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The MMP-9/TIMP-1 Ratio and Concentrations of Osteopontin Are Elevated in Cerebrospinal Fluid of People With Multiple Sclerosis and Decrease After Autologous Hematopoietic Stem Cell Transplantation

Ivan Pavlovic¹  | Ida Erngren² | Kim Kultima² | Anders Larsson² | Malin Müller¹ | Anna Wiberg^{1,3} | Joachim Burman¹

¹Translational Neurology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden | ²Clinical Chemistry, Department of Medical Sciences, Uppsala University, Uppsala, Sweden | ³Clinical Immunology, Department of Immunology, Genetics & Pathology, Uppsala University, Uppsala, Sweden

Correspondence: Joachim Burman (joachim.burman@uu.se)

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ABSTRACT

Objectives: To evaluate the utility of cerebrospinal fluid (CSF) biomarkers—matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinases-1 (TIMP-1), the MMP-9/TIMP-1 ratio, and osteopontin (OPN)—as indicators of blood–brain barrier (BBB) integrity and disease activity in people with relapsing–remitting multiple sclerosis (pwMS), and to assess their changes following autologous hematopoietic stem cell transplantation (aHSCT).

Methods: CSF samples from pwMS treated with aHSCT ($n = 43$) and healthy controls ($n = 32$) were analyzed for MMP-9, TIMP-1, and OPN concentrations using ELISA and electrochemiluminescence assays. Lumbar punctures were performed at baseline and at 1, 2, and 3–5 years post-aHSCT. Biomarker findings were compared with standard CSF parameters, prior treatments, and MRI data.

Results: MMP-9/TIMP-1 ratios and OPN levels were significantly elevated in pwMS compared to controls, particularly in those with gadolinium-enhancing lesions or on first-line therapies. Both biomarkers declined significantly after aHSCT and remained low during follow-up. The MMP-9/TIMP-1 ratio showed superior discriminatory capacity and correlated with inflammatory CSF markers.

Interpretation: CSF MMP-9/TIMP-1 ratio and OPN are elevated in MS and decrease following aHSCT, reflecting reduced inflammation and restored BBB integrity. These biomarkers may support disease monitoring and therapeutic evaluation.

1 | Introduction

The blood–brain barrier (BBB) is essential for maintaining central nervous system (CNS) immune privilege by tightly regulating the exchange of cells and molecules between the blood and

brain parenchyma. In multiple sclerosis (MS), a chronic autoimmune disorder characterized by CNS inflammation and demyelination, BBB disruption facilitates the infiltration of peripheral immune cells, thereby amplifying neuroinflammatory activity and lesion formation [1].

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Historically, the ratio of albumin concentrations in cerebrospinal fluid (CSF) and serum—the albumin quotient (Q_{Alb})—has served as the principal biomarker of BBB integrity [2, 3]. Elevated Q_{Alb} reflects passive diffusion of albumin across a compromised BBB and remains widely used in clinical practice [4]. However, Q_{Alb} lacks specificity to MS pathology, may not detect subtle or dynamic barrier changes, and provides limited mechanistic insight into the molecular drivers of BBB dysfunction.

Recent attention has shifted toward molecular biomarkers that may offer more sensitive and disease-relevant assessments of BBB pathology. Matrix metalloproteinase-9 (MMP-9), its endogenous inhibitor tissue inhibitor of metalloproteinases-1 (TIMP-1), and osteopontin (OPN) have emerged as promising candidates. MMP-9 actively contributes to BBB breakdown by degrading basement membrane components and disrupting tight junctions, and elevated levels have been associated with active MS and increased BBB permeability [5–7]. Notably, the MMP-9/TIMP-1 ratio may better reflect net proteolytic activity at the BBB than either marker alone. High CSF MMP-9/TIMP-1 ratios have been linked to increased lesion burden and disease severity in people with MS [8, 9]. OPN, a multifunctional glycoprotein involved in immune cell recruitment and proinflammatory signaling, is also implicated in MS pathogenesis. It is expressed in active MS lesions, and elevated CSF concentrations have been associated with disease activity, early disability accrual, and cortical atrophy in relapsing–remitting MS (RRMS) [10–12].

Autologous hematopoietic stem cell transplantation (aHSCT) is a potent immune reconstitution therapy increasingly used in highly active RRMS. Long-term remission rates are high, with approximately two-thirds of patients maintaining sustained ‘no evidence of disease activity’ (NEDA) for at least a decade post-transplant [13]. aHSCT thus represents a model of near-complete inflammatory suppression and an opportunity to investigate treatment-induced changes in CSF biomarkers of BBB dysfunction.

In this study, we examined the concentrations of MMP-9, TIMP-1, their ratio, and OPN in CSF from people with RRMS before and after aHSCT. We aimed to assess their utility as dynamic biomarkers of BBB integrity and inflammatory activity, and to evaluate their response to aHSCT over extended follow-up.

2 | Materials and Methods

The study was approved by the Regional Ethical Board of Uppsala (Dnr 2008/182 and 2012/080/1). All participants gave their informed written consent in accordance with the Declaration of Helsinki [14].

This retrospective observational cohort study was performed in compliance with the STROBE guidelines.

2.1 | Participants

Patients scheduled for autologous hematopoietic stem cell transplantation (aHSCT) with a cyclophosphamide-based conditioning regimen at Uppsala University Hospital between December 2011 and February 2020 were invited to participate in the study.

All patients were diagnosed with RRMS according to the 2017 revision of the McDonald criteria [15]. The demographic data of the cohort are presented in Table 1.

Based on prior disease-modifying therapies (DMTs), patients were categorized into three groups: 1st line, 2nd line, and treatment-naïve. First-line treatments included dimethyl fumarate, glatiramer acetate, interferon, teriflunomide, and fingolimod. Second-line treatments comprised rituximab and natalizumab. No evidence of disease activity-3 (NEDA-3) was defined as the absence of new MRI lesions, clinical relapses (CR), or confirmed disability worsening (CDW). CDW required progression in expanded disability status scale (EDSS) scores confirmed at two separate visits. Patients who did not maintain NEDA-3 status during follow-up were considered to have evidence of disease activity (EDA). The proportion of patients maintaining NEDA-3 and conversely exhibiting EDA after aHSCT is shown in Figure S1. One patient exhibited EDA after the final sample was collected and was therefore not included in the Kaplan–Meier curve.

