatic Hydrops

Histopathologic study of the human temporal bone entails microscopic examination and analysis of a series of histologic sections. This is currently the most effective method for observing the pathologic conditions of MD by examining the entire inner ear. Endolymphatic hydrops (EH) has been a very common pathologic finding in temporal bone histopathologic investigations, since it was first recognized and reported by Hallpike and Cairns[47] and Yamakawa[48]. EH is a dilation of the membranous labyrinth by an increase in volume of endolymph, but not perilymph, so it is hyphotetized that a cellular stress can produce and endolymph ionic imbalance and a cochlear-vestibular dysfunction. Paparella[49, 50] reported that the most important histopathologic correlate of MD is endolymphatic hydrops in the cochlea and the saccule, both of which belong to the pars inferior of the temporal bone (**Figure 6**).

MD could begin in the in the spiral ligament inside the cochlea. The endolymph is produced in the stria vascularis in the cochlear duct and, it is reabsorbed in the ES. An interruption of the flow leads to an accumulation of endolymph and therefore, to the development of EH. An increase in the production or a decrease of the absorption of endolymph in the ES caused by an ionic imbalance, genetic mutations, viral infection, dietary factors, autoimmune disease, allergic responses, or vascular irregularities could be a trigger for EHs. This increase of endolymph affects the pressure in the cochlear duct and produces dilation and elongation of the Reissner's membrane[34].

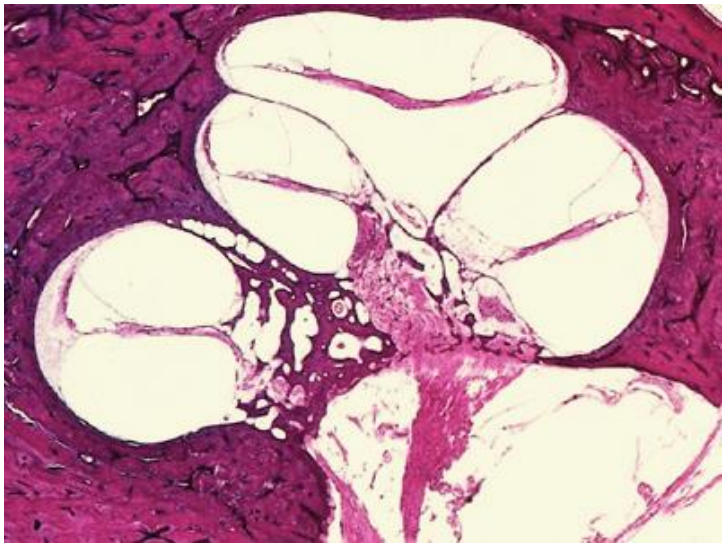


Figure 6. Histology of human cochlea showing EH.
Image taken from Pathology of the Ear (1974)[51].

1.3.1.1. Cellular and molecular alterations caused by EH

From a macroscopic outlook, the mastoid of the affected ear has a smaller size and the vestibular aqueduct is shorter, with narrower periaqueductal and external openings. Besides, significant differences were found in the thickness of the Oval membrane in MD patients when compared to healthy controls. From a microscopic view, significant alterations were also observed in patients with advanced age, such as loss of hair cells, atrophy of supporting cells in the organ of Corti and distortion and atrophy of the tectorial membrane[52].

Spiral ganglion neurons (SGN) are divided into two types: type I neurons (representing 90%) and type II neurons (representing 5-10% of the total ganglion). MD patients present a deterioration of these neurons before any damage in cochlear hair cells and the decrease of inner hair cells is significantly lower than the corresponding loss of SGN[53]. In addition, it is observed a significant decrease in type I afferent nerve endings number and synapses at the base of the inner and outer hair cells in the affected ear of unilateral MD patients[45]. These data support the hypothesis that the loss of SGN could be the initial event in the degenerative cascade in the cochlea which would conclude with the loss of hair cells.

Remarkably, the correlation between inner hair cells loss and EH showed no significant differences[53]. The absence of a connection between the severity of EH and hair cells loss indicates that EH is not directly involved in the origin of the molecular and cellular events. Furthermore, these findings support the idea that EH is a marker of an alteration of the homeostasis in the labyrinth, where one or more unknown factors could cause Meniere's symptoms. Currently, data support the hypothesis that EH is an epiphenomenon associated with a variety of inner ear disorders[46], and genetics and environmental factors contribute to its development.

1.3.2. Genetics

Most MD patients are considered sporadic but, some patients report relatives with a history of SNHL or vertigo. Early studies described that 1/3 of MD patients have a close relative in first degree related to the disease[54]. Additional studies examined family pedigrees, concluding that between 3-14% of MD patients have a genetic predisposition [55, 56]. Autosomal dominant patterns of inheritance with incomplete

penetrance and expressivity (**Figure 7**) have been described in several families[57, 58, 59] but, families with recessive inheritance have also been described[54, 60, 61]**.**

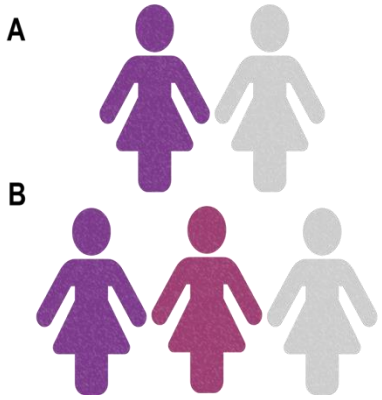


Figure 7: How gene expression affects people.
A. Incomplete penetrance. Both individual have the affected gene but only one shows the phenotype.

B. Incomplete penetrance and expressivity. Three individuals carry out the affected gene but only two show the phenotype. Yet only one of two affected have the complete phenotype.

Recent studies suggest that MD could appear in two forms: clustered in families, Familial MD (FMD) and sporadic MD (SMD). Actually, the frequency of FMD is around 5–15% in European population. This suggests a strong genetic component in the MD etiology[30, 55].

Several methodologies have been adopted trying to find candidate genes associated with MD (**Table 4**). Linkage studies in autosomal FMD have found candidate loci at 5q14–15 in German families[60] and at 12p12.3 in Swedish families[61]. Case-control genetic association studies can establish a relationship between genetic variants that exist in a population and a particular phenotype of a disease. This relationship is only a statistical correlation and not a genetic phenomenon. This approach is the most widely used when searching for genetic assotiations with a disease.

Table 4. Candidate Genes related to MD.

Author/year	Study design	Genes	Ancestry
Koyama et al, 1993[62]	Case-control	*HLA-Cw04DRB1*1602*	Asian
Melchiorri et al, 2002[63]	Case control	*HLA-Cw07*	Caucasian
Lopez-Escamez et al, 2002[64]	Case-control	*HLA-*	Caucasian
Mhatre et al, 2002[65]	Case-control	*AQP2*	American
Lynch et al, 2002[66]	Case-control	*ATQ1*	Australian
Doi et al, 2005[67]	Case-control	*KCNE1 – 3*	Asian
Klar et al, 2006[61]	Familial	*12p12PIK3C2G*	Caucasian
Lopez-Escamez et al, 2007[68]	Case-control	*HLA-DRB1*1101*	Caucasian
Teggi et al, 2008[69]	Case-control	*ADD1*	Caucasian
Kawaguchi et al, 2008[70]	Case-control	*HSPA1A*	Asian
Vrabec et al, 2008[71]	Case-control	*HCFC1*	Caucasian
Lopez-Escamez et al, 2009[72]	Case-control	*PARP-1*	Spanish
Candreia et al, 2010[73]	Case-control	*AQP3*	Caucasian
Maekawa et al, 2010[74]	Case-control	*AQP2*	Asian
Campbell et al, 2010[75]	Case-control	*KCNE1 – 3*	Caucasian
Lopez-Escamez et al, 2010[76]	Case-control	*PTPN22*	Caucasian
Khorsandi et al, 2011[77]	Case-control	*HLA-Cw04*	Caucasian
Hietikko et al, 2011[78]	Familial	*12p12.3*	Caucasian
Furuta et al, 2011[79]	Case-control	*IL1A*	Asian
Lopez-Escamez et al, 2011[80]	Case-control	*CD16A/CD32*	Caucasian
Arweiler-Harbeck et al, 2011[60]	Familial	*Chromosome 5*	Caucasian
Gazquez et al, 2011[81]	Case-control	*NOS1 – NOS2A*	Caucasian / American
Gazquez et al, 2012[82]	Case-control	*MICA-STR A.4*	Caucasian / American
Hietikko et al, 2012[83]	Case-control	*KCNE1*	Caucasian
Yazdani et al, 2013[84]	Case-control	*MIF-173*	Caucasian
Gazquez et al, 2013[85]	Case-control	*MIF, INFG, TFNA*	Spanish / American
Requena et al, 2013[86]	Case-control	*TLR10*	Spanish
Teranishi et al, 2013[87]	Case-control	*Cav1*	Japanese
Cabrera et al, 2014[88]	Case-control	NFKB1	Spanish
Requena et al, 2015[57]	Familial	DTNA; FAM136A	Spanish
Yazdani et al, 2015[89]	Case-control	RANTES	Caucasian
Martin-Sierra et al, 2016[58]	Familial	PRKCB	Spanish
Martin-Sierra et al, 2017[59]	Familial	DPT; SEMA3D	Spanish

1.3.3. Immune hypothesis

1.3.3.1. Autoimmune condition?

The conception that the immune system may have a role in some idiopathic hearing loss and vestibular disorders was introduced during the early decades of the past century by Joannovic[90] and Masugi[91]. In 1958 Lehnhardt[92] suspected that certain cases of sudden bilateral hearing loss could be associated to the production of anti-cochlear antibodies. Kikuchi[93] suggested an autoimmune etiology after observing that surgery on one ear affected the other one. Beickert[94], in 1961, and Terayama[95], 3 years later, both presented data supporting an autoimmune mechanism in experimental guinea pig cochleae. In 1979, McCabe[26] first described patients with bilateral progressive hearing loss that responded to steroid therapy. The clinical presentation of SNHL can be quite variable, often overlapping with other disorders such as MD. Hughes et al.[96] reported that over 52% of patients diagnosed with AIED presented hearing loss and vertigo. This suggests that a continuum might exist between MD and SNHL.

Besides, the prevalence of systemic autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, and systemic Lupus Erythematosus in patients with MD is 3- to 8-fold higher than in the general population[30, 55, 97] (**Table 5**).

Table 5. Prevalence of different autoimmune diseases diagnosed with MD in different populations

Autoimmune diseases	Spanish (%)	UK (%)	Finnish (%)
Rheumatoid arthritis	**1.39**	**21**	4.5
Autoimmune hypothyroidism	1.22	6.8	16.5
Systemic lupus erythematosus	**0.87**	-	-
Ankylosing spondilytis	**0.7**	-	-
Grave's disease	0.7	-	-
Autoimmune thyroiditis	0.7	-	-
Psoriasis	0.7	2	-
Sjogren Syndrome	0.52	-	-
Ulcerative colitis	0.52	5.1	-
Type I diabetes	0.35	-	2.3
Allergy	-	24.9	35.2
Rhinitis or eczema	-	22.2	-
Other Autoimmune diseases	10.95	28.8	14.8
Undetermined	1.57	2.8	-

Numbers in bold represent higher values than in general population.

Today, we have wide evidence of autoimmune mechanisms in some of the inner ear disease entities, including MD, otosclerosis, progressive SNHL and sudden deafness. Approximately 1/3 of MD patients seem to be of an autoimmune origin, though the immunological mechanisms involved are still not clear[98, 99], several theories as to how autoimmune inner ear disease might arise. **Figure 8** shows a suggested diagram as an autoimmune mechanism in MD.

- **Bystander damage:** damage to the inner ear causes cytokines to be released, which provoke (after a delay) additional immune reactions. This theory might explain the relapsing/recurrent course of disorders such as MD.
- **Cross-reactions:** antibodies or rogue T cells cause accidental inner ear damage because the ear shares common antigens with a potentially harmful substance, virus, molds or bacteria that the body is fighting. This is presently the preferred theory of AIED.
- **Intolerance:** the body may not recognize about all of the inner ear antigens. When they are released (perhaps after a surgery or an infection), the body may wrongly attack the “foreign” antigens. In the ear, a mechanism which could be involved in the so called sympathetic cochleo-labyrinthitis has been reproduced in animal models[98].
- **Genetic factors:** genetically controlled features of the immune system could increase or otherwise be associated with increased susceptibility to common hearing disorders, such as MD. Bernstein et al.[99], reported that 44% of MD patients had one particular extended major histocompatibility complex (MHC) haplotype, compared to only 7% of controls. Immune response genes related with SNHL progression like MICA, TLR10 or NFκB have also been reported[82, 86, 88].

According to others studies, autoimmunity seem to be responsible for 6% of unilateral and 16% of bilateral forms of Meniere's disease[100]. This hypothesis is supported by several experimental studies:

- EH can be induced experimentally by injection of antigens or monoclonal antibodies[101]. Inner ear antigens with molecular weights of 28, 42, 58 and 68 kDa could be the main components inducing autoimmune MD in guinea pigs[102].
- The deposition of circulating immune complexes (CIC) might produce inflammation and interfere with the capability of the ES filtering. Several studies demonstrated increased values of CIC in 21% to 96% of MD patients[103].
- Presence of autoantibodies in the sera of MD patients.[104].

▪ The ES is the site of the immune response of the inner ear and is also the site mostly involved in the pathogenesis of MD[105].

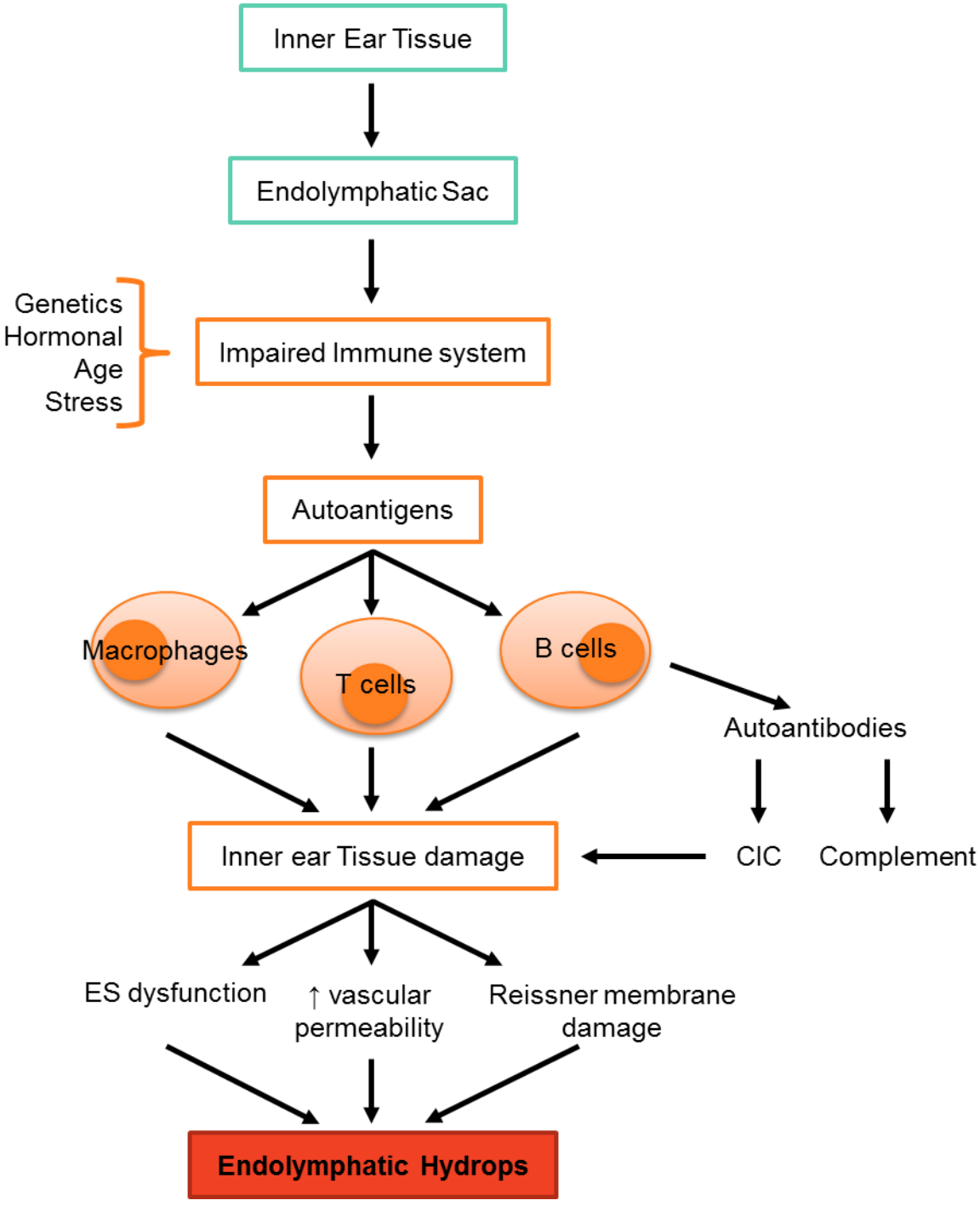


Figure 8. Potential mechanism for autoimmunity in MD[106]

During the last decade, new molecules have been explored to identify the role of cytokines in autoimmune diseases and, new drugs are being developed to interfere with them. It has been shown that about 60% of MD patients have antibodies in their sera against proteins in the inner ear and, there is evidence of the presence of cytokines in the cochlea, including IL-1α, TNF-α, NF-kβ P65, P50 and Ikβα[107]. TNF-α is a cytokine that induces the infiltration of immunocompetent cells into the tissues and amplifies the immune response. Ren et al[108] reported on TNF-α in patients with sudden SNHL and progressive SNHL in 1998. In a small study population of 15 subjects, they found that both TNF-α and IL-6 levels were significantly elevated in patients with sudden SNHL and progressive SNHL compared with controls. This was followed by a preliminary report by Rahman et al[109] on the use of Etanercept, a well-known TNF-α blocker used in autoimmune diseases such as psoriasis and rheumatoid arthritis, for patients with immune-mediated cochleovestibular disorders who did not respond to conventional therapies. They reported a 92% success rate in hearing loss improvement with etanercept therapy.

Animal models of labyrinthitis have been used to investigate the role of TNF-α in recruiting inflammatory cells to the cochlea and its role in resultant hearing loss[110]. While some investigators reported that, in an animal model, etanercept has a protective effect on hearing loss[111], others found it less effective than prednisone, with a greater potential for adverse effects[112].

Human studies performed in patients with sudden SNHL showed that levels of TNF in patients were found to be increased[113], while other studies presented the opposite results[114] . Likewise, the benefit of etanercept has not been consistently demonstrated. Pilot studies conducted by Matteson et al[115] showed that only 30% improved with etanercept therapy; however, 57% experienced no improvement and 13% worsened. In other study, Cohen et al[116] failed to demonstrate any benefit of etanercept compared with placebo in maintaining hearing following benefit from corticosteroids. In 2006, Van Wijk et al [117] suggested that locally administered etanercept into the middle ear allowed patients to be tapered off corticosteroid therapy and preserve their hearing function. None of these trials examined or used TNF-α level as a criterion for study enrollment, but in 2012, Svrakic et al[118] presented a prospective study performed in patients with immune-mediated SNHL (IM-SNHL) where they concluded that TNF-α has the potential of being used as both, a diagnostic marker for IM-SNHL and a prognostic biomarker for corticosteroid response in the disease. In 2015, Pathak et al[119]

demonstrated that in AIED patients, the TNF-α could be attenuated in a subset of patients using N-acetylcysteine (NAC) as it exerts anti-inflammatory effects[120] and has been identified to be otoprotective, and serve as a beneficial adjunctive therapy in hearing restoration. NAC has been shown to have a protective adjunct role in idiopathic sudden hearing loss, where the addition of NAC to corticosteroid therapy resulted in better hearing recovery than with corticosteroids alone[121].

Recently, the critical role of the IL-1 family as regulators of inflammation and immunity has become evident[122]. Early immune system reactions to perceived pathogens drive many of the later adaptive T cell responses that maintain the disease. Expression of IL-1β and IL-1R1 are critical for Th17 cells development[123, 124] and the subsequent expression of IL-17. Absence of IL-1 receptor antagonist (IL-1RA) expression during an immune response, or other molecules that oppose the IL-1β inflammatory cascade, can promote the development of autoimmune diseases. The role of IL-1β in hearing disorders is largely unknown; however, there are examples in both, animal models of AIED and clinical autoinflammatory disorders with associated SNHL[125]. SNHL has been observed as a component of clinical diseases of IL-1β dysregulation, such as neonatal onset multisystemic inflammatory disease syndrome[126] and Muckle-Wells syndrome[127]. Likewise, improvement of SNHL has been observed in response to treatment with the soluble IL-1RA, Anakinra, in Muckle-Wells syndrome and AIED[128, 129].

1.3.3.2. Allergy condition?

The association between MD and allergy was first described in the literature in 1923[130]. Two cases with symptoms consistent with MD were treated with epinephrine and symptoms resolved. Amongst cross-sectional surveys, the prevalence of diagnosed allergy was three times higher in MD patients compared to the general population[37]. More precisely, of those with MD, 58% had a history of allergy and 41% had a positive skin test. Patients with the disease have been shown to have a “sharp immune” reaction. MD patients had elevated IgE levels (43.3%) compared to healthy controls (19.5%)[131]. Savastano et al[132] found increased immune complexes, interleukins, and autoantibodies in patients with MD. Additionally, disease severity was associated with elevated immune complexes as well as an elevated CD4/CD8 ratio.

Derebery and Berliner described three theories connecting allergy to MD that focus on inflammation within the ES[133].

- The ES comprises a fenestrated blood supply that could allow antigen entry inducing mast cell degranulation and inflammation.
- A second proposed mechanism includes CIC entering the ES circulation and the stria vascularis producing inflammation and enlarged permeability as well as fluid balance disruption.
- Viral antigen-allergic interaction. Viruses have been shown to worsen allergic symptoms by enhancing histamine release and may damage epithelial surfaces as well as trigger T-cell migration to the ES[132].

Both, inhalant and food allergens have been associated with MD. **Table 6** shows an epidemiologic association between allergy status and MD. In Derebery's work, gliadin was the most common food allergen in MD patients[37]. More recently, Di Beradino et al[134] performed a study to verify the incidence of gliadin IgE hypersensitivity in MD patients. In a non-controlled study, Topuz showed that prick test may induce aural symptoms such as tinnitus or aural fullness in 62% of patients and an increase of endolymphatic pressure measured by electrocochleography in 77% of cases[135]. This study suggests that certain allergens may induce EH and, also concluded that antigen exposure triggered a histaminergic reaction, leading to inflammation in the ES and potentially manifesting as symptoms of MD.

Table 6. Studies demonstrating an epidemiologic association between allergy status and MD. Modified from Banks et al.[136]

Study	n	Outcome or OR for patients with:
Derebery, 2000[37]	906	Airborne allergy and MD, 2.00 (59.2 %/42 %)
		Food allergy and MD, 2.02 (40.3 %/25 %)
		Positive skin test/ blood test in MD, 2.06 (37 %/22.2 %)
Keles et al., 2004[131]	92	MD and elevated IgE, 2.91 (41.3 %/19.5 %)
		MD and history of allergy, 3.87 (67.3 %/34.7 %)
Sen et al., 2005[137]	208	Migraine and MD vs control OR, 2.89 (39 %/18 %)
		Allergy and MD vs control OR, 3.60 (51.9 %/23 %)
Savastano et al., 2011[132]	250	56 % of MD had elevated CIC
		None in control group
Singh et al., 2011[138]	50	100 % of AR patients had sensorineural hearing loss
		None in control group
Karabulut et al., 2011[139]	89	Hearing loss in AR patients with positive skin test vs AR with negative skin test, 0.23 (39.6 %/74.1 %)
Berardino and Cesarani, 2012[134]	109	57 % of MD patients had positive gliadin prick test
		All 50 control tested were negative

To support the theory that the ES seems to be a target for allergic activity, histamine receptors have been found within the sac. In animal studies, Dagli *et al.* revealed immunohistochemical evidence of H1 and H2 receptors and weakly immunoreactive H3 receptors[140]. Likewise, Takumida *et al.* showed that all four types of histamine receptors (H1R, H2R, H3R, and H4R) are expressed in the mouse inner ear, thus supporting the hypothesis that histamine plays a physiological role in the inner ear[141]. In human studies, Møller et al presented expression of the histamine receptor HRH1 in the epithelial lining of the ES, whereas HRH3 was expressed exclusively in the sub-epithelial capillary network. Receptors HRH2 and -4 were not found[142].

Betahistine is a structural analog of histamine and acts as a weak H1 receptor agonist and strong antagonist on H3 receptors, and has been widely used in Europe as a treatment for MD. Its mechanism is thought to decrease the release of histamine, dopamine, gamma aminobutyric acid, acetylcholine, norepinephrine and serotonin and improve microcirculation in the inner ear[140]. In a retrospective analysis, Lezius *et al.* examined patients with MD who received high doses (288-480 mg/day) of betahistine and found that with increasing dosage of the medication, the number of patients reporting vertiginous attacks was lower[143].

As another marker of allergy, Takeda et al. performed a study examining plasma arginine vasopressin (p-AVP) and endolymphatic volume[144]. P-AVP has been known to be elevated during allergic insults, while endolymphatic volume has been show to increase with rising p-AVP.

Research regarding the effectiveness of anti-allergic treatments in MD subjects has been limited. Patients using allergy immunotherapy and food elimination treatments have reported improvement in symptoms including, decreased severity and frequency of episodes, decreased tinnitus and vertigo when compared to healthy controls[37]. In a prospective study, patients rated symptoms before and after treatment and there was a decreased in severity of symptoms[133]. The authors concluded recommending allergy testing for patients with MD in the following cases: a) history of seasonal or food allergy, b) past childhood or family history of allergy, c) bilateral MD symptoms, or d) development of symptoms within a short time after exposure of food or inhaled allergen.

2. Hypothesis & Goals

2.1 Hypothesis

There is a subset of patients with MD with an alteration in the immune response. We propose that these individuals may have functional allelic variants which may confer susceptibility to autoimmune MD. Moreover, these patients may have specific clinical features (autoinmune background) and elevated proinflammatory cytokines.

Since certain molds are able to induce a proinflamatory response in patients with autoinmune inner ear disorders, we hypothetise that a subset of patients with MD could also have an exacerbated immune response to molds involving cytokine profile and gene expression in immune cells.

2.2 Main goal

The aim of this Thesis is to identify a genetic marker for diagnosis of autoimmune MD and to investigate the role of allergenic extract from *Penicillium* and *Aspergillus* in the immune response in MD.

2.3 Specific goals

1. To define a clinical group of patients with autoimmune MD.

2. To identify allelic variants associated with autoimmune MD that could be used as genetic markers and, to investigate their role in MD inflammatory response.

3. To define the effect of allergenic extracts from *Penicillium* and *Aspergillus* in proinflammatory cytokines and gene expression profile in MD patients PBMCs.

