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Research Article

Utility of Myelin Basic Protein as an Early Prognostic Biomarker in Multiple Sclerosis

Samiie Pouragahi,[1,3,7] Marjan Nassiri-Asl,[2,*] Mohammad Ali Sahraian,[3] Mehdi Sadeghi,[4] Abdolali Banki,[5] Zahra Zamanzadeh,[6] Mitra Ataei,[6] and Mohammad Hossein Sanati[7,*]
[1]Department of Molecular Medicine, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[2]Cellular and Molecular Research Center, Department of Pharmacology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran
[3]Multiple Sclerosis Research Center, Sina Hospital, Department of Neurology, Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Bioinformatics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[5]Department of Neurology, Baqiyatallah University of Medical Sciences, Baqiyatallah Hospital, Tehran, Iran
[6]National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[7]Department of Molecular Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

[*]*Corresponding authors*: Mohammad Hossein Sanati, Department of Molecular Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. Tel: + 98-44580301, Fax: +98-2144580396, E-mail: marjan_nassiriaslm@yahoo.com; Marjan Nassiri-Asl, Cellular and Molecular Research Center, Department of Pharmacology, School of Medicine, Qazvin University of Medical Sciences, Qazvin, Iran. Tel: +98-2833336001, Fax: + 98-2833324971, E-mail: mnassiriasl@qums.ac.ir

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Abstract

Background: Myelin basic protein (MBP), a crucial neuro-autoantigen involved in the maintenance of the myelin sheath, is one of the biomarkers of therapeutic response in multiple sclerosis (MS).
Objectives: The study examines prognostic biomarker and molecular mimicry hypothesis MS etiology by MBP.
Methods: This study is convergence of three arms including in silico and in vitro (bioinformatics) with the in vivo (experimental). A novel methodology combining molecular techniques was used to confirm the antigenic properties of MBP and study its efficiency in increasing the susceptibility to MS. One hundred eighty MS patients and healthy subjects were recruited for the study from Jan 2013 to Feb 2016 in Iran. Age and sex-matched healthy volunteers and patients were analyzed using various quantitative and qualitative molecular laboratory techniques. Peripheral blood mononuclear cells (PBMCs) and plasma was used for the retrieval of MBP and IgG assay, respectively.
Results: The optimum concentration of the MBP epitope for the immune system to react and facilitate prognostication was found to be 50 and 150 μg/mL in MS patients and healthy individuals, respectively ($P < 0.0001^{****}$). Combined results from ELISA and real-time PCR showed that the total IgG and the ratio of gene expression for candidate human MBP epitope was higher in MS patients in all the three groups compared to that in healthy controls $P < 0.0001^{****}$).
Conclusions: Molecular assays in the early stages of the disease could help in elucidating the effectiveness of the MBP as a prognostic factor in MS.

Keywords: Multiple Sclerosis (MS), Myelin Basic Protein (MBP), Real Time PCR, ELISA, Molecular Mimicry

1. Background

Most of the effective biomarkers -antibody, peptides, and proteins- play a role in mediating the effects of genetic/environment etiological factors, and can predict, on a case-to-case basis, the presence of a preclinical state or the progression of the disease (1). The efficiency of therapeutic interventions in patients with progressive multiple sclerosis (MS), in whom disease severity does not correlate as well with clinical symptoms as in patients with early-stage disease, needs to be studied (2). This can lead to the identification of potential tools, based on quantitative and qualitative molecular methods, for the assessment of autoimmune response in neurodegenerative disorders that will be accurate, sensitive, and be able to identify the disease process even in the absence of overt symptoms (2). Most of the identified protein biomarkers are indicative of inflammatory processes and neurodegenerative diseases in general, and are not specific to the processes related to myelin sheath disruption (1). Identification of biomarkers specific to myelin sheath disruption is important as the disruption promotes molecular mimicry (3) and causes neurodegeneration (4).

Myelin, an asymmetric multilamellar membrane with a polyunsaturated lipid-rich structure, has several hundred protein biomarkers (~ 80% of total protein fraction). These proteins and their self-epitopes are susceptible targets for an immune attack (5). Among the myelin-specific proteins, myelin basic protein (MBP) acts as a surrogate endpoint of clinical outcome in MS. It is a highly positively charged (pI > 10.5) peripheral protein that can be intrinsically disordered, and plays a crucial role in the mainte-

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nance and formation of the myelin sheath (6). The immunogenic wealth of MBP (especially its antigenic epitopes) is emphasized not only by its unique structure but also by its relation to the autoimmune demyelinating diseases of the central nervous system (CNS) involving perturbation of the myelin sheath such as MS (7).