2.2 | Collection of Cerebrospinal Fluid

Participants were asked to undergo lumbar puncture before aHSCT, and again at 1, 2, and 5 years post-aHSCT. If a lumbar puncture was not possible, such as during pregnancy, it was scheduled for a later follow-up visit. Additional lumbar punctures were performed for some patients with suspected relapse, to assess disease status. CSF samples from healthy controls were collected at a single timepoint, and all samples were processed according to consensus guidelines [16]. Blood samples were collected simultaneously as part of routine care.

2.3 | Procedures

Autologous hematopoietic stem cells were mobilized with a single dose of 2g/m² cyclophosphamide, followed by 5–10 μg/kg/day filgrastim for 6–7 days. The cells were harvested about 10 days after the start of the mobilization regimen without any ex vivo graft manipulation. Patient conditioning involved cyclophosphamide (200 mg/kg) and rabbit anti-thymocyte globulin (rATG, 6 mg/kg). Prophylaxis against fungal, viral, and bacterial infections was administered during the neutropenic phase, with ongoing prophylaxis for herpes viruses and *Pneumocystis jiroveci* for at least 3 months. A detailed description of the procedure is available elsewhere [13].

2.4 | Biomarker Analysis

Determination of CSF mono- and polynuclear cell counts (MNC and PNC, respectively), CSF concentration of neurofilament light (NfL), both serum and CSF concentrations of IgG, IgM, and albumin concentration were performed as part of routine health care (Table 2). Additionally, the generation of the Q_{Alb} , IgG-, and IgM indices were also performed as part of routine health care. The Q_{Alb} was not calculated for the control group. However, according to Swedish healthcare, the healthy reference values for Q_{Alb} are ≤ 6.8 for individuals under 45 years of age and ≤ 10.2 for those over 45 years of age.

TABLE 1 | Demographics and clinical characteristics of the cohort.

<i>N</i>		RRMS	Healthy controls
		(<i>n</i> = 43)	(<i>n</i> = 32)
Age at inclusion (years)	Mean (SD)	31 (6)	25 (7)
Sex	F/M	28/15	15/17
EDSS	Median [IQR]		
	Baseline	4.0 [2.0–4.0]	NA
	1 year	2.0 [1.0–4.0]	NA
	2 years	2.0 [1.0–4.0]	NA
	3–5 years	2.0 [1.0–4.0]	NA
Annual relapse rate	Mean (SD)	1.4 (1.6)	NA
Disease duration (years)	Mean (SD)	6.8 (5.9)	NA
Number of previous treatments	Median [IQR]	2 [1–3]	NA
Days from baseline MRI to aHSCT	Median [IQR]	40 [39–62]	NA
Days from last relapse to aHSCT	Median [IQR]	207 [102–596]	
All treatments	Naive	5	NA
	1st line	16	NA
	2nd line	22	NA
Gadolinium-enhancing lesions on MRI (%)	1st line	62.5	NA
	2nd line	9.1	NA
NEDA-3/EDA		32/11	NA

Abbreviations: aHSCT, autologous hematopoietic stem cell transplantation; EDA, evidence of disease activity; EDSS, expanded disability status scale; IQR, interquartile range; MRI, magnet resonance imaging; NA, not applicable; NEDA, no evidence of disease activity; SD, standard deviation.

Concentrations of TIMP-1 were analyzed in-house with the Human TIMP-1 DuoSet ELISA Assay (R&D Systems, Minneapolis, MN, USA; Cat. No. D970). The plates were read with a spectrophotometer and a microplate reader using SoftMax Pro 7 software (Molecular Devices, San Jose, CA, USA).

OPN and MMP-9 were analyzed at the Science for Life (SciLife) facility in Uppsala, using a Meso Scale Discovery (MSD) R-PLEX Human Osteopontin Assay (Meso Scale Discovery, Gaithersburg, MD, USA; Cat. No. K151YMR) and MSD R-PLEX Human MMP-9 (total) Assay (Cat. No. K1515QR).

2.5 | Statistics

Statistical analyses were performed in GraphPad Prism version 10.3.1 for macOS (GraphPad Software) [17]. Shapiro–Wilk’s test for normality was used to assess if data fit the Gaussian distribution. The data did not fit a normal Gaussian distribution. Therefore, all paired and unpaired analyses between groups, and correlation analyses were performed with non-parametric tests. The Mann–Whitney *U* test was used to compare two groups, while the Wilcoxon signed-rank test was used for paired analyses. The Kruskal–Wallis test, followed by Dunn’s multiple

comparisons post hoc test, assessed differences between treatment groups at baseline. Correlation matrices were constructed using Spearman’s ranked correlation coefficient to evaluate associations between parameters and analytes. Two-tailed *p* values equal to or below 0.05 were considered significant. Where applicable, *p* values were adjusted for multiple comparisons according to the Benjamini–Hochberg methods. Concentrations are presented as median with interquartile range (IQR). Cox proportional hazard models were built in R (v.4.5.0) and adjusted for age and sex. To fit the MMP-9/TIMP-1 ratio correctly to the Cox proportional hazard model, the values were scaled by a factor of 10³.

Associations were classified according to the British Medical Journal guidelines [18]. Figures were created with GraphPad Prism and graphically processed in Affinity Designer (v. 1.10.5, Serif (Europe) LTD.) [19].

3 | Results

The initial cohort comprised CSF samples obtained from 50 pwMS and 32 healthy controls. In order to be included, patients needed to be represented at baseline and at least at one of the

TABLE 2 | Biochemical characterization of study participants.

