Clinical Immunology 271 (2025) 110413

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Full Length Article

Allergy and autoinflammation drive persistent systemic inflammatory response in Meniere Disease: A longitudinal study

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ARTICLE INFO

Keywords:
Cytokines
Inflammation
IL-1β
IgE
Meniere Disease

ABSTRACT

Background: Meniere disease (MD), an inner ear disorder influenced by genetic and environmental factors, potentially leads to chronic inflammation. This study evaluates whether inflammation in MD patients is driven by allergy or autoinflammation.
Methods: 2-year longitudinal study. Cytokine and chemokine levels were measured in plasma from 72 patients. Functional clusters were identified using weighted-based discriminant and km3d trajectory analyses. THP-1 cells were exposed to patients' plasma to assess macrophage polarization, and qPCR analyzed upstream cytokine release events.
Results: Four groups were identified: 1) Autoimmune (20 %) with high TNF-α ($p = 0.0004$); 2) Allergic (25 %) with elevated IgE ($p < 0.0001$) and M2 polarization; 3) Autoinflammatory (13 %) with increased IL-1β ($p < 0.0001$), activated via CASP1/NLRP3; 4) Low cytokine levels (42 %; cytokines in Q1). Group stability was observed, with 36 % of allergic patients also showing high IL-1β.

Abbreviations: °C, Degrees Celsius; 7-AAD, 7-aminoactinomycin; AIED, Autoimmune inner ear disorder; BCA, bicinchoninic acid; BME, 2-mercaptoethanol; BRCC3, BRCA1/BRCA2-Containing Complex Subunit 3; BSA, Bovine Serum Albumin; CARD8, Caspase Recruitment Domain Family Member 8; CASP1, Caspase 1; CASP8, Caspase 8; CCL2, Chemokine (C—C motif) ligand 2; CCL3, Chemokine (C—C motif) ligand 3; CCL4, Chemokine (C—C motif) ligand 4; cDNA, complementary DNA; DNA, Deoxyribonucleic acid; ELISA, Enzyme-Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GATA3, GATA Binding Protein 3; GSDMD, Gasdermin D; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; IgE, immunoglobulin E; IgG, Immunoglobulin G; IL-10, Interleukin 10; IL-13, Interleukin 13; IL-1β, Interleukin 1 beta; IL2RG, Interleukin 2 receptor subunit gamma; IL-4, Interleukin 4; IL4R, Interleukin 4 Receptor; IL-5, Interleukin 5; IL-6, Interleukin 6; IL-8, Interleukin 8; IL-9, Interleukin 9; INF-γ, Interferon-gamma; INHBA, Inhibin Subunit Beta A; IU, International Units; JAK2, Janus Kinase 2; JAK3, Janus Kinase 3; JNK1, also known as MAPK8, Mitogen-Activated Protein Kinase 8; LPS, Lipopolysaccharide; MAPK3, Mitogen-activated protein kinase 3; MD, Meniere Disease; mL, milliliters; mM, millimolar; Na+, Sodium; NaCl, Sodium chloride; ng, nanograms; NLRP1, NLR Family Pyrin Domain Containing 1; NLRP3, NLR Family Pyrin Domain Containing 3; nm, nanometers; NOD1, Nucleotide-binding oligomerization domain 1; NOD2, Nucleotide-binding oligomerization domain containing 2; PBMC, Peripheral Blood Mononuclear Cells; PBS, phosphate-buffered saline; PC, Principal Component; PCA, Principal Component Analysis; PCR, Polymerase Chain Reaction; PMA, phorbol-12-myristate-13-acetate; qPCR, Quantitative Polymerase chain reaction; RNA, Ribonucleic acid; RT-qPCR, Real-Time quantitative PCR; SD, Standard Deviation; SNHL, Sensorineural Hearing Loss; STAT3, Signal Transducer And Activator Of Transcription 3; STAT6, Signal Transducer And Activator Of Transcription 6; TNF-α, Tumor Necrosis Factor-alpha; WB, Western Blot; WBDA, Weighted-Based Discriminant Analysis; β-Gal, β-Galactosidase Enzyme; μL, microliters; μm, micrometers.

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https://doi.org/10.1016/j.clim.2024.110413
Received 25 July 2024; Received in revised form 18 November 2024; Accepted 21 November 2024
Available online 30 November 2024

Conclusion: Identified immunophenotypes, allergy-driven IgE responses, and IL-1β-mediated autoinflammation indicate that targeting inflammation with biomarkers could optimize MD treatment and outcomes.

1. Introduction

Meniere disease (MD) is a chronic inner ear disorder associated with genetic and environmental factors [1,2]. The phenotype is defined by recurrent episodes of vertigo associated with sensorineural hearing loss (SNHL), tinnitus, and aural fullness with a non-predictable course [3,4]. Most patients progress to chronic imbalance, moderate to severe deafness in the affected ear, and, in many cases, develop persistent and disabling tinnitus [5,6]. The phenotype is variable and may be associated with comorbidities such as migraine, several autoimmune and autoinflammatory disorders, and allergies such as allergic rhinitis or allergic asthma [7–10].

Several triggers, including allergens, infectious agents, or changes in the ionic balance of the endolymph, could induce an inflammatory response in MD that may become persistent over time, leading to a chronic inflammatory process [11].

MD and allergy have been intertwined since 1923 when Duke first described their association [12]. Then, in early 2000, various studies showed a prevalence of diagnosed allergy 3 times higher in those with an MD history compared to the general population and more elevated IgE levels [13,14]. Besides, both inhalants and food allergies have been associated with MD [15]. Since then, a robust association between MD and allergies has been demonstrated [16,17], and reports have described an over-expression of IgE and several type 2-related cytokines in MD patients [18–20]. Of note, the presence of IgE in the ampulla, macula, semicircular canal, and endolymphatic sac by immunohistochemistry and dense deposits of IgE in the utricle of MD patients have been described [19].

Similarly, several studies have described an autoimmune or autoinflammatory role in MD within the past decade [21–26]. Different methylation patterns between MD patients and controls have been reported [25], as well as differentiation of 2 subgroups of MD patients according to the baseline levels of IL-1β (High and low), leading to different immune response profiles to antigens that reflect the functional status of the immune system [22]. Moreover, differential expression of many proinflammatory cytokines such as IL- 1β, CCL3, CCL18, CCL22, CXCL1, and CXCL4 has been able to differentiate between MD and vestibular migraine [23] and migraine [24]. Additionally, Zou et al. found elevated IL-8, HGF, and G-CSF as potential triggers in definite MD [26].