Molecular experimental investigations over the past three decades identified target of autoantigen (8-10), affecting either the CNS or PNS in the myelin sheath. In the CNS, a major autoantigen component of myelin in the adhesion of myelin is maintained by Myelin basic Protein (MBP) (8, 11). MBP is a highly positively charged peripheral protein and, plays a crucial role in the maintenance and formation of the myelin sheath (6). Since MBP has been termed an executive protein (12) and molecular sieve (13). It is necessity/essential for CNS myelin formation, unlike groups such as proteolipid protein (PLP) and myelin-associated protein (MAG) (6). Moreover, MBP is an autoantigens in experimental autoimmune encephalomyelitis (EAE) which can be related to the etiology of Multiple sclerosis (14) unlike myelin oligodendrocyte glycoprotein (MOG) (6).

MS, a complex, chronic, multifactorial disease, is characterized by gene/environment reciprocity, enigmatic etiology, and uncertain prognosis even after 150 years of research (1). It is the most common reason for neuroinflammatory disability, has a 2: 1 gender bias toward young females, may affect single or multiple organ systems, and results in a considerable decrease in the quality of life (15). About 2.5 million people around the world are estimated to be suffering from this disease; rates of MS are higher in regions farther away from the equator (16, 17). The Iran MS Registry, the result of a 10-year national survey, showed that the prevalence was 51.9 per 100,000 persons, female to male ratio was 2.60, and age at disease onset was 27.2 $\pm$ 8.3 years (18-20). MS is more common in between the ages of 20 - 39 years (18, 19). A lack of knowledge of the MS etiology is one of the biggest challenges in therapeutic decision-making (2). It is hypothesized that the increased exposure of MBP-immunodominant epitopes (21, 22) along with the interplay of environmental factors could play a possible role in the disease etiology. Thus, the candidate autoantigen MBP might be of use at various stages of the disease in assessing therapeutic efficacy (2, 23). Few studies have been published on this subject. Previous MBP epitope prediction studies were based on in vitro methods rather than a combination of in silico and in vitro methods (24-27). This study attempts, for the first time, to study the differences among three groups of thirty subjects each; untreated MS, untreated MS with and without family history, and untreated MS at two different time points of diagnosis (early and late). We used quantitative and qualitative molecular techniques to identify the optimum concentration of MBP required to induce an immune response in the early stages of MS. MBP is also considered to be a therapeutic response biomarker. Hence, an effort to combine molecular techniques with a resource-efficient theoretical bioinformatics approach might aid in the development of personalized medicine for MS.

2. Objectives

The present study examines role of MBP as a prognostic biomarker and evaluates the molecular mimicry hypothesis of MS etiology by using MBP to induce the immune system. MBP was used at varying concentrations to assess the optimum concentration of the antigen required to induce cellular immunity and to understand its prognostic value to facilitate early diagnosis in MS.

3. Methods

This study is convergence of in silico and in vitro (bioinformatics) with the in vivo (experimental) which is performed in Tehran, Iran.

3.1. Epitope Synthesis

Synthesis of the epitope was carried out for the 15-amino-acid-long candidate antigenic sequence of MBP in KJ Ross-Peterson APS, chemical research and development laboratory, Denmark. Stock solutions of the synthesized MBP epitope [25 mg/mL] were stored in single-use aliquots at -80°C.

3.2. Subjects

The research population included patients admitted to the Multiple Sclerosis Research Center of selected hospitals in Tehran (Sina hospital, Tehran University of Medical Science, and Baqiyatallah hospital). New cases of MS were recruited as patients. Subjects were also assessed using the expanded disability status scale (EDSS) (28).

All subjects underwent an MRI scan and the reports were reviewed. All patients were examined by a neurologist. Patients who had received either pulse therapy or immunosuppressive drugs prior to the commencement of the study were excluded, since, the interaction between MBP and any related drugs for the treatment of MS might lead to confounding results. Moreover, patients who had not showed symptoms or signs of acute MS, also, patients or healthy volunteers had any acute infections or underwent immunomodulatory treatment during the 10 months prior to the commencement of the study were included.

Plasma and PBMCs were isolated from fresh blood samples obtained from the subjects, and were used for the evaluation of standard biomarkers that indicate the status of the immune system. The diagnosis of MS was based on the revised McDonald diagnostic criteria for definite MS (29-31).

3.2.1. Ethical Standard

The protocol was approved by the local ethics committee of Qazvin University of Medical Sciences (28.20.7934). A written informed consent was obtained from subjects prior to their participation in the study.

3.2.2. Sample Size

One hundred eighty patients and healthy subjects were participated in this study (30 subject in each group) as a sample size formula with a power of 0.0052 and an attrition rate of 20%. Three groups of thirty subjects, untreated MS, untreated MS with and without family history, untreated MS at two different time points from diagnosis (1 - 15 and 15 - 23 days) were recruited for the study (n = 90). Healthy individuals were recruited to serve as controls (n = 90). Age and sex-matched healthy volunteers and patients were analyzed using various quantitative and qualitative molecular laboratory techniques.