Factor	Healthy controls (n = 32)		Baseline		1 year		2 years		3–5 years		
		n		n		n		n		n	
CSF	Osteopontin (ng/mL)	27.1 [20.7–37.6]	43	47.7 [25.9–71.4]	42	30.7 [25.1–40.7]	42	28.3 [20.6–41.9]	29	35.9 [23.6–50.3]	13
	MMP-9 (pg/mL)	118 [93.2–149]	43	473 [179–1270]	42	196 [156–287]	42	196 [131–343]	29	213 [181–344]	13
	TIMP-1 (ng/mL)	72.6 [62.0–89.1]	43	73.0 [56.8–89.5]	42	63.5 [56.3–83.9]	42	69.5 [62.1–85.1]	29	87.5 [62.4–108]	13
Mononuclear cells (10 ⁶ /L)		≤ 5	43	2.0 [1.0–6.0]	41	0.0 [0.0–0.0]	41	0.0 [0.0–0.0]	29	1.00 [0.50–2.50]	13
		≤ 1	43	0.0 [0.0–0.0]	41	1.0 [0.0–2.0]	41	1.0 [0.0–2.0]	29	0.00 [0.00–0.00]	13
		≤ 45	43	38 [26–62]	41	29 [20–39]	41	28 [19–41]	29	35.0 [33.5–44.0]	13
IgM (mg/L)		≤ 560	42	835 [430–2650]	41	440 [300–570]	41	360 [230–550]	29	320 [230–555]	13
		≤ 320	43	220 [150–310]	41	190 [150–280]	41	193 [165–277]	29	256 [180–293]	13
		≤ 14.5	43	9.3 [8.0–11]	41	9.2 [7.9–11]	41	9.4 [8.0–11]	29	9.40 [8.05–10.3]	13
S-IgM (g/L)		≤ 2.1	38	0.9 [0.6–1.4]	37	0.8 [0.5–1.1]	37	0.8 [0.6–1.1]	29	0.64 [0.44–1.00]	13
		≤ 0.6	38	0.1 [0.07–0.3]	37	0.07 [0.05–0.17]	37	0.06 [0.05–0.10]	29	0.07 [0.06–0.09]	13
		≤ 0.6	43	0.7 [0.6–0.9]	41	0.6 [0.5–0.8]	41	0.6 [0.5–0.7]	29	0.58 [0.53–0.75]	13
MMP-9/TIMP-1 (10 ⁻³)		1.66 [1.14–2.14]	43	6.85 [2.79–20.0]	42	3.46 [2.18–4.62]	42	2.68 [1.92–4.62]	29	2.39 [2.00–4.48]	13
		NA	43	4.90 [3.70–8.00]	42	4.85 [3.30–6.48]	42	4.90 [3.70–8.10]	29	6.30 [3.85–7.35]	13
		No	43	40/3	40	34/6	40	27/2	29	9/4	13
IgM OCB (yes/no)		No	40	8/32	37	3/34	37	2/26	28	2/10	12

Note: All concentrations are presented as median with interquartile range. Abbreviations: CSF, cerebrospinal fluid; HC, healthy controls; IgG, immunoglobulin G; IgM, immunoglobulin M; MMP, matrix metalloproteinase; NFL, neurofilament light chain; OCB, oligoclonal bands; P, plasma; S, serum; TIMP, tissue inhibitor of metalloproteinase.

TABLE 3 | Sensitivity, specificity, Youden's index, and likelihood ratios for optimal thresholds of selected biomarkers.

Biomarker	Optimal cutoff	Sensitivity (%)	Specificity (%)	Youden's index	Likelihood ratio
MMP-9 (pg/mL)	> 171	81	88	0.69	6.51
MMP-9/TIMP-1 ratio	> 2.3 × E−3	91	81	0.72	4.84
OPN (ng/mL)	> 43.2	58	91	0.49	6.20

follow-up time points in all biochemical assays. After the exclusion of patients not meeting the criteria, the final number of samples (*n*) included in each group were: *Healthy controls*: 32; *Baseline*: 43; *1-year*: 42; *2-year*: 29; *3–5-year*: 13. The median clinical follow-up time was 5.4 years (IQR, 4.2–6.4).

3.1 | Receiver Operating Characteristic Analysis Asserts the MMP-9/TIMP-1 Ratio to Be Most Valuable in Discriminating Between pwMS From Healthy Controls

A receiver operating characteristic (ROC) analysis was performed, and the area under the curve (AUC) was calculated to compare the capabilities of the biomarkers to discriminate between healthy controls and pwMS at baseline.

The highest AUC was observed in relation to the MMP-9/TIMP-1 ratio (0.92 [95% CI, 0.86–0.98], $p \leq 0.0001$); however, it was only fractionally higher than MMP-9 alone (0.91 [95% CI, 0.84–0.97], $p \leq 0.0001$). The calculated AUC for OPN was 0.72 (95% CI, 0.61–0.84) ($p \leq 0.01$), while TIMP-1 possessed no discriminatory capability of significance. The threshold values yielding the optimal ROC areas with sensitivity, specificity, Youden's index, and likelihood ratio are reported in Table 3.

As the MMP-9/TIMP-1 ratio was stronger than MMP-9 alone in discriminating between pwMS and healthy controls, only the ratio was assessed in the following analyses.

3.2 | Levels of Osteopontin and MMP-9/TIMP-1 Ratio Are Higher in Patients With Multiple Sclerosis Compared With Healthy Controls

Median concentrations of OPN were significantly higher in pwMS at baseline (47.7 ng/mL [IQR 25.9–71.4]) compared with healthy controls (27.1 ng/mL [20.7–37.6]) ($p \leq 0.001$). No significant differences were observed between the two groups in relation to median concentrations of TIMP-1 (baseline: 73.0 ng/mL [56.8–89.5], healthy controls: 72.6 ng/mL [62.0–89.1]). However, the ratio generated between MMP-9 and TIMP-1 was significantly higher among pwMS than among healthy controls, with median ratios of 6.85 E^{-3} [2.79–20.0] and 1.66 E^{-3} [1.14–2.14] ($p \leq 0.0001$), respectively (Figure 1).

The median Q_{Alb} for pwMS at baseline was 4.90 [3.70–8.00]. The healthy reference limit is a quotient ≤ 6.8 for individuals below the age of 45 and a quotient ≤ 10.2 for individuals above the age of 45. At baseline, 16 pwMS had a $Q_{\text{Alb}} > 6.8$, with only one of the 16 pwMS being above 45 years of age. The Q_{Alb} was not calculated for the healthy controls.

3.3 | PwMS With Gadolinium-Enhancing Lesions Have Higher MMP-9/TIMP-1 Ratios and Higher Concentrations of Osteopontin

At baseline, pwMS with gadolinium-enhancing lesions ($n = 14$) had significantly higher median concentrations of OPN (64.2 ng/mL [43.1–93.2]) compared to pwMS without gadolinium-enhancing lesions ($n = 29$) (median 35.8 ng/mL [IQR 22.9–62.4], $p \leq 0.05$) (Figure 2).

