

Communication

Biomarker Identification in Patients with Multiple Sclerosis Treated with Autologous Hematopoietic Stem Cell Transplantation

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Abstract: Introduction: Approximately 80% of individuals with multiple sclerosis (MS) have a positive response to autologous hematopoietic stem cell transplantation (aHSCT). Markers that may predict the transplant outcome are necessary. The objective of this work is to identify markers that may refine the selection of patients with multiple sclerosis who could benefit from aHSCT. Methods: We evaluated the levels of six biomarkers in the peripheral blood of patients with MS before aHSCT. The design of this study is cross-sectional; patients were divided into two transplant-responses-at-12-months groups, responders (Δ EDSS < 0) and non-responders (Δ EDSS > 0). Pre-transplant samples were used to assess the different markers. Results: Thirty-four patients were enrolled: fourteen were non-responders and twenty were responders to aHSCT. Among the evaluated biomarkers, a significant difference was only detected in miR-146a levels, with increased values in the non-responder group. Conclusions: The biomarker miR146a could be useful to evaluate the response to aHSCT in patients with MS.

Keywords: biomarkers; multiple sclerosis; auto-HSCT



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1. Introduction

Multiple sclerosis (MS) is a demyelinating, autoimmune, inflammatory disease of the central nervous system, affecting 50 to 300 individuals out of every 100,000. Worldwide, there are currently approximately 2 to 3 million people with MS [1]. The disease is characterized by the presence of inflammation, gliosis, and demyelination, resulting in its protean clinical presentations [2].

Hematopoietic stem cell transplantation (HSCT) has been employed in the treatment of several malignant diseases such as acute leukemia, chronic leukemia, plasma cell myeloma, lymphoma, myelodysplasia, etc. It has also become a tool for the management of autoimmune diseases such as MS, aplastic anemia, systemic sclerosis, systemic lupus erythematosus, and other conditions. The cells used in HSCT can either be allogeneic or autologous, whereby human stem cells (HSCs) in allogeneic transplantation are obtained from a donor, while patients receive their own HSCs in autologous transplantation. HSCT has been used in the treatment of MS patients since the 1990s [3]. The goal of treatment with autologous hematopoietic stem cell transplantation (aHSCT) in MS is the reduction of autoreactive effector cells with immunoablative agents (conditioning regimen) followed by the infusion of HSCs to modulate the reconstitution of both the immune and

the hematopoietic systems [4]. Post-transplantation, an improvement in immunoregulation is expected and mediated by the generation of regulatory T lymphocytes (Tregs), as well as the reprogramming of residual autoreactive cells towards an anti-inflammatory phenotype [5,6].

We have developed and employed a new non-myeloablative technique to conduct HSCT in patients with MS, and have previously demonstrated its viability [7], safety [8], and usefulness [9]. Accordingly, we have also reported that among patients with MS who receive an aHSCT following our method, approximately 80% develop a positive response (40% stabilization of the neurological condition and 40% improvement) [10], as assessed by the results in the expanded disability status scale (EDSS score).

In this study, we evaluated the levels of six biomarkers in the peripheral blood of patients with MS prior to aHSCT. Each biomarker was selected according to its possible role in MS: microRNAs (miR-146a, miR-155, and miR-326) were selected as immune system dysfunction markers; interleukin-4-induced-1 (IL4I1) was selected as a remyelination marker; neurofilament light chain protein (NFL) was selected as a neuroaxonal injury marker; and the human leukocyte antigen (HLA) allele HLA-DRB1*15 was selected as an overall MS risk marker.

2. Materials and Methods

2.1. Patients and Sample Collection

Individuals with relapsing-remitting (RRMS), secondary progressive (SPMS), or primary progressive (PPMS) courses of MS were referred to our center for HSCT and those with response data at 12 months post-transplant were included in this study. Patients should have a Karnofsky performance status above 70% and an expanded disability status scale (EDSS) score of 8 or below in the two weeks prior to transplantation. None of the patients had received bone marrow-damaging agents before being included in the study and all had a normal complete blood cell count when the mobilization was started. All patients had a wash-out period of at least three months of other immunosuppressive, DMT agents. The protocol for HSCT is registered in [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02674217) identifier NCT02674217.