2. Hipótesis & Objetivos

2.1 Hipótesis

Hay un subgrupo de pacientes con EM que tienen una alteración de la respuesta inmune. Nosotros proponemos que estos individuos pueden tener variantes alélicas funcionales que podrían conferir susceptibilidad a EM autoinmune. Además, dichos pacientes pueden tener características clínicas específicas (antecedentes autoinmunes) y citoquinas inflamatorias elevadas.

Dado que ciertos hongos son capaces de inducir respuesta proinflamatoria en pacientes con trastornos autoinmunes del oído interno, hipotetizamos que un subconjunto de pacientes con EM podría tener una respuesta inmune exacerbada a hongos que implicaría un perfil de citoquinas y expresión de genes en células de inmunidad.

2.2 Objetivo principal

El propósito de esta tesis es identificar un marcador genético para el diagnóstico de la EM autoinmune e investigar el papel de extractos alergénicos de *Penicillium* y *Aspergillus* en la respuesta inmune en la EM.

2.3 Objetivos específicos

1. Definir un grupo clínico de pacientes con EM autoinmune.

2. Identificar variantes alélicas asociadas a EM autoinmune mediante un array de genotipado y que pudieran servir de marcador diagnóstico e investigar su papel en la respuesta inflamatoria de la EM.

3. Definir el efecto de extractos alergénicos de *Penicillium* y *Aspergillus* sobre citoquinas proinflamatorias y perfiles de expresión génica en células mononucleares de pacientes con EM.

3. Materials & Methods

3.1. Materials

3.1.1. Primers

Table 7 shows all pairs of primers used in this thesis. Primers were designed using Primer3 Input v.4.0.0., NCBI/PrimerBLAST and OligoAnalyzer3 were used as quality controls to avoid heterodimers and primers amplifying other regions.

Table 7. Primers used

GENE	Fordward sequence	Reverse sequence	(bp)
HPRT-1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	94
TNFRSF12A	CTGGCTCCAGAACAGAAAGG	GGGCCTAGTGTCAAGTCTGC	157
NFKB1	GAAGCACGAATGACAGAGGC	GCTTGGCGGATTAGCTCTTTT	137
FOS	GGGGCAAGGTGGAACAGTTAT	CCGCTTGGAGTGTATCAGTCA	126
BIRC3	AAGCTACCTCTCAGCCTACTTT	CCACTGTTTTCTGTACCCGGA	79
NFKB1E	TCTGGCATTGAGTCTCTGCG	AGGAGCCATAGGTGGAATCAG	175
FADD	GTGGCTGACCTGGTACAAGAG	GGTAGATGCGTCTGAGTTCCAT	96
ICAM-1	GATTCTGACGAAGCCAGAGG	CCGGGTCTGGTTCTTGTGTA	198
LFA-1	TTGGGGTTTGAAGAAGTCTCAG	GTGCCTCCCATTGAAGATGT	255
TJP-1	GGACCAATAGCTGATGTTGCC	CCACTGGGCATAGTTAAGACGA	213
IL-1β	AATCTCCGACCACCACTACAG	GTTCAGTGATCGTACAGGTGC	217
IL-6	CCACTCACCTCTTCAGAACGA	TGATTTTCACCAGGCAAGTCTC	211
TNF-α	TCTTCTCCTTCCTGATCGTGG	GAGGGTTTGCTACAACATGGG	186
IL-1RN	GGATTCACAAGACGATCTGCC	ATCACCAGACTTGACACAGGA	242

3.1.2. Cell culture media & Reagents

- PBMC and LCL media:
  - RPMI 1640 medium, GlutaMAX™ Supplement (Thermo Fisher Scientific, #61870-044) supplemented with
  - 10% FBS (Biowest, #S181B-500),
  - 1% non-essential amino acids (Thermo Fisher Scientific, # 11140-035)
  - 1% Sodium Pyruvate solution (Biowest, #L0642-500).
  - Ficoll: Lymphosep Lymphocyte Separation Media (BioWest , #L0560-500)
  - Wash buffer: 1xPBS (Dulbecco's Phosphate Buffered saline (10x, Sigma-Aldrich, #1408))
- Epstein-Barr virus (EBV) transduction:
  - RPMI 1640 medium, GlutaMAX™ Supplement (Thermo Fisher Scientific, #61870-044)

- 20% FBS (Biowest, #S181B-500)
- Epstein-Barr virus (ratio 1:1)

- Human TWEAK (Peprotech, #310-06-25UG, Lot# 1007149-1 L1614)
- PrestoBlue™ Cell Viability Reagent (Life Technologies, #A13261, Lot#1767775A)

3.1.3. Mold Extract

Two allergenic extract-mixes of fungi were used:

- Mix *Aspergillus* (ALK Abelló, #1034497), 4 equal parts of oryzae, repens, niger and terreus species.
- Mix *Penicillium* (ALK Abelló, #1034760), 4 equal parts of brevicompactum, expansum, notatum and roqueforti.

The combinations were dialyzed to remove phenol using Tube-o-dialyzer (VWR Intranational) against deionized distilled water. Fungus protein concentration was analyzed by Bradford dye-binding method protein assay (Bio-Rad Laboratories)[145].

3.1.4. Genomic reagents

- TaqMan® Genotyping Master Mix (Life Technologies, #4371357)
- Brilliant III Ultra-Fast SYBR® GreenQPCR Master Mix (Agilent Technologies, #600882, Lot#0006312389)

3.1.5. Genotyping

- **Immunochip** is an Illumina Infinium single-nucleotide polymorphism (SNP) microarray. The probes on this array interrogate 195,806 SNPs and 718 small insertion–deletions of 12 defined autoimmune diseases[146, 147, 148].
- **Taqman assays:** The following TaqMan assays (**Table 8**) were used to validate and estimate the allele frequency of the selected SNP based on the results of the discovery phase performed with the ImmunoChip technology.

Table 8. Taqman assays used

rs	Part#	Assay ID	Company
rs4988957	4351379	C_25607081_10	Life Technologies
rs11465670	4351379	C_31439397_10	Life Technologies
rs4851589	4351379	C_31439315_10	Life Technologies
rs886424	4351379	C_11690723_10	Life Technologies
rs9380217	4351379	C_30244016_10	Life Technologies
rs4947296	4351379	C_29649099_10	Life Technologies
rs1150754	4351379	C_7515494_10	Life Technologies

3.1.6. Kits

- DNA isolation Kit: QIAamp DNA Blood Mini Kit (250) (Qiagen, # 50951106)
- Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, #Q32850)
- RNA isolation Kit: RNeasy Mini Kit (Qiagen, # 74104)
- cDNA synthesis: AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, #600559, Lot#0006310118)
- Milliplex Map Kit: Human Cytokine/Chemokine Magentic Bead Panel – 96-well plate (EMD Millipore, #HCYTOMAG-60K-4)
- Sandwich ELISA Kits (96-well plate)
  - Human IL-1Beta/IL-1F2 Quantikine Elisa Kit (R&D Systems, #DLB50)
  - Human IL-6 Quantikine Elisa Kit (R&D Systems, #D6050)

3.1.7. Whole mount staining LCLs

- Wash buffer: 1x PBS (Dulbecco's Phosphate Buffered saline (10x, Sigma-Aldrich, #1408))
- Fixation:
  - Absolut Methanol (Fisher Scientific, #A452-4)
  - Methanol:DMSO (4:1) (DMSO; Sigma-Aldrich, #D2650)
  - 2% PFA in 0.1% Tween20/PBS (PFA: Sigma-Aldrich, #P6148-500G)
- Rehydration:
  - Methanol in 0.1% Tween20/PBS – 75%, 50%, 25%
  - 0.1% Tween20/PBS (TWEEN20, Sigma-Aldrich,# P7949-100mL)
- Blocking solution: 2% Milk in 0.5% Tween20/PBS
- Staining solution: 1% Milk in 0.5% Tween20/PBS + Antibodies (described below)
- Equilibration: Glycerol (Sigma-Aldrich, #G5516-100ML) – 30%, 50%, 80%
- Mounting:
  - Slides (VWR, #631-1553)
  - 22x22mm Coverslips (VWR, #631-1570)
  - 24x50mm Coverslips (VWR, #631-0901)

3.1.8. Western Blot

- RIPA buffer (Sigma-Aldrich, #R0278)
- Protease Inhibitor (Sigma-Aldrich, #P8340)
- Sample Buffer: NuPAGE® LDS sample buffer (4x) (Life Technologies, #NP0007)

- Electrophoretic Buffer: Tris 3g, Glycine 14.3g, SDS 1g (1x, for 1L)
- Polyacrylamide gels: Mini-Protean TGX gels 4-15%, 10wells-50uL (Bio-Rad,#4561084)
- PVDF membrane: Trans-blot turbo transfer pack PVDF 8.5x13.5cm, 10pk (Bio-Rad, #1704157)
- Ponceau S staining solution (VWR international, #K793-500mL)
- TBS: Tris 0.25M, NaCl 1.5M, KCl 0.027M, pH=7.5 (10x)
- TBS-T: TBS (1x) + 0.005% TWEEN-20 (Sigma-Aldrich, #P7949-100ML)
- Blocking solution: 5% of non-fat dried milk in TBS-T
- Membrane developer: Clarity western ECL substrate (Bio-Rad, #1705060)
- Stripping Buffer: HCl 0.1M
- Ladder: Precision plus protein dual color standards (Bio-Rad, #1610374)

3.1.9. Antibodies

Table 9 shows all antibodies used for western blot and immunocytochemistry.

Table 9. Antibodies used

TECHNIQUE	ANTIBODY	ISOTYPE	COMPANY	CATALOG	LOT #	CONCENTRATION (mg/mL)	DILUTION
Western Blot	chk ∞ GAPDH	IgG	Millipore	AB2302	2580669	1	1:3000
	ms ∞ Fn14	IgG	Santa Cruz Biotechnology	sc-56250	C0116	0.2	1:100
	rbt ∞ NFκB p105/p50	IgG	Abcam	ab7971	GR226290-1	0.2	1:400
	ms ∞ iL6	IgG	R&D Systems	MAB206			1:1000
	ms ∞ iL1-β	IgG	R&D Systems	MAB201			1:1000
	ms ∞ TNF-α	IgG	R&D Systems	MAB610			1:1000
	Peroxisade gt ∞ rbt	IgG	R&D Systems	HAF008	FIN1715091	0.1	1:3000
	Peroxisade rbt ∞ chk	IgG	Sigma-Aldrich	A9046	015M4856V		1:10000
	Peroxisade gt ∞ ms	IgG	R&D Systems	HAF007	FIM2815031	0.1	1:6000
ICC	ms ∞ Fn14	IgG	Santa Cruz Biotechnology	sc-56250	C0116	0.2	1:50
	rbt ∞ NFκB p105/p50	IgG	Abcam	ab7971	GR226290-1	0.2	1:50
	gt∞ ms AF555	IgG (H+L)	Life Technologies	A21422	1608465	2	1:500
	gt∞rbt AF633	IgG (H+L)	Life Technologies	A211071	1744737	2	1:500
	ms∞Actin AF488	IgG	BD Bioscience	558623	5352903		1:100
	Hoechst 33342		Life Technologies	H1399			1:1000

3.2. Methods

3.2.1. Ethics committee

The ethical standards recognized by the Spanish biomedical research law[149] and the principles of the Declaration of Helsinki of 1975 (as revised in 2013)[150] for investigation with humans[150] were followed. The experimental protocols of this study were approved by the Institutional Review Board in all participating hospitals and every patient signed a written informed consent (**Annex 1**).

3.2.2. Subjects recruitment and samples

3.2.2.1. Meniere's disease cohort

A total of 1610 samples of patients with MD according to the diagnostic scale of the AAO-HNS[27] were collected by experts in Otoneurology. Recruitment was carried out in 25 centers distributed over Spain, Portugal and Italy.

Clinical features

Every patient underwent a complete neuro-otological evaluation, including a pure-tone audiometry, an otoscopy, nystagmus examination and a caloric testing. A brain MRI was performed to exclude any other possible cause of neurological symptoms. Patients with simultaneous SNHL in both ears were considered to have synchronic SNHL, while metachronic SNHL was considered if an interval longer than one month between the first and the second ear was observed.

Clinical variables studied were as follow: gender, duration of disease, age of onset, family history of MD, hearing loss at diagnosis, hearing stage defined as four-tone average of 0.5, 1, 2 and 3 kHz according to the AAO-HNS criteria (stage 1, ≤ 25 dB; stage 2, 26-40 dB; stage 3, 41-70 dB and stage 4, >70 dB), type of headache (migraine, tension-type headache), history of autoimmune disease (AD), familial MD was defined according to the criteria established by the Barany Society International Classification for Vestibular Disorders[13], cardiovascular risk factors (high blood pressure, type 2 diabetes, dyslipidemia and smoking), Tumarkin crisis and the functional scale of the AAO-HNS.

Exclusion criteria

We excluded patients with benign paroxysomal positional vertigo, vestibular neuritis, ear surgery, recurrent infection of the middle ear, acoustic neuroma, vestibular schwannoma or any other cause mimicking MD, according to the diagnostic scale of

the AAO-HNS[27]. Patients with unilateral MD or bilateral MD with less than 5 years of evolution were excluded of the study. Finally, other patients were excluded because of inconsistent data in the clinical records.

3.2.3. Genotyping

3.2.3.1. DNA isolation

DNA was isolated from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit, according to the manufacturer's instructions. The concentration of genomic DNA was measured using the Qubit dsDNA BR Assay Kit and concentrations were standardized to 50 ng/mL. DNA quality ratios 260/280 and 230/260, used to determine contamination grade of protein and salt, respectively, were determined by Nanodrop 2000C (ThermoFisher Scientific). All samples had ratios 1.8 - 2.

3.2.3.2. Immunochip and Quality controls

DNA samples were genotyped by the Immunochip, a custom genotyping array which include loci previously associated with twelve autoimmune disorders [151]. Clusters were manually inspected and verified, and SNPs with poor clustering quality metrics were removed (call frequency <0.98, cluster separation <0.4 and GenCall scores <0.15). Further, the SNPs that did not meet the following criteria were excluded: minor allele frequency (MAF) < 5%, Hardy-Weinberg equilibrium < 10^{-4} in controls, non-random differential missing data rate test between cases and controls < 10^{-5} and missing-genotype rate < 0.5%. All markers in chromosome X were also excluded. After QC, 96899 SNVs remained with a MAF > 5% for statistical analysis.

Samples with a genotype success rate of <90% and increased heterozygosity rate (<0.18 and > 0.45) were excluded from the analysis. Finally, genetic outliers determined by PCA were removed from the analysis (> 3 standard deviation around the mean).

3.2.3.3. Taqman SNP assay and Quality controls

The genotyping of our cohort was performed using 96-well plates in an ABI 7500 Fast Real-Time PCR System (Life Technologies) with its standard conditions (**Figure 9**).

The alleles were determined using the SDS 2.2.1 software (Applied Biosystems). We used principal-component analysis (PCA) to identify population substructure.

Furthermore, a representative sample of SNVs genotyped by the Immunochip was validated also by Taqman assays in 165 individuals. The correlation coefficient between Immunochip and Taqman assays was 98%. Genotype calling was performed in all samples with the Genotyping Module (v1.8.4) of the Genome Studio Data Analysis Software. NCBI Build 36 (hg18) mapping was used (Illumina manifest file Immuno_BeadChip_11419691_B.bpm). Data were converted into the human Build hg38 using (https://genome.ucsc.edu/cgi-bin/hgLiftOver).

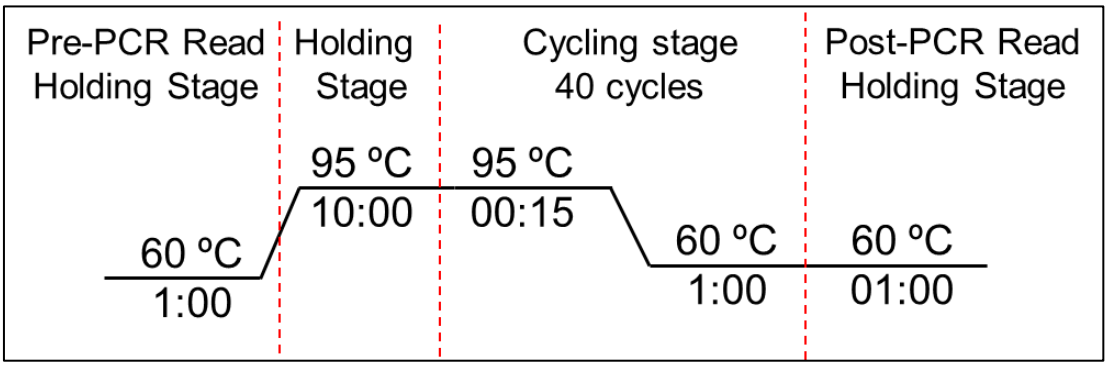


Figure 9. Taqman assay standard conditions for performed in an ABI 7500 Fast Real-Time PCR Systems.

Quality controls (QC) were performed, for each set of samples and SNVs separately, using Genome Studio Data Analysis Software and PLINK software (version 1.07) [152]. After all QC, 189 patients with bilateral SNHL and 735 controls remained for further statistical analyses. We have evaluated the association between each single nucleotide variants (SNV) and patients with unilateral or bilateral MD.

3.2.4. Cell culture

3.2.4.1. Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll gradients and cultured in RPMI 1640 supplemented with 10% (v/v) FBS, non-essential amino acids and sodium pyruvate solution, and plated at $1x10^6$ cells/mL in 6-well plates.

3.2.4.2. Stimulation

In some experiments, fungus extract was added One x 10^6 PBMC per mL was stimulated with 5µg/mL dialyzed mixed fungus. PBMC were incubated during 16 h at 37°C in 7% CO_2 and compared with cultured, unstimulated PBMC. The optimal

concentrations of these mixtures were previously identified by culturing PBMC with increasing concentrations of these reagents to identify the best concentration (mold mix: 5µg/mL) that could be used without distressing cell viability. At the end of all incubations, PBMC were centrifuged, cells harvested and supernatants were collected and stored in -20 °C.

3.2.4.3. Lymphoblastoid cell line

Epstein-Barr transduction and cell line culture

Conditioned lymphoblastoid cell lines (LCL) were obtained from patients with MD according to the haplotype defined by rs9380217/rs4947296. For this purpose PBMCs were isolated using Ficoll gradients. PBMCs were seeded at a density of 5×10^6 cells/mL in RPMI 1640 containing FBS and, EBV at 1:1 ratio was added. Cells were placed in an incubator maintained at 37°C with 7% CO_2 and cultured in RPMI 1640 supplemented with, non-essential amino acids solution and sodium pyruvate solution.

Cell viability & Proliferation Assay

Cell viability and proliferation assays were performed in LCL to investigate the effect of the conditional haplotype. A thousand cells were plated in 96well-plates and incubated at different TWEAK (PeproTech) concentrations to examine the effect over both cell lines (0, 50, 100, 200 and 500ng/mL)[153]. Proliferation rate was measured at 24h, 48h, 72h and 96h. At each time point, 20 µL of PrestoBlue™ (Life Technologies) was added to each well and cultured at 37 °C for 4 h. After that the absorbance of the supernatant was measured at 570 nm in a Tecan Infinite Nanoquant M200 Pro absorbance microplate reader. Blank controls were performed for each measure using medium and PrestoBlue™ (Life Technologies). Cell viability assay was performed using Trypan blue staining (Fisher Scientific). A hundred thousand cells were plated in 12well-plates and incubated at 100ng/mL of TWEAK[154] for 6h, 12h, 24h, 48h, 72h, 48h, and 96h at 37ºC.

3.2.5. Immunoassays

3.2.5.1. Multiplex Assay

Conditioned supernatants were collected and stored at –20ºC until a sufficient number of samples were acquired. Frozen samples were thawed immediately prior to analysis and none of the samples underwent repetitive freeze-thawing cycles prior to analysis. IL-1β, IL-6, IL-1Ra and TNFα levels in conditioned supernatant were quantified

simultaneously with a bead-based multiplex assay (EMD Millipore) using a Luminex 200 (Luminex Corp) and read with Luminex x PONENT 3.1 software (Luminex Corp.).

The minimum detection limit for the assay was 0.14pg/mL and the maximum 10000 pg/mL. Samples with readings below or above these levels were assigned values of 0pg/mL for the minimum value or 10000pg/mL for the maximum value. The percentages of samples with levels below 0.14pg/mL (detection limit for unstimulated samples) were: IL-1β 0.08%, IL-1RA 0.03%, IL-6 0.006% and TNF-α 0%. The percentages of samples with levels above 10000pg/mL (detection limit for samples stimulated with Aspergillus) were: IL-1β 0.006%, IL-1RA 0%, IL-6 0.06% and TNF-α 0.047%; and for samples stimulated with Penicillium were: IL-1β 0.012%, IL-1RA 0%, IL-6 0.05% and TNF-α 0.6%.

For a subgroup of 30 patients we measured the cytokines in duplicate and determined the reliability of the measurements by calculating the Intraclass Correlation Coefficient (ICC). ICC for each cytokine was greater than 80% in samples stimulated with Mix Apergillus: IL-1β 97.8%, IL-1RN 98.2%, IL-6 81% and TNF-α 94%; and in samples stimulated with Mix Penicillium: IL-1β 94.9%, IL-1RN 90.2%, IL-6 84.5% and TNF-α 91.7%. In addition, we had two quality controls for each cytokine, run in duplicate, which were within the expected range with coefficients of variation less than 8%. Based on these data, additional cytokine assays were run in singlicate.

3.2.5.2. ELISA

To validate Multiplex assay results, IL-1β and IL-6 levels were quantified, in conditioned supernatant, using a sandwich ELISA (R&D Systems) as described by the manufacturer's procedure. All samples were run in duplicate to ensure reproducibility. Correlation between Multiplex Bead-Based assay and ELISA was greater than 85% for both cytokines.

3.2.6. Expression analyses

3.2.6.1. RNA isolation

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of the extracted RNA were assessed with Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies).

3.2.6.2. Expression array

Expression levels were measured using the HumanHT-12 v4 Expression BeadChip (Illumina Inc.) with 500 ng of total RNA and processed with the high resolution scanner iScan (Illumina Inc.). Limma R package from Bioconductor was used for expression data analysis, normalization and differential expression analysis

3.2.6.3. Q-RT-PCR

cDNA was synthesized by the QuantiTect Reverse Transcription Kit (Agilent) and Quantitative RT-PCR was performed using SYBR® Green kit (Agilent) and an ABI 7900 HT Fast Real-Time PCR Systems (Life Technologies) following commercial instructions and standard conditions (**Figure10**). Hypoxanthine phosphoribosyl-transferase1 (HPRT-1) was used as housekeeping gene. Biological and technical replications were performed in triplicate to reduce experimental errors. Fold change for each gene was obtained using the comparative CT method. Statistical analyses were performed using Student's t-test and a p-value <0.05 was considered statistically significant.

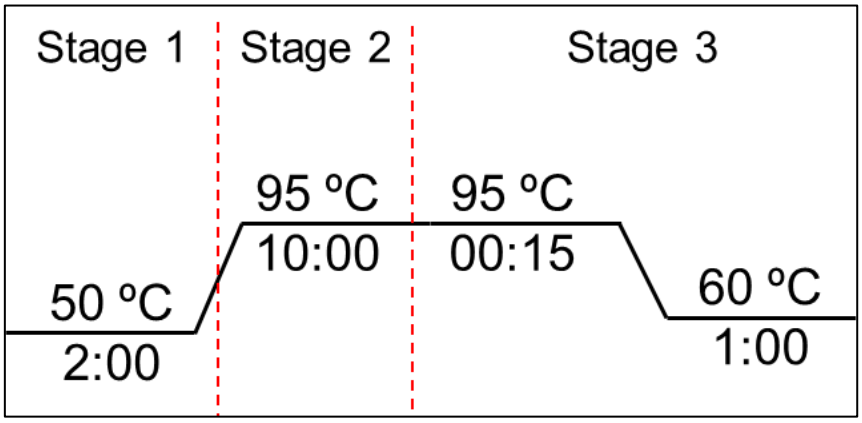


Figure 10. qPCR Standard conditions performed in an ABI 7900 HT Fast Real-Time PCR Systems.

3.2.7. Bioinformatics

Signaling pathway analysis was performed using Ingenuity Pathways Analysis (IPA®, Qiagen, Venlo, Netherlands, http://www.ingenuity.com/products/ipa) software. Core analysis tool was executed using the DEG with an adjusted P value cutoff of 0.001.

Pathway enrichment analysis was performed with MetaCore (GeneGo, https://portal.genego.com/) [155], using the differentially expressed genes (DEG) with the

enrichment P value cutoff of 0.001 and the shortest paths were visualized in Cytoscape ver. 2.7.0 [156].

3.2.8. Proteomics

3.2.8.1. Protein extraction

The protein extraction was carried out by acetone precipitation[157]. Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, California, USA) and total protein was stored at -80ºC.

3.2.8.2. Western-blot

Fifteen µg of total proteins were separated for molecular weight in a poliacrilamyde gel (130V, 1h 30min) and transferred to a PVDF membrane by Trans-Blot® Turbo™ Transfer System (BioRad). To confirm the transference, membranes were incubated in Ponceau S staining solution for 10min and then washed 3x5min with TBS-T. The membranes were then blocked in blocking solution for 1h at room temperature to avoid unspecific unions. The membranes were incubated with primary antibodies overnight at 4ºC. Then, membranes were washed with TBS-T 3x5min, and then incubated with secondary antibodies for 1h at room temperature. Finally membranes were washed with TBS-T 3x5min and developed and images were obtained by the Image Quant LAS4000 (GE Helthcare Life Science). We used ImageJ software (NIH, USA) for the quantification.

3.2.8.3. Whole mount staining of LCLs

NOTE: We did not centrifuge the LCLs. To change buffers, make washes, etc, we left the tube resting so the LCLs would pellet at the bottom of the tube simply by gravity.

- Fixation:
  - Wash hEBs with PBS.
  - Wash with 1mL Methanol:DMSO (4:1) (Freshly made).
  - Remove supernatant and add 1mL of fresh Methanol:DMSO.
  - Leave at 4ºC overnight, with gentle rotation.
  - Next day, let rest to pellet the LCLs. Remove supernatant.
  - Wash with 1mL Methanol. Remove supernatant.
  - Add 1mL Methanol.