No differences were observed between the groups concerning TIMP-1 concentrations. The MMP-9/TIMP-1 ratio, however, was significantly higher in pwMS with lesions at baseline, with a median of 21.7 E^{-3} [10.0–41.7], compared with those without lesions, a median of 3.66 E^{-3} [2.62–15.4] ($p \leq 0.001$).

3.4 | PwMS Receiving 1st Line Treatments Have Higher MMP-9/TIMP-1 Ratios and Higher Concentrations of Osteopontin Than Those Receiving 2nd Line Treatments

To assess if previous DMT use was associated with higher or lower concentrations of the biomarkers, the participants were split into one of three groups based on prior DMT use (naive, 1st line, or 2nd line). The median concentration of OPN was observed to be significantly lower in pwMS receiving 2nd line treatments (27.1 ng/mL [22.5–48.7]) at baseline compared with both untreated pwMS (63.7 ng/mL [48.5–117], $p \leq 0.01$) and those receiving 1st line DMTs (63.9 ng/mL [41.9–94.9], $p \leq 0.05$). No differences were observed in OPN concentrations between pwMS receiving 1st line DMTs and pwMS who were treatment-naive (Figure 3).

The MMP-9/TIMP-1 ratio was observed to be significantly higher in pwMS receiving 1st line DMTs (median ratio 16.9 E^{-3} [IQR 5.79–37.7]) compared to pwMS on 2nd line treatments (median ratio 2.91 E^{-3} [IQR 2.37–6.91]) ($p \leq 0.05$). No differences were observed in MMP-9/TIMP-1 ratios between pwMS receiving 1st line DMTs and pwMS who were treatment-naive, nor between 2nd line treated patients and treatment-naive patients.

There were no statistically significant differences in Q_{Alb} between the treatment groups.

3.5 | Levels of OPN, MMP-9, and MMP-9/TIMP-1 Correlate to Routine CSF Analyses

Spearman's correlation coefficient was applied to construct a correlation matrix with clinical and biochemical variables

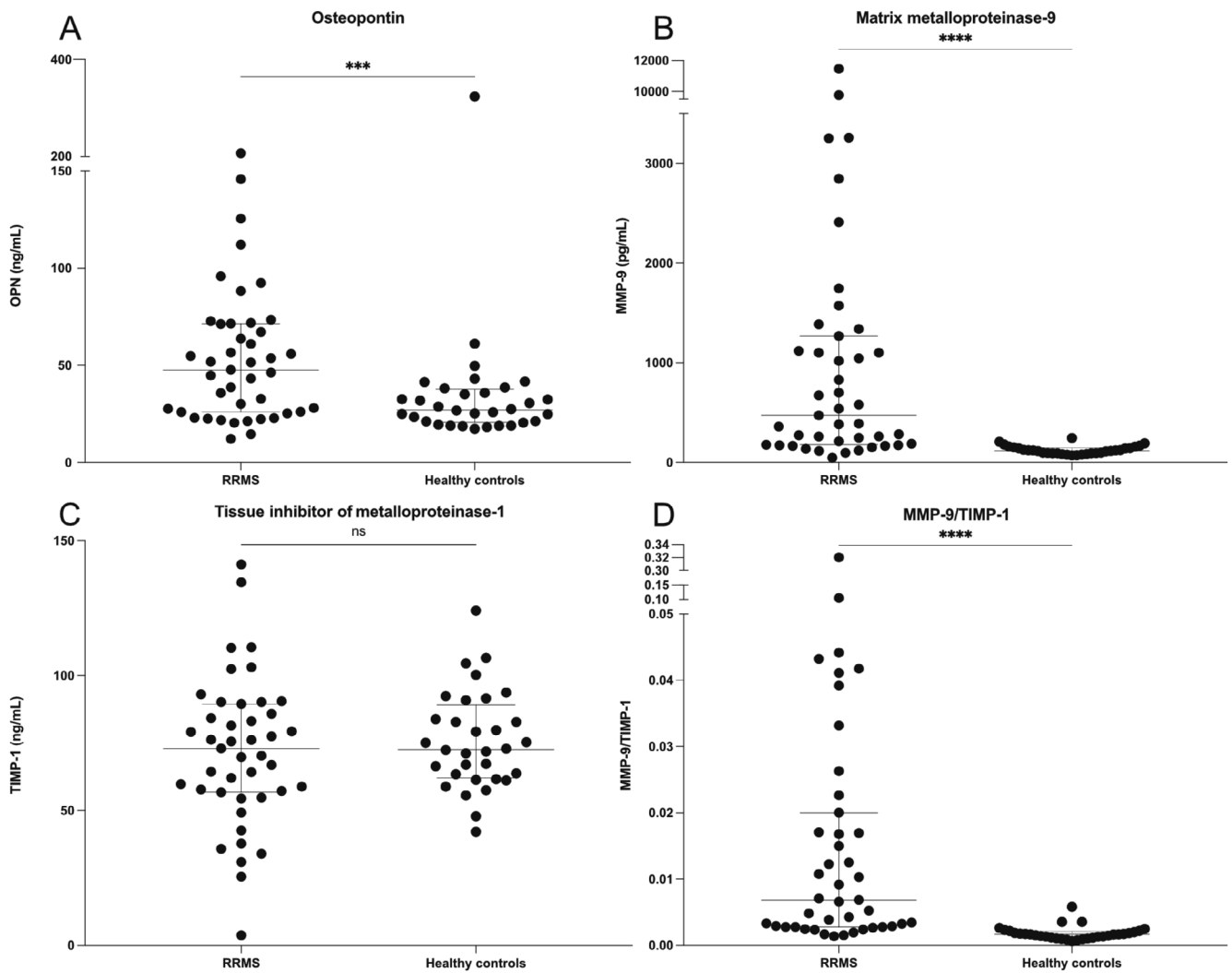


FIGURE 1 | CSF concentrations of osteopontin, MMP-9, TIMP-1, and the MMP-9/TIMP-1 ratio in people with relapsing–remitting MS compared with healthy controls. Levels of osteopontin (OPN) (A), matrix metalloproteinase-9 (MMP-9) (B), tissue inhibitor of metalloproteinase-1 (TIMP-1) (C) and the MMP-9/TIMP-1 ratio (D), were measured in CSF from patients with RRMS (before aHSCt) and healthy controls. The Mann–Whitney U test was used to establish a statistically significant difference between the groups. Each dot represents a separate sample and the error bars mark the median concentration with the interquartile range. ns = not significant, $p < 0.001$ (***), $p < 0.0001$ (****).

available at baseline. The final correlation matrix presented in Figure 4 includes only variables that exhibited at least one significant association with any of the three biomarkers: OPN, MMP-9/TIMP-1 ratio, or Q_{Alb} .