Since both inflammatory premises have been validated in several studies, we hypothesize that patients can be categorized based on their inflammatory response profiles, influenced by allergic and autoinflammatory mechanisms. For this, we performed a longitudinal study measuring cytokines and chemokines previously associated with MD as well as cytokines related to allergy and autoinflammation, such as IgE, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, TNF-α, CCL2, CCL3, and CCL4, to categorize MD patients according to their profile. This classification will enhance the understanding of MD pathophysiology, facilitating targeted therapeutic approaches that address specific inflammatory pathways to improve patient outcomes.

2. Materials and methods

2.1. Participants and ethics

To ensure sufficient statistical power to detect a clinically significant difference, a power calculation was performed before the study. Based on an expected effect size of 0.4, with an alpha level of 0.05 and a desired power of 80 %, a sample size of 76 patients was determined using G*Power [27].

A prospective longitudinal study included 80 patients (> 18 years old) with definite MD according to the 2015 diagnostic criteria [3]. The patients were under no treatment for at least six months before entering the study and during the duration of it. Participants were recruited in 4 neurotology clinics in Spain from 2019 to 2022. All gave their informed consent to take part in this study. Individuals underwent 4 visits during 2 years of follow-up, where blood was drawn, and a complete audiological and vestibular assessment was conducted. The study was conducted according to the 2013 Ethical Principles for Medical Research Involving Human Subjects from the World Medical Association Declaration of Helsinki [28]. The Granada Ethical Review Board for Clinical Research approved the protocol (PE-0356-18).

2.2. Plasma isolation

We used lavender-top tubes (K2EDTA) since they are used for most hematological procedures and are the preferred ones for molecular tests. The tubes were centrifuged at 1600 ×*g* for 10 min at room temperature, and plasma was harvested and stored at −80 °C until used.

2.3. Cytokine measurement

Frozen plasma was thawed immediately before analysis, and no samples underwent more than 2 freeze-thaw cycles. Eight cytokines (IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF-α), and 3 chemokines (CCL2, CCL3, and CCL4) were measured using the Multiplex Bead-Based Kit (EMD Millipore). The measurements followed the manufacturer's protocols, using a Luminex 200 (Luminex Corp.) and read with Luminex x PONENT 3.1 software (Luminex Corp.). Samples with readings below or above the detection limits for the assays were assigned values of 0 pg/mL for the minimum value and 10,000 pg/mL for the maximum value. Two quality controls for each cytokine were run in duplicate.

2.4. IgE measurement

Frozen plasma samples from all participants were thawed immediately before analysis. IgE was measured using the commercially available IgE Human Uncoated ELISA Kit (#88–50,610-22, Thermofisher Scientific), following the manufacturer's protocol. The absorbance was measured at 450 nm with a 570 nm correction using the infinite M200 NanoQuant plate reader (Tecan). All samples were run in triplicates. IgE levels were converted from ng/mL to IU/mL and considered high levels if IgE > 150 IU/mL (360 ng/mL; standard values in the clinic <100 IU/mL [29]).

2.5. RNA isolation and RT-qPCR

Total RNA was isolated from PBMC previously separated using the density gradient separation method (Lymphosep Lymphocyte separation media; Biowest) using the High Pure RNA Isolation Kit (Hoffmann-La Roche) according to the manufacturer's protocol. RNA concentration was measured on a Nanodrop (NanoDrop Technologies), and the quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA was reverse transcribed into complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit (Thermofisher Scientific, Waltham, MA, USA). Then, a quantitative PCR (qPCR) was run using PerfeCTa® SYBR® Green Fast Mix® ROX (QuantaBio) and the QuantStudio 6 Real-Time PCR thermocycler (Thermofisher Scientific). Relative quantification was performed using GAPDH as the

housekeeping gene. Fold changes for each gene were calculated using the $2^{-\Delta\Delta CT}$ method. All primers used may be found in supplementary Table 1.

2.6. THP-1 cell culture and stimulation

THP-1 cell line (ATCC) was cultured under the following growing conditions: RPMI1640 + GlutaMAX™ (Thermofisher Scientific), supplemented with 10 % FBS (Biowest), 1 % non-essential amino acids (Thermofisher Scientific), 1 % pyruvate (Biowest), 1 mM HEPES (Sigma-Aldrich) and 1 % Penicillin/Streptomycin (Thermofisher Scientific).

THP-1 cells in the monocyte state were differentiated into a macrophage-like phenotype using phorbol-12-myristate-13-acetate (PMA; 50 ng/mL final concentration, Merck Millipore) with 2-mercaptoethanol (BME; 0.1 mM, Gibco) for 24 h at 37 °C, 5 % CO_2. The day after, the medium is changed to RPMI1640 supplemented with 0.5 % FBS to induce macrophage polarization. By day 3, positive controls were treated with 50 ng/mL IFN-γ (R&D Systems) and 10 ng/mL LPS (InvivoGen) for M1 and 50 ng/mL IL-4 (R&D Systems) and 50 ng/mL IL-13 (R&D Systems) for M2 polarized macrophages in RPMI1640 supplemented with 0.5 % FBS during 24 h at 37 °C. The conditioned wells were treated with patients' plasma for 1 h followed by 2 h cultured in RPMI1640 supplemented with 0.5 % FBS.

2.7. Flow cytometry

Polarized macrophages derived from THP-1 cells were Trypsinized (TrypLE; Thermofisher Scientific), washed with 1× PBS, resuspended in 100 μL 1× PBS with 0.5 % BSA (Sigma-Aldrich) and 2 % FBS (Biowest) with different antibodies described in supplementary table 2 for 20 min at RT. Cell viability was assessed using 7-amino actinomycin D (7-AAD; 1 μL; BD Biosciences) staining. For flow cytometry validation, compensation controls were performed using single-color stained samples, and appropriate controls for non-specific binding (e.g., isotype controls) were included. A calibration standard was used to ensure the accuracy of the flow cytometer measurements. Data acquisition was carried out on a BD FACSVerse™ flow cytometer (BD Biosciences), with BD FACSuite™ software (BD Biosciences) for acquisition and FlowJo™ v.10 for analysis (FlowJo, LLC-BD Biosciences). Quality control checks included the analysis of fluorescent minus one (FMO) controls to define proper gating strategies and the evaluation of instrument performance through daily calibration to minimize spectral overlap and ensure the reproducibility of results.

