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Role of Cathepsin H, Cathepsin S, Chitinase-3–like protein 1, and other biomarkers in Multiple Sclerosis patients

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ABSTRACT

The study aims to assess the potential of cathepsin S, cathepsin H, and other biomarkers as diagnostic and prognostic indicators in patients with multiple sclerosis. A total of 280 participants aged 25–45 years were collected from Baghdad Medical City/Baghdad/Iraq, between May 2025 and January 2026. They were divided into three groups according to the McDonald criteria: relapsing-remitting (N=70), secondary progressive (N=70), and primary progressive (N=70) MS, based on neurological assessment. Serum and paired CSF samples were analyzed using ultra-sensitive immunoassays for all biomarkers (NfL, GFAP, chitinase-3–like protein 1 (CHI3L1), CXCL13, MMP-9, Cathepsin S, Cathepsin H, NSPs, AAP-1, and CBC) in discriminating relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS). CSF biomarkers consistently outperformed serum markers across all disease subtypes. In RRMS, CSF NfL achieved an AUC compared with serum NfL. Similarly, CSF GFAP yielded an AUC versus serum. CHI3L1, CXCL13, and MMP-9 demonstrated parallel trends, with CSF AUCs ranging versus serum. The highest discriminative accuracy was observed for CSF NfL in PPMS. Composite ROC visualization confirmed the superior performance of CSF biomarkers across all subtypes. CSF-derived biomarkers exhibit higher diagnostic accuracy than serum counterparts, reflecting direct proximity to neuroinflammatory and neurodegenerative processes.

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1. INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disorder that primarily affects the brain and spinal cord. It is caused by the destruction of myelin, a fatty substance that covers and protects the nerve fibers. Thus, the damage disrupts communication between the brain and the body. As the disease progresses, it becomes debilitating (1). MS is a painful and debilitating disease, affecting quality of life. Major MS types are relapsing-remitting MS, primary progressive MS, secondary progressive MS, and progressive-relapsing MS. Thus, a proper MS treatment is crucial. Relapses may be associated with infection or stress. Symptoms may include vision problems, including optic neuritis, loss of strength, weakness, walking or balance problems, sensations like numbness, tingling, or burning, muscle spasms, and bladder/bowel problems (2). Multiple sclerosis is clinically characterized by heterogeneous visual, motor, sensory, fatigue, and cognitive impairment symptoms, which occur variably depending on type, like relapsing remitting multiple sclerosis (RRMS), secondary progressive multiple sclerosis (SPMS), and primary progressive multiple sclerosis (PPMS) (3). More than 2.8 million people suffer from MS all over the globe. Prevalence depends on factors like geographic distribution, genetic susceptibility, and environmental risk factors (3). Its etiopathogenesis is not well understood. However, current data suggest a multi-factorial etiology. In other words, there are genetic susceptibility, autoimmune dysregulation, and environmental triggers. The environmental triggers include vitamin D deficiency, viral infections, and smoking (4). Although disease-modifying therapies have improved, multiple sclerosis is still a leading cause of non-traumatic disability. The most common clinical form of MS, the relapsing-remitting clinical phenotype, can eventually transition to a secondary progressive form with a

disability accrual over time (5). To slow down the progression of the disease and ensure improved outcomes over a longer term, diagnosis and commencement of treatment should be early. The pathogenesis of MS might be affected by the role of proteolytic enzymes like cathepsins and neutrophil serine proteases (NSPs). Cathepsin S is a cysteine protease that plays a crucial role in the processing of MHC class II antigens by cleaving the invariant chain and boosting autoreactive T-cell activation (7). BBB disruption also results from the degradation of extracellular matrix by this. Although Cathepsin H is less studied, it is expressed in neurons and microglia and may amplify proteolytic cascades contributing to neuroinflammation and axonal injury (8). Aside from cathepsins, neutrophil elastase, proteinase 3, and cathepsin G are all released upon degranulation or the formation of a neutrophil extracellular trap (NET). Proteases can cause demyelination by disrupting the integrity of the blood-brain barrier (BBB) and degrading myelin proteins (9). The activity of NSP zymogens is regulated by aminopeptidase AAP-1, which maintains their proteolytic potential. When these systems of proteases are dysregulated, there is an acceleration of lesion formation. This may also lead to disease progression from relapse (10). Despite the availability of disease-modifying therapies (DMTs) that reduce relapse rates and delay disability progression, MS remains a major cause of long-term disability in young adults. Therefore, identifying novel biomarkers and therapeutic targets, including protease-related pathways, is of considerable importance for improving disease monitoring and management strategies (11). The study aims to assess the potential of cathepsin S, cathepsin H, and other biomarkers as diagnostic and prognostic indicators in patients with Multiple Sclerosis.