3.3. PBMC and Plasma Harvesting

The candidate human MBP epitope sequence (MBP 110 - 124, MBP 84 - 98, MBP 304 - 319, common to all the classic and non-classic isoforms) with 100% homology pattern including a panel of molecular mimicry between MBP and microorganism sequences was isolated using first phase outcome, a new methodology based on comprehensive bioinformatic predictions. PBMC and plasma were separated from fresh whole blood specimen collected using a sterile BD Vacutainer® CPT™ cell preparation tube (8 mL draw capacity) with sodium heparin (16 × 125 - mm tube size) (Becton Dickinson and company, Franklin Lakes, N.J., USA), according to the manufacturer's protocol. The plasma was collected from fresh blood at the time of PBMC extraction.

3.4. Cell Culture

For proliferation assessment, PBMCs (150 - 200 μL/well, 0.5 - 0.6 $\times 10^6$ cells/mL) were incubated in a 24-well round bottom microtiter plate (TPP techno plastic products, Switzerland) in Dulbecco's modified eagle medium (DMEM) (Gibco, 1X), supplemented with 10 % (w/v) autologous heat-inactivated fetal bovine serum (Gibco, USA) and antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin) (Sigma-Aldrich, USA). For both MS patients and healthy controls, cells grown in plates were seeded at 37°C in a humidified 5% CO_2 chamber overnight, in preparation for other experiments. In addition, for the first time, the rate of proliferation of fresh human PBMCs was modulated in a dose-dependent manner by stimulating them with varying concentrations of MBP (range: 25 - 150 μg/mL in 25 μg/mL steps). This enabled the assessment of the optimum concentration of the antigen required to induce cellular immunity and facilitated better understanding of the prognosis to improve early diagnosis of MS. The viability (percent of live cells) of the fresh PBMCs was assessed immediately after preparation.

3.5. Real-Time PCR

Total RNA was isolated from CPT-processed fresh PBMCs using Roche's high pure RNA isolation Kit (Roche diagnostics GmbH, Roche applied science, Germany), according to the manufacturer's instructions. The quantity and quality of extracted RNAs were determined by 1% agarose gel electrophoresis and spectrophotometry (Nano drop-1000) and samples stored at -80°C. cDNA synthesis was performed using a RevertAid First Strand cDNA synthesis kit (Thermo scientific, EU, US), according to the manufacturer's instructions (Cat NO # K1622).

Real-time PCR was performed using β-actin as an endogenous reference gene (32), and proliferating cell nuclear antigen (PCNA) for PBMCs treated with MBP as a target gene was measured (33).

Mi-real-time EvaGreen® master (2×) (Biotium, Inc., Hayward, CA, USA) was used according to the manufacturer's instructions. The reaction mixtures contained 10 μL mi-real-time EvaGreen® Master (2×), 0.3 μL of the forward primer (10 μM), 0.3 μL of the reverse primer (10 μM),1 μL of template, and 8.4 μL DEPC water, for a final reaction volume of 20 μL. A final extension step was carried out at 95°C for 2 minutes, 95°C for 15 seconds, 62°C for 8 seconds, 70°C for 10 seconds, and PCR products were stored at 4°C. Real-time PCR was performed using a Rotor gene 6000 fast Real Time PCR system (Corbett Biosystems, Corbett Research, Mortlake, NSW, Australia) which was calibrated.

Primer sets were PCNA F: 5'-AGCACCAAACCAGGAGAAAGT-3' R: 5'-TCACTCCGTCTTTTGCACAG-3', β-actin F: 5'- AGACGCAGGATGGCATGGG-3' R: 5'-GAGACCTTCAACACCCCAGCC-3'.

3.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Since commercially synthesized anti-MBP was not available, a manual protocol was used to detect antibodies against MBP epitopes. MaxiSorp 96-well plates (Nunc, Denmark) were pre-coated with a bicarbonate buffer at 37°C overnight, followed by three washes using phosphate-buffered saline, PBS-Tween containing 0.5% BSA, bovine

serum albumin and 0.05% Tween 20. The carbonate-plated wells were coated with 20 μg/mL of the positive control (MBP antigen) at 4°C and incubated overnight. The wells with MBP antigenic epitope were blocked with 1% BSA in PBS buffer and incubated at 37°C for 2 hours. The plasma-plated wells (1: 10), as primary antibodies, were incubated with 10% BSA in PBS buffer at room temperature for 2 hours or 4°C overnight.