The design of this study is cross-sectional; patients were divided into two transplant response groups: responders and non-responders (Figure 1). Response to HSCT was assessed with the patient-reported outcome in the expanded disability status scale (EDSS) score, which was evaluated before aHSCT and one year later. The inclusion of patients in the transplant response groups was defined by the decrease (responders, $\Delta\text{EDSS} < 0$) or increase (non-responders, $\Delta\text{EDSS} > 0$) in the EDSS score when compared to the pre-transplant EDSS score. Thirty-four patients were included in this study, 23 women and 11 men, with a median age of 48 years (interquartile range [IQR]: 14), and a median disease course of 9 years (IQR: 10). In terms of the type of MS, 19 (55.9%) patients had relapsing-remitting multiple sclerosis (RRMS), 6 (17.6%) had primary progressive MS (PPMS), and 9 (26.5%) had secondary progressive MS (SPMS). Table 1 lists the characteristics of these patients.

Pre-transplant blood samples were used to analyze the different markers. Plasma and DNA samples were obtained to measure the biomarkers of interest; samples stored at $-20\text{ }^{\circ}\text{C}$ were analyzed. The techniques used for marker analysis were qPCR for microRNA measurement (miR-146a, miR155, miR326), SSP-PCR for HLA-DRB1*15, and immunoassay for IL4I1 and NFL.

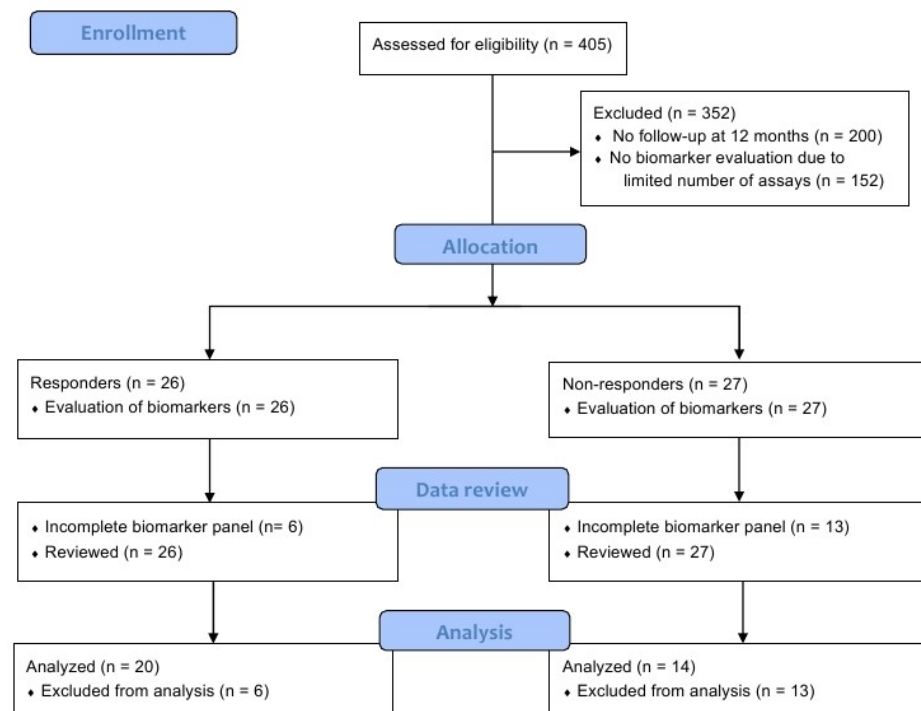


Figure 1. Flowchart of excluded and included patients.

Table 1. Characteristics of these patients.

Parameter	N = 34 Median (IQR)
Age	47.5 (42–56)
Weight	75.4 (66–88.5)
Height	1.7 (1.62–1.75)
Years with disease	8.5 (3–13)
EDSS	4.5 (3–6)
Hemoglobin (g/dL)	14.05 (13.1–15.1)
White blood cell ($\times 10^3/\mu\text{L}$)	5.9 (4.7–6.8)
Platelet ($\times 10^3/\mu\text{L}$)	259.5 (227–311)
Total protein (g/dL)	6.75 (6.4–7.1)
Glucose (mg/dL)	87.45 (84.1–92.9)
Uric acid (mg/dL)	4.615 (3.5–5.81)
Cholesterol (mg/dL)	213 (175–239)
Triglycerides (mg/dL)	87.3 (62.8–127.9)
Iron ($\mu\text{g}/\text{dL}$)	100 (71–124)
Transferrin saturation (%)	27.72 (19.5–33.96)

2.2. miRNAs

The miRNAs were extracted from plasma using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Mexico City, Mexico) and following the manufacturer's instructions. Plasma samples were thawed on ice and centrifuged at 1200 rpm for 5 min; 400 μL of plasma were then used for miRNAs extraction. In this step, 5 μL of MS2 bacteriophage were also added at a sufficient concentration to obtain a Ct of approximately 28. The MS2 bacteriophage is a single-stranded RNA virus used as an external standard that allows the standardization of miRNA levels per sample volume.