- Rehydration:
  - Remove methanol.
  - Add 75% Methanol in 0.1% Tween20/PBS. Incubate 5 min at RT with gentle rotation.
  - Remove supernatant. Add 50% Methanol in 0.1% Tween20/PBS. Incubate 5 min at RT with gentle rotation.
  - Remove supernatant. Add 25% Methanol in 0.1% Tween20/PBS. Incubate 5 min at RT with gentle rotation.
  - Remove supernatant. Add 0.1% Tween20/PBS. Incubate 5 min at RT with gentle rotation.
- Block with 2% Milk in 0.5% Tween20/PBS 5 hours with gentle rotation.
- Remove blocking. Stain overnight with first primary antibodies (Fn14 and NFkB) diluted in 1% Milk in 0.5% Tween20/PBS. Incubate at 4ºC with gentle rotation.
- Next day, remove antibody solution and perform a quick wash with 0.1% Tween20/PBS.
- Wash 5 times with 0.1% Tween20/PBS for at least 1 hour per wash, at RT and with gentle rotation.
- Stain overnight with secondary antibodies, mouse anti-actin conjugated AF488 and nuclear staining (Hoetsch) diluted in 1% Milk in 0.5% Tween20/PBS. Incubate them at 4ºC with gentle rotation.
- Next day, perform washes as described above.
- Fix 1 hour in 2% PFA in 0.1% Tween20/PBS for 1 hour at RT with gentle rotation.
- Equilibration:
  - Add 30% glycerol, mix gently upside down, and leave resting until LCLs reach the bottom of the tube. Remove 30% glycerol.
  - Add 50% glycerol, mix gently upside down, and leave resting until LCLs reach the bottom of the tube. Remove 50% glycerol.
  - Add 80% glycerol, mix gently. Equilibrate overnight at 4ºC.
- Mounting LCLs for Confocal imaging (**Figure 11**):
  - Chambers preparation: on a slide, fix 2 blocks of three 22x22 mm coverslips using transparent nail polish, one at a time. Wait until one layer is dry to add the next.

- Carefully with a plastic Pasteur pipette of wide mouth take the LCLs from the 80% glycerol and deposit them in the chambers, adding 80% glycerol so there are no bubbles.
- Close the chamber with a 22x60mm coverslip, and fix all the sides with transparent polish nail

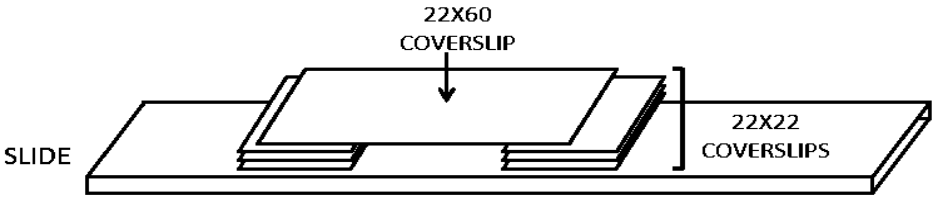


Figure 11. Diagram showing how to assemble the samples

3.2.9. Webtools and Web Resources

Several public Webtools and public databases were used during this thesis. The URL for data presented herein as follows:

Genemania, http://www.genemania.org/

1000Genomes project, http://www.1000genomes.org/dbSNP

CTCFBSDB 2.0, http://insulatordb.uthsc.edu/

HaploReg, http://www.broadinstitute.org/mammals/haploreg/haploreg.php

LocusZoom, http://locuszoom.sph.umich.edu/locuszoom/

Primer3 Input v.4.0.0, http://primer3.ut.ee/

NCBI/Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/

OligoAnalyzer v.3.1, http://eu.idtdna.com/calc/analyzer

IPA®, http://www.ingenuity.com/products/ipa

MetaCore, https://portal.genego.com/

RegulomeDB, http://regulomedb.org/

seeQTL, http://www.bios.unc.edu/research/genomic_software/seeQTL/

3.2.10. Statistical Analysis

A descriptive statistical analysis of all data was performed using SPSS software (SPSS Inc.). Data are shown as means with their standard deviations (SD). Quantitative variables were compared using Student's unpaired T-test. Qualitative variables were compared using cross tables and Fisher's exact test. Nominal p-values <0.05 were considered statistically significant.

A two-step cluster analysis, using log-likelihood distance measures, was carried out as previously described. The final cluster analysis was applied using only four categorical variables: history of autoimmune disease, delayed MD, FMD or sporadic cases, and migraine.

3.2.11. Image Analysis

A laser scanning confocal microscope LSM 710 (Carl Zeiss, Oberköchen, Germany) was used for image collection and the Zeiss browser software program ZEN (black edition) was used to acquire and export the data. All images were taken with the same laser intensity settings on the microscope. Final image processing and labeling were performed with the image analysis program ImageJ (NIH,http://imagej.nih.gov/ij/). Image stacks were exported from ZEN to ImageJ as split channels. In ImageJ, individual clusters were measured. Measurements taken were: Area, Mean density and integrated density. Images were converted to 8bits (range from 0 to 255). Corrected total cell fluorescence (CTCF) was calculated. Data are presented as mean value± standard deviations. Two-tailed t-tests with unequal variances were performed to determine significance.

4. Results

4.1. AIM 1: To define a clinical group of patients with autoimmune MD.

In order to achieve the first goal, we first segregated MD patients in 2 different groups, uni- and bilaterals (UMD and BMD, respectively) and studied them separately. Finally we compared both groups.

4.1.1 BMD features

Three hundred ninety-eight patients with BMD were included in the study. There were 258 sporadic cases (SMD) and 52 individuals with Familial MD (FMD) (20%). Although apparently there were no clinical differences within the phenotype between sporadic and familial cases, FMD had an earlier age of onset (p=0.003) and a higher prevalence of autoimmune comorbidities (AD) (**Table 10**). Thus, the distribution of frequencies for the age of onset showed that the number of patients starting before 40 years old was significantly higher in the FMD (**Figure 11**). **Table 11** lists the autoimmune conditions found, being rheumatoid arthritis the most common in our cohort.

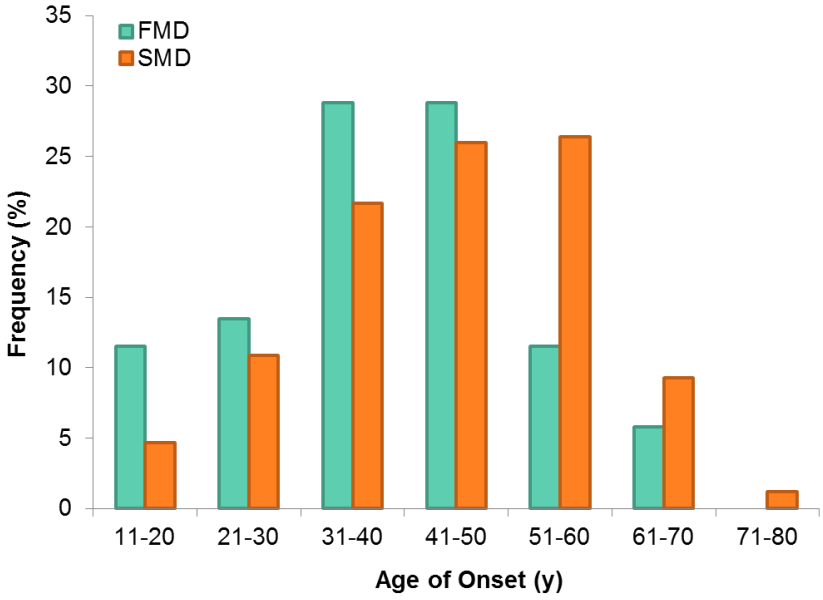


Figure 11. Age of onset in BMD.
Distribution of frequencies in between SMD and FMD

Table 10. Clinical phenotype in SMD and FMD with at least 5 years since the onset of the disease.

VARIABLES	FMD (n=52)	SMD (n=258)	P value
Age, Mean (SD)	55.5 (12.7)	61.5 (11.1)	**0.001**
Gender (%women)	34 (65.4)	147 (57.0)	0.28
Age of Onset (SD)	39 (12.9)	44.8 (13.1)	**0.003**
Age of Onset ≤40, n (%)	28 (53.8)	96 (37.2)	**0.03**
Time Course (years), mean (SD)	16.3 (8.7)	16.3 (9.4)	0.96
Sinchronic, n (%)	11 (21.6)	72 (27.9)	0.39
Metachronic, n (%)	40 (78.4)	186 (72.1)	
Hearing loss at diagnosis, mean (SD)	51.9 (15.5)	56.6 (17.8)	0.092
Headache, n (%)	23 (44.2)	92 (36.1)	0.27
Migraine, n (%)	13 (25.0)	44 (17.3)	0.24
Rheumatoid history, n (%)	10 (20.4)	25 (9.8)	**0.048**
Hearing stage, n (%)			
1	0 (0.0)	4 (1.6)	0.58
2	9 (17.6)	35 (13.6)	
3	28 (54.9)	131 (51.0)	
4	14 (27.5)	87 (33.9)	
Cardiovascular Risk			
High Blood Pressure, n (%)	13 (26.5)	93 (39.7)	0.1
Dyslipemia, n (%)	21 (42.0)	111 (47.6)	0.53
Type 2 Diabetes, n (%)	12 (24.0)	41 (17.4)	0.32
Smoking, n (%)	15 (30)	53 (21.5)	0.2
Tumarkin Crisis, n (%)	17 (35.4)	63 (25.5)	0.16
Functional Scale, n (%)			
1	9 (17.6)	53 (21.3)	0.81
2	15 (29.4)	71 (28.5)	
3	10 (19.6)	58 (23.3)	
4	7 (13.7)	35 (14.1)	
5	7 (13.7)	25 (10.0)	
6	3 (5.9)	7 (2.8)	

P values in bold are statistically significant

Table 11. AD and other rheumatoid conditions observed in BMD patients.

Autoimmune diseases	N
Rheumatoid Arthritis	10
Fibromyalgia	6
Arthrosis	5
Ankylosing spondylitis	5
Psoriasis	4
Hypothyroidism	3
Sjogren Syndrome	3
Type 1 Diabetes	2
Rosacea	2
Graves-Basedow disease	2
Systemic Lupus Erythematous	2
Psoriatic arthritis	1
Autoimmune inner ear disease	1
Polymyalgia rheumatica	1
Inflammatory Bowel Disease	1
Cogan Syndrome	1
Hip synovitis	1
Carpal tunnel syndrome	1
Undetermined	10

Next, we compared the clinical features in patients with SMD and FMD stratified according to the presence or absence of autoimmune comorbidities. In SMD, headache and migraine were most commonly observed in patients with autoimmune background (62.5% and 33%, respectively) compared with patients without AD (33% and 16%), suggesting a potential association between migraine and autoimmunity in patients with sporadic BMD (**Table 12**).

We then compared patients according to their onset of hearing loss (HL) (**Table 13**). One hundred three (26%) subjects developed simultaneous HL in both ears (synchronic HL, either symmetric or asymmetric), while 291 (73%) patients started with HL in one ear and developed the HL in the contralateral one (metachronic HL). **Figure 12** compares the distribution of frequencies for the age of onset in patients with synchronic or metachronic HL. There were no clinical differences between them, but the incidence of headache was most commonly observed in synchronic HL ($p=0.0004$) and the worst hearing stage was observed in patients with metachronic HL ($p=0.004$).

Table 12. Clinical features of SMD and FMD stratified by the presence of AD

	Sporadic MD			Familial MD		
VARIABLES	**AD+ (n=25)**	**AD- (n=230)**	**P-value**	**AD+(n=10)**	**AD- (n=39)**	**P-value**
Age, Mean (SD)	61.7 (9.1)	61.6 (11.2)	0.94	56.5 (13.8)	55 (12.4)	0.74
Gender (%women)	18 (72.0)	128 (55.7)	0.14	6 (60.0)	26 (66.7)	0.72
Age of Onset (SD)	43.4 (11.0)	45.2 (13.2)	0.5	35.9 (12.3)	40.5 (13.0)	0.31
Age of Onset ≤40, n (%)	11 (44.0)	82 (35.7)	0.51	6 (60.0)	19 (48.7)	0.73
Time Course (years), mean (SD)	17.4 (8.7)	16.1 (9.6)	0.52	20.7 (8.9)	14 (7.0)	**0.01**
Hearing loss at diagnosis, mean (SD)	57.5 (18.3)	56.7 (17.8)	0.83	52.3 (15.2)	52 (15.9)	0.96
Headache, n (%)	15 (62.5)	77 (33.5)	**0.007**	8 (80.0)	14 (35.9)	**0.03**
Migraine, n (%)	8 (33.3)	36 (15.7)	**0.044**	5 (50.0)	7 (17.9)	**0.05**
Hearing stage, n (%)						
1	0 (0.0)	4 (1.7)	0.37	0 (0.0)	0 (0.0)	0.32
2	5 (20.0)	30 (13.1)		1 (11.1)	8 (20.5)	
3	9 (36.0)	119 (52.0)		4 (44.4)	23 (59.0)	
4	11 (44.0)	76 (33.2)		4 (44.4)	8 (20.5)	
Cardiovascular risk factors						
High Blood Pressure, n (%)	13 (59.1)	80 (37.7)	0.07	2 (20.0)	10 (27.0)	1
Dyslipemia, n (%)	12 (50.0)	97 (46.9)	0.83	4 (40.0)	16 (42.1)	1
Type 2 Diabetes, n (%)	8 (33.3)	33 (15.8)	**0.046**	5 (50.0)	7 (18.4)	0.09
Smoking, n (%)	6 (28.6)	47 (21.0)	0.41	3 (30.0)	12 (30.8)	1
Tumarkin Crisis, n (%)	6 (27.3)	57 (25.3)	0.8	5 (50.0)	12 (32.4)	0.46
Functional Scale, n (%)						
1	4 (17.4)	48 (21.3)	0.94	2 (20.0)	7 (17.9)	**0.007**
2	7 (30.4)	64 (28.4)		4 (40.0)	11 (28.2)	
3	6 (26.1)	52 (23.1)		1 (10.0)	8 (20.5)	
4	2 (8.7)	33 (14.7)		0 (0.0)	6 (15.4)	
5	3 (13.0)	22 (9.8)		0 (0.0)	7 (17.9)	
6	1 (4.3)	6 (2.7)		3 (30.0)	0 (0.0)	

P values in bold are statistically significant

Table 13. Clinical features in BMD according to the onset of HL.

VARIABLES	Synchronic (n=103)	Metachronic (n=291)	P-value
Age, Mean (SD)	61 (11.0)	60.1 (11.9)	0.49
Gender (%women)	63 (61.2)	161 (55.3)	0.36
Age of Onset (SD)	46.1 (12.8)	43.5 (13.2)	0.07
Age of Onset ≤40, n (%)	39 (37.9)	118 (40.5)	0.73
Time Course (years), mean (SD)	14.4 (8.9)	16.2 (8.9)	0.08
Family history, n (%)	39 (39.8)	119 (43.0)	0.64
FMD, n (%)	11 (13.3)	40 (17.7)	0.39
Hearing loss at diagnosis, mean (SD)	55.1 (17.0)	55.9 (17.0)	0.71
Headache, n (%)	55 (53.4)	96 (33.3)	**0.0004**
Migraine, n (%)	25 (24.3)	49 (17.0)	0.11
Rheumatoid history, n (%)	15 (15.0)	35 (12.2)	0.49
Hearing stage, n (%)			
1	1 (1.0)	6 (2.1)	**0.004**
2	27 (26.5)	34 (11.7)	
3	42 (41.2)	152 (52.4)	
4	32 (31.4)	98 (33.8)	
Cardiovascular Risk			
High Blood Pressure, n (%)	47 (51.1)	109 (39.9)	0.068
Dyslipemia, n (%)	53 (55.2)	121 (45.1)	0.097
Type 2 Diabetes, n (%)	13 (13.5)	50 (18.5)	0.35
Smoking, n (%)	22 (21.8)	68 (24.5)	0.68
Tumarkin Crisis, n (%)	24 (25.8)	69 (24.9)	0.89
Functional Scale, n (%)			
1	14 (14.0)	73 (26.0)	0.11
2	29 (29.0)	77 (27.4)	
3	26 (26.0)	55 (19.6)	
4	12 (12.0)	40 (14.2)	
5	16 (16.0)	27 (9.6)	
6	3 (3.0)	9 (3.2)	

P values in bold are statistically significant

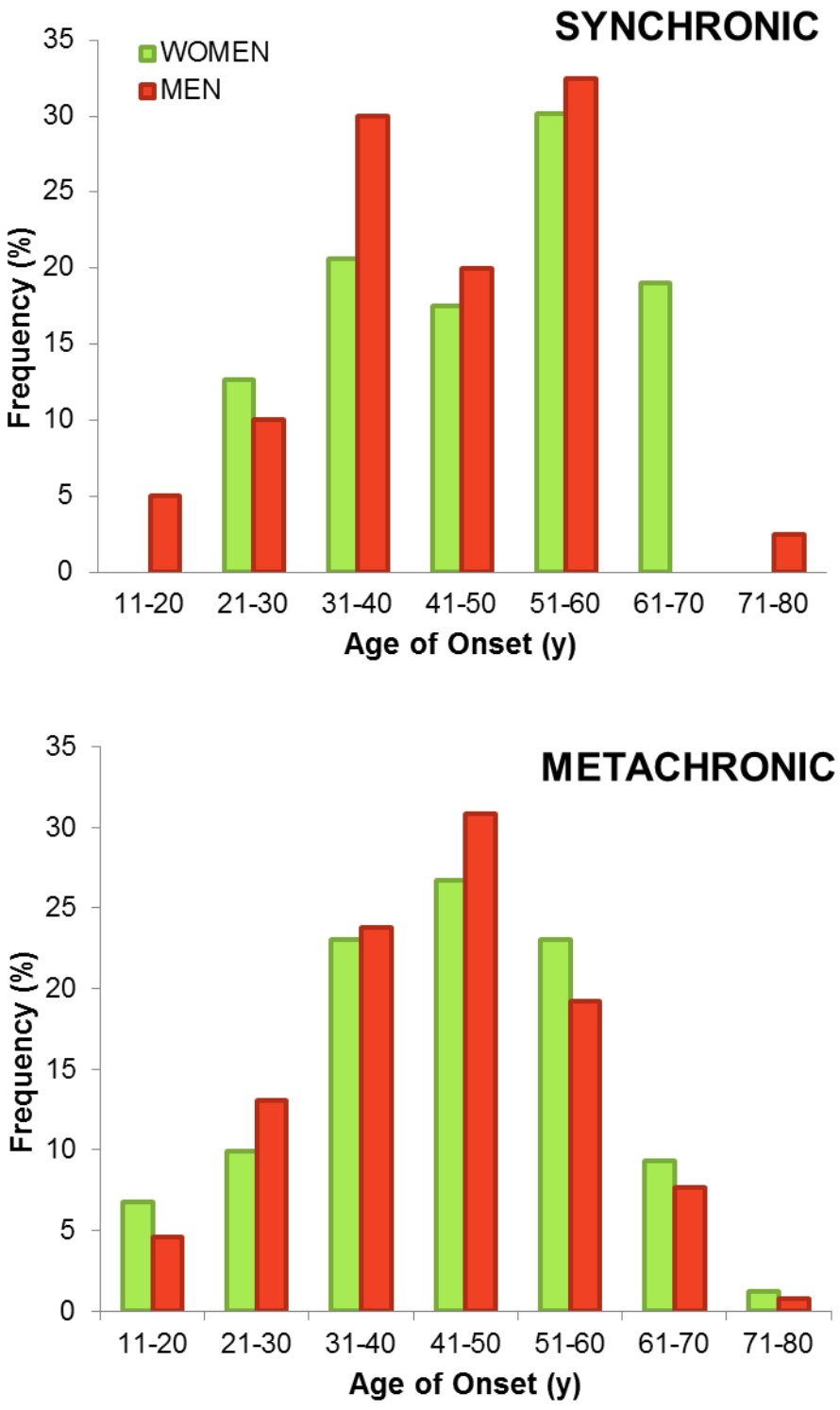


Figure 12. Age of onset in BMD according to their type of HL

4.1.2 UMD features

Figure 13 shows a flowchart with the patients included in this study. Our cohort (**Figure 14**) consisted of 852 SMD and 122 individuals with FMD (12.5%), and the familial history was not available in 14 cases. We observed significant differences in hearing thresholds at diagnosis, which was 51±18 dB HL in SMD and 31±28 dB HL in FMD ($p= 2.4x10^{-11}$). Furthermore, the prevalence of AD was higher in SMD (p= 0.029, **Table 14**). **Table 15** lists the 183 patients showing a comorbid AD found in SMD and FMD, being autoimmune hypothyroidism the most common disease in our cohort.

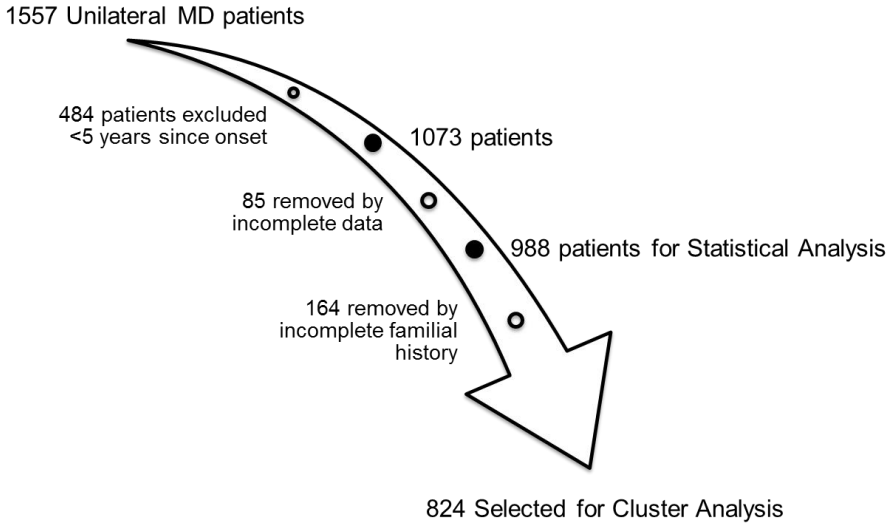


Figure 13. Flowchart showing selected patients with UMD for this study.

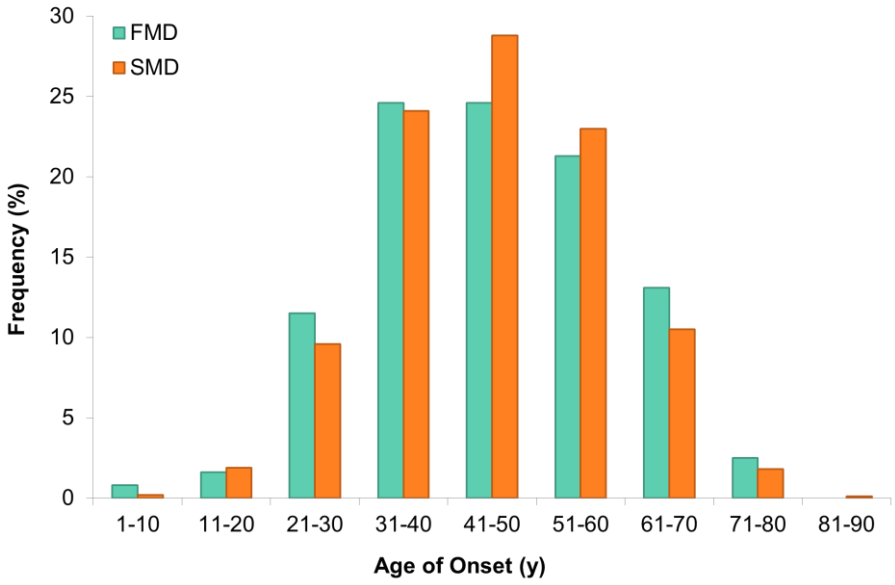


Figure 14. Age of onset in UMD. Distribution of frequencies in between SMD and FMD

Table 14. Clinical features in FMD and SMD with at least 5 years since the onset of the disease.

VARIABLES	FMD (n=122)	SMD (n=852)	P value
Age, Mean (SD)	59.3 (12.3)	57.8 (12.1)	0.2
Gender (%women)	74 (60.7)	478 (56.1)	0.38
Age of Onset (SD)	45.3 (13.6)	45.6 (12.5)	0.82
Age of Onset ≤40, n (%)	47 (38.5)	306 (36.0)	0.62
Time Course (years), mean (SD)	14.0 (9.2)	11.7 (7.2)	**0.011**
Hearing loss at diagnosis, mean (SD)	31.3 (28.0)	50.9 (17.8)	**2.40E-11**
Delayed MD, n (%)	12 (9.9)	61 (7.5)	0.37
Headache, n (%)	49 (40.2)	267 (32.1)	0.08
Migraine, n (%)	25 (20.7)	149 (18.0)	0.45
Autoimmune disease, n (%)	13 (11.0)	146 (19.4)	**0.029**
Hearing stage, n (%)			
1	3 (2.5)	64 (7.8)	**0.045**
2	19 (15.6)	171 (20.9)	
3	70 (57.4)	430 (52.5)	
4	30 (24.6)	154 (18.8)	
Cardiovascular Risk			
High Blood Pressure, n (%)	41 (33.6)	286 (34.2)	0.92
Dyslipidemia, n (%)	55 (45.5)	307 (37.2)	0.09
Type 2 Diabetes, n (%)	14 (11.7)	84 (10.1)	0.63
Smoking, n (%)	23 (19.5)	185 (22.3)	0.55
Tumarkin Crisis, n (%)	22 (18.8)	150 (18.1)	0.9
Functional Scale, n (%)			
1	52 (45.2)	188 (23.0)	**7.00E-06**
2	24 (20.9)	251 (30.7)	
3	13 (11.3)	207 (25.3)	
4	16 (13.9)	107 (13.1)	
5	9 (7.8)	54 (6.6)	
6	1 (0.9)	11 (1.3)	

P values in bold are statistically significant

Table 15. AD and other rheumatoid conditions observed in patients with UMD

Autoimmune diseases	N
Hypothyroidism	18
Fibromyalgia	9
Rheumatoid Arthritis	8
Psoriasis	6
Arthrosis	4
Sjogren Syndrome	4
Ulcerative colitis	4
Thyroiditis	4
Ankylosing spondylitis	3
Type 1 Diabetes	3
Systemic Lupus Erythematous	3
Polymyalgia rheumatica	2
Raynaud's phenomenon	2
Rosacea	1
Graves-Basedow disease	1
Psoriatic arthritis	1
Carpal tunnel syndrome	1
Uveitis	1
Multiple sclerosis	1
Behçet disease	1
Paget disease	1
Choroiditis	1
Hyperthyroidism	1
Pneumonitis	1
Antiphospholipid syndrome	1
Vitiligo	1
Sclerodermia	1
Crest syndrome	1
Celiac disease	1
Oligoarthritis	1
Autoimmune hepatitis	1
Primary biliary cirrhosis	1
Undetermined	94

We analyzed patients with SMD according to the presence or absence of AD. We found an earlier age of onset (p=0.007), worse HL at diagnosis (p=0.035), worse score in the vertigo functional scale ($1.14x10^{-7}$) and a higher prevalence of migraine (36%, $p=2.44x10^{-8}$) in patients with sporadic UMD and AD when they were compared with cases without AD (**Table 16**).

We also compared the clinical features of patients with delayed MD (N=75, 7.6% patients) with the rest of patients with non-delayed MD (N=913, 92.4% patients, **Table 17**). Hearing loss at diagnosis was worse in patients with delayed MD compared with non-delayed MD (p=0.006). Of note, there was no difference in the prevalence of vascular risk factors between both groups.