2.8. Data analysis

Descriptive statistical analysis for clinical data was performed using GraphPad Prism 9.0 (GraphPad Software Inc., Insight Partners, NYC, NY, USA). Data are shown as means ± standard deviation (SD). Quantitative variables were compared using the pair-wise non-parametric Mann-Whitney *U* test when comparing 2 groups and the Kruskal-Wallis test for comparisons of at least 3 groups. Qualitative variables were compared using Chi-square and Fisher's exact test. Nominal p-values < 0.05 were considered statistically significant. Spearman correlation was used to find correlations between continuous variables and Point-Biserial correlation between dichotomous and continuous variables. To evaluate the relationship between biomarkers and clinical outcomes, we employed a variety of statistical models. A multinomial model was conducted to assess the impact of markers on different categorical responses (AMD, IgE, IL1B, and Low). Additionally, bivariate analyses were performed using ANOVA without repeated measures.

To account for potential confounding factors in the analysis, we used multiple linear regression. Based on prior literature and the study design, a set of potential confounders, including age, sex, disease onset, disease duration, migraine, and autoimmunity, were identified. The dependent variables analyzed included cytokine levels and measures of disease severity, specifically hearing thresholds and the frequency of vertigo attacks in the past six months. These models allowed us to control for these factors while assessing the primary relationships of interest. Additionally, a logistic regression model was conducted to estimate the coefficients of the markers between groups. The bootstrap method was applied to provide more reliable coefficient estimates due to the sample size.

To determine high levels of cytokines, the upper (Q3) quartile was calculated, taking as high levels as the values > Q3 and low levels as the rest. We used typical clinical thresholds (100 IU/mL) plus 50 % to determine IgE levels. Therefore, we describe 3 potential groups using clinical and quantitative data, such as autoimmune comorbidities and levels of IgE and IL-1β.

We used multivariate methods to investigate the relations between all variables in a single dimension. We performed a Weighted based discriminant analysis (WBDA) using Euclidean distance by applying the WeDiBaDis R package [30] to 1) investigate if our defined groups (Autoimmune, Allergic, and Autoinflammatory) could be classified according to the selected conditions and 2) explore if the uncategorized patients fit into any of the groups. Furthermore, we used the unsupervised km3d method based on K-means from the KmL3d R package to cluster joint trajectories of cytokine levels over time by maximization of the Calinski and Harabatz criterion [31,32]. This method allowed for the identification of distinct groups of patients with similar longitudinal profiles of cytokine expression. To assess the robustness of the clusters, we evaluated the stability of the clusters through repeated runs ($n = 100$) and examined intra-cluster variance.

3. Results

3.1. Patients' demographics

Initially, 80 patients were enrolled in the study; however, due to dropouts, 72 patients were included in the final analysis.

Table 1 shows the clinical features of the 72 MD patients included in this study. Patients with MD may be classified into different clinical subgroups according to several comorbidities, such as the familial history of MD (Group 3 = 14.5 %), migraine (Group 4 = 19.5 %), or autoimmune disorders (Group 5 = 19.4 %). Still, most patients in our cohort belong to the MD clinical subtype 1 (40.8 %).

Table 1
Demographic variables in patients with Meniere Disease.

Variable	MD
Age (mean ± SD)	53 ± 11
Years of Evolution (mean ± SD)	13 ± 10
Age of onset (mean ± SD)	40.4 ± 12.8
Sex (% Female)	66 %
Laterality (%UMD)	82.90 %
Affected Ear (%Left)	54.70 %
Familial MD (%)	21 %
Headache (%)	50 %
Migraine (%)	25 %
Hearing Threshold (mean ± SD)	52.18 ± 22.5
Functional Level Scale (%)	
1	23.1
2	12.8
3	14.4
4	23.1
5	17.9
6	5.1
MD clinical subgroup (%)	
1	40.8
2	3.9
3 (FMD)	14.5
4 (MD + Migraine)	19.7
5 (AMD)	19.4

Table 2
Demographic variables in MD patients segregated according to their sub/phenotype.

Variables	AMD (n = 14)	IgE↑ (n = 18)	IL1β↑ (n = 9)	Low (n = 30)	p-value
Age (SD)	51.1 (9.2)	52.9 (14.5)	50.7 (15.2)	55.0 (10.1)	0.46
Onset (SD)	35 (10.2)	41.7 (15.3)	32.4 (11.3)	43.4 (11.4)	0.053
Disease Duration in years (SD)	16 (7.7)	11.5 (12.9)	18.1 (15.4)	11.6 (7.4)	0.093
Sex (Female%)	71.4	61.1	66.7	73.3	0.85
Affected Ear (UMD%)	71.4	77.7	100	86.6	0.29
Affected Ear (Left%)	50	64.3	66.7	53.8	0.83
FMD (%)	14.3	33.3	22.2	20	0.62
Headache (%)	61.5	46.7	33.3	50	0.64
Migraine (%)	28.6	16.7	22.2	26.7	0.85
Autoimmune Disease (%)	100	0	0	0	<0.001
Vertigo Attacks in The Past 6 Months	1.46 (3.6)	1.6 (3.0)	2.2 (4.1)	1.1 (2.2)	0.76
Hearing Threshold (SD)	57.1 (21.0)	51.2 (26.0)	51.3 (25.8)	50.7 (20.6)	0.56

3.2. Autoimmune, Allergic, and Autoinflammatory MD

To ensure comparability between groups, baseline characteristics, including age, sex, onset, disease duration and relevant clinical markers, were compared (Table 2). No significant differences were observed between the groups at baseline, suggesting that the groups were well-matched for key variables.

An analysis of potential confounding factors revealed no significant differences between groups in key sociodemographic and clinical variables, including sex, age, migraine history, age at diagnosis, or other relevant factors. As a result, the differences and relationships observed with the analyzed cytokines and chemokines are unlikely to be influenced by these variables.

First, we selected MD patients with a comorbid autoimmune disorder (19.7 %; 14/71), as these are defined as autoimmune MD (AMD). The most common autoimmune disorders within our sample were rheumatoid arthritis, psoriasis, and hypothyroidism.

However, we found no differences when comparing patients with and without an autoimmune disease, except that 14 % of patients with an autoimmune disorder were familial cases. We then compared all measured cytokines and found significant differences in IL-8 ($p = 0.027$) and TNF-α ($p = 0.0004$) (Fig. 1A).