Using miRNA TaqMan assays kits (Thermo Fisher Scientific, Mexico City, Mexico), we initially synthesized cDNA from the extracted RNA with specific primers for each miRNA, and they were subsequently quantified by real-time-PCR according to the manufacturer's

instructions, on a Rotor-Gene Q 5plex (Qiagen, Mexico City, Mexico). In conjunction with the miRNA analysis protocol, quantification of bacteriophage MS2 was performed as an external reference, with the TaqPath 1-Step Multiplex Master Mix plus 5 µL RNA.

Subsequent analysis was performed with the Δ CT method of the miRNA and MS2 CTs.

2.3. DRB1*15

The DNA samples were used to determine the presence of the DRB1*15 allele. The allele was analyzed with the ARMS-PCR technique. PCR reactions were prepared in a total volume of 50 µL with the following components and using Thermo Fisher Scientific Taq DNA polymerase according to the manufacturer's instructions: 10x PCR buffer, 2 mM dNTPs, 0.2 µL of Taq DNA polymerase, 3 ng of DNA, 5 µM of each of the primers, and water for DNA synthesis. The primer sequences for DRB1*15 (all available in the IMGT/HLA database release 3.38) were as follows: forward, 5'-CACGTTTCCTGTGGCAGCCTAAGAG-3' and reverse, 5'-CGCGGCCTGCTCCAGGAT-3'. Amplification was performed in a GeneAmp PCR System 9600 (Applied Biosystems, Mexico City, Mexico) using the following protocol: 95°C for 1 minute; 35 cycles at 95°C for 20 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and 72°C for 2 minutes.

After amplification, a 2 µL aliquot underwent gel electrophoresis on 4.5% polyacrylamide gels stained with ethidium bromide and were photographed.

2.4. NFL and IL4I1

The NFL and IL4I1 biomarkers were evaluated with commercial immunoassay kits according to the manufacturer's recommendations (MyBioSource, San Diego, CA, USA. NFL: MBS167931 and IL4I1: MBS2515832). Plasma samples were added to the wells of plates coated with antibodies against each biomarker. The antigens bind to the antibodies during incubation and are detected with a peroxidase-conjugated antibody. To visualize the bound conjugate, the samples were incubated with a chromogenic substrate solution (TMB). Subsequently, the incubation process was stopped for color measurement at a wavelength of 450 nm. The amount of sample needed was 40 µL for NFL and 100 µL for IL4I1.

2.5. Data Analysis

The Kolmogorov–Smirnov test was used to evaluate the normality of the distribution of the data to be analyzed. Most of the parameters in our study do not conform to a normal distribution, so comparisons between groups were analyzed with the Mann–Whitney U-test. The χ^2 test was used for the analysis of categorical variables. Results with p values < 0.05 were considered statistically significant. Statistical analysis was performed using SPSS 25 software (IBM Corp., Published 2017. IBM SPSS Statistics for Windows, version 25.0. Armonk, NY, USA: IBM Corp.) and GraphPad Prism 9 (GraphPad Prism version 9 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Clinical Characteristics and Blood Work Results

Thirty-four (34) patients were enrolled. Fourteen (41%) were non-responders and twenty were responders (59%). The comparison of the aHSCT response groups, using the Mann–Whitney U statistical test, is shown in Table 2. These results show a statistically significant increase in the non-responder group in the following variables: hemoglobin, (median = 14.9 g/dL vs. median = 13.5 g/dL) $U = 81.5$, $p = 0.036$; serum iron levels (median = 122.5 µg/dL vs. median = 91 µg/dL), $U = 39$, $p = 0.012$; and transferrin saturation (median = 33.94% vs. median = 25.5%), $U = 30$, $p = 0.005$. In addition, an increase in

platelet count was observed in the positive-response group (median = $278.5 \times 10^3/\mu\text{L}$ vs. median = $229 \times 10^3/\mu\text{L}$), $U = 197.5$, $p = 0.043$.