Table 16. Clinical features of SMD and FMD stratified by the presence of AD

	Sporadic MD			Familial MD		
VARIABLES	**AD+ (n=146)**	**AD- (n=607)**	**P-value**	**AD+ (n=12)**	**AD- (n=107)**	**P-value**
Age, Mean (SD)	56.8 (11.4)	58.2 (12.1)	0.19	54.7 (13.7)	59.8 (12.1)	0.16
Gender (%women)	83 (56.8)	337 (55.5)	0.78	8 (61.5)	64 (61.0)	1.0
Age of Onset (SD)	43.2 (10.7)	45.9 (12.6)	**0.007**	42.4 (16.4)	46 (12.9)	0.35
Time Course (years), mean (SD)	13.5 (8.0)	11.5 (7.1)	**0.009**	12.9 (6.6)	13.6 (8.8)	0.77
Hearing loss at diagnosis, mean (SD)	53.7 (16.8)	50.3 (17.8)	**0.035**	48 (20.5)	28.7 (28.4)	**0.007**
Delayed MD, n (%)	7 (4.9)	50 (8.8)	0.17	1 (7.7)	11 (10.5)	1.0
Headache, n (%)	67 (45.9)	184 (30.5)	**5.90E-04**	4 (30.8)	43 (41.0)	0.56
Migraine, n (%)	52 (35.6)	86 (14.4)	**2.44E-08**	2 (15.4)	23 (22.1)	0.7
Hearing stage, n (%)						
1	4 (2.8)	46 (7.7)		1 (7.7)	1 (1.0)	
2	27 (18.6)	131 (21.8)	0.11	3 (23.1)	15 (14.3)	0.25
3	83 (57.2)	315 (52.5)		6 (46.2)	63 (60.0)	
4	31 (21.4)	108 (18.0)		3 (23.1)	26 (24.8)	
Cardiovascular risk factors						
High Blood Pressure, n (%)	59 (40.7)	202 (33.9)	0.15	2 (15.4)	36 (34.3)	0.22
Dyslipidemia, n (%)	62 (42.5)	224 (38.3)	0.39	5 (38.5)	47 (44.8)	0.77
Type 2 Diabetes, n (%)	9 (6.2)	66 (11.1)	0.09	3 (25.0)	9 (8.6)	0.11
Smoking, n (%)	27 (18.5)	146 (24.5)	0.13	2 (15.4)	21 (20.4)	1.0
Tumarkin Crisis, n (%)	30 (20.7)	115 (19.5)	0.73	3 (23.1)	19 (18.6)	0.71
Functional Scale, n (%)						
1	11 (7.7)	115 (19.6)		3 (23.1)	48 (48.0)	
2	39 (27.5)	199 (34.0)		3 (23.1)	21 (21.0)	
3	67 (47.2)	131 (22.4)	**1.14E-07**	2 (15.4)	11 (11.0)	0.061
4	18 (12.7)	87 (14.8)		3 (23.1)	12 (12.0)	
5	7 (4.9)	44 (7.5)		1 (7.7)	8 (8.0)	
6	0 (0.0)	10 (1.7)		1 (7.7)	0 (0.0)	

P values in bold are statistically significant

Table 17. Clinical features of patients with and without delayed MD

VARIABLES	DMD (n=75)	NO DMD (n=913)	P-value
Age, Mean (SD)	55.7 (12.8)	58.1 (12.0)	0.1
Gender (%women)	41 (54.7)	526 (57.5)	0.63
Age of Onset (SD)	45.6 (13.1)	45.4 (12.5)	0.89
Age of Onset ≤40, n (%)	24 (32.0)	334 (36.6)	0.46
Time Course (years), mean (SD)	10.3 (6.1)	12.2 (7.7)	**0.036**
Hearing loss at diagnosis, mean (SD)	54.9 (24.1)	47.4 (20.3)	**0.006**
Headache, n (%)	25 (33.8)	306 (34.5)	1.0
Migraine, n (%)	12 (16.2)	169 (19.2)	0.64
Family history, n (%)	27 (37.0)	341 (38.8)	0.8
FMD, n (%)	12 (16.4)	109 (12.7)	0.37
Autoimmune disease, n (%)	8 (11.3)	156 (19.5)	0.11
Hearing stage, n (%)			
1	2 (2.8)	66 (7.5)	0.08
2	19 (26.8)	175 (19.8)	
3	31 (43.7)	473 (53.6)	
4	19 (26.8)	168 (19.0)	
Cardiovascular Risk			
High Blood Pressure, n (%)	18 (24.7)	307 (34.1)	0.12
Dyslipidemia, n (%)	33 (45.2)	325 (36.9)	0.17
Type 2 Diabetes, n (%)	6 (8.3)	86 (9.7)	0.84
Smoking, n (%)	17 (23.0)	191 (21.9)	0.88
Tumarkin Crisis, n (%)	17 (23.9)	161 (18.0)	0.21
Functional Scale, n (%)			
1	12 (18.8)	233 (26.4)	0.16
2	21 (32.8)	253 (28.7)	
3	11 (17.2)	209 (23.7)	
4	12 (18.8)	112 (12.7)	
5	8 (12.5)	61 (6.9)	
6	0 (0.0)	14 (1.6)	

P values in bold are statistically significant

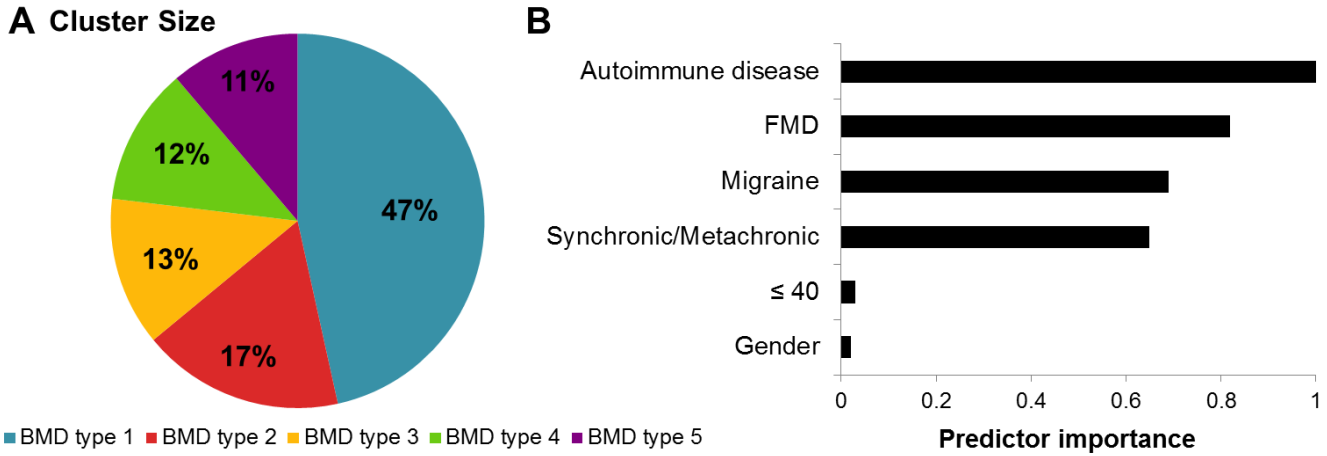
4.1.3 Cluster Analysis

We performed cluster analysis to identify groups of patients with common clinical features in BMD. **Figure 15** shows the size of the clusters, the relevance of predictors and the contribution of each predictor to define the cluster. The best predictors for clustering were autoimmune history, FMD, migraine and the onset of HL (synchronic/metachronic). Ninety-five patients remained unclassified because of incomplete clinical data.

We have defined five clusters for BMD and ranked them according to its relative frequency (**Figure 16**). Cluster 1 is the most common, including 46.5% of patients and it is defined by metachronic HL without migraine, SMD and no AD. Cluster 2 (17.5%) includes patients with synchronic HL, SMD, no migraine and no AD. Cluster 3 (12.9%) includes patients with FMD without migraine in 82% of patients. Cluster 4 (11.9%) consists of patients with migraine and SMD. Cluster 5 (11.2%) groups all patients with AD, being 71% SMD and 29% FMD.

Table 18 shows the five groups found and the major clinical differences amongst groups. Comparing the age of onset by groups, we observed that groups 3-5 have earlier onsets than groups 1 and 2 (p=0.0003). The type of HL, FMD, migraine and AD strongly vary between groups and these variables are the basis to assign a given patient to each cluster.

Figure 15. Summary of cluster analysis in BMD. (**A**) Pie chart showing 5 groups of clinical variants in BMD. (**B**) Bar chart ranking the importance of predictors to define the groups. (**C**) Classification of BMD in five clinical variants according to its observed frequency and lead predictor: Type 1, metachronic SNHL; type 2, synchronic SNHL; type 3, FMD; type 4, migraine; type 5, AD.



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VARIABLES	BMD type 1	BMD type 2	BMD type 3	BMD type 4	BMD type 5
N, (%)	141 (46.5)	53 (17.5)	39 (12.9)	36 (11.9)	34 (11.2)
METACHRONIC HL (%)	100	0	82.1	77.8	61.8
SYNCHRONIC HL (%)	0	100	17.9	22.2	38.2
MIGRAINE (%)	0	0	17.9	100	38.2
AUTOIMMUNE DISEASE (%)	0	0	0	0	100
FMD (%)	0	0	100	0	29.4
SMD (%)	100	100	0	100	70.6

Figure 16. Schematic diagram of BMD 5 groups. Circle areas are proportional to the frequency observed in each group.

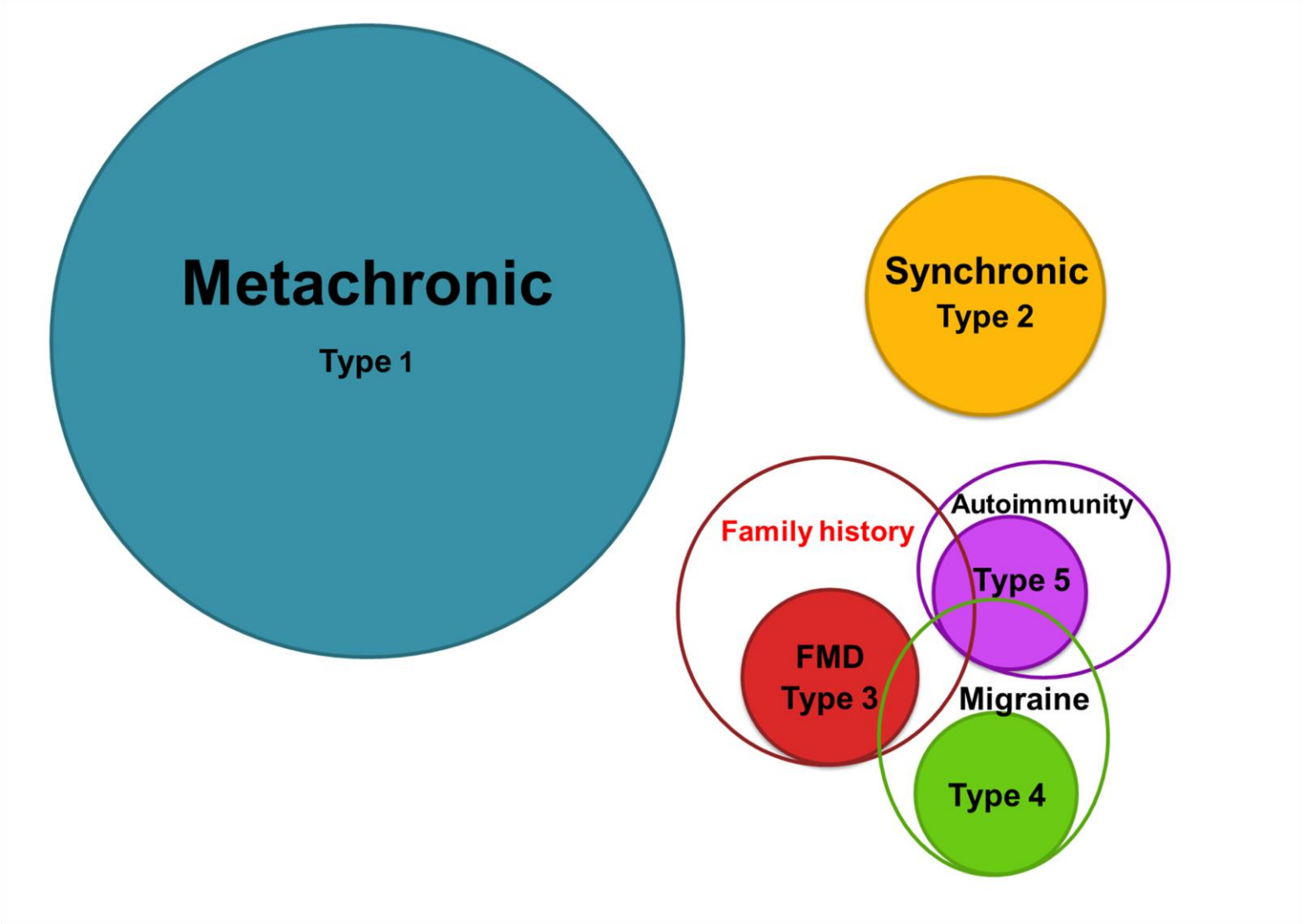


Table 18. Clinical variants in BMD defined by two-step cluster analysis

VARIABLES	BMD type 1 (n=141)	BMD type 2 (n=53)	BMD type 3 (n=39)	BMD type 4 (n=36)	BMD type 5 (n=34)	Pvalue
Group predictor	**Metachronic SNHL**	**Synchronic SNHL**	**FMD**	**Migraine**	**AD**	
Age, Mean (SD)	63.3 (11.0)	62.4 (9.5)	54.7 (13.2)	54.1 (11.5)	59.7 (11.1)	**0.00001**
Gender (%women)	73 (51.8)	30 (56.6)	26 (66.7)	25 (69.4)	24 (70.6)	0.11
Age of Onset (SD)	46.4 (13.1)	47.9 (12.0)	40 (14.5)	**37 (12.5)**	39.8 (11.3)	**0.0003**
Age of Onset ≤40, n (%)	46 (32.6)	15 (28.3)	19 (48.7)	21 (58.3)	16 (47.1)	**0.011**
Sinchronic, n (%)	0 (0.0)	53 (100.0)	7 (17.9)	8 (22.2)	13 (38.2)	**$3.39x10^{-42}$**
Metachronic, n (%)	141 (100.0)	0 (0.0)	32 (82.1)	28 (77.8)	21 (61.8)	
Family history, n (%)	18 (12.8)	7 (13.2)	39 (100.0)	7 (19.4)	19 (55.9)	**$1.81x10^{-27}$**
FMD, n (%)	0 (0.0)	0 (0.0)	39 (100.0)	0 (0.0)	10 (29.4)	**$4.10x10^{-53}$**
Headache, n (%)	22 (15.6)	20 (37.7)	14 (35.9)	36 (100.0)	23 (67.6)	**$4.88x10^{-20}$**
Migraine, n (%)	0 (0.0)	0 (0.0)	7 (17.9)	36 (100.0)	13 (38.2)	**$1.21x10^{-44}$**
Rheumatoid history, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	34 (100.0)	**$2.44x10^{-64}$**
Cardiovascular Risk Factors						
High Blood Pressure, n (%)	46 (34.3)	23 (50.0)	10 (27.0)	11 (34.4)	15 (46.9)	0.15
Dyslipemia, n (%)	58 (45.3)	26 (53.1)	16 (42.1)	13 (43.3)	15 (45.5)	0.86
Type 2 Diabetes, n (%)	23 (17.8)	9 (18.4)	7 (18.4)	1 (3.2)	12 (36.4)	**0.019**
Smoking, n (%)	31 (22.6)	10 (18.9)	12 (30.8)	6 (17.1)	9 (29.0)	0.53

P values in bold are statistically significant

We also carried out a cluster analysis to identify subgroups of patients with common clinical features in UMD. Two hundred forty-nine patients remained unclassified because of incomplete clinical data (25% of our cohort). **Figure 17** shows the size of clusters and the contribution of each predictor to define the cluster. The predictors were ranked according to its importance to define each cluster, being delayed MD and FMD the most relevant (**Figure 18**).

We have identified five clusters for UMD. Cluster 1 is the most common, including 53.4% of patients and it is defined by SMD without migraine, delayed MD and no AD. Cluster 2 (8.4%) groups all patients with delayed MD, being 83% SMD and 17% FMD. Cluster 3 (12.7%) includes patients with FMD, without AD in most cases (90%) and no migraine in 78% of them. Cluster 4 (14.9%) includes patients with SMD and migraine, without delayed MD or AD in 40% of cases. Cluster 5 (10.6%) consists of SMD patients with AD, without migraine or delayed MD.

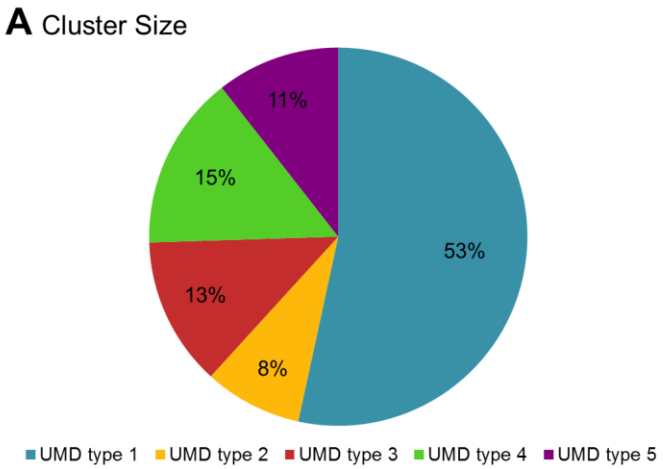
Table 19 shows the five groups found and the main clinical differences within all groups. We observed that cluster 4 has an earlier age of onset than the rest of groups ($p=0.001$). Moreover, according to the functional scale of the AAO-HNS we observe that groups 4 and 5 have more vertigo attacks ($9.33x10^{-11}$) than the other groups. Delayed MD, FMD, migraine and AD strongly differ amongst groups and these variables are enough to assign a given patient to each cluster.

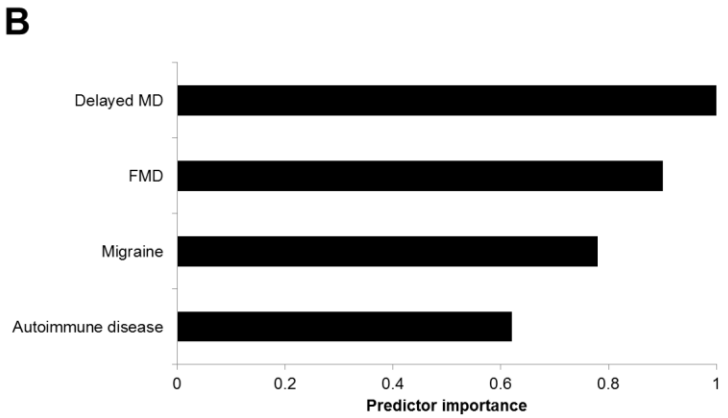
As a final point, we compared clinical features between UMD and BMD included in clusters 3-5 (**Table 20**). Thereby, individuals with BMD in cluster 3 (FMD) showed an earlier age of onset ($p=0.03$), worse hearing loss at diagnosis ($1.04x10^{-8}$) and worse score in the AAO-HNS functional scale ($p=0.02$) than patients with UMD type 3. However, patients with UMD in cluster 3 presented an AD in 11.4% of cases unlike BMD patients.

Patients with BMD in cluster 4 (migraine) showed worse hearing stage ($p=0.03$) and more Tumarkin crisis ($p=0.04$), probably associated with a longer duration of the disease ($p=0.004$). However, patients with UMD type 4 presented a comorbid AD in 40.7% of cases ($p=2.16x10^{-7}$). This finding was not observed in patients with BMD and migraine, where none showed AD.

Finally, patients with UMD in cluster 5 (AD) had a worse score on the functional scale ($p=0.002$), while patients with BMD in cluster 5 also had a longer disease duration ($p=0.001$) and it was associated with migraine in 38% ($p=9.46x10^{-9}$), type 2 diabetes in 36% ($7.6x10^{-5}$) and FMD in 29% ($1x10^{-6}$) of cases.

Figure 17. Summary of cluster analysis in UMD. (A) Pie chart showing five groups or clinical variants in UMD. (B) Bar chart ranking the importance of predictors to define the groups. (C) Classification of UMD in 5 clinical variants according to its observed frequency and lead predictor: Type 1, classical MD; Type 2, Delayed MD; Type 3,FMD; Type 4, migraine; Type 5, AD.





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VARIABLES	UMD type 1	UMD type 2	UMD type 3	UMD type 4	UMD type 5
N, (%)	440 (53.4)	69 (8.4)	105 (12.7)	123 (14.9)	87 (10.6)
SMD (%)	100	82.6	0	100	100
FMD (%)	0	17.4	100	0	0
Delayed MD (%)	0	100	0	0	0
Migraine (%)	0	17.4	21.7	100	0
Autoimmune Disease (%)	0	11.6	11.4	40.7	100

Figure 18. Schematic diagram of all subgroups observed in UMD. Circle areas are proportional to the frequency observed in each group.

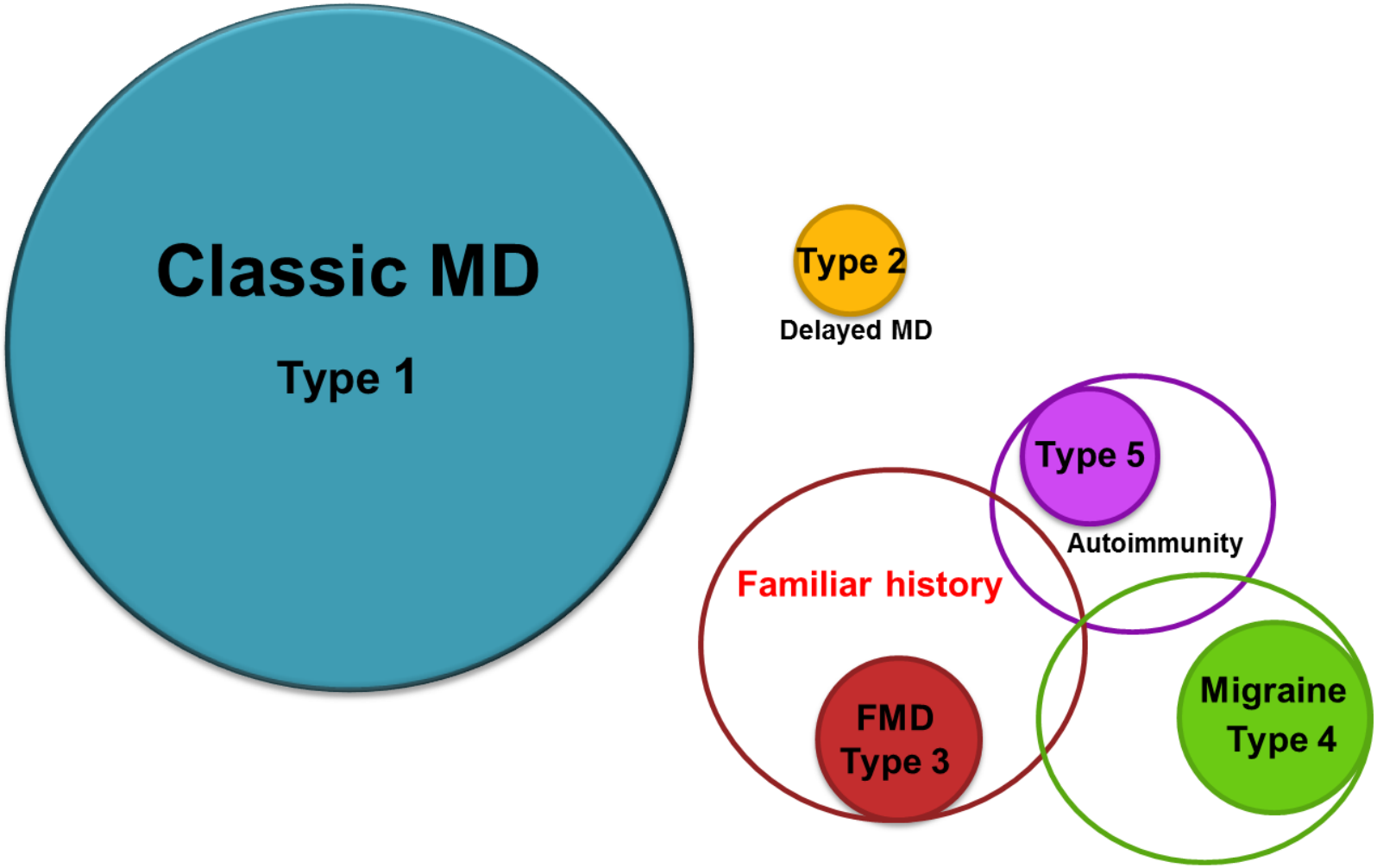

Table 19. Comparison of the main variables in each clinical subgroups defined by two-step cluster analysis in UMD.