Next, we investigated the allergic component among the remaining patients ($n = 57$) using an ELISA assay to measure IgE levels. Among these patients, 31.5 % had high IgE levels (IgE↑ >150 IU/mL; 18/57) in all 4 visits, none of whom had a prior history of airborne or food allergies, and only 3 patients had either allergic rhinitis or asthma. Additionally, to discard seasonal allergies, a regression analysis was used to test if the season significantly predicted IgE levels; the overall regression was not statistically significant ($p = 0.08$; supplemental Fig. 1). Meanwhile, 46.5 % (27/57) of patients had low levels of IgE in all visits (IgE↓ < 105 IU/mL). We compared both groups (IgE↑ and IgE↓) and found that the levels of IL-10 ($p < 0.0001$), IL-13 ($p < 0.0001$), IL-4 ($p < 0.0001$), IL-5 ($p = 0.001$), IL-6 ($p < 0.0001$) and IL-8 ($p < 0.0001$) were all significantly different (Fig. 1B). Moreover, the correlation matrix found a strong positive correlation among these cytokines within the IgE↑ group (Pearson $r > 0.64$; Fig. 1C). Additionally, we compared IgE↑ patients against the rest of the patients ($n = 53$) and found higher levels of IL-10 ($p = 0.0002$), IL-13 ($p < 0.0001$), IL-4 ($p = 0.0002$), IL-5 ($p = 0.02$), IL-6 ($p < 0.0001$), IL-8 ($p < 0.0001$).

Finally, the remaining MD patients ($n = 39$) were classified according to IL-1β levels to define patients with autoinflammatory phenotype. Thus, we compared patients according to their IL-1β basal levels. We observed that 23 % of these patients had persistently high levels of IL-1β (IL-1β↑ > 9.7 pg/mL, 9/39) in at least 2 visits separated by at least 6 months; moreover, when we compared patients with IL-1β↑ against the rest of the patients with low levels of IL-1β ($n = 30$), we also found high levels of IL-10 ($p = 0.02$), IL-4 ($p = 0.002$), IL-5 ($p = 0.002$) (Fig. 1D).

Interestingly, when we compared the significantly different cytokines within the autoimmune, allergic, and autoinflammatory phenotypes, we found that only TNF-a, IgE, and IL-1β significantly differed among the 3 groups (Fig. 1E).

Additionally, a multinomial model was conducted to evaluate the impact of markers on different categorical responses (AMD, IgE, IL1B, and Uncategorized). The predictive markers found to be significant for each group included IgE ($p < 0.001$), which showed a significant effect on the response variable, indicating a strong relationship with the response categories. Additionally, IL-5 ($p = 0.0015$), IL-1β ($p = 0.0482$), TNF-α ($p = 0.0248$), and IL-8 ($p = 0.0352$) were also significantly associated with the response variable. Other variables, despite their significance in the univariate model, did not provide sufficient evidence to be considered significant in the multivariate model due to collinearity, such as IL-4 or IL-10.

3.3. Age, Disease duration, and Hearing thresholds correlate to cytokine levels

To explore the relationships between clinical variables and cytokine levels, a correlation matrix was generated, revealing significant associations among the measured factors. When assessing age, we found CCL3 ($r = -0.34$, $p = 0.003$) and IL-1β ($r = -0.28$, $p = 0.02$) negatively correlated to age, but TNF-α ($r = 0.27$, $p = 0.046$) was positively correlated to age. Disease duration was negatively correlated to IL-6 ($r = -0.27$, p = 0.02) and IgE ($r = -0.28$, $p = 0.017$) but positively correlated to CCL4 (r = 0.27, $p = 0.03$) and hearing thresholds ($r = 0.26$, $p = 0.027$). Furthermore, hearing thresholds were negatively correlated to cytokines levels such as IL-13 (r = −0.28, $p = 0.018$), IL-4 (r = −0.28, p = 0.018), IL-5 ($r = -0.32$, $p = 0.006$) and IL-6 ($r = -0.256$, p = 0.03). Additionally, TNF-α was positively correlated to migraine ($r = 0.25$, $p = 0.038$) and negatively to FMD (r = −0.28, p = 0.02).

3.4. Cytokine-based discriminant analysis separates groups of MD patients

Thirty-one patients (43 %) remained uncategorized. Therefore, we performed a WBDA according to our previously defined groups (Autoimmune, Allergic, and Autoinflammatory). The accuracy was 45 % with a Cohen's Kappa coefficient (k) of 0.175. While the sensitivity for each group was 17 %, 70 %, and 46 %, respectively, the specificity was 61 %, 29 %, and 45 %, respectively, with a predicted value of 27 %, 61 %, and 35 %, respectively. We then conducted the WDBDA using the 2 groups selected according to IgE and IL-1β levels with an accuracy of 74 % (k = 0.473) (Fig. 2A). The sensitivity and specificity increased to 72 % and 77 % in the allergic group and 77 % and 72 % in the autoinflammatory group, with a predicted value of 83 % and 64 %, respectively. With that in mind, we explored whether the uncategorized patients (group 4) fit into the allergic or autoinflammatory groups. This model had an accuracy of 64 % (k = 0.43). In this case, the sensitivity and specificity for the unknown groups were 68 % and 60 %, while the predictive value was 77 % and 73 % F1-score. As we see in Fig. 2B, the uncategorized individuals could not be reclassified as allergic or autoinflammatory; they seem to be their group with low cytokine levels (Low Group).

3.5. Trajectory analysis segregates three groups of MD patients

To combine longitudinal data, we used trajectory analyses using all cytokines and visits to improve clustering. Patients were followed for 2 years with visits every 6 months approximately (4 visits in total). Fig. 2C shows 3 diverging trajectories along the 4 visits. Group A, comprising