2. METHOD

Study Design and Participants

This case-control study was conducted in the Department of Neurology, Baghdad Medical City, Iraq, from May 2024 to May 2025. A total of 280 participants aged 25–45 years were included. Out of them, 210 patients were diagnosed with clinically definite Multiple Sclerosis (MS) as per the 2017 McDonald criteria. According to neurologic evaluation, patients were classified into clinical subtypes (N=70) relapsing-remitting, (N=70) secondary progressive, and (N=70) primary progressive MS. The 70 healthy volunteers in the control group were age and sex matched, with no history of a neurological disease, autoimmune disease, or chronic inflammatory disease. The following exclusion criteria were applicable for both groups: absence of infection (in the past 4 weeks), malignancy, chronic hepatitis or kidney problems, pregnancy, and the use of corticosteroids and/or disease-modifying therapies in the last three months.

Clinical and Demographic Assessment

Demographic characteristics (age, sex, BMI, smoking status) and clinical variables (disease duration, relapse history, and treatment status) were documented. Neurological disability in MS patients was evaluated using the Expanded Disability Status Scale (EDSS).

Blood Sample Collection

Serum

About 10 ml of Peripheral venous blood was obtained from each participant under aseptic conditions using Vacutainer tubes without anticoagulant. Samples were allowed to clot in an upright position at room temperature for 30–60 minutes before centrifugation at 3000 rpm for 15 minutes. The supernatant serum was carefully aliquoted into low protein-binding polypropylene tubes to avoid repeated freeze–thaw cycles. Aliquots were stored at –20 °C until further analysis.

Cerebrospinal Fluid (CSF)

Using sterile conditions, a lumbar puncture at the L3/L4 or L4/L5 interspace collected cerebrospinal fluid. We collected about 3–7 mL of CSF in polypropylene tubes. The first 1–2 mL of CSF was discarded to minimize blood contamination; the subsequent fractions were collected into polypropylene tubes. Blood samples were visually examined for the presence of blood. The blood samples were gently mixed. Then, centrifugation at 2000 x g for 10 minutes was performed to effectively separate and remove cellular components from the blood samples. In low protein-binding cryovials, the supernatant was distributed in 0.25–1.0 mL aliquots with sufficient headspace and was immediately frozen at –30 °C until analysis.

All samples were processed according to standardized protocols to preserve biomarker stability, and detailed records of collection time, processing time, and storage conditions were maintained.

Statistical analysis

All data were analyzed using SPSS Ver. 31 from IBM. Continuous variables were recorded as means ± standard deviations (SD). For normally-distributed continuous variables, group comparisons were made using one-way analysis of variance (ANOVA), and appropriate post-hoc tests are reported. The Pearson formula was used to compute relationships among continuous variables. Receiver operating characteristic (ROC) analyses were

performed to detect the biomarker as diagnostic for the disease. A $p \leq 0.05$ was used to determine significance in statistical analyses.