Notably, no statistically significant correlations were found between these biomarkers and several parameters, including EDSS, age, and disease duration.

3.6 | CSF OPN Concentrations and the MMP-9/TIMP-1 Ratio Decrease After Intervention With aHSCt

Median baseline concentrations of the biomarkers were 47.7 ng/mL [IQR 25.9–71.4] for OPN, 473 pg/mL [179–1270] for MMP-9, and 73.0 ng/mL [56.8–89.5] for TIMP-1. The median baseline MMP-9/TIMP-1 ratio was 6.85×10^{-3} [2.79–20.0], and the median Q_{Alb} was 4.90 [3.70–8.00].

After intervention with aHSCt, long-term decreases in OPN concentrations, the MMP-9/TIMP-1 ratios, and a short-term decrease in the Q_{Alb} were observed (Figure 5). Median OPN concentrations decreased from 47.7 ng/mL [IQR 25.9–71.4] at baseline to 30.7 ng/mL [25.1–40.7] ($p < 0.001$) by the 1-year follow-up, to 28.3 ng/mL [20.6–41.9] by the 2-year follow-up ($p < 0.001$), and to 35.9 ng/mL [23.6–50.3] by the last follow-up ($p < 0.05$).

Concentrations of TIMP-1 were not statistically different between the different timepoints. However, the ratio generated from MMP-9 and TIMP-1 decreased over time. The median ratio decreased from 6.85×10^{-3} [2.79–20.0] at baseline to 3.46×10^{-3} [2.18–4.62] by the first follow-up ($p < 0.001$). The ratio further decreased to 2.68×10^{-3} [1.92–4.62] by the second follow-up ($p < 0.001$) and to 2.39×10^{-3} [2.00–4.48] by the 3–5-year follow-up ($p < 0.01$).

The median Q_{Alb} decreased from 4.90 [IQR 3.70–8.00] at baseline to 4.85 [3.30–6.48] by the 1-year follow-up ($p < 0.05$). No

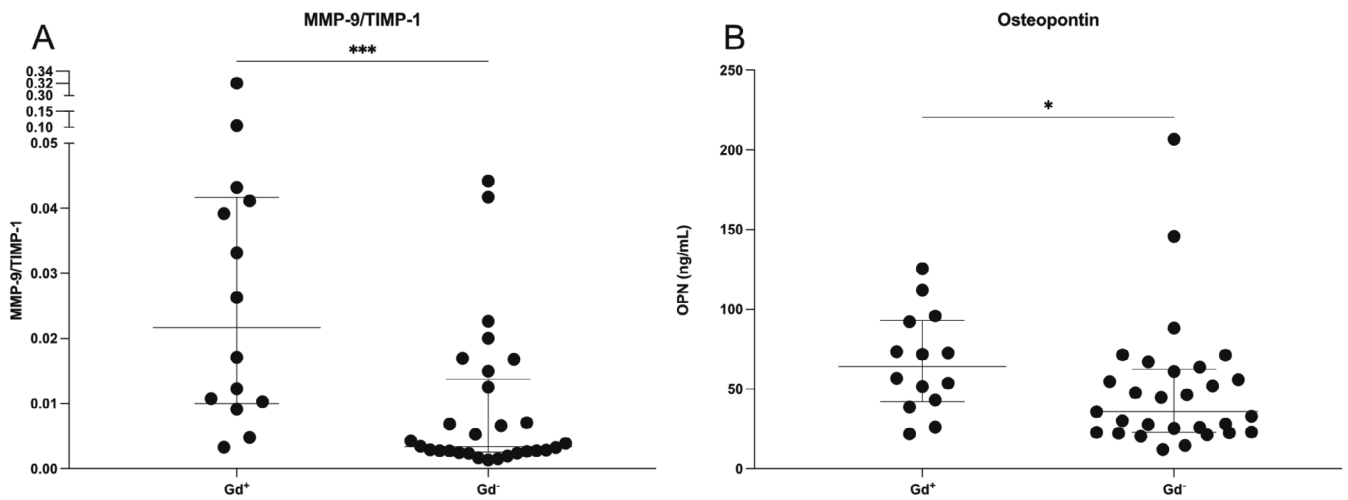


FIGURE 2 | MMP-9/TIMP-1 ratio and concentrations of osteopontin in people with MS with and without gadolinium-enhancing lesions. Patients with gadolinium-enhancing lesions at baseline had higher MMP-9/TIMP-1 ratios (A), and higher concentrations of osteopontin (OPN) (B) in comparison with patients with no gadolinium-enhancing lesions. The Wilcoxon signed-rank test was used to establish a statistically significant difference between the groups. Each dot represents a separate sample and the error bars mark the median concentration with the interquartile range. Gd⁺ = presence of gadolinium-enhancing lesions, Gd⁻ = absence of gadolinium-enhancing lesions, $p < 0.05$ (*), $p < 0.001$ (***)