Table 2. Results of Mann–Whitney U-test on the significance of differences in clinical characteristics and blood work results between response groups. * variables with statistically significant difference ($p < 0.05$).

	Non-Responders ($n = 14$)	Responders ($n = 20$)	p Value
Age	48 (8)	47.5 (17.5)	0.986
Weight	72.5 (31.5)	76.5 (18.55)	0.691
Height	1.7 (0.15)	1.7 (0.105)	0.231
Years of disease	9 (9)	6.5 (11)	0.323
EDSS	3.5 (3)	4.75 (2.5)	0.129
Hemoglobin (g/dL)	14.9 (1.3)	13.55 (1.6)	0.036 *
White blood cell ($\times 10^3/\mu\text{L}$)	5.2 (2.1)	6.05 (1.65)	0.112
Platelets ($\times 10^3/\mu\text{L}$)	229 (80)	278.5 (81.5)	0.043 *
Total protein (g/dL)	6.95 (0.8)	6.7 (0.5)	0.743
Glucose (mg/dL)	87.3 (11.3)	87.45 (7.8)	0.931
Uric acid (mg/dL)	4.73 (2.69)	4.575 (2.245)	0.717
Cholesterol (mg/dL)	186 (39)	225.5 (74)	0.09
Triglycerides (mg/dL)	76 (42.7)	105 (72.6)	0.066
CRP (mg/dL)	0.76 (1.38)	1.525 (2.745)	0.18
Iron ($\mu\text{g}/\text{dL}$)	122.5 (42)	97.5 (49)	0.012 *
Iron binding capacity (mg/dL)	340.5 (50)	360.5 (47.5)	0.12
Transferrin saturation (%)	33.945 (19.65)	25.5 (12.705)	0.005 *
Albumin (gr/dL)	4.35 (0.54)	4.25 (0.54)	0.742
PPMS	3	3	
RRMS	7	12	
SPMS	4	5	

3.2. DRB1*15

Of the 34 patients screened, 23 (67%) were positive for DRB1*15, and according to the response group, 13 pertained to the non-responders and 10 to the responders. χ^2 analysis between transplant response groups yielded a $p = 0.618$ and a Cramer's V of 0.088 which indicates that there is no relationship between response and the presence of HLA DRB1*15.

3.3. NFL and IL4I1

When evaluating between-transplant response groups, no between-group differences were observed for these biomarkers (Figure 2A,B). However, when evaluating samples from patients with more extreme responses, defined as an EDSS rate of change > 1 in the response group and < -1 in the non-responder group, differences between response groups were observed (Figure 2C,D).

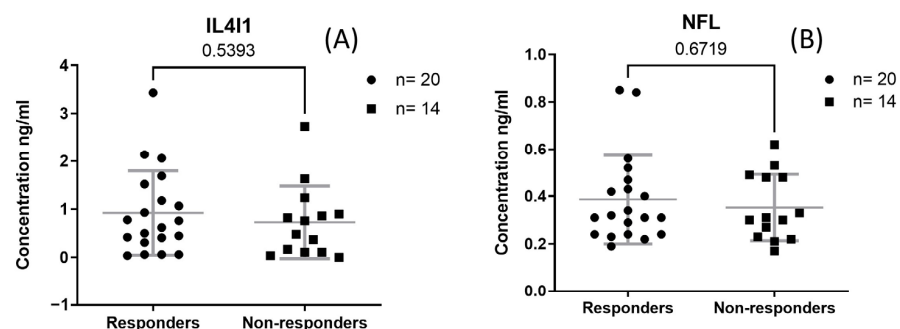


Figure 2. Cont.