Anti-human IgG (whole molecule) peroxidase produced in goat (IgG fraction of antiserum antibody, 1: 1000) (Sigma-Aldrich, USA) was added for 2 - 3 hours at room temperature. Approximately 100 μL of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, USA) 0.4 M was added to each well as the liquid substrate system for peroxidase substrate, and the plates were incubated at 37°C in the dark on the shaker for 15 - 45 minutes. IgG antibodies against the selected epitope were detected by measuring absorbance using a Varioskan Flash microplate reader (Labsystem Multiskan MS Primary Eia, Version 1.2 - 0.0, Helsinki, Finland) at 450 nm which was calibrated. Poly IgG anti-MBP antibodies (1: 10) were used as a negative control. MBP epitope incubated in the absence of IgG served as an additional negative control (Blank). This test was performed three times in triplicates.

The concentration of IgG in the samples was calculated with the help of the standard curve using the kit (R and D, US), according to manufacturer's recommendations. For additional information related to IgG assessment and the rate of PCNA expression, the line slope equation and R squared (R^2) were measured in different groups.

3.7. Immunoblotting/Dot Blotting

Pure synthesized MBP epitope was spotted onto 0.45 μm pore-size nitrocellulose membranes and air-dried in a BioRad biodot system. Membranes were blocked with a blocking buffer with 5% BSA (PAA laboratories GmbH, Colbe, Germany) and 5% skimmed milk powder (Heirler Cenovis GmbH, Radolfzell, Germany) in PBS for 2 h at room temperature on the shaker and incubated with Poly IgG anti-MBP antibodies (at 1: 1, 1: 10, and 1: 100 dilutions) for 1 hour at 37°C on the shaker. Anti-human IgG (whole molecule) peroxidase produced in goat (IgG fraction of antiserum antibody diluted in PBS, 1:1000) (Sigma-Aldrich, USA) was added and incubated for 1 - 4 hours at 37°C. Detection was performed by adding 1 cc of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, USA) on the shaker for 10 - 15 minutes. Membranes were stopped with blocking buffer or DEPC water.

3.8. Immunocytochemistry (ICC)

PBMCs were incubated overnight with purified MBP epitope at 37°C in CO_2 and were fixed with 3% - 4% paraformaldehyde (PFA) at 4°C for 15 minutes. Permeabilization was performed by incubating the samples for 5 minutes in PBS containing 0.2% Triton X-100. Cells were incubated in 10% goat serum (diluted in 1% BSA in PBST) in a humidified chamber for 1 hour at room temperature, with the primary antibody (diluted in 2% BSA in PBS) for 45 minutes at room temperature, and with the secondary antibody, anti-human IgG (whole molecule) peroxidase produced in goat (IgG fraction of antiserum antibody, 1: 200), in 1% BSA for 45 minutes at room temperature in the dark. Cells were counterstained for with 0.1 - 1 mg/mL (200 μL) propidium iodide (PI, a fluorescent molecule that can be used to stain cells) for 1 minute. Coverslip was applied and sealed to prevent drying and movement under microscope (Neu-Isenburg, Germany) with a confocal microscope (Nikon A1R MP).

3.9. Statistics Analysis

Statistical analysis, including the preparation of graphs and charts, was carried out using GraphPad Prism 6.0 (San Diego, CA, USA). Categorical variables were reported as numbers and percentages, while continuous variables were reported as mean $\pm$ standard deviation (SD). Student's independent t-test was performed to determine the statistical significance of the difference between two independent groups. Statistical differences among multiple groups were tested by one way analysis of variance (ANOVA) followed by the Tukey-Kramer test for post-hoc analysis. $P < 0.05$ was set as the threshold for statistical significance. Relative Expression Software Tool (REST), RG-version 3 (REST, 2005) was used to analyze the real-time PCR data following the -$\Delta\Delta$Ct method (Pfaffl method) (34).

4. Results

Sequences were first isolated following epitope prediction in the theoretical arm of this study. It was observed that the epitope sequences of MBP were of variable lengths that ranged from (110 - 124), (7 - 21), (38 - 52), and (156 - 170) (Figure 1). However, all the sequences (110 - 124, 7 - 21, 38 - 52, 156 - 170, and 217 - 231) are common epitopic antigens belonging to either the classic or non-classic MBP sequence isoforms, based-on priority antigenicity. In addition, the collections of viral and bacterial protein families with 100% pattern homology showed cross-reactivity with the isolated candidate epitopes on the MBP sequence and other hypothetical protein family sequences of the microorganisms involved in MS. It was found that fifteen different proteins from sixteen bacterial species and thirty-nine proteins from forty-three viruses showed cross-reactivity with

the isolated candidate epitopes on the MBP sequence. The total number of protein families present in all microorganisms was low.