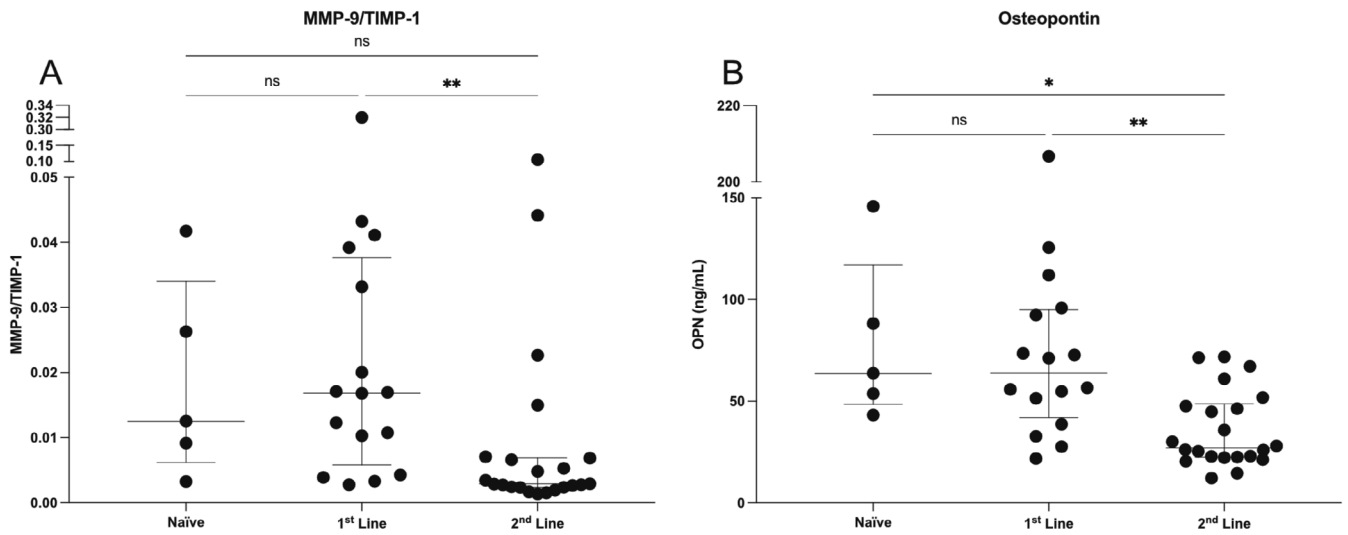


FIGURE 3 | CSF MMP-9/TIMP-1 ratios and osteopontin concentrations by DMT class at baseline. Patients were divided into three groups based on their last treatment before intervention with aHST: Treatment-naive patients and patients treated with either 1st or 2nd line treatment. The Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to establish a statistical significance differences in CSF MMP-9/TIMP-1 ratios (A), and osteopontin (OPN) concentrations (B) at baseline based on DMT class. Each dot represents a separate sample and the error bars mark the median concentration with the interquartile range. ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**)

differences of statistical significance were observed between the baseline and the remaining two timepoints in relation to the Q_{Alb} .

3.7 | PwMS With Evidence of Disease Activity After aHST Had Higher MMP-9/TIMP-1 Ratios

Patients with EDA ($n = 11$) had higher MMP-9/TIMP-1 ratios at the 1-year follow-up (6.03×10^{-3} [3.43–7.90]) than patients maintaining NEDA-3 ($n = 31$) throughout follow-up (3.05×10^{-3} [1.88–3.93], $p \leq 0.01$). There were no other statistically significant differences between patients with EDA and NEDA-3 at any other timepoint or

in relation to Q_{Alb} and OPN. Cox proportional hazard models were constructed to assess the predictive capabilities of the concentrations at baseline and at 1 year after aHST for future EDA. The models revealed the 1-year MMP-9/TIMP-1 ratios to be predictive of loss of NEDA over the remaining follow-up period with an age- and sex-adjusted hazard ratio of 1.48 (95% CI 1.19–1.86), $p \leq 0.001$. No other biomarker was predictive of future EDA.

One individual had clearly elevated MMP-9/TIMP-1 ratio at the 2-year follow-up, with an MMP-9/TIMP-1 ratio 13 times higher than the median for the respective timepoint. This patient had an ongoing MRI-verified relapse at the scheduled 2-year follow-up.

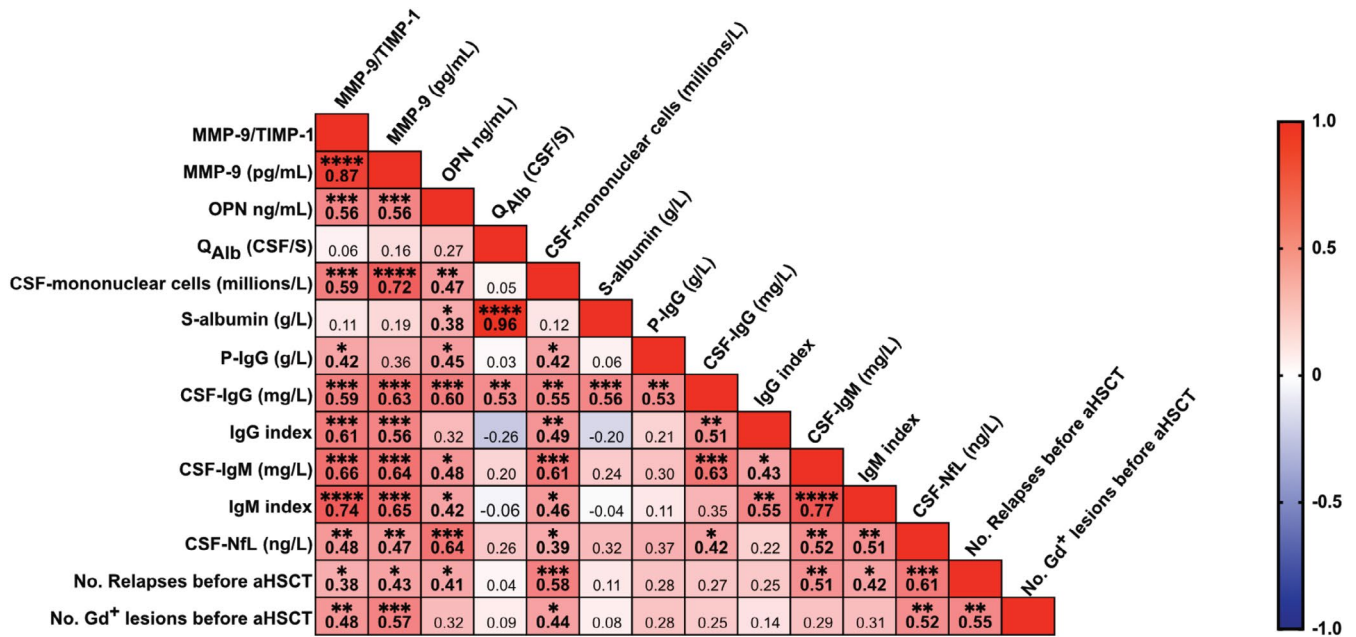


FIGURE 4 | Heat map showing a correlation matrix of CSF analyses. Spearman's correlation coefficient was utilized to assess the co-variation of the MMP-9/TIMP-1 ratio, osteopontin (OPN), albumin quotient (Q_{Alb}) at baseline along with other parameters routinely analyzed in healthcare. Bold text signifies correlation of statistical significance and the asterisks signify the Benjamini–Hochberg adjusted p values. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

