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Cross-reactivity between *Mycobacterium avium subspecies paratuberculosis* 4027 peptide and Human IRF5 may contribute to Multiple Sclerosis in Iranian patients

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ABSTRACT

Background: The etiology of Multiple sclerosis (MS) is complicated and can be affected by several environmental factors, such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in genetically predisposed individuals. The link between MAP and MS depends on host genetic and epigenetic aspects and population-based features that require further investigation. We aimed to study the possible role of MAP in triggering MS using molecular and serological methods.

Materials and methods: This case-control study examined 200 blood samples (100 MS patients and 100 HCs) to search for the MAP-specific IS900 gene. In addition, ELISA was conducted to determine the humoral response against MAP_4027$_{18\text{-}32}$ and its human IRF5$_{424–434}$ peptide homolog.

Results: The frequency of MAP detection based on the molecular method in MS patients and HCs was 48 % and 13 %, respectively ($p < 0.0001$). The presence of antibodies against MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ was 55 % and 65 % in MS patients versus 9 % and 7 % in HCs, respectively ($p < 0.0001$). A good correlation was observed between MAP_4027 and IRF5 antibodies ($r = 0.5782$, $p < 0.0001$), indicating that the same antibodies recognized common peptide epitopes.

Conclusion: Our research revealed a significant association between MAP and MS, highlighting the possible role of MAP as an important infection trigger factor of MS. It is hypothesized that cross-reactivity between MAP4027 and IRF5 may dysregulate immune homeostasis.

1. Introduction

Multiple sclerosis (MS) is a neurological illness that affects the central nervous system (CNS) and is characterized by an autoimmune process that leads to nontraumatic neurological disabilities. The histological signs of MS include leukocyte infiltration, demyelination, neurodegeneration, and reactive gliosis, which cause gradual declines in motor, sensory, and cognitive abilities [1,2]. MS impacts women about 2 or 3 times higher than men [3]. There are four recognized MS subtypes, including clinically isolated syndrome

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(CIS), relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS) [3]. CIS describes the first instance of neurologic symptoms brought on by inflammation and demyelination in the central nervous system. The most prevalent disease course, RRMS, exhibits clearly defined bouts of advancing neurologic symptoms and is the first diagnosis for about 85 % of the patients. Typically, SPMS appears to be 10–15 years after the start of RRMS, evolving gradually from sporadic relapses to slowly progressing illnesses [3]. Primary progressive (PPMS), a less prevalent subtype, is characterized by a progressive decline in neurological function when the disease first manifests without signs of remission [4]. The prevalence of MS is rising worldwide, and 5 to 300 MS cases per 100,000 persons were reported in 2021 [5]. The studies revealed a high prevalence rate of MS in Iran (100 per 100, 000 people in 2021) [6].

The fundamental cause of MS has yet to be well known. This disorder is complicated and can be affected by several environmental factors, such as ultraviolet B light (UVB) exposure, vitamin D, obesity, smoking, and infections in genetically predisposed individuals [7–11]. Relapses in MS is associated with an influx of T cell and macrophage entering the CNS, suggesting that peripheral cells may be drawn to the CNS due to recurring antigen drainage [12–14]. The previous studies imply microbial infections, including Epstein–Barr virus (EBV), endogenous retroviruses, *Chlamydia pneumonia,* and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections trigger autoimmunity, resulting in clinical disease symptoms. In addition, a strong connection between HLA-DRB1 and MS has been explained [15]. The DRB1*1501-DQB1*0602 haplotype was reported as one of the important haplotypes in the Iranian MS population [16].