This was also confirmed by the molecular mimicry between MBP epitope sequences with other microorganisms assumed to be related to MS. To our knowledge, these findings have been reported here for the first time. The synthetized candidate epitope of MBP peptide has the following analytical properties: Net Weight (25.7 mg), molecular weight (1797.11 Da), and purity of 95.86%.

Ninety consecutive untreated MS patients in three groups (F/M = 18/12), with a mean age of 26.63 ± 6.47 year and 90 gender- and age-matched healthy volunteers in three groups (F/M = 18/12), with a mean age of 26.93 ± 6.42 year, were enrolled in this study. The expanded Kurtzke's expanded disability status scale (EDSS) criteria was evaluated (mean entry range = 1.8 ± 0.05).

The demographic details of the MS patients and healthy volunteers in the two groups are presented in Table 1.

The results of the effectiveness of different concentrations of MBP in inducing PBMCs revealed that the optimum concentration of the MBP epitope for inducing the immune system and for ascertaining the prognosis is 50 and 150 μg/mL in MS patients and healthy individuals, respectively (Figure 2A and 2B).

The ratio of total IgG level to the human MBP epitope level in MS patients was significantly higher than that in healthy individuals ($P < 0.0001$) (Table 2). Furthermore, the ratio of gene expression in PBMC treated with MBP in MS patients was significantly higher than that in the case of healthy controls ($P < 0.0001$) (Table 2).

The total IgG level in the plasma specimens of MS patients with and without family history was significantly higher than that in healthy controls ($P < 0.0001$) (Table 3). The level of gene expression in PBMC treated with MBP in patients with and without family history was significantly higher than that in healthy individuals ($P < 0.0001$) (Table 3).

The ratio of total IgG to candidate human MBP epitope in untreated MS patients at two different time points after diagnosis (early and late) was higher than that in healthy individuals ($P < 0.0001$) (Table 4). Moreover, the ratio of gene expression in PBMC treated with MBP in MS patients at 1 to 15 days and at 15 to 23 days after diagnosis was higher than that in healthy individuals ($P < 0.0001$) (Table 4).

The optimum concentrations of IgG against the MBP epitope was calculated with the help of the standard curve generated by the ELISA ($y = 0.1027 \ln(x) + 0.0887$ and $R^2 = 0.9832$) (Figure 3). The results of ICC experiments involving PBMCs treated with MBP (50 μg/well) revealed that the retrieved candidate epitope of MBP (arrowheads) could be generated in the PBMCs of patients with MS (Figure 4A and 4B). This MBP epitope was not observed in the healthy volunteers (Figure 4C and 4D).

5. Discussion

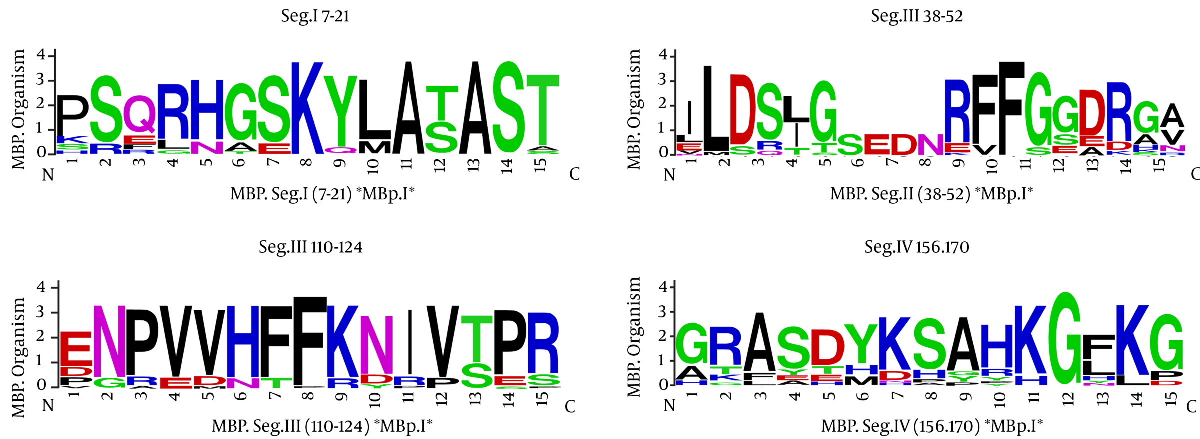
The main outcome of this study that employed a combination of quantitative molecular biology techniques and bioinformatics tools on two types of samples (PBMCs and plasma) revealed differences between patients and healthy individuals. However, there is no significant difference between MS patients with family history and no family history at the IgG level and gene expression.