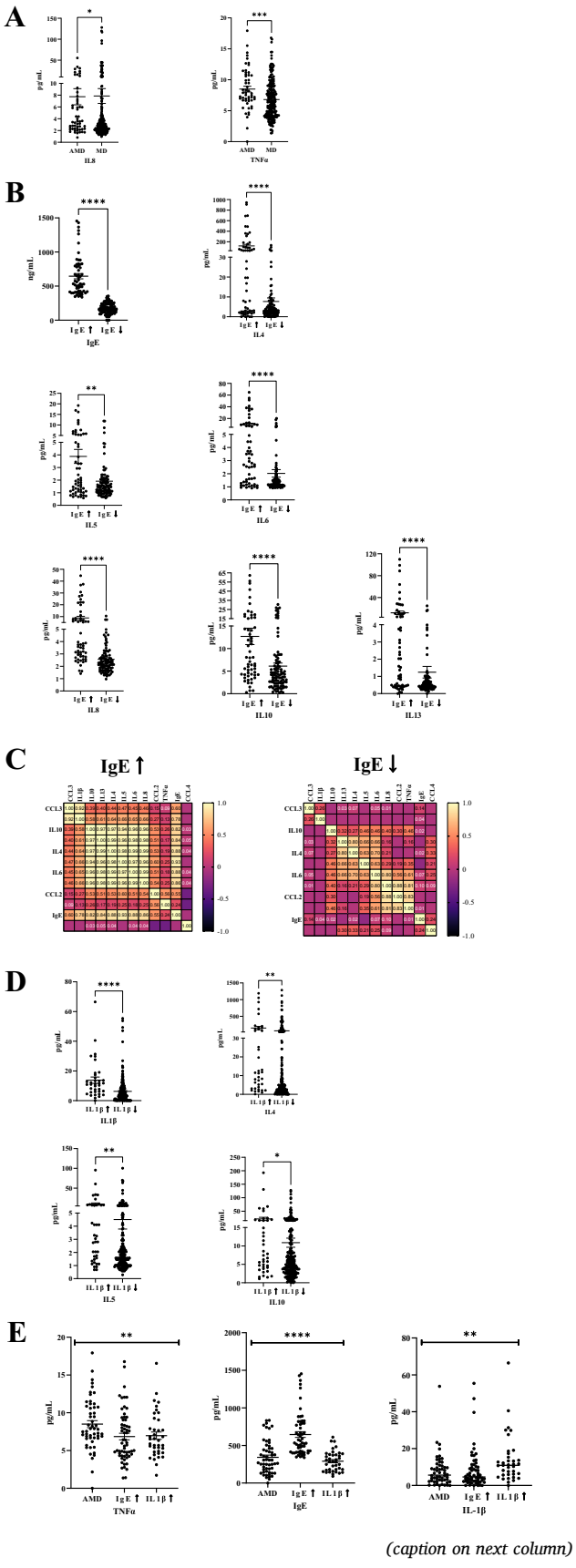

Fig. 1. Cytokine profile according to the pre-defined groups (AMD, Allergy, and Autoinflammatory). (A) IL-8 ($p = 0.027$) and TNF-α ($p = 0.0004$) levels are higher in the AMD group compared to the rest of the patients. (B) When comparing IgE↑ vs IgE↓, the levels of IL-10 ($p < 0.0001$), IL-13 ($p < 0.0001$), IL-4 ($p < 0.0001$), IL-5 ($p = 0.001$), IL-6 ($p < 0.0001$) and IL-8 ($p < 0.0001$) are significantly different. (C) Correlation matrixes in the IgE↑and IgE↓groups. (D) IL-10 ($p = 0.02$), IL-4 ($p = 0.002$), IL-5 ($p = 0.002$) and IL-1β ($p < 0.0001$) were all elevated in the IL-1β↑ group. (E) When comparing all 3 groups simultaneously, only 3 cytokines remained significantly different: TNF-α, IgE, and IL-1β. Of note, each was elevated in their pre-defined group.

58 % of patients, had all cytokines low (non-immune phenotype), group B (23 %) had all cytokines high except for TNF-a and CCL2, and group C (19 %) had high IgE but all cytokines low. Taking our pre-defined groups, we found that 81 % of group A were patients with low levels of cytokines, and groups B and C were comprised mainly of allergic patients, with the difference of group C with 36 % of patients previously defined as autoinflammatory (Fig. 2D).

3.6. Allergy downstream cascade initiates M2/M1 macrophage polarization

We treated THP-1 cells (monocyte cell line) with patients' plasma from the IgE↑ and Low groups and observed their polarization towards M1 ($CD80^+$) or M2 ($CD32^+$) macrophages. We found significant differences in the expression of $CD32^+$ and the double (+) staining, higher in the IgE↑ group ($p = 0.0002$ and $p = 0.0022$, respectively; Fig. 3A, B). The ratio of $CD32^+$ cells in IgE↑ vs. Low was 2:1, and the proportion of $CD80^+CD32^+$ cells when comparing the High vs. the Low Group was 1.5:1. Both $CD80^+$ and double (−) groups had a ratio of 1:1.

To better understand what pathway had been activated and could trigger this cytokine release, we performed qPCR of several potential transcription factors critical to the IL-4 activation (Fig. 3C). However, none were statistically significant when compared against the Low Group.

3.7. IL-1β seems to be activated through the NLRP3 inflammasome

We found that IL-1β levels increase over time (as shown in Supplementary Fig. 1; $p = 0.04$), but the years of evolution within the groups Low and IL-1β↑were not significant ($p = 0.4$, Table 2).

We stimulated M0 macrophages with IL-1β↑ and IgE↑ patients' plasma to study their polarization capacity (Fig. 4A). We observed a similar M1 ($CD80^+$) polarization but a lower capacity regarding M2 ($CD32^+$) in the IL-1β↑ group.

To understand the molecular pathways activated by the IL-1β↑ group we compared gene expression by qPCR in PBMCs RNA from IL-1β↑ and Low groups and found statistically significant differences in the expression of CASP1, GSDMD, IL1B, NLRP3, and NFKB1 (Fig. 4B).

4. Discussion

Clinical allergy is caused by dysregulated type 2 immunity, classically characterized by high levels of immunoglobulin E (IgE) and various cytokines, such as IL-4, IL-5, IL-9, and IL-13 [33]. IgE is central to acute allergic reactions and chronic inflammatory allergic diseases [34]. By contrast, autoinflammatory diseases are a group of clinical syndromes portrayed by constitutive overactivation of innate immune pathways. These results in increased production of or responses to monocyte- and neutrophil-derived cytokines such as interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α). Conventionally, type 2 immune cells and autoinflammatory effectors were considered to neutralize each other. However, growing evidence suggests that, in some contexts, autoinflammatory pathways and cytokines may enhance type 2 immune responses. Conversely, type 2 immune cells and cytokines may regulate autoinflammatory responses in complex and context-dependent