MAP is an intracellular pathogen with a macrophage tropism and a causative organism of severe enteritis in dairy cattle (John's disease) [17]. MAP contamination in the environment is pervasive, as the milk, liver, manure, and other products of the infected animals can contaminate the soil, water (where it resists chlorination), and different environments [18,19]. The prevalence of paratuberculosis in Iran compared to developed countries showed higher contamination, especially in herd tank milk (59 % versus 28 %) [20]. It was proposed that MAP transmission from livestock to people primarily happens via the fecal-oral route because humans are not the natural host of MAP [21,22]. Following the colonization of MAP within the host body, molecular mimicry may influence the host's immune system due to the presence of peptide sequences that resemble those found in host cells [23,24]. Multiple investigations, including meta-analysis, have demonstrated that individuals with Crohn's disease have higher levels of MAP DNA and anti-MAP antibodies than healthy controls in Spain, the UK, and the USA [25–28]. MAP has also been linked to type 1 diabetes (T1D) [29], rheumatoid arthritis (RA) [30], Hashimoto's thyroiditis (HT) [31], and MS [9], although the role of this bacterium in the pathological process has often been controversial. Some investigations have discovered MAP in MS patients [9,32]. In Sardinia, where MAP is endemic, the relationship between MAP and MS was first identified [33]. The genetic susceptibility to MS in Italy is associated with five DRB1*-DQB1* haplotypes [34].

In recent years, the incidence of MS has been rising in Iran, consistent with worldwide trends. This disorder has a significant impact on the socioeconomic condition of the patients. Paratuberculosis is also becoming more prevalent, and it could put people at risk of MAP infection in different ways, which in turn may contribute to autoimmune disorders like MS. Considering the importance of this problem requires further study. Moreover, Due to the limited research on the relationship between MAP and MS in the Middle East and Iran, we aim to investigate the role of MAP in the development of MS.

2. Materials and methods

2.1. Subjects

This case-control study included two hundred participants, including 100 patients (80 RRMS and 20 SPMS patients), 79 females and 21 males with (median age of 36.99 ± 9.88), and 100 healthy controls (HCs), including 85 females and 15 males with (median age of 39.21 ± 10.8) (Table 1). The time of recruiting and sampling participants was between 2022 and 2033, and all participants were from the Golestan province of Iran, located southeast of the Caspian Sea.

Neurologists diagnosed MS patients according to the criteria of McDonald 2017 [35]. The patients did not have any disease or

Table 1
Demographic and clinical characteristics of groups.

		MS[a] n = 100	HCs[b] n = 100	Total n = 200	*P*-Value
Age	Mean ± SD[c]	36.99 ± 9.88	39.21 ± 10.8	38.24 ± 10.43	0.785
Gender	Female	79 (79 %)	85 (85 %)	164 (82 %)	0.269
	Male	21 (21 %)	15 (15 %)	36 (18 %)	
BMI[d]	Mean ± SD	28.37 ± 5.65	25.55 ± 2.93	25.85 ± 4.12	0.963
Blood type	A	17 (17 %)	18 (18 %)	35 (17.5 %)	0.268
	B	15 (15 %)	12 (12 %)	27 (13.5 %)	0.079
	AB	5 (5 %)	7 (7 %)	12 (6 %)	0.934
	O	22 (22 %)	53 (53 %)	75 (37.5 %)	0.005
Type of MS	Relapsing-Remitting	80 (80 %)	–	–	–
	Secondary progressive	20 (20 %)	–	–	–

[a] MS; Multiple Sclerosis.
[b] HCs; Healthy Controls.
[c] SD; Standard Deviation.
[d] BMI; Body mass index.

history of other autoimmune diseases. Also, the control subjects had no known infectious or autoimmune disease history. The exclusion criteria for the patients were any known autoimmune disease or cancer. The HCs were selected among individuals who did not have any known disorders and were matched with the patients in the case of gender. All subjects accepted their informed consent before sampling was taken. The ethical committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1401.238) confirmed the study protocols. Table 1 displays the demographics and characteristics of the patients and HCs.

2.2. Blood sample collection and processing

Five ml of blood was taken from each group; DNA extraction was done using a whole blood extraction kit (GeneAll® Exgene™ Blood SV, Korea), and then DNA samples were kept at −70 °C for PCR test.