VARIABLES	UMD type 1 (n=440)	UMD type 2 (n=69)	UMD type 3 (n=105)	UMD type 4 (n=123)	UMD type 5 (n=87)	P value
Group predictor	**Classical MD**	**Delayed MD**	**FMD**	**Migraine**	**AD**	
Age, Mean (SD)	59.1 (12.2)	56.5 (12.7)	60.0 (11.7)	54.2 (10.9)	56.9 (11.0)	**3.09E-04**
Gender (%women)	237 (53.9)	36 (52.2)	64 (61.0)	86 (69.9)	46 (52.9)	**0.02**
Age of Onset (SD)	46.3 (12.6)	46.2 (13.1)	46.0 (13.0)	**41.2 (11.1)**	44.1 (10.3)	**0.0012**
Age of Onset ≤40, n (%)	153 (34.8)	20 (29.0)	39 (37.1)	**61 (49.6)**	27 (31.0)	**0.01**
Time Course (years), mean (SD)	11.9 (7.1)	**10.6 (6.3)**	13.9 (12.6)	12.6 (7.9)	12.5 (7.8)	**0.04**
Hearing loss at diagnosis, mean (SD)	50.1 (16.8)	54.7 (24.9)	**28.7 (27.5)**	52.0 (17.5)	52.9 (18.1)	**7.74E-24**
Delayed MD, n (%)	0 (0.0)	**69 (100.0)**	0 (0.0)	0 (0.0)	0 (0.0)	**4.86E-177**
Headache, n (%)	83 (18.9)	23 (33.3)	42 (40.0)	123 (100.0)	15 (17.2)	**3.18E-62**
Migraine, n (%)	0 (0.0)	12 (17.4)	23 (21.7)	**123 (100.0)**	0 (0.0)	**4.33E-138**
Family history, n (%)	90 (20.7)	24 (34.8)	105 (100.0)	59 (48.4)	47 (54.7)	**3.14E-50**
FMD, n (%)	0 (0.0)	12 (17.4)	**105 (100.0)**	0 (0.0)	0 (0.0)	**2.04E-159**
Autoimmune disease, n (%)	0 (0.0)	8 (11.6)	12 (11.4)	50 (40.7)	**87 (100.0)**	**1.54E-110**
Hearing stage, n (%)						
1	31 (7.1)	2 (3.0)	2 (1.9)	9 (7.4)	3 (3.4)	0.14
2	84 (19.3)	18 (26.9)	16 (15.2)	25 (20.5)	19 (21.8)	
3	246 (56.4)	28 (41.8)	62 (59.0)	64 (52.5)	46 (52.9)	
4	75 (17.2)	19 (28.4)	25 (23.8)	24 (19.7)	19 (21.8)	
Functional Scale, n (%)						
1	88 (20.5)	12 (20.3)	48 (47.1)	16 (13.6)	6 (6.9)	**9.33E-11**
2	143 (33.3)	20 (33.9)	22 (21.6)	34 (28.8)	24 (27.6)	
3	96 (22.3)	9 (15.3)	12 (11.8)	**41 (34.7)**	**42 (48.3)**	
4	61 (14.2)	11 (18.6)	13 (12.7)	17 (14.4)	10 (11.5)	
5	35 (8.1)	7 (11.9)	6 (5.9)	7 (5.9)	5 (5.7)	
6	7 (1.6)	0 (0.0)	1 (1.0)	3 (2.5)	0 (0.0)	

P values in bold are statistically significant

Table 20.Comparison of clinical features between UMD and BMD

VARIABLES	TYPE 3			TYPE 4			TYPE 5		
	UMD (n=105)	**BMD (n=39)**	**P value**	**UMD (n=123)**	**BMD (n=36)**	**P value**	**UMD (n=87)**	**BMD (n=34)**	**P value**
Age, Mean (SD)	60.0 (11.7)	55.0 (12.3)	**0.03**	54.2 (10.9)	54.1 (11.5)	0.97	57 (11.0)	60.3 (10.9)	0.13
Gender (%women)	64 (61.0)	26 (66.7)	0.57	86 (69.9)	25 (69.4)	1	46 (52.9)	24 (70.6)	0.1
Age of Onset (SD)	46.0 (13.0)	40.5 (13.0)	**0.03**	41.2 (11.1)	37 (12.5)	0.054	44.1 (10.3)	41.5 (11.8)	0.24
Time Course (years), mean (SD)	13.9 (8.7)	14.0 (7.0)	0.97	12.6 (7.9)	17.3 (10.6)	**0.004**	12.5 (7.8)	18.2 (8.8)	**0.001**
Hearing loss at diagnosis, mean (SD)	28.7 (27.5)	52.0 (15.9)	**1.04E-08**	52.0 (17.5)	55.9 (16.1)	0.27	52.8 (18.1)	55.0 (17.0)	0.57
FMD, n (%)	100	100		0	0		0 (0.0)	10 (29.4)	**1.00E-06**
Headache, n (%)	42 (40.0)	14 (35.9)	0.7	123 (100.0)	36 (100.0)		15 (17.2)	23 (67.6)	**3.80E-07**
Migraine, n (%)	23 (21.9)	7 (17.9)	0.82	123 (100.0)	36 (100.0)		0 (0.0)	13 (38.2)	**9.46E-09**
Autoimmune disease, n (%)	12 (11.4)	0 (0.0)	**0.040**	50 (40.7)	0 (0.0)	**2.16E-07**	87 (100.0)	34 (100.0)	
Hearing stage, n (%)									
1	2 (1.9)	0 (0.0)		9 (7.4)	0 (0.0)		3 (3.4)	0 (0.0)	
2	16 (15.2)	8 (20.5)	0.72	25 (20.5)	6 (16.7)	**0.03**	19 (21.8)	6 (18.2)	0.12
3	62 (59.0)	23 (59.0)		64 (52.5)	15 (41.7)		46 (52.9)	13 (39.4)	
4	25 (23.8)	8 (20.5)		24 (19.7)	115 (41.7)		19 (21.8)	14 (42.4)	
Cardiovascular Risk									
Type 2 Diabetes, n (%)	9 (8.6)	7 (18.4)	0.13	7 (5.7)	1 (3.2)	1	5 (5.7)	12 (36.4)	**7.60E-05**
Smoking, n (%)	20 (19.4)	12 (30.8)	0.18	20 (16.5)	6 (17.1)	1	19 (21.8)	9 (29.0)	0.46
Tumarkin Crisis, n (%)	16 (15.7)	12 (32.4)	0.053	25 (20.5)	13 (38.2)	**0.04**	22 (25.6)	11 (34.4)	0.36
Functional Scale, n (%)									
1	48 (47.1)	7 (17.9)		16 (13.6)	3 (8.6)		6 (6.9)	6 (18.2)	
2	22 (21.6)	11 (28.2)		34 (28.8)	8 (22.9)		24 (27.6)	11 (33.3)	
3	12 (11.8)	8 (20.5)	**0.02**	41 (34.7)	9 (25.7)	0.27	42 (48.3)	7 (21.2)	**0.002**
4	13 (12.7)	6 (15.4)		17 (14.4)	8 (22.9)		10 (11.5)	2 (6.1)	
5	6 (5.9)	7 (17.9)		7 (5.9)	4 (11.4)		5 (5.7)	3 (9.1)	
6	1 (1.0)	0 (0.0)		3 (2.5)	3 (8.6)		0 (0.0)	4 (12.1)	

P values in bold are statistically significant

4.2. AIM 2: To identify allelic variants associated with autoimmune MD that could be used as genetic markers using nd, to investigate their role in MD inflammatory response.

In order to achieve our second goal, we first performed an immunochip in 924 MD patients and controls. We carried out taqman assays in 1095 individuals to validate the Single nucleotide variants (SNV) that reached significant association in phase I. Additionally, we selected homozygous individuals for the significant SNV and performed gene expression studies in PBMCs obtained from these patients.

4.2.1. BMD is associated with a locus in the classical class I MHC subregion

Table 21 compares the clinical features of 1410 patients with uni- and bilateral SNHL in MD. Patients with bilateral SNHL had a longer duration of the disease (P =1.5 x 10^{-6}), worse HL at diagnosis (P= 2.5 x 10^{-4}), worse hearing stage (P = 2 x 10^{-6}), higher frequency of AD (P= 4 x 10^{-3}), and higher frequency of migraine (P= 6 x 10^{-3}).

Although no significant association was found in patients with UMD, two genomic regions at chromosome 2 and 6 reached confirmatory significance (P values <10^{-6}) in BMD patients (**Figure 19**). To perform the replication, we selected representative TagSNVs according to the results of the discovery phase in both regions (**Table 22**). The meta-analysis confirmed a suggestive significant association with a locus in the classical class I subregion of the MHC ~ 9 kb at 6p21.33 (31081878-31090401), being the leading SNV rs4947296; OR= 1.91 (1.50 - 2.44), P=4.3x10^{-7}. This association was confirmed by genotyping rs9380217, a close proxy SNV in the same region in complete linkage disequilibrium (LD=1), in the same set of samples (OR= 1.92 (1.50-2.50), P= 6.4 x 10^{-7}). Conditional regression analysis showed no independent associated signals in chromosome 6, and the association between this locus and BMD remained robust when it was adjusted to any variant in the region.

The risk haplotype was found in 13% of patients with BMD and 17% of BMD with an associated AD (OR= 2.59 (1.51-4.43), P=1.5 x 10^{-3}), suggesting a significant enrichment in patients with a comorbid AD.

Subsequently, according to this association (rs9380217/ rs4947296), we defined the homozygous risk haplotype as TT/CC and the protective haplotype as CC/TT for further studies.

Table 21. Clinical features of sporadic MD patients.

VARIABLES	BILATERAL (n = 389)	UNILATERAL (n = 1031)	P value
Age of onset, mean (SD)	45.23 (13.6)	47.24 (12.1)	0.263
Gender (% women)	59.3	57.5	0.598
Time course (years), mean (SD)	15.16 (9.1)	10.35 (± 7.7)	**$1.50x10^{-06}$**
Affected ear (%)		Right (50.9)	
Hearing loss at diagnosis, mean (SD)	56.96 (16.7)	48.66 (19.1)	**$2.47x10^{-04}$**
Migraine, n (%)	70 (18.4)	131 (12.8)	**0.006**
History of autoimmune disease, n (%)	63 (19.2)	119(12.8)	**0.004**
Smoking, n (%)	81(21.3)	254(24.3)	0.422
Hearing stage, n (%)			
1	7 (2.0)	117 (12.2)	**$5.00x10^{-06}$**
2	52 (14.9)	232 (24.2)	
3	178 (51.0)	471 (49.1)	
4	112 (32.1)	140 (14.6)	
Hearing stage, mean (SD)	3.13 (0.7)	2.66 (0.8)	**$2.00x10^{-06}$**
Turmakin crisis, n (%)	85 (29.5)	126 (16.9)	**$8.00x10^{-06}$**
Functional scale, n (%)			
1	37 (12.6)	167 (12.6)	**0.008**
2	91 (31.0)	332 (37.1)	
3	74 (25.2)	185 (20.7)	
4	46 (15.6)	126 (14.1)	
5	37 (12.6)	71 (7.9)	
6	8 (2.7)	13 (2.7)	

P values in bold are statistically significant

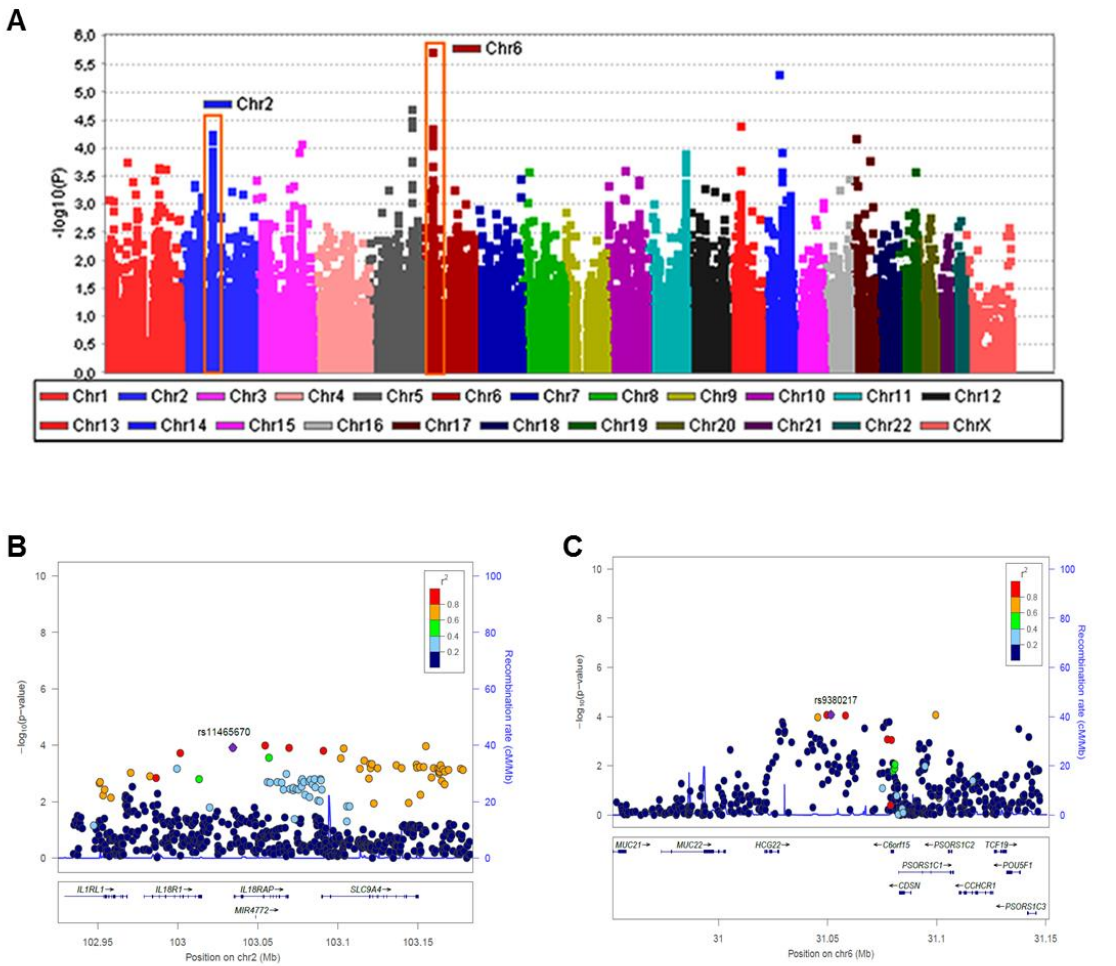

Figure 19. Two loci associated with bilateral MD. (**A**) Manhattan plot for the association study findings from Immunochip genotyped BMD. (**B**) Association area at the region on chromosome 2. (**C**) Association area at the region on chromosome 6. Both (**B**) and (**C**) -logP values of SNVs associated with bilateral SNHL are shown on the left Y axis and the recombination rates expressed in centimorgans (cM) per Mb, are shown on the right Y axis. Positions in Mb are on the X axis (NCBI Build GRCh38). Linkage disequilibrium for each SNV with the top SNV, displayed as a large purple diamond, is indicated by its color. The plots were drawn using LocusZoom tool (http://locuszoom.sph.umich.edu/locuszoom/).

Table 22. Meta-analysis of BMD associated loci**.**

SNV					Phase 1 (n=924)				Phase 2 (n=1095)				Meta-analysis (n=2019)			
Chr.	**Pos.**	**Rs**	**Ref.**	**Alt.**	**RAF_C**	**RAF_N**	**OR (95%)**	**P value**	**RAF_C**	**RAF_N**	**OR (95%)**	**P value**	**RAF_C**	**RAF_N**	**OR (95%)**	**P value**
2	102351615	rs4988957	C	T	0.414	0.358	1.27 (0.99-1.62)	5.24E-02	0.381	0.355	1.07 (0.93-1.23)	1.88E-01	0.406	0.351	1.16 (1.05-1.83)	2.91E-03
2	102417980	rs11465670	T	C	0.156	0.093	1.81 (1.29-2.54)	5.02E-04	0.087	0.083	1.04 (0.73-1.49)	4.41E-01	0.121	0.087	1.38 (1.10-1.73)	4.52E-03
2	102460685	rs4851589	A	G	0.337	0.279	1.32 (1.02-1.69)	3.35E-02	0.247	0.258	0.96 (0.79-1.16)	3.61E-01	0.299	0.267	1.12 (0.99-1.27)	4.53E-02
6	30814225	rs886424	C	T	0.102	0.051	2.11 (1.39-3.21)	3.55E-04	0.082	0.071	1.14 (0.81-1.62)	2.55E-01	0.095	0.067	1.41 (1.09-1.83)	7.73E-03
6	**31083776**	**rs9380217**	**C**	**T**	**0.159**	**0.069**	**2.55 (1.81-3.58)**	**3.40E-07**	**0.108**	**0.078**	**1.43 (1.00-2.06)**	**5.52E-02**	**0.132**	**0.074**	**1.92 (1.50-2.50)**	**6.40E-07**
6	**31090401**	**rs4947296**	**T**	**C**	**0.164**	**0.067**	**2.72 (1.93-3.82)**	**3.15E-08**	**0.108**	**0.078**	**1.42 (0.99-2.04)**	**5.80E-02**	**0.132**	**0.073**	**1.91 (1.50-2.44)**	**4.30E-07**
6	32082981	rs1150754	C	T	0.117	0.061	2.04 (1.38-3.01)	2.66E-04	0.089	0.067	1.16 (0.81-1.65)	2.35E-01	0.096	0.071	1.36 (1.05-1.77)	1.34E-02

Column labels are as follow: chromosome (Chr.), SNV genomic position (Pos.), SNV identifier (Rs), reference (Ref.) or Alternative (Alt.) allele on Human Genome Reference GRCh38. Risk allelic frequency in cases (RAF_C) and controls (RAF_N), odds ratio (OR)
Bold values represent statistical significance

4.2.2. Rs4947296 regulates PBMC gene expression in the TWEAK/ Fn14 pathway.

We compared gene expression profile of PBMCs from six patients according to the homozygous haplotypes defined by rs9380217/rs4947296. We demonstrated that this region is an expression quantitative trait locus (eQTL) in PBMC, showing significant differences in the expression levels of 973 genes (adjusted $P<0.001$, **Figure 20A**; **Annex 2**). Selecting those differential expressed genes (DEG) according to the haplotype, pathway analysis performed by IPA® software, predicted several candidate pathways (**Table 23**). The TWEAK/Fn14 pathway, which was the most significant, showed 16 DEG (48.5%; $P = 3.65 \times 10^{-10}$). Moreover, the eQTL was also associated with the TNFR2 signaling pathway with 13 dysregulated genes (46.4%; $P= 3.25 \times 10^{-8}$).

The enrichment analysis of canonical pathways in MetaCore (adjusted $P<0.001$) also resulted in several TNF-related pathways for apoptosis and inflammation that contained TWEAK/Fn14 subpathway (**Table 24**). To gain insight into the possible molecular interactions that mediate TWEAK signaling to NFκB, we extracted three enriched canonical pathways "Apoptosis and survival Apoptotic TNF-family pathways", "Signal transduction NF-κB activation pathways" and "Apoptosis and survival Anti-apoptotic TNFs-NF-κB-Bcl-2 pathway" from MetaCore. The shortest paths from Fn14 (*TNFRSF12A*) to *NFKB1* genes were extracted (**Figure 20B**) and visualized in Cytoscape v.2.7.0[156]. These shortest paths involved several DEG including *BIRC3* and *NFKBIE*. Although *FADD* was not amongst these shortest paths, it is along the *TNFRSF10A*-induced path that feeds into the TWEAK/Fn14 path, suggesting its complementary role in the TWEAK/Fn14 pathway.

We also validated gene expression profiles of *NFKB1*, *TNFRSF12A*, *BIRC3*, *FADD*, *NFKBIE* and *FOS* genes in patients PBMCs by quantitative RT-PCR (qPCR), according to the selected haplotype (**Figure 20C**).

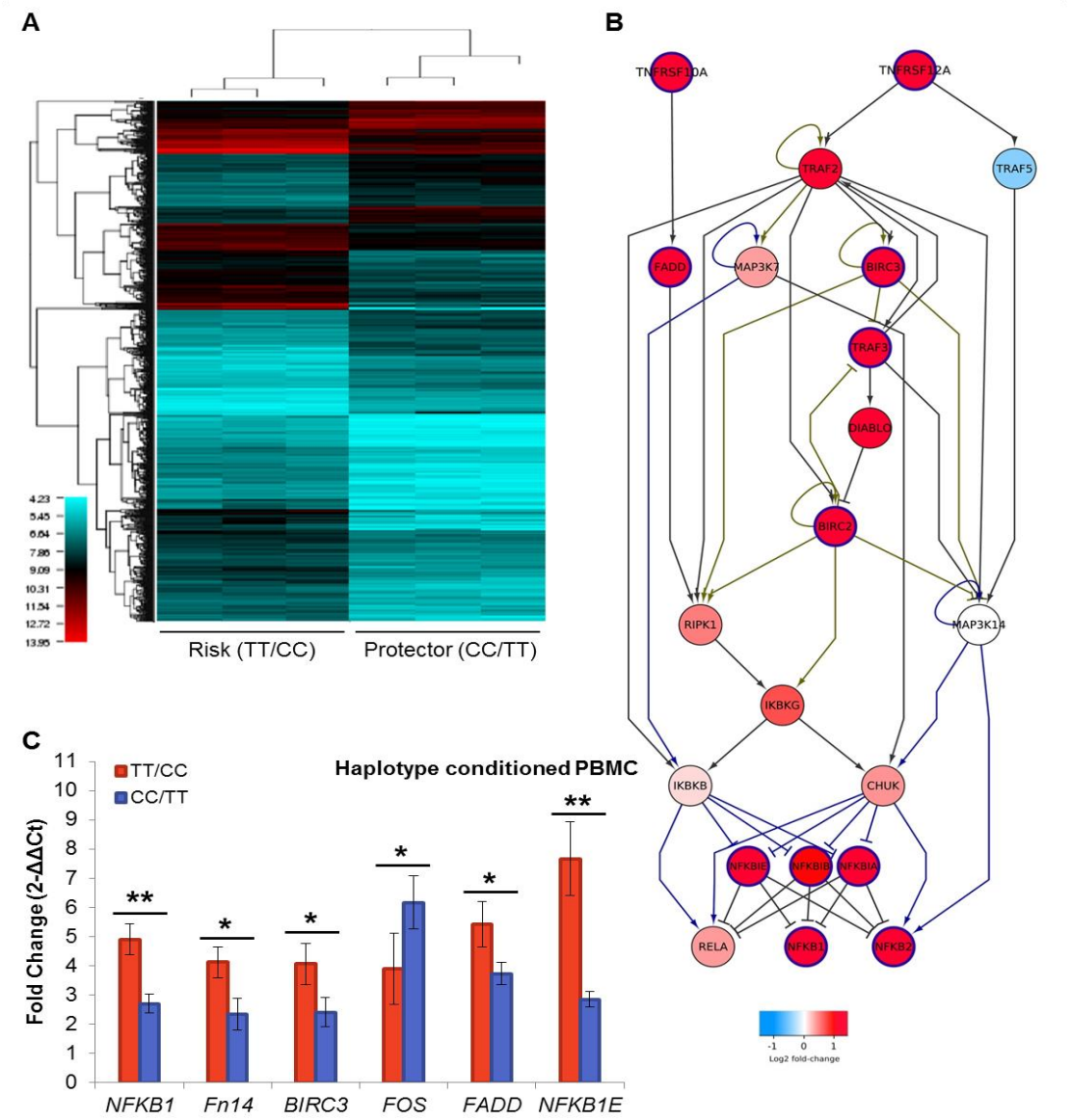

Figure 20. Gene expression in conditioned PBMC. (**A**) Heatmap of 973 DEG in PBMCs. The samples (column) were clustered into two groups according to its haplotype (rs9380217/ rs4947296): three cases with TT/CC haplotype (risk haplotype) and two cases/one control with CC/TT haplotype (protective haplotype). (**B**) The shortest path from Fn14 (*TNFRSF12A*) to *NFKB* genes. The network was retrieved from three MetaCore pathways Log fold-change is color-coded, red nodes indicate UP-regulated genes and blue nodes indicate DOWN-regulated genes. Activation interactions are indicated by arrow heads, while inhibitory interactions are indicated by blunted heads. (**C**) qPCR validation of DEG in the TWEAK/Fn14 pathway (*NFKB1, Fn14, BIRC3, FADD, NFKBIE and FOS*). **P*<0.03, ***P*<0.0005.

Table 23. Significant canonical Pathways (p<0.0001) retrieved from IPA® based on DEG when comparing the Risk group with the Protective group.

Pathway name	Total Molecules	P value	Molecules In our Data	UP Regulated	DOWN Regulated
TWEAK Signaling	33	$3.65x10^{-10}$	16	14	2
TNFR2 Signaling	28	$3.25x10^{-08}$	13	11	2
Death Receptor Signaling	69	$8.22x10^{-07}$	22	19	3
Protein Ubiquitination Pathway	254	$3.20x10^{-06}$	41	31	10
NRF2-mediated Oxidative Stress Response	177	$3.38x10^{-06}$	32	19	13
CD27 Signaling in Lymphocytes	51	$3.38x10^{-06}$	15	11	4
Induction of Apoptosis by HIV1	59	$5.20x10^{-06}$	16	15	1
TNFR1 Signaling	47	$6.06x10^{-06}$	14	12	2
Lymphotoxin β Receptor Signaling	54	$7.39x10^{-06}$	15	15	0
Aldosterone Signaling in Epithelial Cells	151	8.2510^{-06}	28	20	8
Aryl Hydrocarbon Receptor Signaling	135	$8.47x10^{-06}$	26	14	12
Type I Diabetes Mellitus Signaling	106	$1.20x10^{-05}$	22	17	5
Tec Kinase Signaling	155	$1.38x10^{-05}$	28	15	13
Role of NFAT in Regulation of the Immune Response	166	$1.88x10^{-05}$	29	13	16
Role of PKR in Interferon Induction and Antiviral Response	40	$2.58x10^{-05}$	12	12	0
Unfolded protein response	53	$2.77x10^{-05}$	14	12	2
Role of RIG1-like Receptors in Antiviral Innate Immunity	41	$3.39x10^{-05}$	12	10	2
PKCθ Signaling in T Lymphocytes	113	$1.04x10^{-04}$	21	10	11
PI3K Signaling in B Lymphocytes	123	$1.29x10^{-04}$	22	13	9
B Cell Activating Factor Signaling	40	$1.34x10^{-04}$	11	9	2

Table 24. Significantly enriched canonical pathways from MetaCore based on DEG between the Risk and Protective groups

Pathway name	Total	P value	FDR	Molecules In our Data
Apoptosis and survival_Apoptotic TNF-family pathways	42	4.984×10^{-09}	2.977×10^{-06}	15
Development_Notch Signaling Pathway	43	7.252×10^{-09}	2.977×10^{-06}	15
Signal transduction_NF-kB activation pathways	51	1.316×10^{-08}	3.602×10^{-06}	16
Development_NOTCH1-mediated pathway for NF-KB activity modulation	34	2.057×10^{-08}	4.223×10^{-06}	13
Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	42	4.517×10^{-08}	7.416×10^{-06}	14
CFTR folding and maturation (normal and CF)	24	3.538×10^{-07}	4.841×10^{-05}	10
Apoptosis and survival_Role of IAP-proteins in apoptosis	31	6.267×10^{-07}	7.351×10^{-05}	11

4.2.3. TWEAK induces cluster formation and proliferation in haplotype-selected Lymphoblastoid Cell Lines (LCL)

LCLs proliferate forming clusters with rosette morphology as a result of the expression of adhesion molecules such as LFA-1(leukocyte function antigen 1 encoded by *ITGB2* gene) also known as CD11a/CD18 and its ligand, ICAM-1 (intercellular adhesion molecule 1, CD54) in their plasma membrane. Remarkably, the size of the clusters presented significant differences according to the haplotype, being smaller the risk haplotype (TT/CC: 30968.88 ± 1960.45 μm^2; CC/TT: 103921.33 ± 12720.92 nm, $P= 5x10^{-7}$) (**Figure 21**).

When we treated the cells with TWEAK at 250 ng/mL concentration, we observed a significant increase in the size of the clusters in the TT/CC LCLs, which was not observed in the protective haplotype (TT/CC: 125609.84 ± 17502.21 μm^2, $P=2x10^{-6}$; CC/TT: 136132.42 ±14785.38 μm^2, P=0.02), This experiment demonstrated that TWEAK induces a significant aggregation of LCLs in the carriers of the risk haplotype.