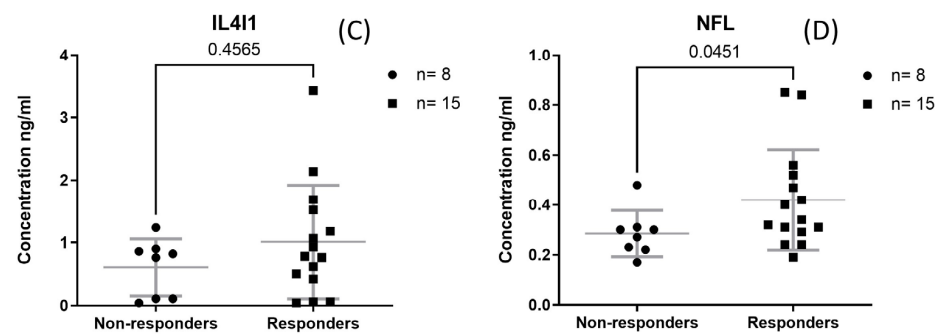


Figure 2. ELISA results for IL4I1 and NFL in transplant response groups. In sections (A,B), the classification of response groups was made based on the difference between EDSS scores before transplantation and one year after transplantation: below zero in the “non-responders” group and above zero in the “responders” group. In contrast, the classification of response groups in sections (C,D) was based on the EDSS score difference: below one in the “non-responder” group, and above one in the “responder” group.

3.4. miRNAs

As there is no consensus on the normalization that could allow the relative quantification of microRNAs, we preferred to use a constant amount of MS2 bacteriophage per plasma volume to normalize the expression levels per unit of volume.

When performing the analysis of these miRNAs by response group, differences were found mainly in 146a and 326 (Figure 3). If only samples from patients with responses > 1 or < -1 were considered, miR-146a yielded the greatest difference between response groups (Figure 3).

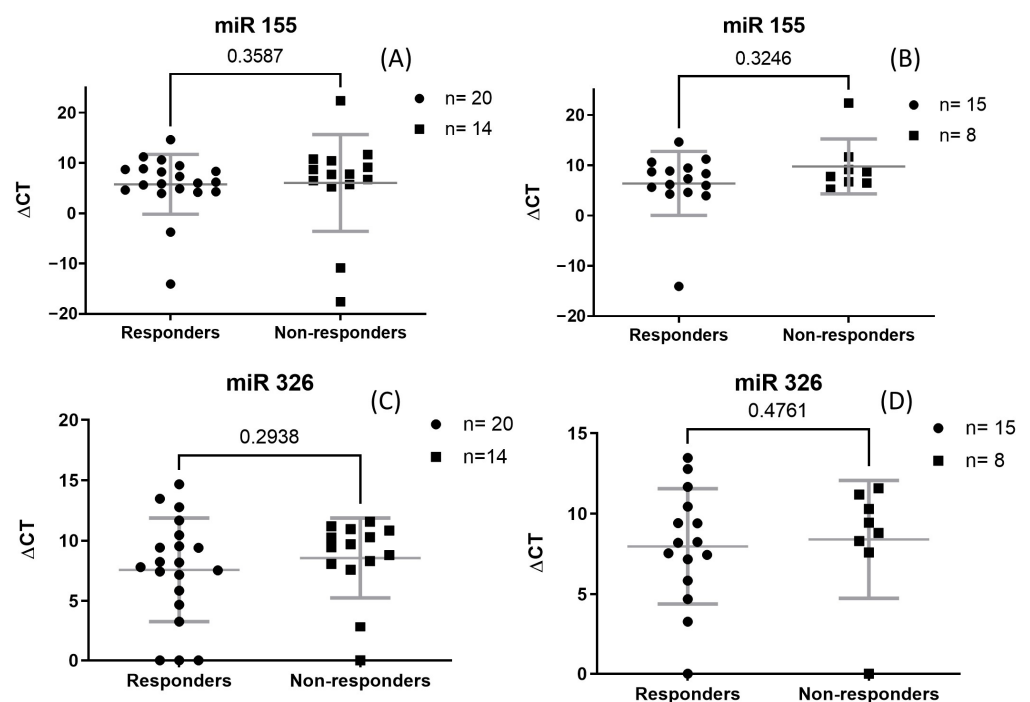


Figure 3. Cont.

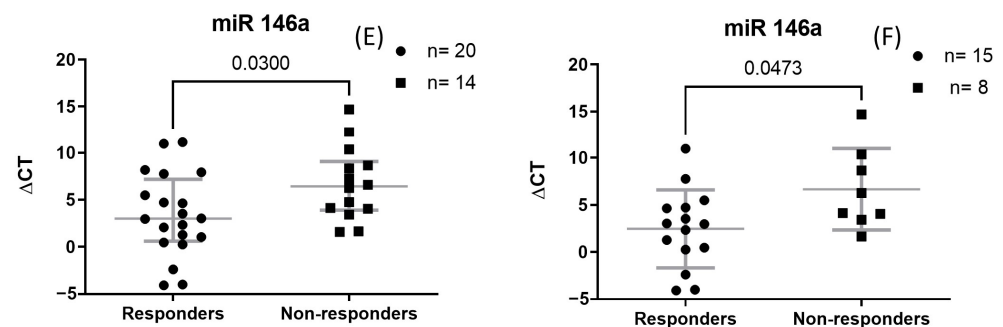


Figure 3. miRNA results in transplant response groups. In sections (A,C,E), the classification of response groups was made based on the difference between EDSS scores before transplantation and one year after transplantation: below zero in the “non-responder” group and above zero in the “responder” group. In sections (B,D,F), the response groups were classified according to the EDSS score difference: below one in the “non-responder” group, and above one in the “responder” group.