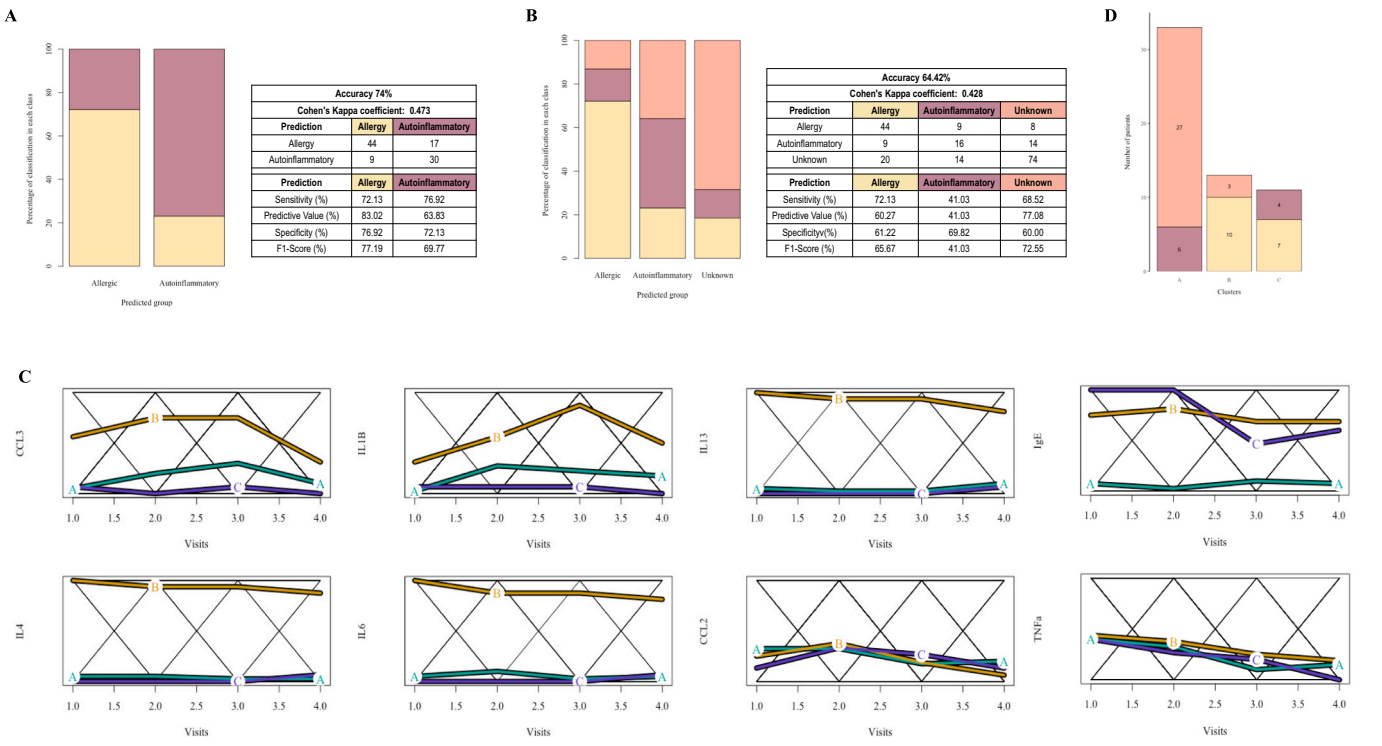

Fig. 2. WBDA and trajectory analysis. (A) The prediction model takes our 2 immuno-phenotype known groups according to IgE and IL-1β levels, finding a better prediction with an F1 score of 77 % in the allergic group and 70 % in the autoinflammatory group. (B) WBDA according to pre-defined groups: Autoimmune, Allergy, Autoinflammatory and Unknown. We tried to categorize the Unknown group into one of the abovementioned groups. We found that it was a different group (F1-Score of 73 %) representing the LOW levels of cytokines group. (C) Three clusters were segregated according to all their levels of cytokines. Group A had all cytokines LOW, Group B had all cytokines HIGH, and Group C had only high levels of IgE. (D) Number of patients included in each group: Group A = 33, Group B = 13, and Group C = 11. The colors represent to which pre-defined group these patients were previously assigned. Yellow = Allergy, Red = Autoinflammatory, and Orange = LOW. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

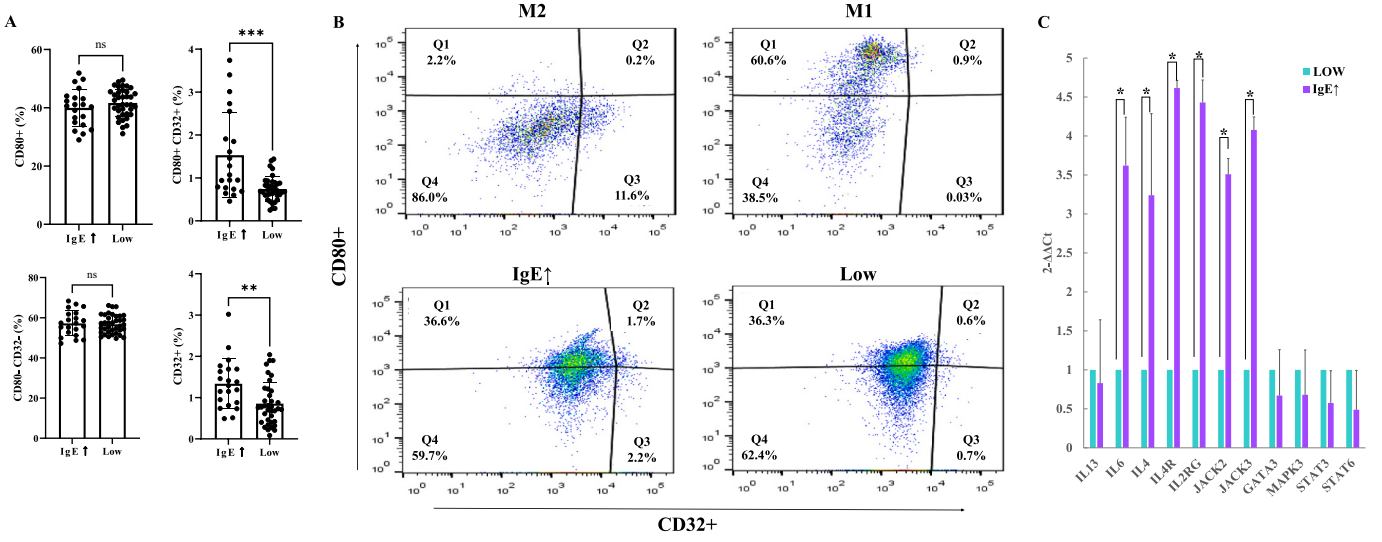

Fig. 3. (A) M0-derived THP-1 cells stimulated with MD patients' plasma. We compared $CD80^{+}$, $CD32^{+}$, double (+), and double (−) expressing cell percentages between IgE↑ and Low groups. (B) Flow cytometry graphs of CD80+, CD32+, double (+), and double (−) cells in M1 and M2 derived macrophages and IgE↑and Low groups. (C) Representation of the $2^{-\Delta\Delta Ct}$ qPCR results.

manners [35].

Our longitudinal data shows MD patients who have not received treatment in the previous 6 months, suggesting that their condition is not active. Nonetheless, 20 % of patients had a comorbid autoimmune condition, 25 % showed persistent high levels of IgE and the suite of cytokines associated with an allergic response, 13 % were characterized by elevated IL-1β associated with several inflammatory cytokines in at least 2 visits, and 42 % of patients have normal basal levels of cytokines.