The sera were separated by centrifuge at 3000 RPM for 10 min, then transferred to a sterile 1.5 mL microtube and stored at −20 °C for ELISA.

2.3. Nested-PCR

Nested PCR determined MAP frequency. The specific primers for amplifying the IS900 MAP gene are listed in Table 2 [36]. The first round of PCR was performed using DreamTaq PCR Master Mix 2X (Ampliqon, Denmark) containing Taq polymerase, dNTPs, MgCl2, and appropriate buffer. Each PCR microtube contained 12.5 μl of reaction mixture consisting of 9.5 μl of master mix, 0.25 μl of each forward and reverse primer solution (at a final concentration of 10 pmol) (Pishgam, Tehran, Iran), five μl of DNA with a concentration of 80 ng/μL and nuclease-free water to complete the final volume. In the second round, 2.5 μl of the PCR product of the first step was used, but the values of the remaining items were the same as in the first round. The first round of temperature cycling includes amplification consisting of initial denaturation at 95 °C, 300 s, followed by 30 cycles of denaturation (95 °C, 40 s), annealing (58 °C for 55 s), and extension (72 °C for 1 min), with a final extension step (72 °C, 7 min). In the continuation of the temperature cycle of the second round, the same as the first round was performed. Amplified DNA was examined by electrophoresis using 1.5 % gel. The positive control in the PCR test was the clinical sample in which both PCR and culture were positive. The negative control in the PCR test was ultrapure water instead of sample DNA.

2.4. Serological assay

Synthetic peptides derived from MAP antigen ($MAP\_4027_{18–32}$) [AVVPVLAYAAARL-LL] and human homologs ($IRF5_{424–434}$) [VVPV—AARL-LLE] were used in the study. The peptide sequences of MAP_402718–32 and IRF5424–434 were obtained from GenBank and aligned to determine their homology. The peptides were synthesized commercially (Biomatik, Wilmington, Delaware, 19809, USA) with a purity >90 % and kept frozen in single-use aliquots [10 mM] at −70 °C [37]. Indirect Enzyme-Linked Immunosorbent Assays (ELISA) were conducted to detect specific Abs for all the synthetic peptides.

Plates from 96 wells were overlaid overnight with 2 μg/mL of each peptide in a 0.05 M solution of carbonate-bicarbonate, pH 9.6 at 4 °C. The plates were washed five times with 0.05 % Tween-20 (in PBS) and blocked with 5 % gelatin in PBS for 2 h at room temperature (25 °C). Serums (1:50 dilution) were added and incubated for 1 h at 37 °C. Secondary antibody conjugated with horseradish peroxidase (HRP) rabbit anti-human immunoglobulin G polyclonal Ab (1:3500). The plates were washed during each incubation. Horseradish peroxidase was detected with 3,3′, 5,5′-tetramethyl benzidine (TMB). The optical density (OD) was read at a wavelength of 405 nm using an ELISA plate reader (BioTek ELx800, USA). There were 100 μL/well incubation volumes in total. Normalization was performed with a positive control induced in each test, and each sample was rubbed in duplicate. The mean signal of an immobilization peptide with secondary Ab alone was used to calculate background activity. Additionally, as a positive control, we used a highly reactive serum from a positive patient in all ELISA plates, whereas as a negative control, we substituted the patient's serum with PBS and then proceeded to add the secondary antibody.

2.5. Statistical analysis

The statistical analysis was done using SPSS16 and GraphPad Prism 9.0 software to describe the investigated variables in the control and patient groups from the statistical indices. A significance level of 5 % will be considered. For qualitative data analysis, the Chi-Square test was used. For quantitative data, after checking the normality of the data with the Shapiro-Wilk test, the independent *t*-test and Mann-Whitney were used for normal and non-normal data, respectively.