3. RESULTS AND DISCUSSION

The demographic and clinical characteristics of the study population are summarized in Table 1. The mean age differed significantly among the groups ($p < 0.001$). Patients with RRMS (32.4 ± 5.6 years) and controls (33.1 ± 5.2 years) were significantly younger compared with patients with SPMS (38.2 ± 4.8 years) and PPMS (40.1 ± 4.5 years). Sex distribution was comparable across groups ($p = 0.88$). The body mass index (BMI) did not differ significantly between patients and controls ($p = 0.42$). Similarly, smoking status showed no significant variation across the study groups ($p = 0.21$). As expected, disease duration was significantly longer in progressive forms of MS. Patients with SPMS had the longest disease duration (10.2 ± 3.8 years), followed by those with PPMS (8.9 ± 3.5 years); in contrast, RRMS patients had the shortest duration (4.5 ± 2.1 years) ($p < 0.001$). Relapse history also varied significantly ($p < 0.001$). A relapse history was present in 52 of 70 RRMS patients (74.3%), 40 of 70 SPMS patients (57.1%), and only 28 of 70 PPMS patients (40.0%). Treatment status differed significantly between groups ($p < 0.001$), with the majority of RRMS patients (85.7%) receiving disease-modifying therapies compared with 71.4% of SPMS patients and 64.3% of PPMS patients. The Expanded Disability Status Scale (EDSS) score showed a progressive increase across disease subtypes ($p < 0.001$). RRMS patients had a mean EDSS of 2.5 ± 1.2 (range 0–4.0), SPMS patients had 5.0 ± 1.5 (range 4.0–7.0), and PPMS patients had the highest mean score of 6.5 ± 1.2 (range 5.5–8.0).

Table 1. Demographic and Clinical Characteristics of Study Participants

Variable	RRMS (N=70)	SPMS (N=70)	PPMS (N=70)	Controls (N=70)	P-value
Age (years)	32.4 ± 5.6[a]	38.2 ± 4.8[b]	40.1 ± 4.5[b]	33.1 ± 5.2[a]	<0.001
Sex (M/F)	28/42	30/40	32/38	29/41	0.88
BMI (kg/m²)	24.8 ± 3.2	25.5 ± 3.5	25.7 ± 3.1	24.6 ± 3.0	0.42
Smoking status (Yes/No)	15/55	18/52	20/50	12/58	0.21
Disease duration (years)	4.5 ± 2.1[a]	10.2 ± 3.8[b]	8.9 ± 3.5[b]	–	<0.001
Relapse history (Yes/No)	52/18[a]	40/30[b]	28/42[c]	–	<0.001
Treatment status (Yes/No)	60/10[a]	50/20[b]	45/25[c]	–	<0.001
EDSS score	2.5 ± 1.2[a] (0–4.0)	5.0 ± 1.5[b] (4.0–7.0)	6.5 ± 1.2[c] (5.5–8.0)	–	<0.001

The concentrations of neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), chitinase-3-like protein 1 (CHI3L1/YKL-40), CXCL13, and matrix metalloproteinase-9 (MMP-9) were measured in both serum and cerebrospinal fluid (CSF) across study groups (Table 2). Neurofilament light chain (NfL): Serum and CSF NfL levels were significantly higher in MS patients compared with controls ($p < 0.001$). The highest concentrations were observed in PPMS patients (38.0 ± 6.5 pg/mL in serum; 230 ± 45 pg/mL in CSF) and SPMS patients (34.1 ± 7.2 pg/mL in serum; 210 ± 40 pg/mL in CSF), both significantly greater than RRMS patients (22.5 ± 5.8 pg/mL in serum; 120 ± 30 pg/mL in CSF). Controls had the lowest values (12.8 ± 3.6 pg/mL serum; 60 ± 15 pg/mL CSF). Glial fibrillary acidic protein (GFAP): Similar trends were observed for GFAP. Serum GFAP was significantly elevated in SPMS (250 ± 50 pg/mL) and PPMS (270 ± 55 pg/mL) compared with RRMS (180 ± 35 pg/mL), while controls demonstrated the lowest levels (120 ± 30 pg/mL). In CSF, GFAP concentrations increased progressively with disease severity (RRMS: 90 ± 20 pg/mL, SPMS: 145 ± 30 pg/mL, PPMS: 160 ± 35 pg/mL, controls: 55 ± 15 pg/mL) ($p < 0.001$). CHI3L1/YKL-40: Serum levels of CHI3L1 were significantly higher in progressive MS subtypes (70 ± 18 ng/mL in SPMS; 75 ± 20 ng/mL in PPMS) than in RRMS (45 ± 12 ng/mL) ($p < 0.001$). CSF concentrations followed a similar pattern, with PPMS patients having the highest levels (40 ± 10 ng/mL) compared