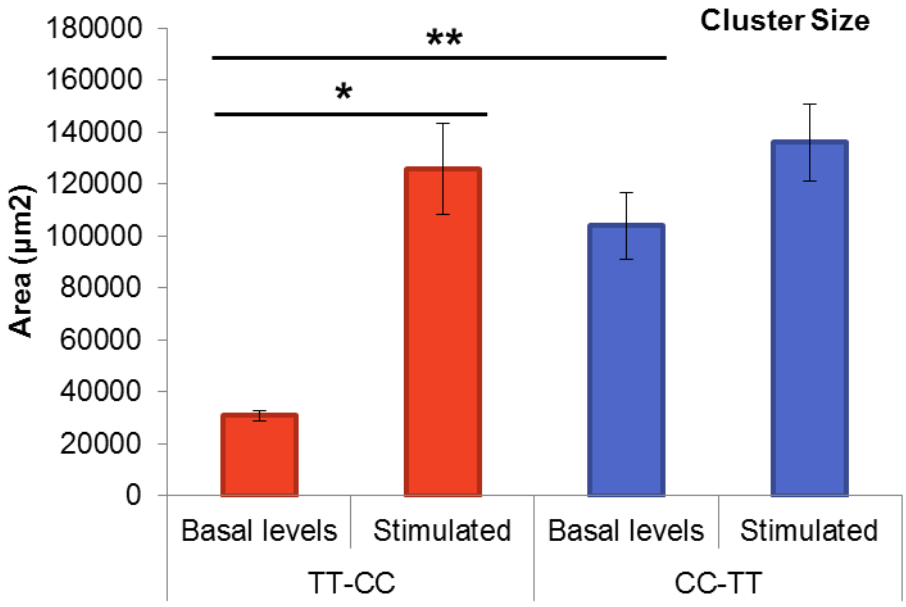

Figure 21. Cluster size within LCLs with and without stimulation

***P*= $5x10^{-7}$, **P*=0.02

Then, we compared the effect of TWEAK in the proliferation of the selected LCLs. Thus, 250 ng/mL TWEAK increased proliferation rate after 48h in both cell lines (TT/CC *P*=0.017, CC/TT, *P*=0.013; **Figure 22**). This effect suggested the activation of the non-canonical NF-κB signaling pathway via Fn14 receptor that we confirmed showing an increased expression of *TNFRSF12A* and *NFKB1* genes (**Figure 23**).

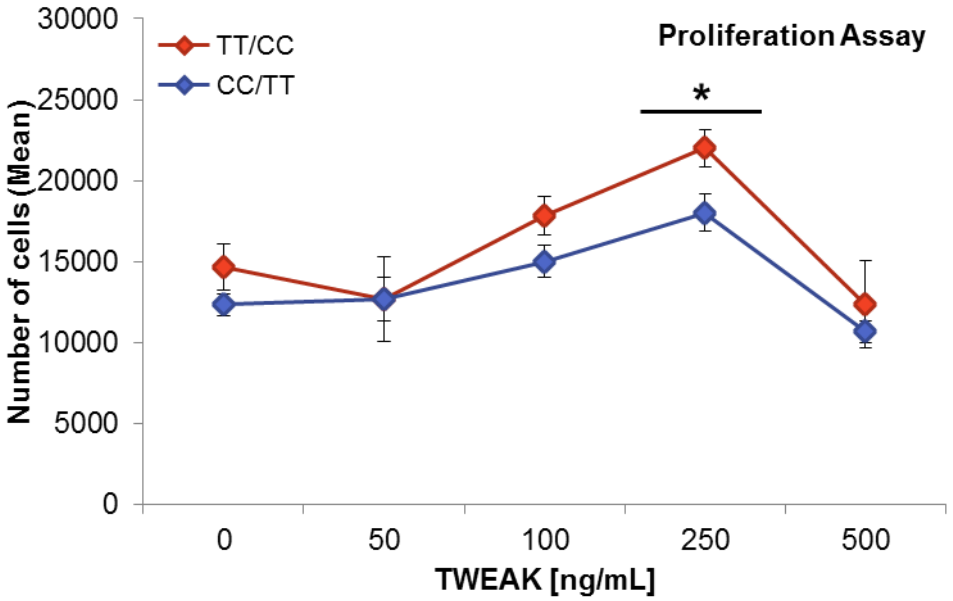

Figure 22. Proliferation assay of homozygous lymphoblastoid cell lines (LCL) treated with TWEAK (0, 50, 100, 250 and 500 ng/mL) and measured using PrestoBlue™. **P*<0.05. Comparisons between groups were achieved using a two-sided student's t-test.

To further investigate whether the difference in the cluster formation was related with the differential expression of cell adhesion molecule genes, we measured mRNA levels of three cell surface markers in LCLs: the integrin LFA-1, as well as its ligand ICAM-1 and, tight junction protein ZO-1 (*TJP1 gene*), which interacts directly with actin. We found a significant increase in the expression of *ITGB2* ($p=5x10^{-6}$; **Figure 23**) and in *TJP1* ($P=3.2x10^{-5}$) in the TT/CC haplotype LCLs, which was not observed in the protective haplotype. The differences in clusters size, *ITGB2* and *TJP1* expression, according to the haplotype, were consistent with the hypothesis that this eQTL could regulate lymphoblasts adhesion and proliferation.

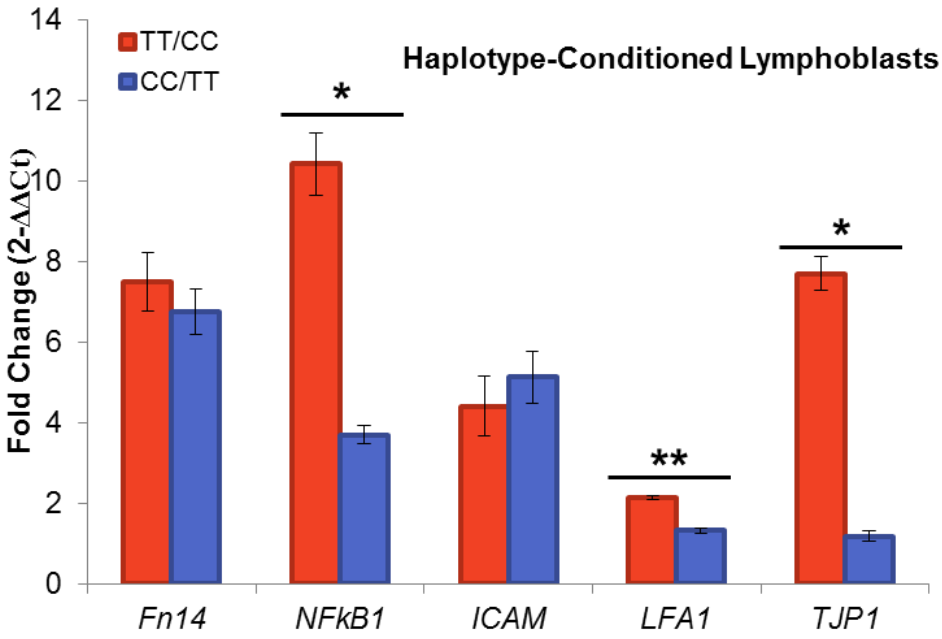

Figure 23. Relative gene expression according to the haplotype, after treatment with 250 ng/mL of TWEAK, P value: Fn14 = 0.44; NFKB1 = 1.36x10-4; ICAM = 0.91; LFA-1 = 5x10-6; TJP1 = 3.2x10-5. *P<1x10-4, **P<5x10-6

4.2.4. The risk haplotype up-regulates the translation of NFkB in LCL

Non-stimulated TT/CC cells (risk haplotype) showed a higher expression of *TNFRSF12A* and *NFKB1* (3.6 ± 0.7 and 2.7 ± 0.7 fold higher than CC/TT, respectively), confirming the previous results obtained in selected PBMCs. When we stimulated both cell lines with TWEAK 250ng/mL, we found no significant differences for *TNFRSF12A*; however, the expression of *NFKB1* was significantly increased (TT/CC: 10.4 ± 0.8; CC/TT 3.7 ± 0.2, $P=1.4x10^{-4}$).

Then, we performed immunocytochemistry to quantify *TNFRSF12A* and *NFKB1* expression at protein level in LCLs by confocal microscopy (**Figure 24**). At basal levels, we found significant differences in the translation of Fn14 between both cell lines (TT/CC: 78.5±9.6; CC/TT 48.3±3.7, $P=10^{-3}$), but no differences were found for NFκB (TT/CC: 56.2±4.2; CC/TT 45.2±3.3, P=0.06). However, TWEAK up-regulated the translation of NFκB significantly in the risk LCLs (TT/CC: P=0.01; CC/TT: P=0.04), but it had no effect on Fn14 in either LCLs (TT/CC: P=0.77; CC/TT: P=0.29).

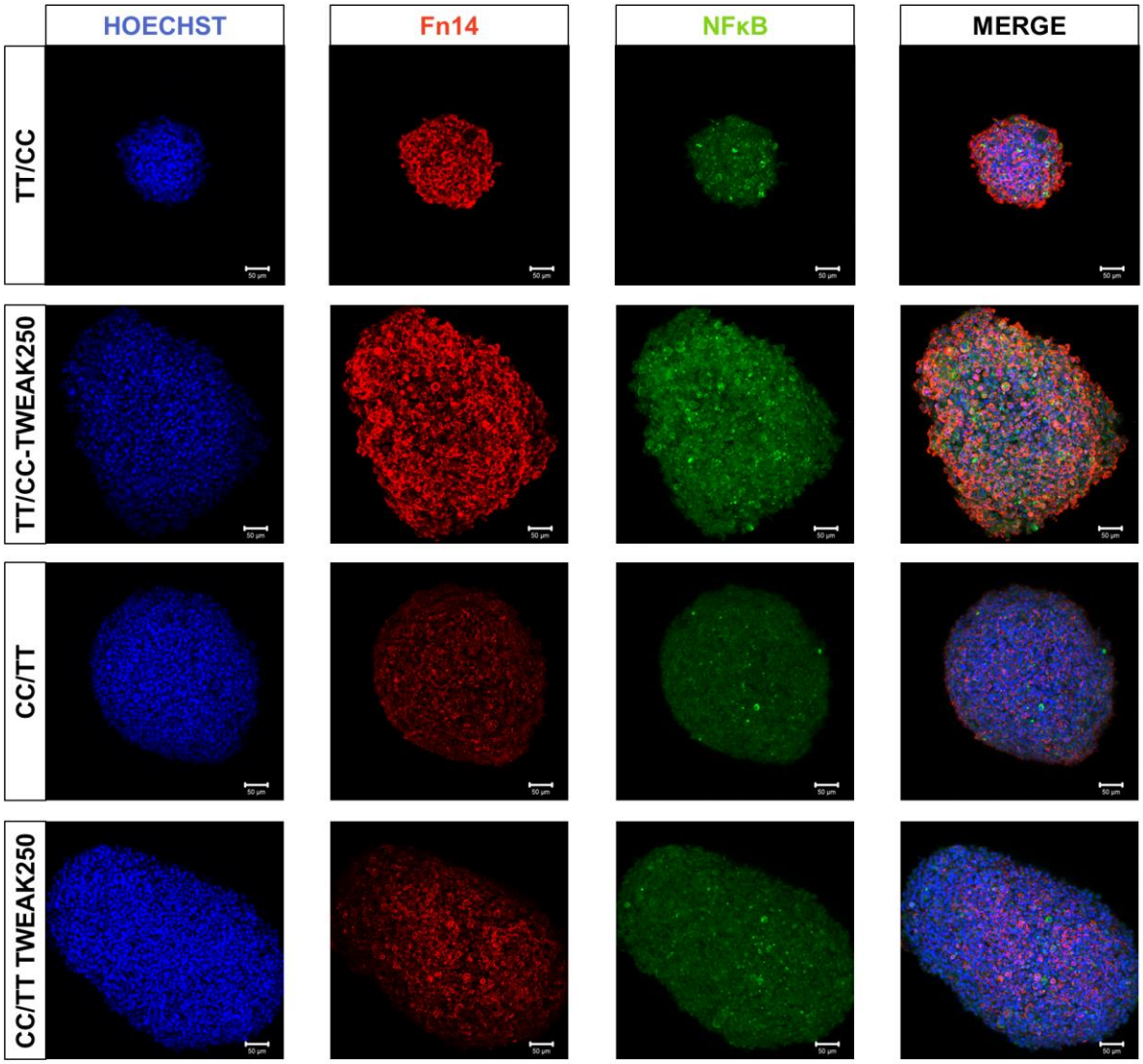

Figure 24. Fn14 and NFκB expression in homozygous LCLs.

Confocal microscopy images showing representative clusters of LCLs with Fn14 and NFκB immunolabelling un-stimulated and after treatment with 250ng/mL of TWEAK. Scale bar = 50μm

4.3. AIM 3: To define the effect of allergenic extracts from *Penicillium* and *Aspergillus* in proinflammatory cytokines and gene expression profiles in PBMCs from MD patients

In order to achieve our third goal, PBMCs from MD patients were stimulated with both, *Penicillium* and *Aspergillus* allergenic extracts. After 16h overnight, supernatant was collected, cells harvested and RNA and proteins were isolated.

4.3.1. Clinical features

Table 25 compares the clinical features of all 113 MD patients with UMD and BMD. Patients with bilateral SNHL had a longer duration of the disease ($P=8.0 \times 10^{-4}$), worse hearing stage ($P=3.0 \times 10^{-2}$), higher cardiovascular risk factors, as they have higher frequency of high blood pressure ($P= 4 \times 10^{-3}$) and type 2 diabetes ($P=2.4x10^{-2}$), and a higher frequency of Tumarkin Crisis ($P= 6 \times 10^{-3}$).

4.3.2. MD patients have higher basal levels of cytokines when compared to healthy control

Basal levels of proinflammatory cytokines in the supernatant of PBMCs patients and healthy controls were tested by Bead-based multiplex immunology assays. When compared to controls, MD patients had 2.8 fold higher expression of IL-1β ($P=3.8x10^{-4}$); 3.8 fold higher IL-1RA ($P=2.74x10^{-4}$); 7.1 fold higher IL-6 ($P=7.83x10^{-4}$) and 2.6 fold more TNF-α ($P=3.5x10^{-5}$) (**Figure 25A**).

4.3.3. MD patients have higher basal levels of IL-1β and TNF-α

We observed two different subgroups of patients, ones with high basal levels of cytokines and the others with low basal levels of cytokines. When compared to controls, 23% of MD patients had significant high levels of IL-1β (**Figure 25B**) (IL-1β > 5pg/mL; P= 2.73x10-7; 10.6 fold higher), 36% of patients had high levels of TNF-α (**Figure 25C**) (TNF-α>10pg/mL; P=8.86x10-8; 5.2 fold higher) and 20% had high levels of TNF-α but low levels of IL-1β (**Figure 25D**) (TNF-α > 10pg/mL; P=1.24x10-4; 2.4 fold higher). Of note, IL-1RA was increased in most patients with MD, with the only exception of those with low levels of IL-1β and TNF-α. When we compared patients

with high levels of cytokines with patients with low levels of cytokines, we found a ratio of 15.6 (P=1.62x10-7) and 6.6 (P=7.15x10-7) for IL-1β and TNF-α, respectively.

Table 25. Clinical features of MD patients

VARIABLES	UNILATERAL (n=73)	BILATERAL (n=40)	P-value
Age, Mean (SD)	59 (12.3)	63 (12.8)	0.08
Gender, n (%women)	46 (63.0)	21 (52.5)	0.32
Age of Onset (SD)	48.2 (13.8)	48.4 (15.7)	0.96
Time Course (years), mean (SD)	8.3 (7.8)	14.1 (9.8)	**0.0008**
High Basal Levels of IL-1β	13 (19.4)	12 (29.3)	0.25
High Basal Levels of TNF-α	11 (16.4)	10 (24.4)	0.33
High Basal Levels of IL-1β and TNF-α	24 (35.8)	22 (53.7)	0.07
Family history, n (%)	19 (26.8)	10 (27.0)	1
Familial Meniere Disease, n (%)	6 (9.0)	4 (11.1)	0.74
Hearing loss at diagnosis, mean (SD)	47.5 (17.0)	54.5 (12.7)	0.06
Headache, n (%)	28 (41.8)	14 (35.9)	0.68
Migraine, n (%)	14 (20.6)	7 (17.9)	0.81
Rheumatoid history, n (%)	10 (17.5)	6 (17.6)	1
Hearing stage, n (%)			
1	7 (9.7)	2 (5.3)	**0.03**
2	25 (34.7)	6 (15.8)	
3	32 (44.4)	19 (50.0)	
4	8 (11.1)	11 (28.9)	
Cardiovascular Risk factors			
High Blood Pressure, n (%)	10 (16.1)	16 (44.4)	**0.004**
Dyslipemia, n (%)	9 (19.6)	12 (40.0)	0.07
Type 2 Diabetes, n (%)	2 (3.5)	7 (20.0)	**0.02**
Smoking, n (%)	10 (15.6)	7 (18.4)	0.79
Tumarkin Crisis, n (%)	6 (9.2)	12 (32.4)	**0.006**
Functional Scale, n (%)			
1	11 (18.3)	4 (10.5)	0.24
2	23 (38.3)	10 (26.3)	
3	12 (20.0)	12 (31.6)	
4	10 (16.7)	6 (15.8)	
5	4 (6.7)	4 (10.5)	
6	0 (0.0)	2 (5.3)	

P values in bold are statistically significant

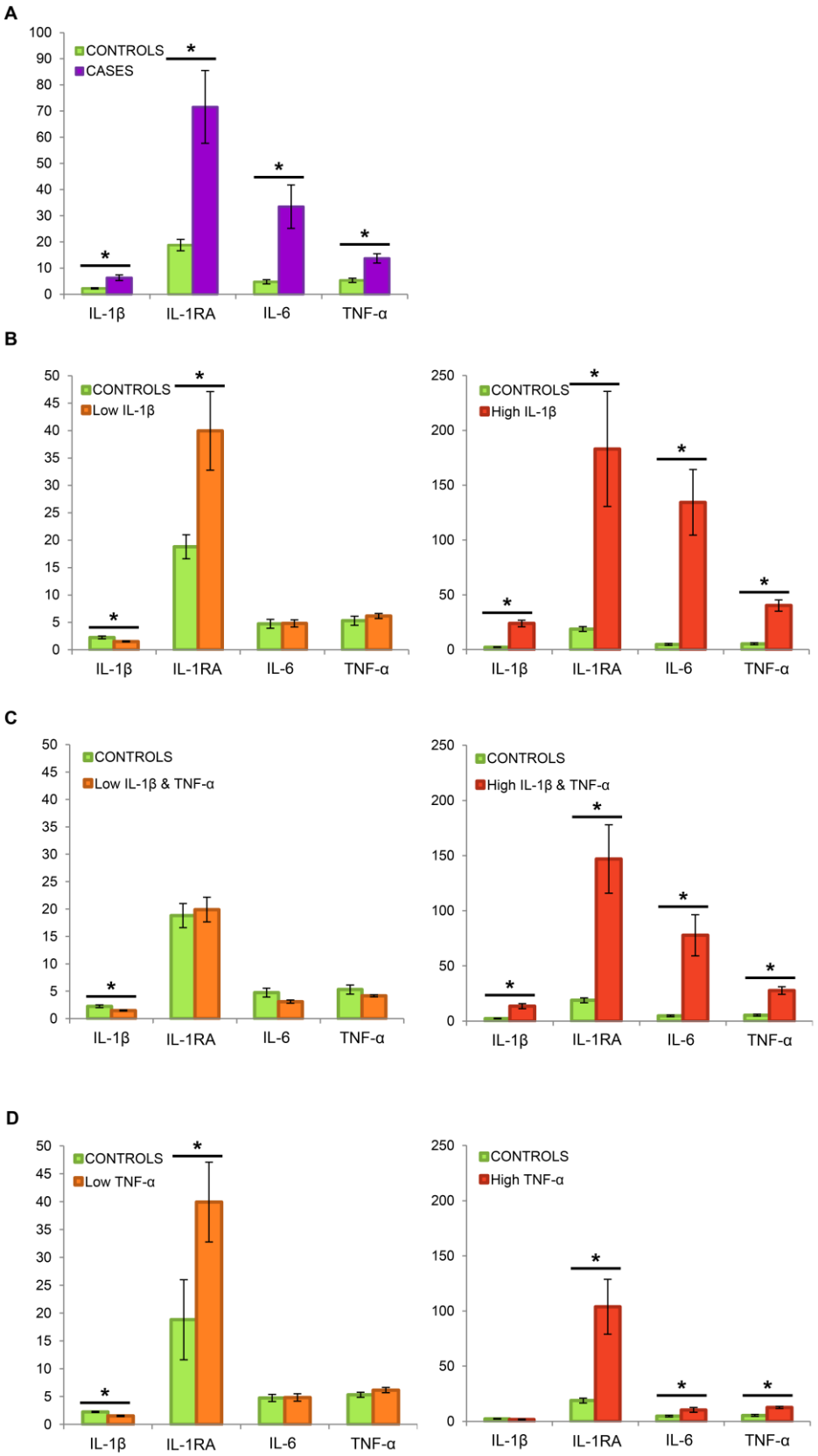

Figure 25. PBMC cytokines release at basal levels. (**A**) All cases and controls. (**B**) High/Low basal levels of IL-1β. (**C**) High/Low basal levels of IL-1β and TNF-α. (**D**) High/Low basal levels of TNF-α. *significant P-values.

4.3.4. *Aspergillus* and *Penicillium* induce IL-6 and TNF-α production in MD patients

MD patients and healthy controls PBMCs were stimulated with mold protein extracts (5µg/mL) for 16 hours. These stimulated PBMCs were compared to un-stimulated PBMCs. PBMC of MD patients showed 1.4 ($P=1.37x10^{-3}$) and 2.8 ($P=6.7x10^{-8}$) fold higher expression of IL-6 and TNF- α in response to *Aspergillus* mix, and 1.5 ($P= 6.3x10^{-5}$) and 2.6 ($9.0x10^{-7}$) fold higher expression of IL-6 and TNF- α in response to *Penicillium* mix*,* respectively (**Figure 26**).

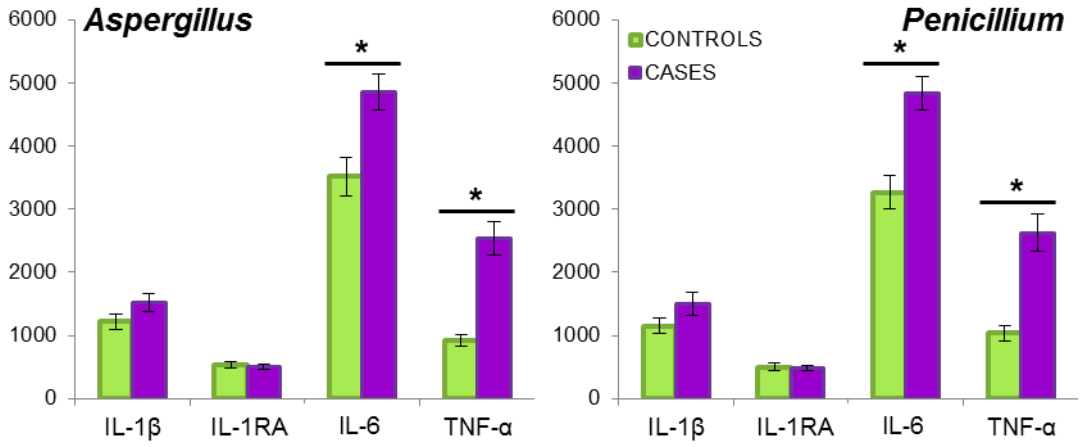

Figure 26. PBMC cytokines release when stimulated with *Aspergillus* or *Penicillium in* all cases and controls

We stratified patients according to their levels of cytokines to compare the response to molds. For those patients with high basal levels of IL-1β we observed 1.7 ($P=7.4x10^{-4}$) and 3.2 ($P=2.9x10^{-3}$) fold higher expression of IL-6 and TNF-α in response to *Aspergillus* mix, respectively, and 1.8 ($P= 1.1x10^{-4}$) fold higher expression of IL-6 in response to *Penicillium* mix (**Figure 27A**). Patients with low levels of IL-1β presented much higher levels of TNF-α (2.7, $P=5.0x10^{-6}$) in response to *Aspergillus,* and, 1.5 ($P=1.6x10^{-3}$) and 2.7 ($P=6x10^{-6}$) fold higher IL-6 and TNF-α respectively, in response to *Penicillium* (**Figure 27B**).

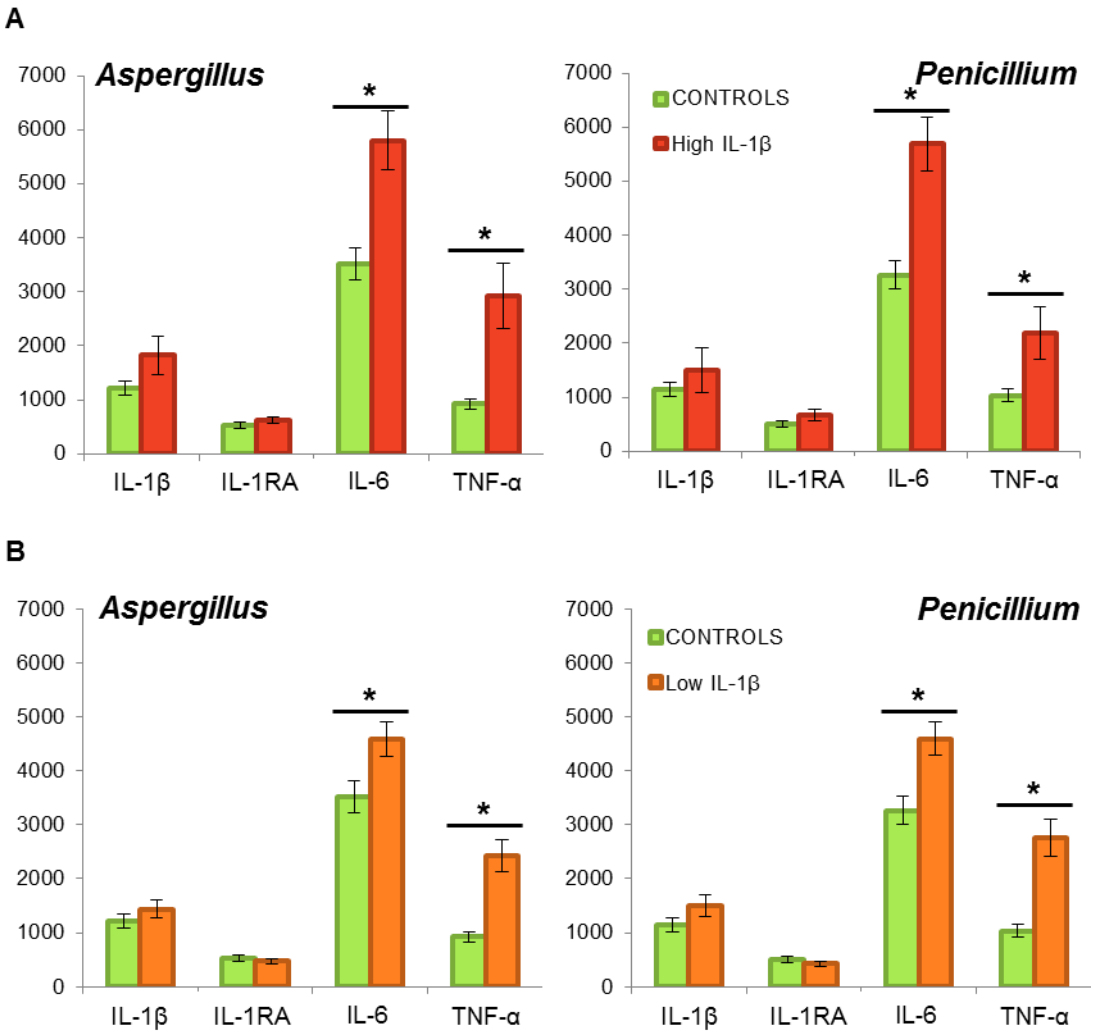

Figure 27. PBMC cytokines release when stimulated with *Aspergillus* or *Penicillium.*
(**A**) High basal levels of IL-1β stimulated PBMC.
(**B**) Low basal levels of IL-1β stimulated PBMC.