4. Discussion

In the logistic regression analysis, the variables pertaining to iron metabolism were those with the highest predictive value in the different groups evaluated.

Iron is an essential mineral for neuronal development and the myelination process; however, it is transported, stored, and handled with great care to avoid iron-mediated toxicity through the Fenton and Haber–Weiss reactions. At birth, there is low to nil iron concentrated in the brain as assessed by magnetic resonance imaging; its concentration increases rapidly between youth and middle age, and thereafter remains relatively constant [11]. In MS, normal-appearing white matter has been reported to contain lower iron levels when compared to lesion areas in which there is significant iron accumulation [12]. In chronic lesions, there is a subset of dormant and inactive lesions in which reactive astrocytes accumulate iron. It is possible that, although astrocyte iron accumulation is protective in the short term, the incorporation of increasing amounts of free iron over time due to continuous inflammatory activity and the constant destruction of iron-loaded oligodendrocytes and macrophages eventually depletes the antioxidant defenses of astrocytes, thus leading to their death [13]. In this study, iron levels, transferrin saturation, and iron binding capacity were analyzed, and we observed that transferrin saturation levels were higher among patients with a poor response to transplantation; we speculate that body iron may be increased in these patients and that hence, iron levels may also be increased in the CNS; this suggests that in patients who do not respond to transplantation, failure is associated with greater neuronal injury, and iron could be an important factor in the process.

Another marker of neuronal damage is NFL, which is an important part of the cytoskeleton of myelinated axons. According to Cai et al., NFL levels are elevated during all phases of MS and tend to decrease toward normal values during intervention with disease-modifying therapies [14]. This suggests that NFL may be a useful biomarker to monitor MS activity, progression, and treatment efficacy. We found slightly elevated levels of NFL in the transplant responder group, suggesting that patients with a better response to transplantation would have a greater degree of neuronal injury than non-responders. This could result from the analysis of the three types of MS equally, or from the degree of MS activity when the blood sample was collected.

The relationship of the HLA-DRB1*15 allele with MS has been extensively documented. In a review published by Schmidt et al., 72 papers published between 1993 and 2004 were analyzed and revealed that in most studies, the frequency of DRB1*15:01 was higher in cases than in controls [15]. The studies in which DRB1*15:01 was not associated with MS were mainly conducted in non-European populations [16]. We expected the presence of the DRB1*15:01 allele to have some association with the response to transplantation, as

there are reports in which it has been associated with an improved response to glatiramer acetate treatment [17], and improved short-term disability with steroid use [18]. However, in this sample, the presence of the allele had no effect or association with the response to transplantation. As for the 15:02 allele, although four of the five positive patients were in the transplant response group, the sample of positive patients is too small to determine whether there is a solid association with the response to transplantation.

In terms of the IL4I1 and NFL biomarkers, we observed an increase in their levels in the transplant response group. IL4I1 was selected as a biomarker of remyelination due to the previous studies published by Psachoulia et al., who worked with the MS animal model and reported that IL4I1 modulates inflammation by regulating T lymphocyte differentiation and proliferation, allowing the formation of a favorable environment for remyelination in central nervous system tissue [19]. The regulatory effect of IL4I1 on B lymphocytes [20] and CD8 T lymphocytes [21] has also been studied. In this study, we found that the median IL4I1 is slightly elevated in transplant responders, suggesting that the inflammatory process due to MS in this group of patients is better contained than in the non-responder group. Aside from the relationship between NFL and MS, elevated serum levels of this biomarker have also been reported in neurodegenerative diseases such as Alzheimer's disease, Guillain-Barré syndrome, and amyotrophic lateral sclerosis [22].