Table 2
Primer sequences and amplicon size.

Gene	Primer 5′–3′	Amplicon size (bp)
L	F: CTTTCTTGAAGGGTGTTCGG R: ACGTGACCTCGCCTCCAT	402
AV	F: ATG TGGTTGCTGTGTTGGATGG R: CCGCCGCAATCAACTCCAG	298

3. Results

3.1. Demographic information

Analysis of demographic data showed that BMI was higher in MS patients (28.37 ± 5.65) than in HCs (25.55 ± 2.93) (p-value = 0.963). Also, the blood group analysis showed that blood group O was significantly lower in the patients than HCs (22 % vs. 53 %), respectively (p-value = 0.005).

3.2. MAP frequency by Nested-PCR assay

MAP IS900 was detected in 48 % of MS patients (36 % RR and 12 % SP) and 13 % of HCs. The findings indicated that MAP frequency was significantly higher in MS patients than HCs ($p < 0.0001$, Table 3.)

3.3. Seropositivity against MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$

Considerable differences were detected between the MS patients and HCs in the positivity of anti-MAP Abs and its human homolog. The positive percentage of antibodies against MAP_4027$_{18\text{-}32}$ in MS patients (55 %) was higher than in HCs (9 %) ($p < 0.0001$) (Fig. 1 A). Anti-IRF5$_{424–434}$ Abs were detected in 65 % of MS patients and 7 % in HCs ($p < 0.0001$, Fig. 1Bc). Additionally, results demonstrated that MAP_4027$_{18\text{-}32}$ and its homolog IRF5$_{424–434}$ antibodies were positive in (56.2 % and 62.5 %) of RRMS and (50 % and 75 %) of SPMS, respectively. The difference between these two groups was not significant.

The percentage of MS and HCs with positive results for both MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ were 49 % and 11 %, respectively ($p < 0.0001$) (Fig. 1C). This status was determined in 48.8 % of RRMS and 50 % of SPMS (p value = 0.920).

In addition, MS patients and HCs with at least one positive result for MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ were 71 % and 11 %, respectively. The statistical significance of this difference between patients and HCs was assessed (p-value = <0.0001). Also, this positivity was observed in 70 % of RRMS and 75 % of SPMS (p-value = 0.659) (Fig. 1 D).

The distributions of optical density (OD) of Abs against MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ are shown in Fig. 2. In HCs, the OD ranges of MAP_4027$_{18\text{-}32}$ were from 0.1 to 1.1 (Mean ± SD, 0.314 ± 0.19), whereas, in MS patients, they were between 0.1 and 1.9 (Mean ± SD, 0.669 ± 0.43) (Fig. 2 A). Similarly, for IRF5$_{424–434}$, the findings showed that the OD of the patients ranged from 0.2 to 1.5 (Mean ± SD, 0.829 ± 0.37) higher than those of HCs (from 0.1 to 1.1) (Mean ± SD, 0.313 ± 0.17) (Fig. 2 B).

3.4. Examination of correlation between anti-MAP4027 and anti-IRF5 antibody ODs

Fig. 3 revealed a relationship between the antibody response to MAP4027 and IRF5 peptides in MS patients and HCs. We discovered a modest correlation between MAP_4027$_{18\text{-}32}$ with IRF5$_{424–434}$ (r = 0.5782, $p < 0.0001$, Fig. 3).

4. Discussion

MS is characterized by demyelination, axonal damage, and brain and spinal cord gliosis. In this way, it causes irreparable damage to the patient's physical health, leading to mental and psychological damage. In addition, because the drugs used to treat this disease are immunosuppressive, people are at risk of reactivating latent pathogens, worsening asymptomatic chronic infections, and contracting new infections, especially tuberculosis, hepatitis B, and herpes viruses [38].