4 | Discussion

This study investigated CSF biomarkers of BBB dysfunction in MS, focusing on MMP-9, its inhibitor TIMP-1, the MMP-9/TIMP-1 ratio, and OPN. We demonstrate that both the MMP-9/TIMP-1 ratio and OPN levels are elevated in pwMS compared to healthy controls, particularly among individuals with gadolinium-enhancing lesions or receiving first-line therapies. Following aHSCT, these biomarkers decreased and remained low, consistent with durable suppression of CNS inflammatory activity.

The MMP-9/TIMP-1 ratio exhibited the strongest discriminatory performance, surpassing MMP-9 and OPN individually. This aligns with previous reports suggesting that the ratio better reflects proteolytic activity at the BBB than either marker alone [8, 9]. Notably, patients with first-line DMTs and gadolinium-enhancing lesions exhibited the highest biomarker levels, indicating that these markers reflect ongoing inflammation despite prior treatment.

Q_{Alb} , traditionally used to assess BBB permeability, offers limited sensitivity to early or localized BBB dysfunction [3, 4]. It provides only a static, non-specific readout of passive leakage, and may be confounded by systemic factors [4, 20, 21]. In contrast, the MMP-9/TIMP-1 ratio and OPN offer mechanistic insight into active BBB disruption and neuroinflammation. MMP-9 contributes directly to BBB breakdown via basement membrane degradation and tight junction disruption [5], and is upregulated in active MS [6, 7]. Elevated ratios have been linked to increased lesion load and new enhancing lesions on MRI [22, 23]. Moreover, TIMP-1-resistant isoforms of MMP-9, shown to be elevated in MS, may skew the ratio further toward a pro-inflammatory profile [24].

OPN, a glycoprotein involved in T cell recruitment and chronic inflammation, is consistently upregulated in MS lesions and correlates with disease activity [10–12, 25–27]. Our findings support prior reports showing elevated CSF OPN in RRMS, particularly during relapse, and its association with clinical and radiological activity. While serum OPN levels show limited correlation with CNS inflammation, CSF levels more accurately reflect local neuroinflammatory processes [28].

Interestingly, we found no significant correlation between Q_{Alb} and either the MMP-9/TIMP-1 ratio or OPN. This divergence likely reflects distinct biological processes: Q_{Alb} represents passive leakage, while the MMP-9/TIMP-1 ratio reflects enzymatic disruption and OPN reflects cytokine-driven inflammation. The lack of association between Q_{Alb} and our biomarkers reinforces the concept that they represent complementary but non-overlapping facets of BBB dysfunction. The differential dynamics of these biomarkers may also reflect their functional roles: the MMP-9/TIMP-1 ratio appears to track acute, lesion-associated inflammation and BBB breakdown, while OPN may indicate more chronic, diffuse inflammatory states. The tighter distribution of MMP-9/TIMP-1 values in patients without gadolinium-enhancing lesions further supports this interpretation. Further reinforcing this hypothesis, a markedly increased MMP-9/TIMP-1 ratio but normal OPN level was observed in one patient with several gadolinium-enhancing lesions at the 2-year follow-up.

Importantly, both the MMP-9/TIMP-1 ratio and OPN decreased significantly after aHSCT and remained suppressed over 3–5 years of follow-up in patients maintaining NEDA-3. These findings suggest that the biomarkers not only reflect current disease activity but also respond robustly to effective immunoablation. Participants were already receiving DMTs at baseline, and