to SPMS (35 ± 8 ng/mL), RRMS (18 ± 5 ng/mL), and controls (10 ± 3 ng/mL). CXCL13: Serum CXCL13 levels were markedly increased in progressive forms (SPMS: 85 ± 20 pg/mL; PPMS: 95 ± 22 pg/mL) relative to RRMS (50 ± 15 pg/mL) and controls (20 ± 8 pg/mL). CSF levels were similarly elevated, with PPMS patients showing the highest concentrations (70 ± 18 pg/mL), followed by SPMS (60 ± 15 pg/mL), RRMS (25 ± 8 pg/mL), and controls (10 ± 5 pg/mL) ($p < 0.001$). MMP-9: Serum and CSF MMP-9 concentrations were significantly higher in SPMS and PPMS compared with RRMS and controls ($p < 0.001$). Serum MMP-9 reached 240 ± 50 ng/mL in PPMS and 220 ± 45 ng/mL in SPMS, compared with 150 ± 35 ng/mL in RRMS and 100 ± 25 ng/mL in controls. In CSF, MMP-9 levels were highest in PPMS (150 ± 35 ng/mL) and SPMS (135 ± 30 ng/mL), compared to RRMS (75 ± 20 ng/mL) and controls (40 ± 12 ng/mL).

Table 2. Serum and CSF Biomarker Levels in MS Subtypes and Controls

Biomarker	Sample	RRMS (N=70)	SPMS (N=70)	PPMS (N=70)	Controls (N=70)	P-value
NfL (pg/mL)	Serum	22.5 ± 5.8[a]	34.1 ± 7.2[b]	38.0 ± 6.5[b]	12.8 ± 3.6[c]	<0.001
NfL (pg/mL)	CSF	120 ± 30[a]	210 ± 40[b]	230 ± 45[b]	60 ± 15[c]	<0.001
GFAP (pg/mL)	Serum	180 ± 35[a]	250 ± 50[b]	270 ± 55[b]	120 ± 30[c]	<0.001
GFAP (pg/mL)	CSF	90 ± 20[a]	145 ± 30[b]	160 ± 35[b]	55 ± 15[c]	<0.001
CHI3L1 / YKL-40 (ng/mL)	Serum	45 ± 12[a]	70 ± 18[b]	75 ± 20[b]	30 ± 8[c]	<0.001
CHI3L1 / YKL-40 (ng/mL)	CSF	18 ± 5[a]	35 ± 8[b]	40 ± 10[b]	10 ± 3[c]	<0.001
CXCL13 (pg/mL)	Serum	50 ± 15[a]	85 ± 20[b]	95 ± 22[b]	20 ± 8[c]	<0.001
CXCL13 (pg/mL)	CSF	25 ± 8[a]	60 ± 15[b]	70 ± 18[b]	10 ± 5[c]	<0.001
MMP-9 (ng/mL)	Serum	150 ± 35[a]	220 ± 45[b]	240 ± 50[b]	100 ± 25[c]	<0.001
MMP-9 (ng/mL)	CSF	75 ± 20[a]	135 ± 30[b]	150 ± 35[b]	40 ± 12[c]	<0.001

As shown in Table 3, Serum Cathepsin S levels were significantly elevated in SPMS (18.7 ± 4.1 ng/mL) and PPMS (20.1 ± 4.0 ng/mL) compared with RRMS (12.5 ± 3.2 ng/mL) and controls (9.8 ± 2.5 ng/mL; $p < 0.001$). A similar pattern was observed in CSF, where SPMS (9.2 ± 2.0 ng/mL) and PPMS (10.5 ± 2.3 ng/mL) patients exhibited higher concentrations than RRMS (5.8 ± 1.6 ng/mL) and controls (3.6 ± 1.0 ng/mL; $p < 0.001$). For Cathepsin H, serum levels were increased in SPMS (15.0 ± 3.5 ng/mL) and PPMS (16.5 ± 3.8 ng/mL) relative to RRMS (10.2 ± 2.8 ng/mL) and controls (8.5 ± 2.0 ng/mL; $p < 0.001$). Likewise, CSF Cathepsin H was significantly higher in SPMS (8.0 ± 2.0 ng/mL) and PPMS (9.0 ± 2.5 ng/mL) compared with RRMS (4.5 ± 1.2 ng/mL) and controls (3.0 ± 0.8 ng/mL; $p < 0.001$). NSP concentrations followed the same trend. Serum levels were markedly elevated in SPMS (2.0 ± 0.5 µg/mL) and PPMS (2.3 ± 0.6 µg/mL), compared with RRMS (1.2 ± 0.4 µg/mL) and controls (0.8 ± 0.3 µg/mL; $p < 0.001$). In CSF, SPMS (1.2 ± 0.3 µg/mL) and PPMS (1.4 ± 0.4 µg/mL) showed significantly higher levels than RRMS (0.6 ± 0.2 µg/mL) and controls (0.3 ± 0.1 µg/mL; $p < 0.001$). Similarly, AAP-1 concentrations were elevated across progressive MS phenotypes. Serum levels were significantly increased in SPMS (38 ± 8 ng/mL) and PPMS (42 ± 10 ng/mL), compared with RRMS (25 ± 6 ng/mL) and controls (18 ± 5 ng/mL; $p < 0.001$). In CSF, SPMS (22 ± 5 ng/mL) and PPMS (25 ± 6 ng/mL) patients had higher values compared to RRMS (12 ± 3 ng/mL) and controls (6 ± 2 ng/mL; $p < 0.001$).