The MS group included 80 % RRMS and 20 % SPMS patients in this study. The number of RRMS was higher in our study; it is the initial diagnosis for approximately 85 % of patients and is the most common type of MS [1–3]. Also, our SPMS patients had a longer disease duration than the RRMS [1–3]. The previous data indicated that this type typically occurs about a decade before the onset of SPMS. According to previous research, MS affects women at a rate approximately two to three times higher than men [3], and in this project, most patients were women (79 %). In a similar study, the number of females was more than that of males in MS patients [37].

A more accurate understanding of the factors that trigger MS can prevent the irreversible consequences of this disease. As mentioned, MAP is one of the underlying environmental factors of this disease. MAP primarily affects ruminants but can sometimes infect humans via the fecal-oral route. It may be an infectious agent contributing to MS in genetically predisposed individuals. This

Table 3
MAP frequency in MS patients and HCs.

		Nested-PCR		p-value
		Positive	Negative	
[a]MS Patients	Relapsing-Remitting	36 (36 %)	44 (44 %)	0.230
	Secondary progressive	12 (12 %)	8 (8 %)	
	Total	48 (48 %)	52 (52 %)	<0.0001
[b]HCs		13 (13 %)	87 (87 %)	

[a] MS; Multiple Sclerosis.
[b] HCs; Healthy Controls.