Due to several mechanisms, exacerbated inflammation may occur without clear symptoms. Subclinical inflammation can persist, affecting inner ear tissues without noticeable symptoms, and elevated levels of inflammatory markers in remission might indicate ongoing molecular

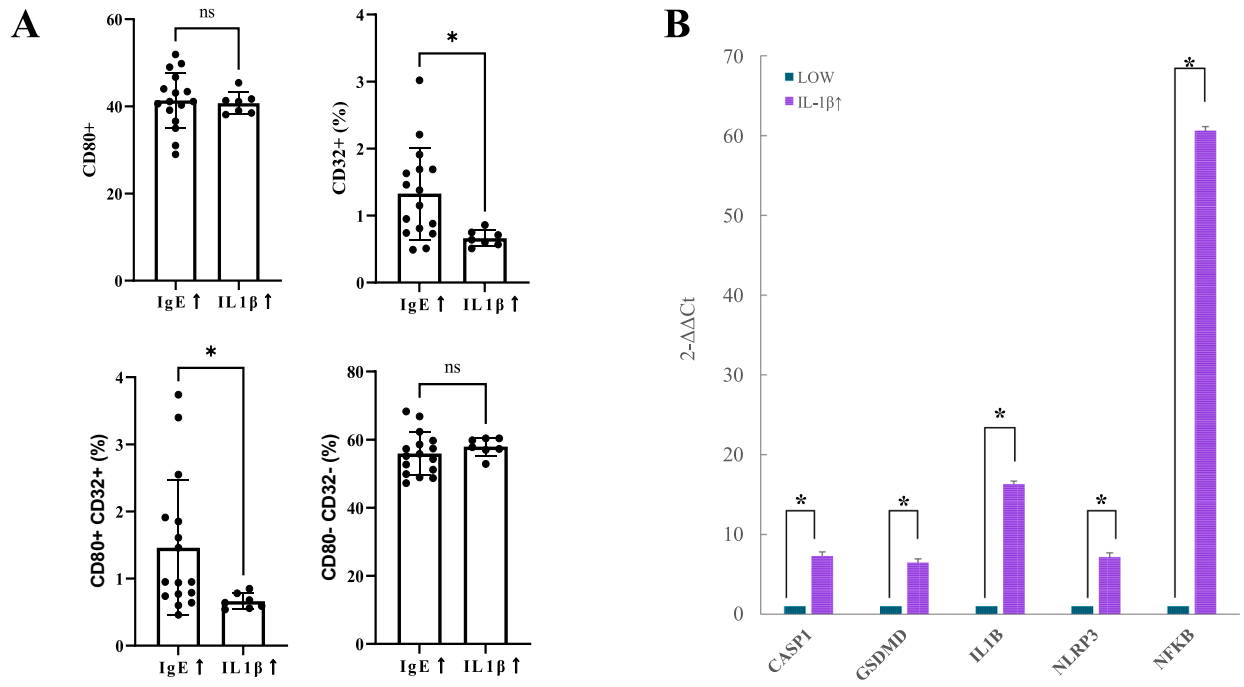

Fig. 4. (A) M0-derived THP-1 cells stimulated with MD patients' plasma. Comparison of $CD80^+$, $CD32^+$, double (+), and double (−) expressing cell percentage between Groups High and IL-1β↑. *$p < 0.05$. (B) Representation of the $2^{-\Delta\Delta Ct}$ qPCR results from patients' PBMC comparing patients from the Low Group against patients with only high levels of IL-1β (IL-1β↑).

immune activity, highlighting MD's complex nature.

Historical and contemporary studies have proposed that allergies may contribute to MD, given that patients with MD often have a high prevalence of comorbid allergies and positive allergy immunology markers such as IgE deposits in vestibular end organs [36]. Elevated IgE levels suggest an allergic component in MD, linking immune responses to the disease's pathogenesis. Clinically, this implies that allergic reactions may exacerbate MD symptoms, such as vertigo and hearing loss, by promoting inflammation in the inner ear, and anti-allergy treatments have shown beneficial effects in both patients and animal models. Recently, a study described increased levels of IgE, IL-4, IL-5, IL-10, and IL-13 in serum samples from MD patients compared to controls [19]. Moreover, Flook et al. identified a subgroup of patients with an altered immune response involving IgE and IL4 leading to persistent inflammatory status [20]. Both studies and the current data support the idea that 24 % of MD patients will have type 2 inflammation.

IL-4 is a critical regulatory cytokine during the immune response, particularly important in allergy and asthma. When resting T cells are antigen-activated and expand in response to Interleukin-2 (IL2), they can differentiate as Type 1 (Th1) or Type 2 (Th2) T helper cells. IL-4 influences the Th1 or Th2 fate. Th2 cells secrete IL-4, which both stimulates Th2 in an autocrine fashion and acts as a potent B cell growth factor to promote humoral immunity [37]. Of note, IL-13 is an immunoregulatory cytokine secreted by activated Th2 cells. It is a crucial mediator in the pathogenesis of allergic inflammation. IL-13 shares many functional properties with IL-4, and IL-13 receptors are expressed in human B cells, basophils, eosinophils, mast cells, endothelial cells, fibroblasts, monocytes, macrophages, respiratory epithelial cells, smooth muscle cells, but unlike IL-4, not in T cells. Thus, IL-13 does not appear necessary in the initial differentiation of CD4 T cells into Th2 cells; instead, it is essential in the effector phase of allergic inflammation [38].

To study the potential upstream events driving the release of allergy-related cytokines, we investigated the expression patterns of several receptors and known Th2 response genes. IL4R, IL2RG, JACK2, and JACK3 were significantly higher in the allergy group compared to the Low group, but GATA3, MAPK3, STAT3, and STAT6 were not found to be significant. So, to further validate our TH2 response hypothesis, we studied the ability to polarize macrophages using plasma from MD patients.

Macrophages adopt various functional phenotypes in response to the microenvironment, typically becoming polarized to M1 (classically activated, also known as proinflammatory) or M2 (alternatively activated) subtypes [39,40]. While classically activated M1 macrophages are induced by TH1 cytokines like interferon γ (IFNγ) or interleukin 1β (IL-1β), alternatively activated M2 macrophages are triggered by TH2 cytokines such as IL-4 and IL-13, inducing an anti-inflammatory phenotype by signaling through IL4R alpha in a STAT6-dependent manner [41,42]. Thus, in our study, we have studied the potential of patients' plasma to polarize M0 macrophages derived from THP-1 cells into M1 or M2 macrophages, finding that MD patients with high levels of the allergy cocktail cytokines were able to polarize to M2 macrophages when compared to patients with low levels of cytokines. However, the group of patients with high levels of IL-1β did not have that ability.