The increasing rate of PCNA gene expression in treated PBMCs with MBP as demonstrated by real-time PCR technique and the increase in IgG anti-MBP antibody in ELISA demonstrated that the MBP-epitope might be a marker of early MS disease onset. It might also be helpful in the differential diagnosis in the early-stage of MS disease. From the results of the immunocytochemistry experiments, it can be deduced that epitope-MBP could serve as a good lead in MS prognosis and differentiate between patients and controls in the onset of the disease. The primary objective of this study was to induce an immunity reaction by auto antigenic MBP-epitope. To this end, fresh blood samples of MS patients who had never been treated with any medication, immunosuppressive drugs, and pulse therapy was collected. Other published studies have reported collection of blood samples after one to few years of the onset of the disease (26, 35-37). However, in the present study, the time of sample collection was one to few days after the diagnosis of the disease.

Majority of the mechanisms of MS take place mainly within the PBMC and the CNS. Hence, the other objectives of this study were to facilitate a method that would ease the process of sample collection for both the donors and researchers. The use of fresh blood sample instead of cerebrospinal fluid (CSF) results in a more sensitive experimental observation even though CSF is routinely used for clinical laboratory purposes. Furthermore, the collection of fresh blood as a sample source yields better results in comparison to the collection of subdivided individual cell types (PBMC and plasma) per the CPT-BD protocol.

In a related study, sera (less invasive) has been used instead of CSF (more invasive) (35). Mameli and colleagues (26) have used subjects who were untreated only 2 - 4 weeks before the commencement of the study. MBP sequences were bovine- recombinant and the epitope sequence was 14-amino-acid long. ELISA was performed using a commercial ELISA kit that included alkaline phosphatase-conjugated goat anti-human IgG polyclonal Ab as secondary antibody and paranitrophenyl phosphate as the

Figure 1. Sequences Isolated for Classic and Non-Classic Myelin Basic Protein (MBP) Isoforms



The major sequences as a prediction of myelin-specific proteins such as MBP isolated four candidate epitope MBP sequences at ranges of (110 - 124), (7 - 21), (38 - 52), and (156 - 170) with 15 amino acid lengths based on antigenic priority.

Table 1. The Demographic Characteristics of the Patients in the Untreated MS Patient and Control Groups[a]

Demographic Characteristics	Control (n = 90)	MS Patient (n = 90)	Statistical Test	P Value
Age, y	26.63 ±6.47	26.93 ± 6.42	t-test	< 0.05
Gender				
Male	19	19	t-test	< 0.05
Female	12	12		
BMI	20 ± 1.303	20 ± 1.593	t-test	< 0.05
Systolic	12 ± 1.53	12 ± 1.32		
Diastolic	8 ± 1.34	8 ± 1.24		
Weight	65 ± 3.21	77 ± 3.53		
Height	164 ± 3.41	178 ± 4.10		
History	-	-		

[a]Values are expressed as mean ± SD.

Table 2. Efficacy of Human Myelin Basic Protein (MBP) Epitope in Inducing Gene Expression in Combination With IgG Assay

Group	Mean ± SD		Statistical Test	P Value
	Ig.G	**Gene Expression**		
Control	5.205 ± 1.398	2.061 ± 0.2509	t-test, n = 30	< 0.0001****
untreated MS Patient	231.3 ± 27.98	96.65 ± 8.370		

substrate (26). Multiple sclerosis (MS) is a degenerative disorder rather than solely an autoimmune disease and its etiology remains enigmatic and disputed (5). Based on recent studies, classification of MS has changed and it is now considered to be an outside-in and an inside-out model of autoimmune disease and degenerative disorder (2, 7). Under such circumstances, a neuro-auto antigenic biomarker in the myelin sheath such as the MBP is important for understanding the disease etiology and for obtaining accurate diagnosis. It will also lead to a prompter prog-