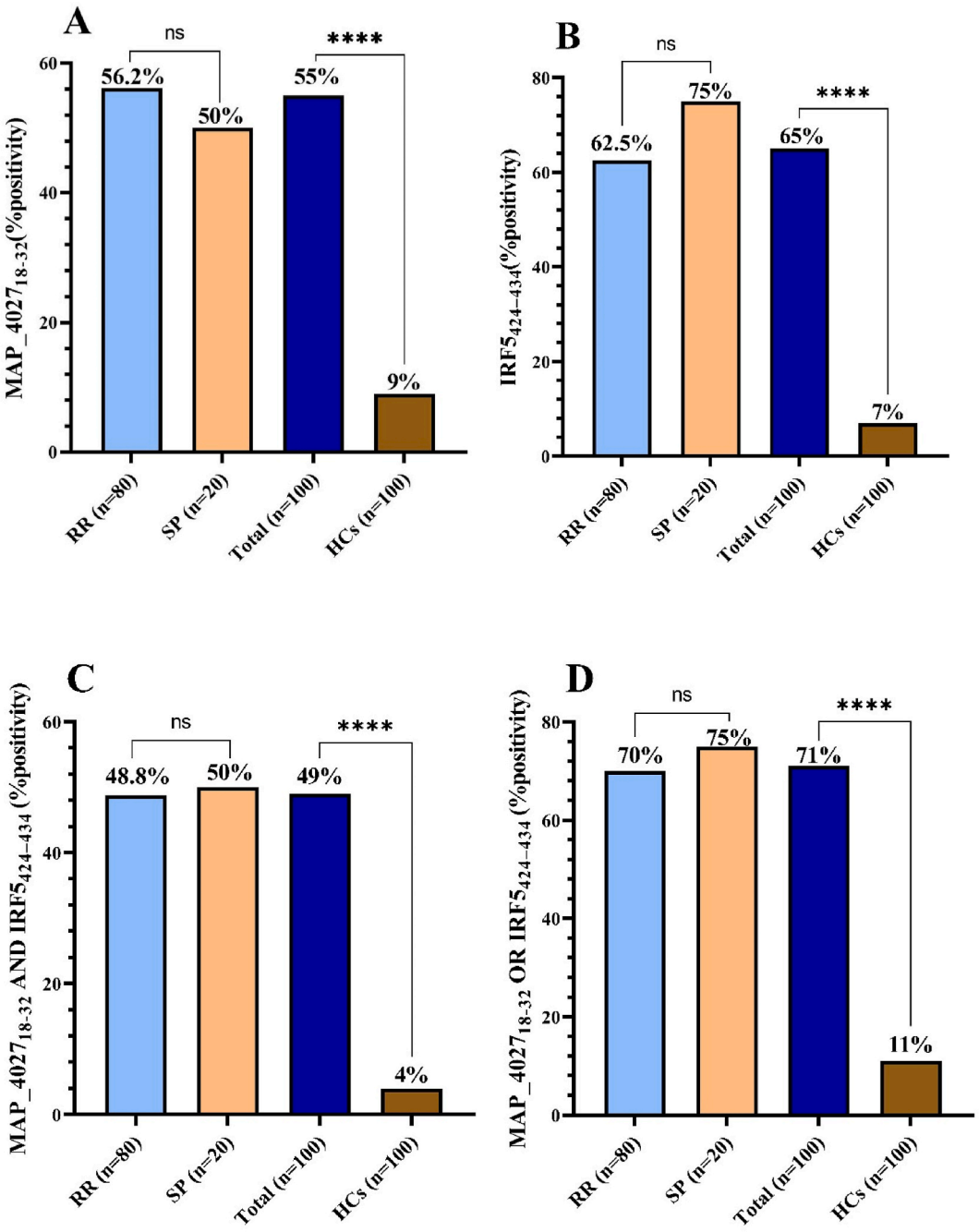

Fig. 1. Prevalence of Antibodies against MAP_4027$_{18-32}$ and IRF5$_{424-434}$ antigens in Multiple Sclerosis (MS) patients (Relapsing-remitting Multiple Sclerosis (RRMS) and Secondary Progressive Multiple Sclerosis (SPMS)) and healthy controls (HCs). The percent of positive is shown by bars. *P-values* are demonstrated above the summary. (A) MAP_4027$_{18-32}$ (B) IRF5$_{424-434}$ (C) MAP_4027$_{18-32}$ and IRF5$_{424-434}$ (D) MAP_4027$_{18-32}$ or IRF5$_{424-434}$. (ns; not significant).

bacterium can stimulate humoral and/or cellular immunity to target epitopes through different techniques, such as molecular mimicry, based on amino acid sequence or structural motif homology between microbial epitopes and self-protein [39,40]. The studies about the link between MAP and MS were conducted in some regions; however, this relationship in the Middle East area, as well as the Iranian people, had yet to be seen. Therefore, it is necessary to explore different populations to understand better the range of infection and involvement in MS.

In our study, MAP frequency was seen significantly more in the blood samples of MS patients than in the HCs. In an investigation conducted by Cossu et al. in Italy in 2011, using the PCR test, MAP was detected in 42 % of MS patients and only 12.5 % of the control