As mentioned above, inflammation plays a vital role in wound healing. Monocytes/macrophages and leukocytes coordinate debris removal and initiate wound healing by attracting endothelial cells, epithelial cells, and fibroblasts. However, if chronic inflammation occurs, this process can become dysregulated, leading to pathological wound repair, accumulation of permanent fibrotic scar tissue at the injury site, and failure to restore normal function to the tissue [43,44]. Nowadays, the role of cytokines as fibrotic mediators has become well-established. Initially, several signaling cascades are modified by changes in the microenvironment involving Th1 and Th2 cytokines being released from damaged epithelial or endothelial cells but also because of fibrocyte recruitment and differentiation, activation, and proliferation of pericytes via epithelial to mesenchymal transition (EMT) or endothelial to mesenchymal transition (EnMT).

Cochlear dysfunction, therefore, can be caused by dysfunction of spiral ligament fibrocytes (SLFs), a primary cell type in the lateral wall, essential for maintaining endocochlear potential and blood flow [45]. SLF dysfunction due to chronic inflammatory responses driving fibrosis can consequently result in impaired hearing. Oxidative stress has also been linked to sensorineural disorders like MD [46–48]. Reactive

Oxygen species (ROS) can damage cochlear structures, while inflammation may exacerbate cellular stress and auditory dysfunction.

NLRP3 and IL-1β expression is reduced in M2 cells [49], and inhibition of NLRP3 inflammasome drives M2 polarization [50]. Interestingly, NLRP3 activation can also cause M2 polarization via the upregulation of IL-4 in an inflammasome-independent process [51]. NLRP3, but not the inflammasome adaptor ASC or caspase-1, may promote the polarization of M2 macrophages by up-regulating the expression of IL-4, thereby contributing to its regulation of asthma. Although NLRP3-controlled M1/M2 polarization is essential, an accurate understanding is yet to be elucidated. Furthermore, a study reveals that the downregulation of serum/glucocorticoid-inducible kinase 1 (SGK1) is linked to the activation of the NLRP3 inflammasome in vestibular macrophage-like cells from MD patients. This activation leads to increased production of IL-1β, which subsequently damages inner ear hair cells and the vestibular nerve [52].

Taking everything into account, we can say that heightened levels of inflammatory cytokines indicate ongoing inflammation, even during periods of clinical remission. We have a group of MD patients with an allergy-type response with high levels of IL-4, IL-13, and IgE, capable of polarizing macrophages into M2-type, a second group with a classical proinflammatory response activated via inflammasome, led by IL-1β with enormous amounts of NFKB and a third group with no disturbance in their cytokine levels. These results emphasize the importance of targeting inflammation to prevent disease progression and symptom flare-ups.

However, some limitations should be noted. The small sample size limits the generalizability of our findings, and the study's observational nature does not allow for the establishment of causality. In a recent study, Xie et al., found causal correlations between inflammatory cytokines, like IL-10 and neurotrophin-3 and MD [53].

Larger, prospective studies are needed to confirm these results and explore the dynamic relationship between cytokines, macrophage polarization, and MD progression over time. Additionally, while we observed alterations in cytokine profiles, the mechanisms underlying these changes remain poorly understood and require further investigation to elucidate the molecular pathways at play.

Despite these limitations, the clinical implications of this study are noteworthy. Monitoring cytokine profiles, including IgE levels and inflammatory markers such as IL-4, IL-13, and IL-1β, may offer valuable insights into disease status and help identify patients at risk of exacerbations or progression to more severe forms of MD. This could enable personalized treatment strategies, particularly for patients with an allergy-driven inflammatory response. Given the role of inflammation in MD, therapies targeting specific inflammatory pathways could prevent disease progression, mitigate symptoms such as vertigo and hearing loss, and improve overall patient outcomes. Additionally, the inability of patients with high IL-1β to induce M2 polarization suggests a dysregulated immune response that may drive the chronic inflammation observed in these patients. The relationship between macrophage polarization, chronic inflammation, and inner ear dysfunction is a potential therapeutic target that warrants further investigation.

Looking forward, future research should focus on large longitudinal studies that track changes in cytokine levels and macrophage polarization over longer time periods to better understand how these immune mechanisms evolve and contribute to MD's clinical course. Additionally, interventional studies are needed to assess the effectiveness of anti-inflammatory treatments and immunomodulatory therapies in altering the disease trajectory, particularly in patients with allergy-associated inflammation.

In conclusion, this study provides valuable insights into the immunological mechanisms underlying MD and highlights the potential of biomarkers and targeted therapies in managing inflammation and improving disease outcomes. Although further validation in larger cohorts is necessary, our findings open avenues for novel therapeutic strategies aimed at controlling inflammation in MD patients, ultimately enhancing clinical management and patient quality of life.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2024.110413.

Funding

This work was supported by Instituto de Salud Carlos III (FEDER Funds from the EU;ISCIII; Grant# PI20–1126), the Andalusian Health Department (Grant #PI027–2020), and the Meniere's Society, UK (Grant CLINMON-2). LF is supported by ISCIII Sara Borrell Fellowship (CD20/0153), the F18/00228 funded MF, and PPC is funded by the Andalusian Health Government (Grant RH-0150-2020).

CRediT authorship contribution statement

Lidia Frejo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francisca E. Cara:** Writing – review & editing, Investigation, Formal analysis. **Marisa Flook:** Writing – review & editing, Methodology, Investigation. **Paula Robles-Bolivar:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Alba Escalera-Balsera:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Maria Alharilla Montilla-Ibañez:** Writing – review & editing, Resources. **Emilio Dominguez-Duran:** Writing – review & editing, Visualization, Resources. **Marta Martinez-Martinez:** Writing – review & editing, Visualization, Resources, Conceptualization. **Patricia Perez-Carpena:** Writing – review & editing, Visualization, Resources, Conceptualization. **Jose Antonio Lopez-Escamez:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

We want to acknowledge all the patients participating in the study. We would also like to recognize Alberto M. Parra Perez for suggestions with discriminant analysis, the technical support of Estrella Martinez, Jesus Vela, and Andreina Peralta with blood samples, and Ana Ibañez from the Fundamental Biology Core facility at CIBM for helping with the Luminex readings.

Data availability

Data will be made available on request.

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