Patients who presented high basal levels of TNF-α had 1.7 ($P=2.6x10^{-5}$) and 2.9 ($P=1.42x10^{-4}$) fold higher expression of IL-6 and TNF-α in response to *Aspergillus*, respectively. In addition, they had 1.9 ($P= 1.0x10^{-6}$) and 2.3 ($P=1.36x10^{-3}$) fold higher expression of IL-6 and TNF-α, in response to *Penicillium,* respectively (**Figure 28A**). *However,* patients with low levels of TNF- α presented much higher TNF-α release in response to both *Aspergillus* (2.7, $P=8.0x10^{-5}$) and *Penicillium* (2.75, $P=8.8x10^{-5}$; **Figure 28B**).

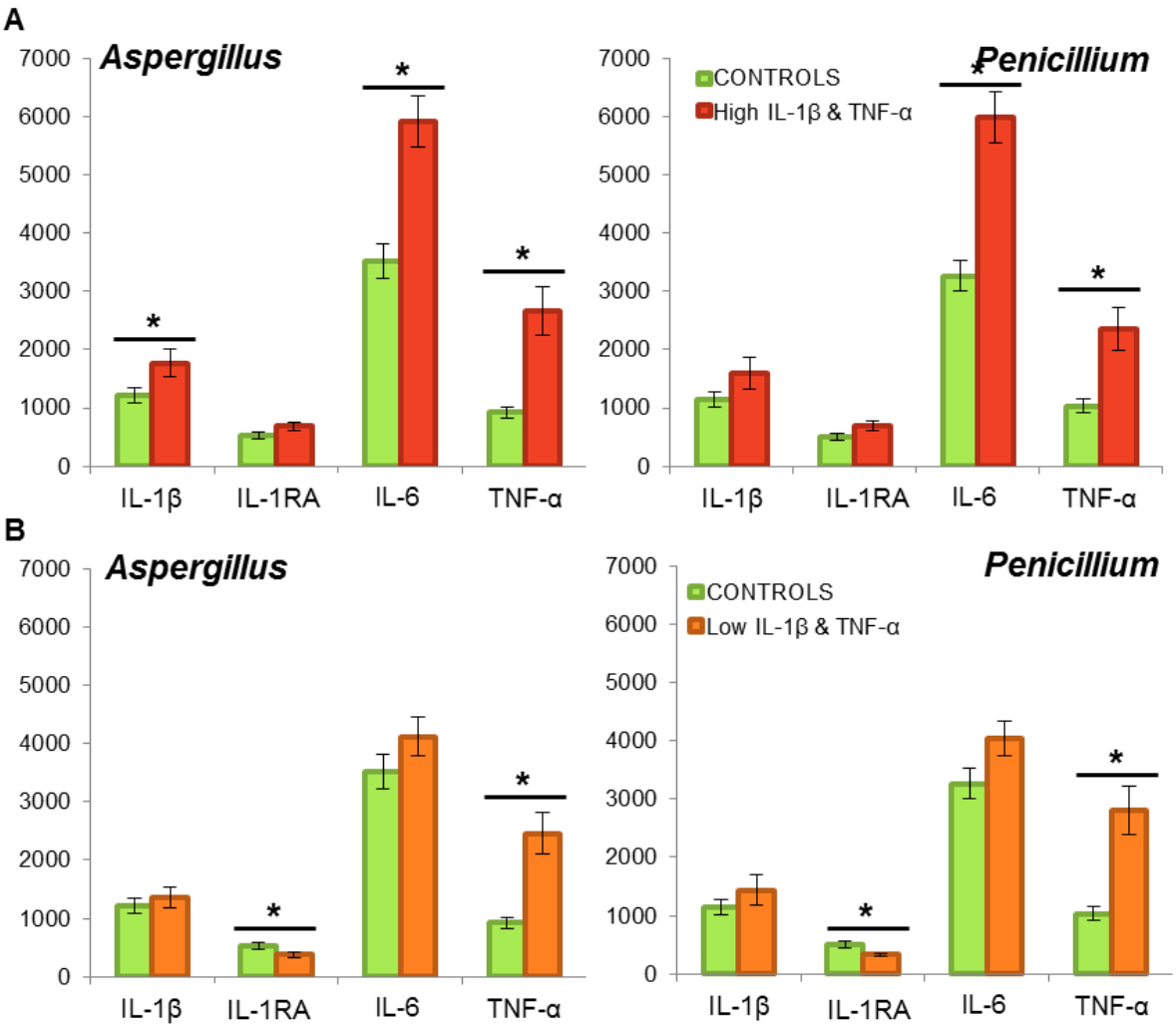

Figure 28. PBMC cytokines release when stimulated with *Aspergillus* or *Penicillium.*

(**A**) High basal levels of IL-1β and TNF-α stimulated PBMC.

(**B**) Low basal levels of IL-1β and TNF-α stimulated PBMC.

Finally, patients with high basal levels of TNF- α and low levels of IL-1β when exposed to *Aspergillus* or *Penicillium* mix only had much higher levels of IL-6 (1.8-2 fold change) (**Figure 29A**). However, patients with low levels of TNF-α, showed much higher levels of TNF-α when they were stimulated with either mold extract (3-fold change, $P=6x10^{-6}$) (**Figure 29B**).

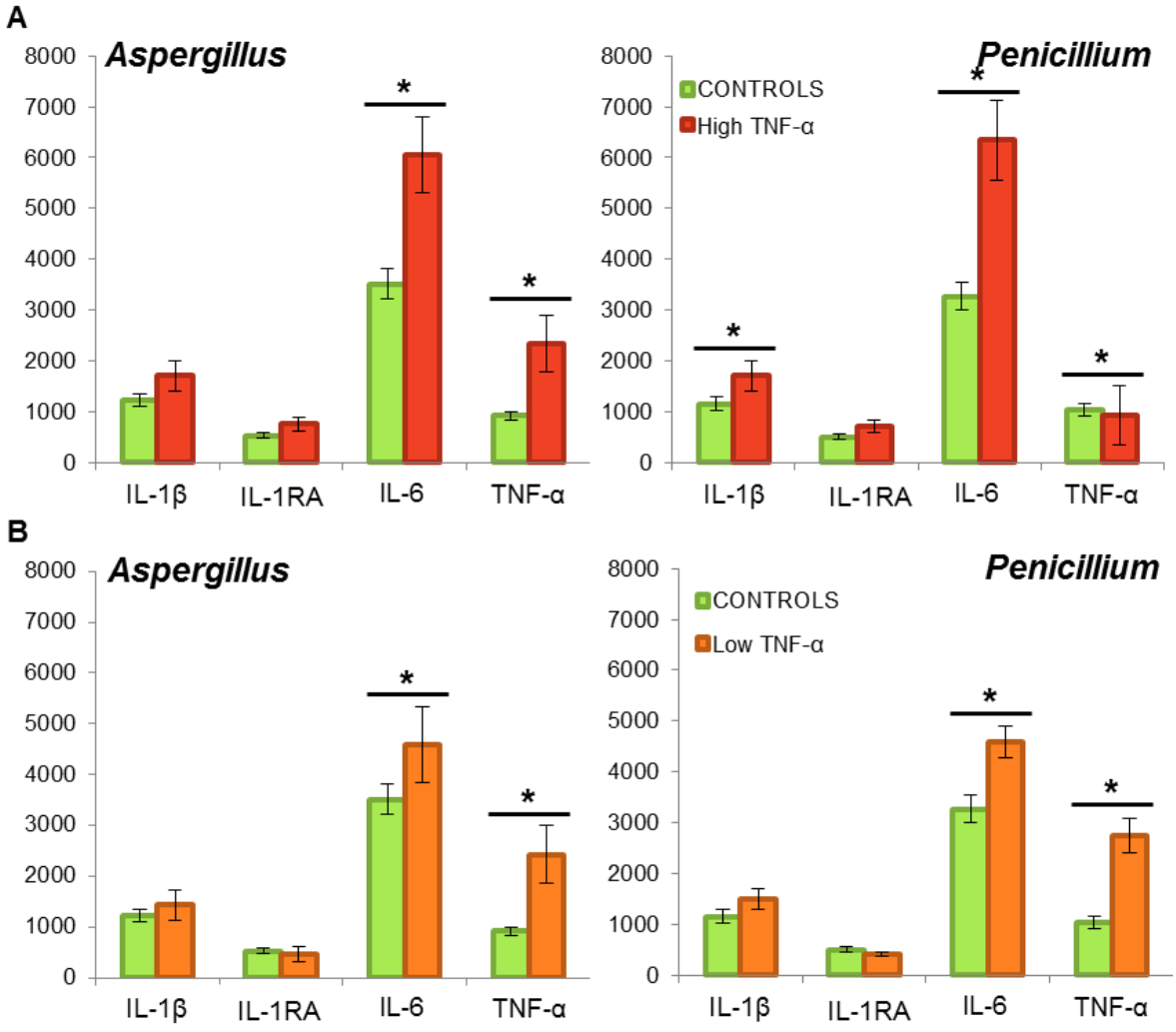

Figure 29. PBMC cytokines release when stimulated with *Aspergillus* or *Penicillium.*

(**A**) High basal levels of TNF-α stimulated PBMC.

(**B**) Low basal levels of TNF-α stimulated PBMC.

4.3.5. PBMCs gene expression at basal levels

We compared gene expression profiles in non-treated PBMCs from patients with high and low levels of cytokines. We performed a Core analysis from IPA® software using all genes from both expression profiles. The Upstream analysis within the Core analysis identifies upstream regulators that are predicted to be activated or inhibited based on an activation z-score. Our data revealed that 660 upstream regulators (**Annex 3**), interacting with around 5000 molecules. Based on regulators effects and

the pathways retrieved (**Annex 4**), we could predict the involvement of inflammatory response, apoptosis, activation of CD4+ T –lymphocytes and B lymphocytes,

When compared to healthy controls we found that most pathways activated in those patients with high levels of cytokines were inhibited in the patients with low levels of cytokines (**Figure 30**). So, patients with high basal levels of cytokines have increased gene expression levels than healthy controls, while patients with low levels of cytokines have also low gene expression profiles.

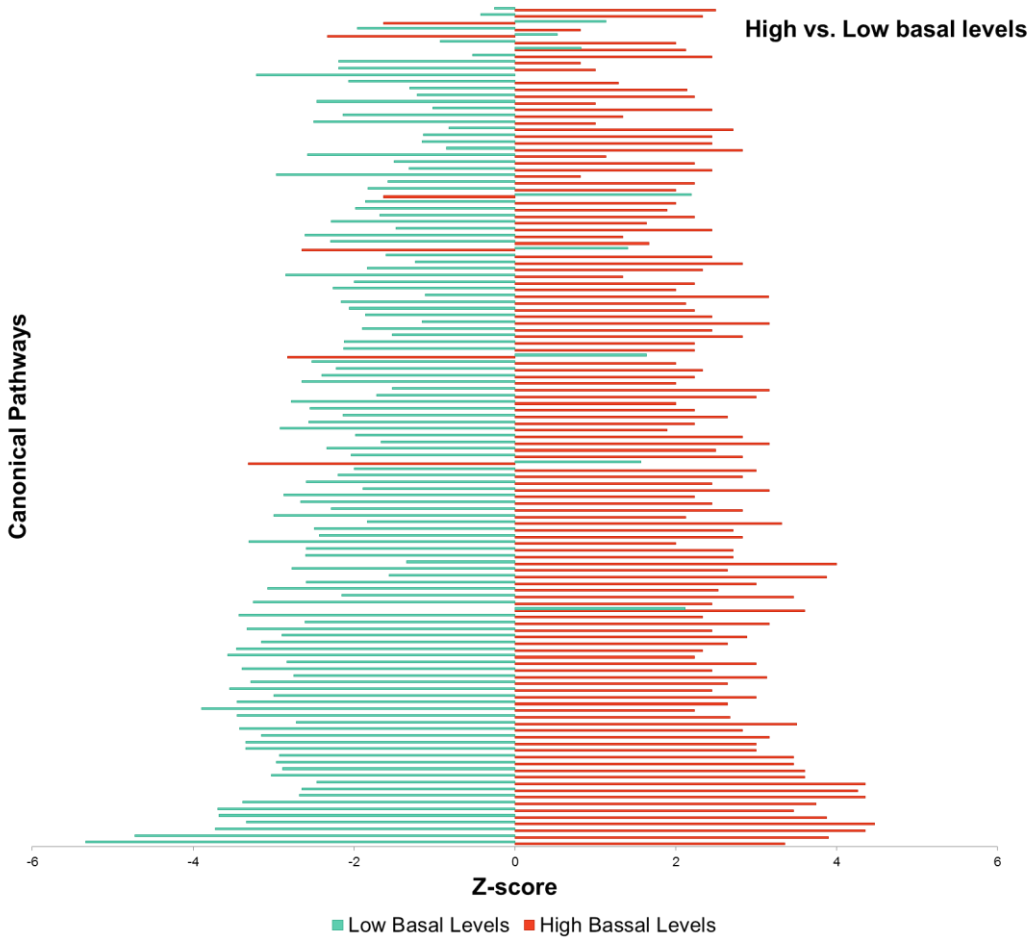

Figure 30. Comparison of canonical pathways retrieved by IPA® between high and low levels of cytokines.

4.3.6. Mold-induced gene expression

First, we carried out a correlation plot to determine if both, stimulation with *Penicillium* and *Aspergillus* were comparable or they led to a different gene expression profile. **Figure 31** shows high correlation between *Penicillium* and *Aspergillus* stimulations (over 60% in most cases), so we considered that both allergenic extracts induced similar gene expression in PBMCs and data were merged for further analyses**.** Then, we compared the gene expression profile of stimulated and un-treated PBMCs from 12 MD patients and 10 healthy controls. A total of 25921 probes showed significant differences in their expression levels (adjusted P<0.001,). We selected DEG between stimulated and non-stimulated PBMC, and extracted network interactions amongst all genes within significant gene sets, revealing a number of highly interconnected modules (**Figure 32**)**.** To add further annotation-based evidence for functional relationships between these genes, we performed pathway enrichment analysis by IPA® software, using the DEG with an enrichment P value cutoff <0.001. Several enriched predicted pathways were retrieved (**Annex 5**).

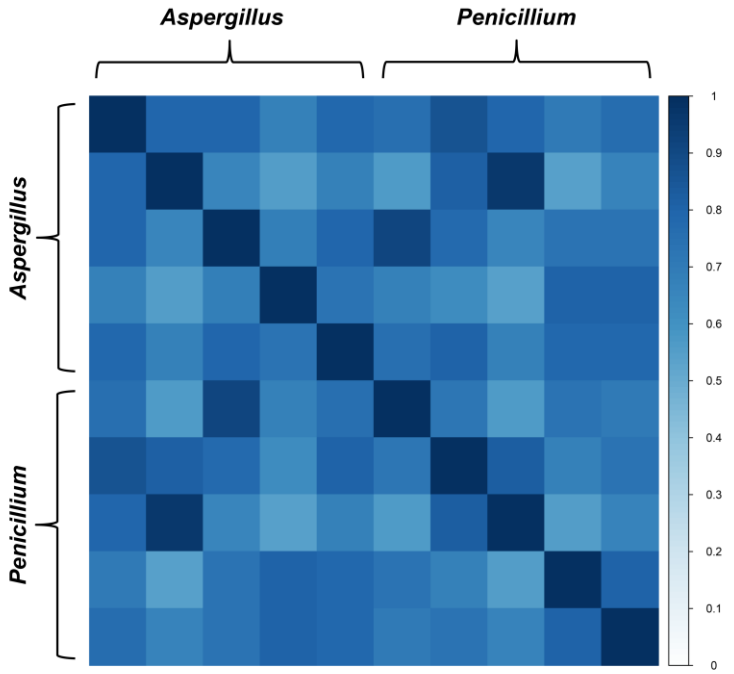

Figure 31. Correlation plot performed with stimulated samples. To figure out how stimulation affected gene expression in both, *Aspergillus* and *Penicillium* stimulated samples we performed interclass correlation coefficient after expression data analysis and normalization. Correlation between samples was greater than 60% in most cases.

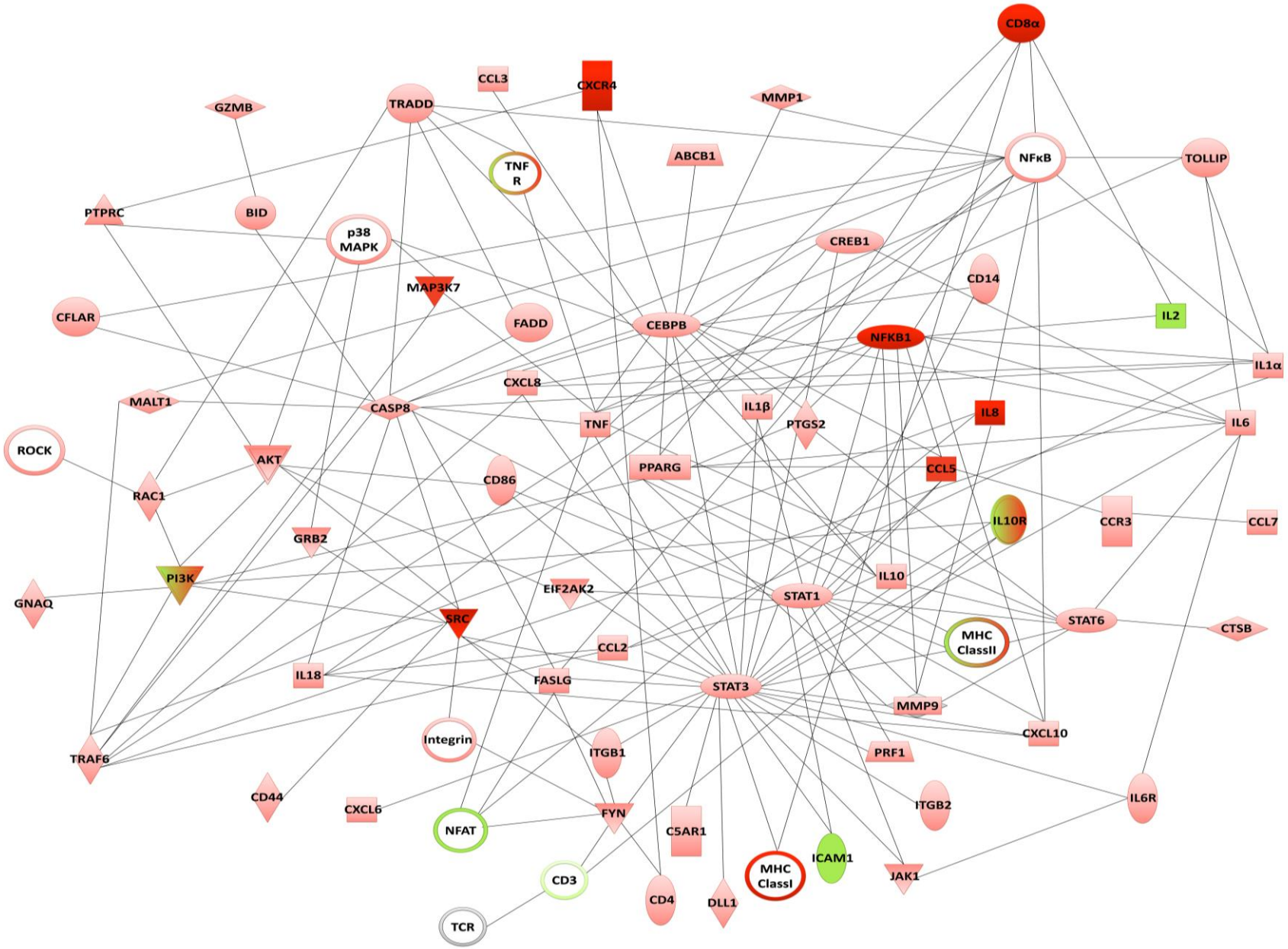

Figure 32. Network obtained after comparing the gene expression profile of stimulated and non-treated PBMCs. Genes in red were up-regulated, while genes in green were down-regulated

4.3.7. MD patients and healthy controls show a different response to mold stimulation

To further characterize the role of mold in inflammatory response, MD patients PBMCs were cultured and their responses to mold were directly compared with stimulated PBMC from healthy volunteers. Although there was no significant differences in the gene expression profile for most of genes in PBMC from controls and MD subjects, a small number of genes were differentially expressed when we compared both groups (**Table 26**). The IPA Network Generation Algorithm created a network containing 7 'focus genes' that were DEG between MD patients and healthy controls. An additional 27 intermediate molecules called 'non-focus genes' were added to the network. **Figure 33** shows the nearest connections between the DEG and non-focus genes suggesting molecular and cellular functions related with the immune response such as cellular movement, cellular assembly and organization, cell morphology or connective tissue development and function.

Table 26. Differentially expressed genes when we compared stimulated Cases and Controls

GeneID	UP/DOWN Regulated	Fold Change	P value
HTRA1	UP	5.364	1.60E-05
LAT2	UP	5.400	1.46E-05
MMP1	UP	5.455	1.27E-05
NRP1	UP	6.160	2.20E-06
SLMO1	UP	5.430	1.36E-05
WNT5A	UP	5.699	6.90E-06
ZNF543	UP	5.496	1.15E-05
PPP2R1B	UP	5.239	2.20E-05
SF1	DOWN	-5.225	2.28E-05

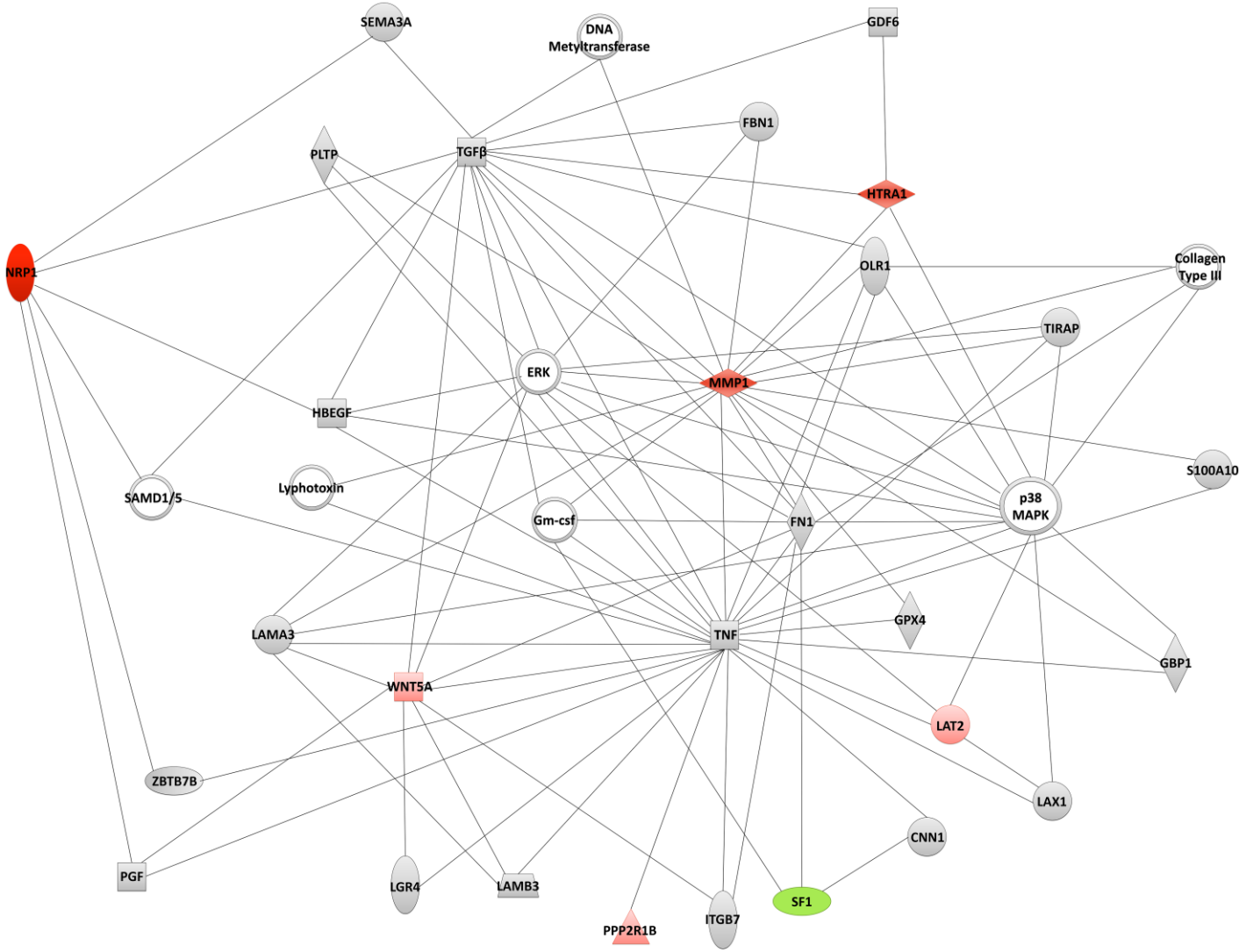

Figure 33.DEG in MD patients exhibit a high degree of network interactions.