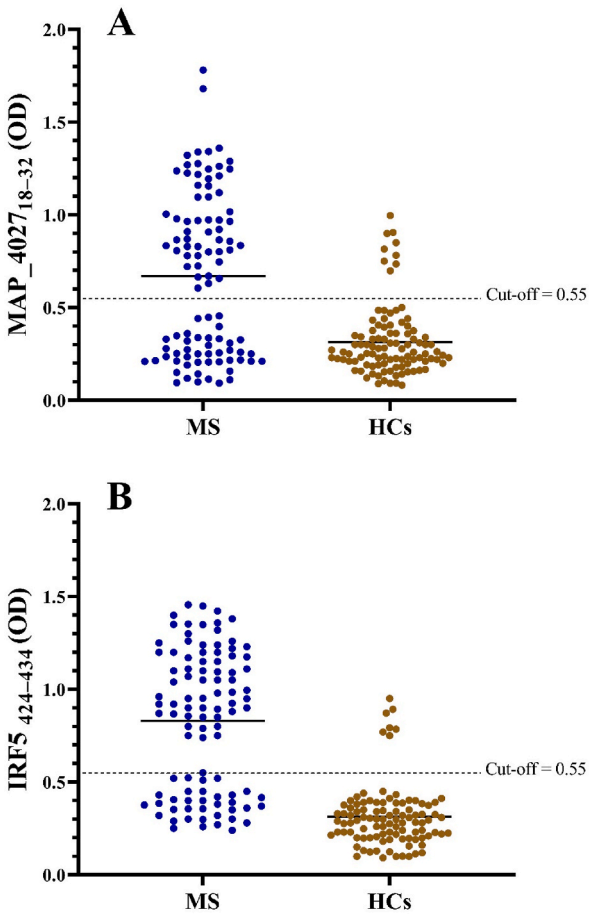

Fig. 2. The distribution of Optical density (OD) of ELISA results for MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ in Multiple Sclerosis (MS) patients and healthy controls (HCs). The dotted line shows the receiver operating characteristic (ROC) analysis determined positive levels.

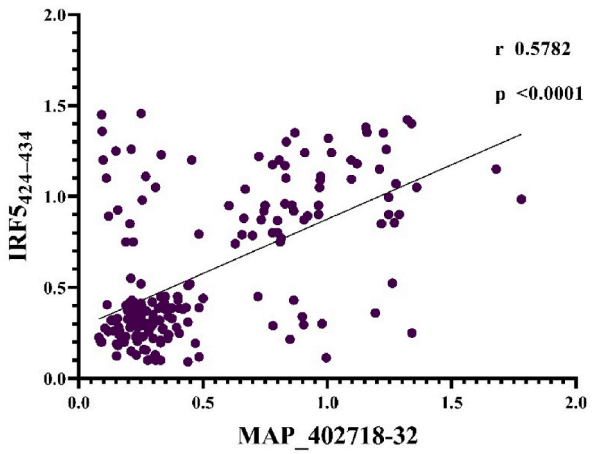

Fig. 3. Scatter plot showing the correlation between MAP_4027$_{18\text{-}32}$ and IRF5$_{424–434}$ peptides in Multiple Sclerosis (MS) patients and healthy controls (HCs).

group [33]. Also, another study conducted by Cossu et al., in 2012 identified MAP DNA in 27.5 % of MS patients and 6.3 % of healthy controls [41]. These reports showed the importance of MAP in the development of MS.

MAP can imitate molecules by using different peptides and escapes from the immune system. One of these antigens is MAP_4027,

the homolog of IRF5 in the human host [42]. IRF5 is a molecular switch determining the stimulation or inhibition of inflammation, which emphasizes the protein's function in an autoimmune condition [43]. IRF5 is involved in the control and synthesis of cytokines essential in physiology [44]. In some studies, IRF5 polymorphism has been linked to MS and affects IFNβ therapy's pharmacological and clinical results [45–47]. In this study, the positive percentage of anti-MAP_4027 antibodies was higher in Iranian MS patients than in the control group ($p < 0.0001$). Also, the level of antibodies against its human homologs, $IRF5_{424–434,}$ was higher in the patients than in HCs ($p < 0.0001$). These findings might explain that MAP infections in MS may have caused specific humoral immunity to react against the IRF5 host protein and alter the immune response. Thus, the destruction of myelin may trigger the immune response by generating antibodies against cached antigens.

MS usually begins with an RRMS course. This relapsing-remitting course is often followed by a phase of worsening neurological function independent of the relapse, called SPMS [48]. In this study, the percentage of positive PCR and antibodies against the $MAP\_4027_{18\text{-}32}$ peptide group were higher in RRMS than in SPMS type. In contrast, the antibody against its human homolog, $IRF_{5424\text{-}434}$, although not statistically significant, appeared to be more prevalent in SPMS than in RRMS. This hypothesis can be associated with the epitope spreading theory. Additionally, it may show that, firstly, active MAP could be presented in MS patients, especially with earlier stages of disease and antibodies against MAP produced. After that, in the later stages, antibodies against human homologous peptides developed. In this regard, considering that the two epitopes have amino acid sequence similarities and considering the strong correlation observed in the antibody titer through correlation analysis, one would anticipate similar antibody titers. However, this hypothesis needs a larger sample size and cohort study design.