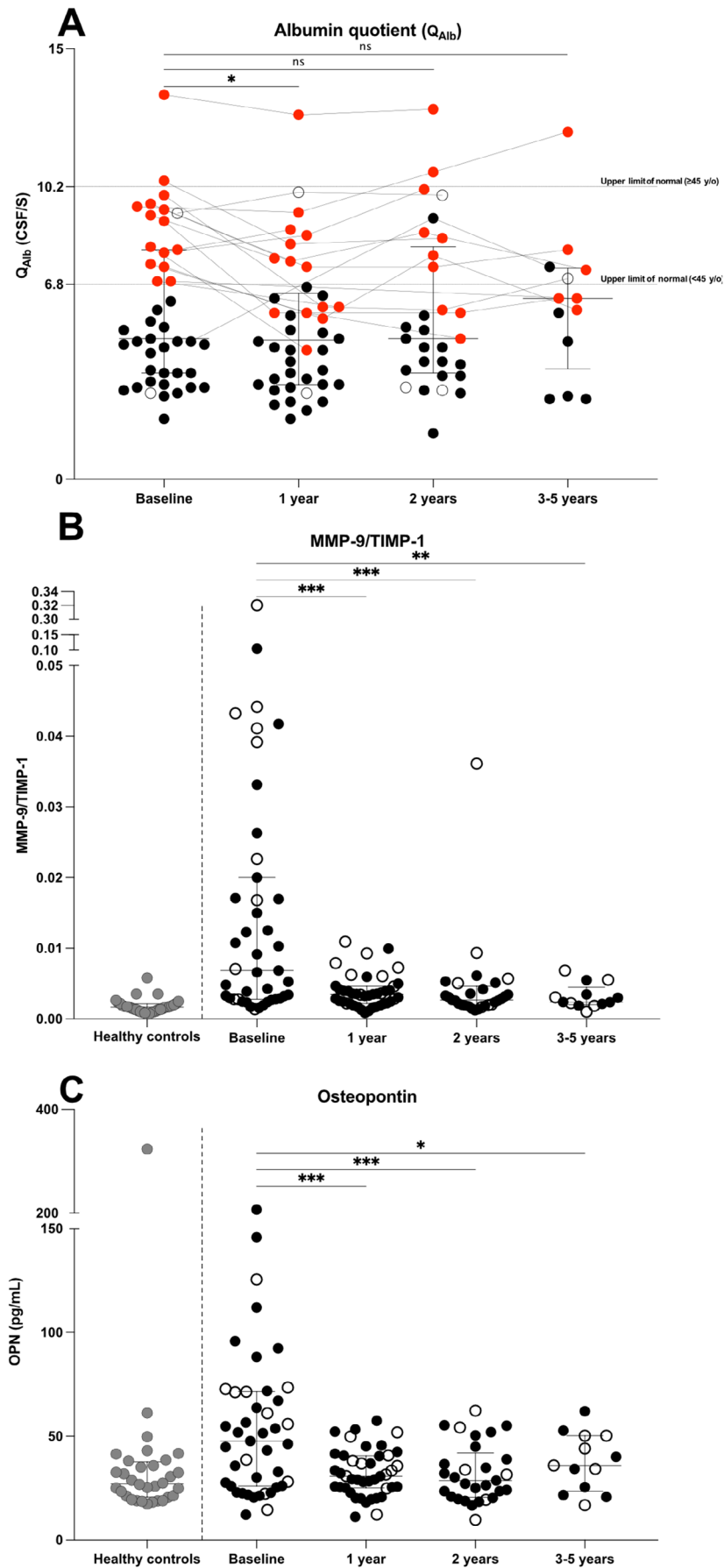


FIGURE 5 | Legend on next page.

FIGURE 5 | Biomarkers of the blood–brain barrier before and after aHSCT. (A) Age-related healthy reference values acquired at Uppsala University Hospital’s Clinical Chemistry lab (dotted lines) for the albumin quotient, ≤ 6.8 for people < 45 years of age and ≤ 10.2 for people ≥ 45 years of age. Red dots signify patients whose baseline sample was above the reference value of their respective age. White dots signify patients ≥ 45 years of age at the time of sampling. Black dots signify patients < 45 years of age at baseline with baseline values < 6.8 . Full lines denote paired samples with a baseline value > 6.8 with one exception where the patient’s baseline was below the healthy upper limit but both follow-up samples were above the limit, this patient is denoted by black dots and a full line pairing the samples. (B) CSF MMP-9/TIMP-1 ratio, and CSF concentrations of osteopontin (OPN) (C), before and after intervention with autologous hematopoietic stem cell transplantation (aHSCT). Data from HC (marked in gray in B and C) are included as a reference. People with MS are shown with data obtained before (baseline) and at follow-up timepoints (1 year, 2 years, and 3–5 years) post-aHSCT. One individual had clearly elevated MMP-9/TIMP-1 ratio at the 2-year follow-up, with an MMP-9/TIMP-1 ratio 13 times higher than the median for the respective timepoint (B). This patient had an ongoing MRI-verified relapse at the scheduled 2-year follow-up. In contrast, the same individual had normal values of OPN (C). Each dot represents a separate sample and the error bars mark the median concentration with the interquartile range. Black dots signify patients maintaining NEDA-3 throughout the follow-up period, while patients who experience new evidence of disease activity are depicted with white dots with black outlines. p values adjusted for multiple comparisons with the Benjamini–Hochberg method are signified by asterisks. ns = not significant, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)

the further reductions observed post-aHSCT further highlight the potency of this intervention.

While the baseline concentrations of the biomarkers were not predictive of future EDA, the 1-year MMP-9/TIMP-1 ratios were associated with a HR of 1.48 for future EDA. This suggests that the MMP-9/TIMP-1 ratio may be used for risk assessment and possibly to stratify patients for adjuvant therapy after aHSCT.

Compared to conventional DMTs, aHSCT appears to exert a more profound and sustained effect on BBB-related biomarkers. Previous studies have shown that natalizumab, an $\alpha 4$ -integrin inhibitor, reduces CSF MMP-9 and OPN levels over 12 to 60 weeks of treatment, in both relapsing–remitting and progressive MS disease courses [29, 30]. Similarly, interferon-beta has been associated with reduced serum MMP-9, increased TIMP-1, and an overall decrease in the MMP-9/TIMP-1 ratio [8, 22]. However, these effects often depend on treatment adherence and are probably reversible upon discontinuation. In contrast, our study demonstrates that aHSCT not only leads to a more pronounced decline in both MMP-9/TIMP-1 ratio and OPN levels but that these reductions are sustained for up to five years post-intervention. This prolonged suppression of inflammatory biomarkers suggests that aHSCT induces a durable immune reset, consistent with its superior clinical outcomes in patients with active MS. The normalization of these biomarkers in patients maintaining NEDA-3 also supports their potential utility in evaluating treatment response and long-term disease remission.

4.1 | Limitations

This study has several limitations. The cohort size was modest, and not all patients were represented at every follow-up timepoint, limiting power for longitudinal subgroup analyses. CSF sampling is inherently invasive, and although it offers a direct window into CNS pathology, repeated sampling limits feasibility. Biomarkers were analyzed using different platforms (ELISA and electrochemiluminescence), which may introduce inter-assay variability. Finally, our focus on CSF concentrations, while biologically informative, may limit comparison with studies analyzing serum or plasma levels.

4.2 | Conclusion

Our findings support the MMP-9/TIMP-1 ratio and OPN as dynamic biomarkers of BBB dysfunction and neuroinflammation in MS. Both were elevated in active disease and declined following aHSCT, paralleling clinical remission. The MMP-9/TIMP-1 ratio, in particular, appears sensitive to acute inflammatory activity and may outperform traditional markers such as Q_{Alb} in reflecting BBB pathology. These results provide mechanistic insight into aHSCT efficacy and suggest potential applications for these biomarkers in disease monitoring and therapeutic evaluation.

Author Contributions

I.P., M.M., A.W., and J.B. contributed to the conception and design of the study; I.P., I.E., K.K., and A.L. contributed to the acquisition and analysis of data; I.P. and J.B. contributed to drafting the manuscript and/or figures.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** NEDA-3 maintenance after intervention with aHST. Kaplan–Meier curve presenting the proportion of patients did not maintain NEDA-3 during the entirety of clinical follow-up ($n = 11$) divided by reason for evidence of disease activity (EDA), categorized as new events in one or more of the following: confirmed disability worsening (CDW), clinical relapses (CR) and new lesions on magnetic resonance imaging (MRI).