When measuring miRNAs, we faced the challenge of standardization. Unfortunately, despite the existence of different studies on the expression of miRNAs, there are still no universal reference genes that allow for adequate comparisons and experiment reproducibility in different populations; most probably, they will not exist, as clearly observed in the case of the so-called housekeeping genes. For a given gene to be used as a reference, one must demonstrate that it has a sufficiently constant expression level between comparison conditions, samples, or groups. The simplest approach when deciding which is the ideal reference gene is to review many previously performed studies in similar populations and choose the most frequently used reference gene. The main hindrance when selecting the reference gene in this way is its proclivity to modify its expression in different experimental conditions.

In this study, we decided to use the MS2 bacteriophage as an external reference. Since endogenous reference genes can present important variations depending on the type of sample or disease, we considered that the use of exogenous reference nucleic acids (armed RNA or RNA virus particles) added to the sample at the time of extraction could be more useful for the normalization of results, although they do not undergo modification between sample collection and extraction. We observed that in our sample, there are differences in the three miRNAs between response groups, particularly in miR-146a. This miRNA is one of the most studied miRNAs and plays a critical role in the regulation of immune system cells. Suppression of miR-146a expression can increase the production of Tregs, but alters their functions, subsequently leading to the failure of immune tolerance through tissue infiltration and extensive lymphocyte activity. miR-146a is highly expressed in Tregs and in signal transducer and activator of transcription 1 (STAT1) targets (such as T helper type 1 (Th1) cells). Thus, it may selectively control the suppression of Treg-mediated inflammation-dependent interferon-gamma (IFN- γ) and Th1 responses. The skipping of miR-146a in B lymphocytes results in the formation and spontaneous progression of germinal center responses, the production of high-affinity autoantibodies, and an increased incidence of autoimmune phenomena [23]. Among the miRNAs analyzed, miR-146a was the only one that yielded statistically significant differences between transplant response groups. Several studies have reported that miR-146a levels are often elevated in MS patients. A recent study reported that CSF miR-146a levels were elevated in MS patients compared to healthy controls; furthermore, miR-146a levels normalized 12 months after aHSCT with

a conditioning lymphoablative protocol that uses a greater amount of cyclophosphamide than the protocol employed in this study. In our cohort, we observed that patients who prior to HSCT had higher plasma levels of miR-146a had a worse response 12 months after transplantation. A study including the analysis of this biomarker after transplantation is needed to assess whether there are differences between the different aHSCT protocols [24].

This study has some limitations due to its design; the project is cross-sectional and is vulnerable to selection bias. While the use of ELISA remains a fundamental method for biomarker detection, biomarker measurement could have been performed with platforms such as SIMOA and Lumipulse which in recent years have been used more frequently in the clinical setting due to their higher sensitivity and specificity [25]. Since most of the patients who underwent transplantation are foreigners, there is no access to subsequent blood samples to reanalyze the biomarkers; the EDSS value at 12 months is mainly self-assessed, and among patients with an EDSS difference above +1, the differences between biomarkers are larger, but this limits the sample size. However, our study findings offer useful information and insights into patients with MS who underwent aHSCT.

5. Conclusions

Serum iron levels, hemoglobin levels, transferrin saturation, and the platelet counts before aHSCT are different between individuals with MS that respond or do not respond to aHSCT; these variables, coupled with miR-146a, may be of use to evaluate patients' response to aHSCT.

Author Contributions: Conceptualization, A.R.-A., S.J.G.-E. and M.M.G.-P.; methodology, S.J.G.-E., M.M.G.-P., V.R.-N. and S.S.-O.; formal analysis, S.J.G.-E. and M.M.G.-P.; investigation, S.J.G.-E., M.M.G.-P. and G.J.R.-A.; data curation, S.J.G.-E., M.M.G.-P., V.R.-N. and S.S.-O.; writing—original draft preparation, S.J.G.-E. and M.M.G.-P.; writing—review and editing, S.J.G.-E., G.J.R.-A. and M.M.G.-P.; supervision, S.J.G.-E.; funding acquisition, S.J.G.-E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Centro de Hematología y Medicina Interna, Clínica Ruiz (approval number: CEI-03-09-22-2; approval date: 9 March 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, M.M.G.-P., upon reasonable request. The data is not publicly available due to privacy and confidentiality agreements, as they contain sensitive information that could compromise the privacy of research participants.

Conflicts of Interest: Author Guillermo José Ruiz Argüelles was employed by the company Centro de Hematología y Medicina Interna, Clínica Ruiz. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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