Genes in red represent UP-regulation, while genes in green are DOWN-regulated

5. Discussion

5.1. MD has an extended phenotype beyond the inner ear.

Meniere disease is not a single disease. It is a clinical syndrome with may present additional symptoms and co-morbidities. The epidemiological association of MD with migraine and several AD, including rheumatoid arthritis or psoriasis, support the hypothesis of an extended phenotype, beyond audio-vestibular symptoms[30]. The diagnostic criteria for MD formulated by the Classification Committee of the Bárány Society state that bilateral involvement is determined by hearing loss ≥ 35 dB defined in the audiogram. So the criteria for definite UMD are based on the observation of recurrent attack of vertigo associated with low-to-middle frequency SNHL and fluctuating aural symptoms (hearing loss, tinnitus or aural fullness) in the involved ear[13]. So, if the absolute thresholds for bone-conducted sound must be ≥ 35 dB HL at each of to contiguous frequencies below 2000 Hz (250, 500 or 1000 Hz) in both ears, and the patient has experienced two or more spontaneous episodes of vertigo each lasting 20 minutes to 12 hours associated with fluctuating aural symptoms, the diagnosis of definite BMD is established. The notes added to the definition also describe a second clinical variant when the patient develops simultaneous bilateral SNHL (symmetric or asymmetric) [13, 158], but no further clinical information was included in the 2015 definition. Although these criteria increase the accuracy in clinical diagnosis for UMD, e.g. isolated high-frequency hearing loss with vestibular episodic symptoms was excluded [159], they do not consider some comorbidities commonly observed in some patients such AD or migraine.

5.2. UMD and BMD are heterogeneous disorders

Our study demonstrates that UMD and BMD are heterogeneous disorders. We have used two-step cluster analysis since it allows the inclusion of quantitative and categorical variables to identify clusters of patients with common symptoms[160] such as: FMD, AD, migraine, delayed MD or the type of onset for HL. According to these predictors we have identified 5 clinical subgroups in both, BMD and UMD patients with strong potential etiological implications. Three of these subgroups can be found in both (type 3 FMD, type 4 MD with migraine and type 5 MD with AD). These clinical variants observed in UMD and BMD confirm previous epidemiological studies[30], but they also

suggest a separate role for genetics and autoimmunity as factors contributing to the development of MD. The role of migraine also deserves attention, but further studies are needed in this specific subgroup to clarify the relationship UMD/BMD-migraine-AD.

Previous studies in patients with BMD were focused in the diagnosis by electrocochleaography or magnetic resonance imaging (MRI) [161, 162, 163], but they did not consider the comorbidities commonly observed such as migraine or AD in some cases. The phenotyping of a patient with an episodic vestibular syndrome should not be limited to the description of the inner ear symptoms, skipping crucial information such as the familiar history of MD or migraine. Furthermore, the comorbidities of migraine or AD may explain the perception of MD as a continuum which overlaps with migraine [164] or autoimmune innear ear disease [13, 165, 166].

5.3. Clinical subgroups in BMD

The most remarkable finding in our cluster analysis is that the five groups of patients identified do not overlap themselves, and each of them has a set of features able to define the group.

BMD type 1 is the most common clinical variant and it includes patients with MD in one ear (unilateral MD) and they develop the hearing loss in the contralateral ear moths or even years later (conversion from UMD to BMD). The mean age of onset was 46 years old, comparable to BMD type 2, but it is significantly higher than it was observed in the rest of the groups (Types 3, 4 or 5). BMD type 1 has no familial or autoimmune history, and patients do not have migraine, so further studies are required to investigate other concurrent comorbidities to determine contributing factors.

BMD type 2 is the second most frequently observed clinical variant and fluctuating bilateral SNHL loss may resemble AIED, since simultaneous SNHL with vestibular symptoms can occur in 50% patients with AIED [165]. However, these patients do not have any AD, FMD or migraine. Interestingly, BMD type 2 patients show a high vascular risk profile, since 50% of them show high blood pressure and 53% have dyslipemia. When we compared these frequencies with BMD type 1, (which do not differ in age or sex profile to BMD type 2), they were not significantly different ($p=0.078$), but further studies should assess the role of vascular risk factors in labyrinthine microcirculation in MD.

Comparing the hearing stage for the worst ear, it seems to be worse in BMD type 1 (metachronic SNHL) than in type 2 (synchronic SNLH). Since both groups do not differ for the age of onset, duration of disease or gender distribution, we cannot determine the reason for the severe SNHL in the first ear in BMD type 1.

BMD type 3 includes all patients with familiar history of MD, and we could subtype them in two subgroups (3A with migraine, 82%, and 3B without migraine 18%). These findings confirm the early description of families with MD co-segregating migraine and MD [167] and the more recent description of FMD without migraine [56, 60, 78]. According to this subtyping for FMD, there will be two types of families with MD, with and without migraine, and they reflect the clinical heterogeneity in FMD. The families include patients with UMD and BMD, so epigenetic factors may influence uni or bilateral involvement. Most of the described families have an autosomal dominant pattern of inheritance and the participation of several genes indicate a genetic heterogeneity in FMD [56, 57].

BMD type 4 is associated with migraine in all cases, but they do not have FMD. This group may overlap with vestibular migraine (VM) and it may share common pathophysiological mechanisms [168]. Patients with MD may show migraine symptoms also during the attacks of vertigo[164], and this finding could make difficult the differential diagnosis of MD and VM. A VM+MD overlap syndrome has been described in several studies (Cha, Neff and Staab), but the clinical differences with MD plus migraine and the progression of hearing loss in these patients has not been investigated. MRI may be useful in the diagnostic evaluation of patients with the spectrum of MD/VM (MD with concurrent migraine or VM with auditory symptoms) [169].

BMD type 5 might be considered as autoimmune MD, since all patients present another concurrent AD. Yet, this group is quite heterogeneous because includes patients with SMD (71%) and FMD (29%), migraine (38%) and both synchronic (38%) and metachronic SNHL (62%). Here, there is a clinical overlap with AIED when these patients develop vestibular symptoms[26], but additional clinical and immunological studies are needed for a better understanding of autoimmune MD.

5.4. Clinical subgroups in UMD

UMD type 1 or “classic MD” is the most common clinical variant and includes patients with SMD without migraine or AD. The mean age of onset was 46 years old,

comparable to the age of onset observed in the rest of the groups, with the exception of UMD type 4 (with migraine), which usually starts 5 years earlier. UMD type 1 has no FMD or AD, and patients do not have migraine, so further studies are required to investigate this large subgroup of SMD to determine its contributing factors.

UMD type 2 is defined as delayed MD (previously known as DH) and it is a rare condition. No other subgroup presented any patient with delayed MD, so this single predictor defines the group. Most of patients were SMD and do not have migraine (83%). AD was only observed in 10% of patients, as expected in the general population. Interestingly, UMD type 2 patients do not show a particular vascular risk profile since the observed frequencies for high blood pressure and dyslipidemia were not significantly different from those observed in UMD type 1, (which do not differ in age or sex profile to UMD type 2). Although the etiology of delayed MD is not known, a potential viral infection of the spiral ganglion in the cochlea, a secondary involvement of the vestibular ganglion or the labyrinth are tentative hypotheses[170], given the lack of genetic or vascular risk factors in this subgroups.

UMD type 3 includes all patients with FMD. The mean hearing thresholds observed at diagnosis in FMD were around 30 dB HL, suggesting that subclinical fluctuating hearing loss may go unnoticed in familial recurrent vertigo. We have subtyped them in two subgroups (**UMD type 3A** without migraine, 78%, and **UMD type 3B** with migraine 22%). FMD is a rare Mendelian disorder and most of the families show an autosomal dominant inheritance with incomplete penetrance and variable expressivity. So far, six genes have been identified in FMD by exome sequencing: *COCH, DPT, DTNA, FAM136A, PRKCB and SEMA3D* [57, 171, 172]. Of note, these findings resemble BMD type 3 where most of the families do not present migraine[56, 78]. According to this subtyping for FMD, there will be two types of FMD, with and without migraine, and they will mirror genetic heterogeneity in autosomal dominant FMD. The co-segregation of migraine and MD in some families with MD, suggests that some of the allelic variants conferring risk for migraine could be enriched in these families with MD and migraine[173, 174]. The families may include patients with UMD or/and BMD, so epigenetic factors may influence uni- or bilateral involvement[56, 57].

UMD type 4 is defined as SMD and migraine, and it was found in 15% of cases. Although most of the patients do not have an autoimmune background, there is a 41% of UMD type 4 with another AD. These individuals with SMD plus migraine and AD represent a challenge for the treatment. As in BMD, this group may overlap with VM

and could share common pathophysiological mechanisms[168]. Further clinical characterization of patients with MD, migraine and autoimmune background are needed, since potential diagnostic and treatment implication may arise.

UMD type 5 could be considered as autoimmune MD, since all patients have other concurrent AD and, all of them are SMD without migraine. Since the age of onset is earlier, the lack of migraine in a patient with an early onset of clinical symptoms of MD should be considered a red flag to rule out other subclinical autoimmune conditions.

Our clinical study has several limitations. Despite of our efforts to improve phenotyping in patients with BMD and UMD, we could not classify 95 patients with BMD and 249 patients with UMD in any cluster and they were excluded of the model. Actually, the largest groups (BMD type 1 and UMD type 1) remain poorly characterized, since they are not associated with any particular clinical feature or etiological factor. The role of allergy in MD deserves more research efforts, since a high prevalence of sensitization to inhalant or food allergies have been reported in MD [38, 134, 175]. Finally, UMD type 3 and type 5 overlap in 10% of cases (FMD with AD without migraine).

However, the recognizing of different subgroups of patients and the definition of clinical variants in BMD and UMD is the first step to improve the selection of patients for genetic and immunological studies, but also for randomized clinical trials (RCT). Most of the RCT performed in MD, were not able to demonstrate any effects of diuretics [40] or betahistine [176] and had limited effectiveness for intratympanic gentamicin [177] or steroids [178], and these results could be explained by a biased selection of patients with different etiologies. Further phenotyping of these clinical variants are needed for a better understanding of the clinical heterogeneity observed in uni- and bilateral MD.

Since the prevalence of BMD is around 25% in our cohort and BMD type 5 is found in 11% of cases, we could estimate that the prevalence of BMD type 5 would be around 1/40000 individuals. Our results confirm previous studies that supported a significant association between BMD, migraine and ADs [30, 97].

5.5. A locus at 6p21.33 suggests an association with BMD

The SNV rs4947296 has been previously described as one the most strongly SNV associated with Behcet's disease ($P < 10^{-12}$) in a GWAS conducted in Korean, Japanese, and Han Chinese populations [179, 180, 181], as well as associated with Graves'

disease in Chinese population [182]. *In silico* analysis predicts transcription factors binding sites for the CCAAT/enhancer-binding protein beta (CEBPB) and the CCCTC-binding factor (CTCF) in our locus with a regulatory role in the immune response. CTCF is required for the expression of the MHC class II genes and it is involved in their topological organization[183]. CEBPB regulates the expression of genes involved in immune and inflammatory responses, including cytokines like IL-6, IL-4, IL-5 and TNFα [184]. This study confirms that the haplotype rs4947296 is a trans-eQTL (expression quantitative trait locus), regulating gene expression in the TWEAK/Fn14 pathway (members of the TNF ligand/receptor families, respectively) in LCL, and these findings support an abnormal immune response in BMD pathophysiology. Additionally, this pathway could be likewise involved in Behcet and Graves' disease and it could be a potential target for therapy in these disorders. So, pleiotropy, which happens when one gene influences two or more seemingly unrelated phenotypic qualities, is a common finding in tras-eQTL for autoimmune disorders [185, 186].

5.6. rs4947296 regulates NFκB-mediated inflammation in BMD LCLs

TWEAK is a multifunctional cytokine that regulates multiple cellular responses, including inflammation, cellular adhesion, proliferation and apoptosis [187, 188]. TWEAK triggers signals through its receptor, Fn14, which is highly expressed in epithelial cells and induced in several human diseases[189]. Increased levels of TWEAK and/or Fn14 have been also found to be associated with the pathogenesis of rheumatoid arthritis (RA) [190], systemic lupus erythematosus (SLE) [191], multiple sclerosis (MS) [192] or neuroinflammation [153]. The binding of TWEAK to Fn14 induces both, an acute activation of the canonical NFκB pathway and a prolonged activation of the non-canonical NFκB pathway[187]. Besides, the non-canonical NF-κB pathway plays a key role in immunity and immune-mediated disorders as SLE[187]. Our findings using homozygous LCLs demonstrate that this eQTL up-regulates the expression and translation of NFkB in lymphoid cells and induces NFkB-mediated inflammation in patients with BMD.

The non-canonical NFκB pathway relies on the phosphorylation-induced p100 processing, which is triggered by signaling from a subset of TNFR members, including Fn14, TNFR2, BAFFR, CD40, LTβR, and RANK[193]. Most of these signals are regulatory elements of the immune response and support the hypothesis that the allelic variants of immune response genes can modify the clinical course in MD. Previous

studies have suggested that variants in *TLR10* and *NFKB1* genes are modifiers of hearing outcome in patients with BMD [86] or UMD [88] respectively, but the relationship between TLR10 and NFkB-mediated inflammation in MD is not known.

5.7. Potential mechanisms of inflammation in MD

The risk haplotype could be used as predictor for bilateral SNHL or autoimmune mechanism in MD and our findings support an NFκB-mediated inflammation in MD.

Even though TWEAK might induce the abnormal activation of this pathway in MD, the site of inflammation remains unknown. An interesting hypothesis to explore is an inflammatory damage of the blood-labyrinth barrier (BLB), given the role of TWEAK in maintaining the blood-brain barrier (BBB) permeability and regulating the structure and function of the neurovascular unit [194] (**Figure 34**). Recent evidences support a role for TWEAK/Fn14 pathway in compromising the BBB in neuropsychiatric SLE[195], TWEAK/Fn14 interactions increase the accumulation of inflammatory cells in the choroid plexus, disorganizing the BBB integrity, gaining neuronal damage and inducing neuronal death in vitro by NFκB signaling pathway [196, 197], but the role of TWEAK/Fn14 in the regulation of inner ear fluids in the BLB is yet unexplored.

A second hypothesis is that inflammation may occur in the ES, since proteomic studies have found high contents of immunoglobulins in the ES [198]. The endo- and perilymph homeostasis is maintained at the cochlea and vestibular semicircular canals at multiple sites including the spiral ligament, the stria vascularis , the cochlear and vestibular non-sensory epithelial cells, and the ES. The sac is a small organ located in the posterior cranial fossa and has a crucial role, not only in the maintenance of endolymph composition, but also in the innate immune response[199]. We hypothesize that, after exposure to an environmental trigger, the carriers of the risk haplotype could have an abnormal NFκB-mediated inflammatory response at the ES, causing an ionic imbalance in the endolymph leading to the accumulation of it at the cochlear duct.

A third hypothesis would involve the increase of NFκB in fibrocytes within the spiral ligament (SL) and the spiral limbus after a stress stimuli and the release of proinflammatory cytokines. Genetic mutations involving spiral ligament cells may lead to SNHL[200, 201, 202]. Immune-mediated and acoustic trauma-mediated HL may result

from the vulnerability of type I and type II fibrocytes to acoustic trauma and systemic inflammatory stress, respectively [107].

The last hypothesis is based on the function of cell adhesion molecules in the sensorineural epithelia of the cochlea. The strict compartmentalization in the inner ear is necessary for normal hearing and is achieved by interactions between tight junctions (TJs) of the reticular lamina (RL), which consists of a mosaic of hair cells and supporting cells [203]. An outstanding example for these interactions is established by the tight junction proteins (TJP), TJP1, TJP2, and TJP3 that connect with the cytoplasmic domains of different integral membrane proteins such as occludins and claudins [204]. Tjp1 was shown to directly interact with F-actin, building a molecular bridge between integral membrane proteins like tricellulin (encoded by the *TRIC* gene) and the cytoskeleton, and human mutations in *TRIC* lead to a truncated form of tricellulin which fails to interact with Tjp1 leading to deafness [205]. Other members of the TJPs have been described to be involved in some types of deafness. Thus, a mutation in *TJP2* was linked to progressive NSHL DNFA51 [206]. These findings made us think that the carriers of the risk haplotype could have an abnormal expression of cell adhesion molecules which might compromise the permeability of the reticular lamina causing an ionic imbalance that entails a faster progression of hearing impairment.

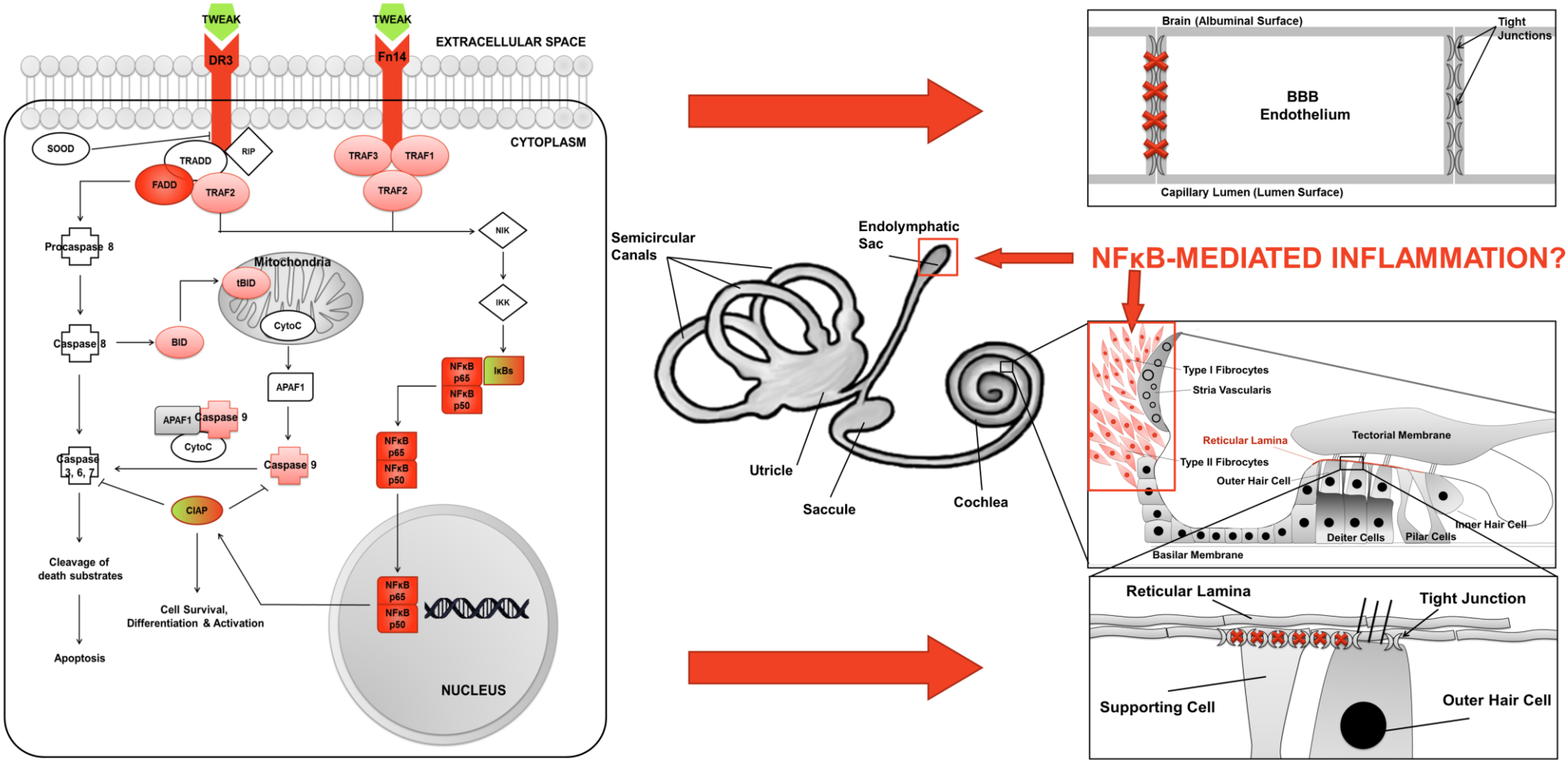

Figure 34. Potential mechanisms of inflammation in MD.

Potential sites of inflammatory damage: the BBB, the ES,

the SL and the RL in the neurosensory epithelium of the cochlea.

5.8. MD type 1 presents higher basal levels of cytokines

These experiments have found that basal levels of proinflammatory cytokines are increased in both, patients with UMD and BMD. This cytokine profile may represent two subgroups of patients with MD with intrinsic differences in the immune response or two functional states of the immune system in patients with MD.

As mentioned above, MD type 1 is the most common clinical variant in both, UMD and BMD and, it includes patients without migraine, AD or FMD. They include three other categories with genetic background (FMD or MD type 3), patients with MD plus migraine (MD type 4) and association with other AD (MD type 5). Thus, most of the patients with increased IL-1β were classified as MD type 1 (73.1%), 11.5% were MD type 4 and 7.7% were MD type 3 and type 5. Patients with high levels of TNF-α were mostly MD type 1 with a 68.2%, MD type 4 and 5, both with a 13.6% and finally MD type 3 was 4.5%. Finally, patients with both IL-1β and TNF-α increased were in a 70.2% MD type 1, 12.8% MD type 4, 10.6% MD type 5 and 6.4% MD type 3.

The genes in the IL-1 complex code for three cytokines: IL-1α, IL-1β and IL-1RA. The severity of a given infection is influenced by the balance between the levels of IL-1β and IL-1RA. In healthy individuals, IL-1RA is readily detectable in plasma while IL-1 β levels are usually undetectable [207]. Remarkably, IL-1RA levels were found to be elevated in most patients with MD and 71% were MD type 1. Since TLR10 is an anti-inflammatory pattern-recognition receptor able to up-regulate IL-1RA [208], and the TLR10 allelic variant rs11096955 has been associated with faster hearing loss progression in bilateral MD [86], the activation of TLR10 could explain the increased levels of IL-1RA found in patients with MD. Moreover, individuals bearing the TLR10 variant rs11096957 displayed increased levels of IL-1β, TNF-α and IL-6 upon ligation of TLR2 [208].

5.9. IL-6 and TNF-α in MD

Our experiments demonstrate for the first time that protein extracts from *Aspergillus* and *Penicillium* induce a proinflammatory response involving TNF-α and IL-6. Previous studies have established a relationship between mold extracts and proinflammatory cytokines in AIED[165]. So, when PBMC from patients with AIED were exposed to mold resulted in an up-regulation of IL-1β mRNA expression, and enhanced IL-6 and IL-1β

secretion, suggesting that mold acts as a PAMP in a subset of these patients. Since most of our patients with MD were classified as MD type 1 and did not have a comorbid AD, and we also excluded patients with response to inhalatory allergens in the prick test (including *Penicillium* and *Aspergillus),* the elevation TNF-α and IL-6 observed in MD patients PBMCs after exposure to *Penicillium* and *Aspergillus* cannot be explained by another autoimmune condition or previous sensitization to other allergens.

Furthermore, it is well established that molds can cause respiratory symptoms and may worsen the course of bronchial asthma, which could lead to increase secretion of TNF-α, IL-6, IFN-γ or IL-1β [209, 210].

5.10. NRP-1, MMP1 and WNT5A may have a role in MD

Several genes that were modulated in these experiments have been involved in the host cell-fungus interaction in other studies. Thereby, *NRP-1* gene*,* encoding neuropilin-1 has been previously described to be significantly increased in peripheral blood monocytes in a chronic model of allergic asthma [211]. Semaphorin 3A binds to neuropilin-1 and play a regulatory role in immune responses and have a demonstrated effect on the course of rheumatoid arthritis (RA), where their altered expression on T cells was shown to correlate with the progression of RA [212], as well as in systemic lupus erythematosus [213]. Wnt signaling controls a variety of developmental processes such as proliferation, migration, and differentiation. Wnt signaling pathways have been divided into two broad categories: a) the canonical pathway, and b) the non-canonical pathways, which involves all *β*-catenin-independent Wnt-induced signaling events by Wnt ligands including *WNT5A [214].* Wnts have been shown to induce inflammatory genes such as interleukins and matrix metalloproteinases (MMPs), such as MMP1. So, MMP1 expression is induced by WNT5A in endothelial cells [215], suggesting that Wnt signaling is involved in inflammatory regulation [216]. Besides, *WNT5A* seems to enhance maturation of mast cells via the canonical pathway, increasing histaminergic response [217].

All these data may explain that some patients respond to drugs with antihistaminic effects, such as betahistine, but most patients do not show a response different from placebo[176]. Moreover, since patients using immunotherapy have reported an improvement of the duration and frequency of the vertigo episodes compared to controls [218], the assessment of allergic response in patients with MD, and the use of

antihistaminic or immunotherapy as part of the treatment plan to control vertigo attacks should be considered[219].

6. Further directions

The recognition of different subgroups of patients in UMD or BMD is the first step not only to improve the selection of patients for genetic and immunological studies, but also for conducting randomized clinical trials (RCT). Most of the RCT performed in MD were not able to demonstrate any effects of diuretics [40], betahistine or steroids [178]. These results could be explained by a biased selection of patients with different etiologies. Thus, further phenotyping of these clinical variants are needed for a better understanding of the clinical heterogeneity observed in MD, including

- Phenotyping of MD type 4 to differentiate it from vestibular migraine
- Better understanding of mechanisms in familial MD (Type 3)
- Deciphering alternative mechanisms in autoimmune MD (Type 5)

Our study also supports a regulatory effect in the immune response and suggests that Fn14 receptor or NFκB could be candidate targets for drug therapy for the carriers of the risk haplotype in MD. Future preclinical studies and clinical trials will be needed to demonstrate any potential benefit such as:

- To perform the diagnostic studies in other populations like Asian or american.
- To determine the functional effect of TWEAK in the tight junctions and NFkB activation in a conditional epithelial cellular model, like a549.
- To perform human ES transcriptome to determine the genes and biochemical pathways involved in the immune response within the inner ear sac.

Finally, we observed that basal levels of proinflammatory cytokines are increased in some MD patients and that after stimulation with either *Penicillium* or *Aspergillus* an increase of TNF-α and IL-6 occurs. The next steps should be:

- To perform longitudinal experiments in the same patients, to assess if the increase in the cytokines is persistent or fluctuating over time.
- To evaluate the effect of molds stimulation in other diseases with overlapping symptoms with MD such as vestibular migraine.
- To define gene expression profile and epigenetic markers in PBMCs during crisis and quiescent phase.
- Try to further investigate the downstream effects of IL-6 and TNF-α to define candidate targets for therapy.
- To define additional cytokines in separated immune cell populations to figure out which cell types are driving the response to molds.

7. Conclusions

1) Cluster analysis defines 5 clinical subgroups in UMD and BMD and it extends the phenotype beyond audiovestibular symptoms.
2) The SNV rs4947296 is a trans eQTL and it regulates gene expression in the TWEAK/Fn14 pathway in PBMCs and LCLs from MD patients
3) Two subgroups of patients can be defined according to their basal levels of cytokines.
4) *Aspergillus* and *Penicillium* trigger the release of IL-6 and TNF-α in a subset of patients with MD.

7. Conclusiones

1) Clúster análisis define 5 subgrupos clínicos en EM unilateral y bilateral y extiende el fenotipo más allá de los síntomas audiovestibulares.
2) El SNV rs4947296 es un trans-eQTL que regula la expresión génica en la vía de TWEAK/Fn14 en células mononucleares y linfoblastoides en EM.
3) Dos subconjuntos de pacientes se pueden definir según su expresión basal de citoquinas.
4) *Aspergillus* y *Penicillium* desencadenan la liberación de IL-6 y TNF-α in a en un grupo de pacientes con EM.

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