One study demonstrated that antibodies against $MAP\_106c_{121–132}$ were higher in MS patients than in unspecified neurological disease (UND). Additionally, this peptide was seen in the CSF of 46 % of MS patients, and none of the UND controls. Also, antibodies against IRF peptide were found in the CSF of 44 % of MS patients and 11 % of controls and the sera of 63 % and 9 % of MS patients and the control subjects [32]. In another study, more antibodies against $MAP2694_{259–303}$, $MAP\_0106c_{121\text{-}132,}$ and $MAP\_4027_{18\text{-}32}$ were reported in Japanese MS patients than in the controls [49].

Additionally, in our study, the OD ranges of $MAP\_4027_{18\text{-}32}$ and $IRF5_{424–434}$ antibodies in the MS patients (0.669 ± 0.43 and 0.829 ± 0.37, respectively) were significantly higher than the HCs (0.314 ± 0.19 and 0.313 ± 0.17, respectively). Mameli et al. also showed that the OD measured against the $MAP\_4027_{18\text{-}32}$ peptide in the MS group ranged from 0 to 0.9, while in the non-inflammatory neurodegenerative disease (NIND) and unspecified neurological disease (UND) groups were 0–0.6 and 0 to 0.5, respectively. Their data explained higher rates of OD in the MS group than in the other groups [32].

This study observed a good correlation between MAP_4027 and IRF5 antibodies ($r = 0.5782$, $p < 0.0001$), confirming that the same antibodies recognized common peptide epitopes. In a survey conducted in Italy, they observed a correlation between two peptides, $IRF5_{424–434}$ and $MAP\_4027_{18–32}$, in serum ($R^2 = 0.47$; $p < 0.0001$) and in CSF ($R^2 = 0.40$; $p < 0.0001$) [32]. These results showed the possible cross-reactivity between these two peptides; however, this data needs further investigation to be approved. Further research on animals has shown that oral administration of MAP activated mucosal immunity and worsened acute EAE in C57BL/6 J mice via altering immune cell traffic from secondary lymphoid organs to the central nervous system [50]. As a result, it is probable that MAP contributes to the pathogenesis of MS and worsens the patient's condition.

In our previous investigation, we MAP DNA and antibodies in autoimmune disorders like RA (not yet published), and HT in this area [51,52]. These findings indicated that people in this region are repeatedly exposed to MAP, and it emphasizes the importance of the healthcare system being aware of this problem.

The limitation of this study was the retrospective nature of the survey, that restricts some data availability. Therefore, designing cohort studies for a larger sample size, especially with different groups of all MS types and stages, could be helpful.

In conclusion, these findings showed that MAP could be involved in the development of MS; however, the relationship between MAP and MS may be a phenomenon that depends on the population and is heavily reliant on many genetic and non-genetic elements. On the other hand, simultaneous response to MAP peptide and its homologs, known as IRF5, implies the importance of cross-reactivity as an important mechanism of MAP pathogenesis in MS development. Finding Antibodies against MAP in the serum of MS patients might be considered as an extra criterion essential to comprehending the pathogenesis of MS. Further studies should examine other processes of cellular as well as humoral immune responses for better understanding to explore this hypothesis across many nations and continents.

Ethics statement

The study was conducted following the Declaration of Helsinki and approved by the Ethics Committee of Golestan University of Medical Sciences (IR.GOUMS.REC.1401.044).

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Negar Asgari: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ezzat Allah Ghaemi:** Writing – review & editing, Validation, Formal analysis, Conceptualization. **Mohammad Hasan Naeimi:** Writing – review & editing, Data curation. **Alireza Tahamtan:** Writing – review & editing, Conceptualization. **Leonardo Antonio Sechi:** Writing – review & editing,

Conceptualization. **Samin Zamani :** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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