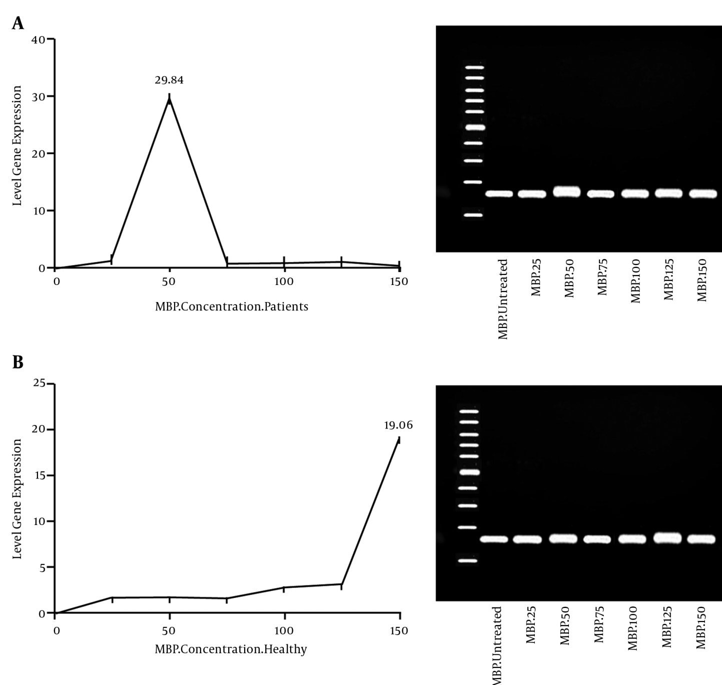


Figure 2. Efficacy of optimum concentration of myelin basic protein (MBP) to induce immune reaction in MS patients and healthy individuals with a significant difference. A, patients with MS; B, healthy individuals. t-test, n = 30, $P < 0.0001^{****}$.

Table 3. Efficacy of Human Myelin Basic Protein (MBP) Epitope in With and Without Family History MS Patients and Healthy Individuals to Confirm Gene/Environment Reciprocal Bias to Environment Element

Group	Mean ± SD			Statistical Test	P Value
	Control	Untreated MS Patient			
		With family history	without family history		
Ig.G	5.205 ± 1.398	261.6 ± 55.99	216.2 ± 31.84	Tukey-Kramer, n = 30	$< 0.0001^{****}$
Gene Expression	2.061 ± 0.2509	103.7 ± 12.30	93.13 ± 11.07		

Table 4. Effectiveness of Candidate Myelin Basic Protein (MBP) Epitope in Early Onset of the Disease

Group	Mean ± SD			Statistical Test	P Value
	Control	Untreated MS Patient			
		Early Diagnosis	Late Diagnosis		
Ig.G Assay	5.253 ± 1.254	284.7 ± 48.16	132.0 ± 15.68	Tukey-Kramer, n = 30	$< 0.0001^{****}$
Gene Expression	1.976 ± 0.1670	132.0 ± 17.53	72.33 ± 4.78		

nosis along with improving experimental and laboratory tests. This enquiry is a new approach to the causes that evoke an immune response in MS. However, very few studies have studied this subject comprehensively. Further, most MBP-epitope sequences were neither isolated from human species nor were previously tested in human MS patients. In addition, the sample type collected included CSF and sera.

In this double-phase project, none of the patients had ever been treated with any medication. The isolation of human epitope-MBP of 15-amino-acid length and its synthesis was done by theoretical and experimental phase, respec-

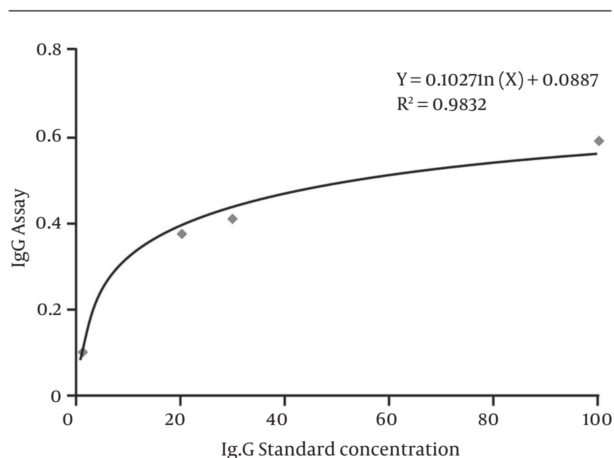


Figure 3. Standard Curve for Finding Concentration of Myelin Basic Protein (MBP)

tively. Since positive control was not available commercially, the prediction of human candidate MBP-epitope as a positive control was done in the first (theoretical) phase, and manual ELISA setup was performed in the next (experimental) phase. However, system setup outcome for ELISA, the solutions, and dilutions differed from other recent studies. Anti-human IgG (whole molecule)-peroxidase produced in goat, IgG fraction of antiserum Ab (1: 1000) and 2, 2'-azino-bis- (3-ethylbenzthiazoline-6-sulfonic acid) were used as secondary antibody and substrate in the study. In addition, the subsequent quantitative molecular tests, anti-MBP epitope IgG formation, proliferation operation that involved RNA extraction, cDNA synthesis, and real-time PCR were performed.

The other panel of studies refers to the qualitative tests that were performed either on the other myelin protein such as PLP or on the MBP (37). Furthermore, samples were collected from the spinal cord (38), brain, or the optical nerve (37). The results of the experiments evaluating the reactivity of the MBP epitope with different dilutions of plasma demonstrated that the optimum concentration could be 1: 10 dilution. Moreover, cells from MS patients reacted to the MBP epitope by producing anti-MBP IgG while this was not seen in samples from healthy individuals. The results of the dot blot revealed that the efficacy of skimmed milk powder as a blocking material is more than BSA. The results of the precise ICC demonstrated that the candidate MBP epitope was found in PBMCs of MS patients but not in case of healthy individuals.

DAPI (4', 6-diamidino-2-phenylindole) and Alexa Fluor® 594-conjugated wheat germ agglutinin (WGA) are generally used for membrane staining. However, in this study, propidium iodide (PI), a membrane-impermeant dye that is generally excluded from viable cells and is absorbed more by the PBMC, was used as a membrane staining dye.

A novel methodology combining molecular techniques was used to confirm the antigenic properties of MBP and study its efficiency in increasing the susceptibility to MS. The consolidated methodology is cost and time effective.

The strong point between the present study (in silico) and other experimental studies is the convergence of the results of the first phase (bioinformatics) with the last phase (experimental). In the first phase, epitope prediction and molecular mimicry, a favored hypothesis in MS (10), was performed by comprehensive bioinformatics studies. In the last phase, the results of the functional theoretical methodology and molecular testing data provided a solid basis and validated the reliability of the consolidated method. The focus of the experimental phase included performance evaluation of good lead biomarkers, confirmation of molecular mimicry, and MS prognosis in early stage of disease onset carried out by the synthesis of purified human candidate MBP epitopes.

In addition, it is suggested that the candidate MBP might also be considered as a surrogate endpoint for assessing therapeutic efficacy. It can also serve as a diagnosis test for susceptibility to MS in the early stage. These results also support the molecular mimicry hypothesis in MS, which posits MS as a multifactorial disorder with a bias toward environment factors.

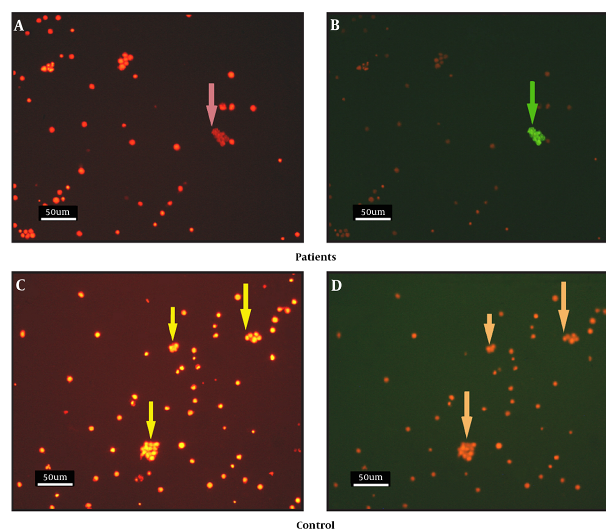
There were some limitations to this study. Since commercially synthesized anti-MBP was not available, a manual protocol was used to detect antibodies against MBP epitopes in ELISA techniques, and real-time PCR technique. In addition, the concentration of MBP between patients and controls was not assessment. Few number of MS patients who had never been treated with any medication, immunosuppressive drugs, and pulse therapy was another limitation of this study.

Future research in the field of MS biomarkers could use bioinformatics by exploiting the relationship between structure and function of antigenic peptides. Bioinformatics could also be used for the identification of detailed auto-antigenic myelin-specific proteins (candidate antigenic sequences of MBP) for personalized treatment in MS.

5.1. Conclusions

We combined quantitative molecular biology and bioinformatics tools to assess the efficacy of MBP as a biomarker of MS. Our results suggest that the MBP epitope may represent a marker of the early stage of MS and, candidate MBP may be considered as a biomarker for assessing therapeutic efficacy.

Figure 4. Effective Candidate Myelin Basic Protein (MBP) Epitope in Patients With MS, as Generated Using ICC Data



The results of ICC experimental observation in treated the human PBMCs with MBP (50 μg/well), displayed that, retrieval candidate eoitope-MBP (arrowheads) could be generated in human PBMCs of patients with MS. this eoitope-MBP was not observed in the healthy volunteers (right). Scale bar: 50 Um.

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Footnotes

Authors' Contribution: Samiie Pouragahi was a study investigator, and active participation in the data collection, analysis, and interpretation of results, and wrote the primary draft; Marjan Nassiri-Asl was involved in the interpretation of results, and review and critique of the manuscript; Mohammad Hossein Sanati proposed the idea, and revised the primary draft; Mohammad Ali Sahraian, Mehdi Sadeghi contributed to the design of the study, and data collection; Abdolali Banki contributed in data collection; Zahra Zamanzadeh and Mitra Ataei were investigators. All the authors read and approved the final manuscript.

Conflict of Interest: The authors declare that they have no competing interest in this study.

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