Table 3. Serum and CSF for Cathepsin S, H, NSPs, and AAP-1 Levels in MS Subtypes and Controls

Biomarker	Sample	RRMS (N=70)	SPMS (N=70)	PPMS (N=70)	Controls (N=70)	P-value
Cathepsin S (ng/mL)	Serum	12.5 ± 3.2[a]	18.7 ± 4.1[b]	20.1 ± 4.0[b]	9.8 ± 2.5[c]	<0.001

Cathepsin S (ng/mL)	CSF	5.8 ± 1.6[a]	9.2 ± 2.0[b]	10.5 ± 2.3[b]	3.6 ± 1.0[c]	<0.001
Cathepsin H (ng/mL)	Serum	10.2 ± 2.8[a]	15.0 ± 3.5[b]	16.5 ± 3.8[b]	8.5 ± 2.0[c]	<0.001
Cathepsin H (ng/mL)	CSF	4.5 ± 1.2[a]	8.0 ± 2.0[b]	9.0 ± 2.5[b]	3.0 ± 0.8[c]	<0.001
NSPs (μg/mL)	Serum	1.2 ± 0.4[a]	2.0 ± 0.5[b]	2.3 ± 0.6[b]	0.8 ± 0.3[c]	<0.001
NSPs (μg/mL)	CSF	0.6 ± 0.2[a]	1.2 ± 0.3[b]	1.4 ± 0.4[b]	0.3 ± 0.1[c]	<0.001
AAP-1 (ng/mL)	Serum	25 ± 6[a]	38 ± 8[b]	42 ± 10[b]	18 ± 5[c]	<0.001
AAP-1 (ng/mL)	CSF	12 ± 3[a]	22 ± 5[b]	25 ± 6[b]	6 ± 2[c]	<0.001

As shown in Table 4, Hemoglobin, glucose, ALT, and creatinine levels showed no statistically significant differences among the groups ($p > 0.05$), indicating preserved baseline hematological and biochemical status in all participants. In contrast, significant alterations were observed in inflammatory and immune-related markers. WBC count was significantly elevated in SPMS (7.5 ± 1.8 ×10³/μL) and PPMS (7.8 ± 1.7 ×10³/μL) compared with RRMS (6.8 ± 1.5 ×10³/μL) and controls (6.5 ± 1.4 ×10³/μL; $p < 0.001$). Lymphocyte percentage was reduced in SPMS (28 ± 5%) and PPMS (27 ± 5%) compared with RRMS (32 ± 6%) and controls (35 ± 7%; $p < 0.001$). Markers of systemic inflammation, including CRP and ESR, were markedly elevated in progressive forms of MS. SPMS (7.8 ± 3.0 mg/L) and PPMS (8.5 ± 3.2 mg/L) patients had significantly higher CRP levels compared with RRMS (4.5 ± 2.0 mg/L) and controls (2.5 ± 1.0 mg/L; $p < 0.001$). Similarly, ESR values were increased in SPMS (25 ± 8 mm/h) and PPMS (27 ± 9 mm/h) relative to RRMS (18 ± 6 mm/h) and controls (12 ± 4 mm/h; $p < 0.001$). Taken together, these results demonstrate that while general biochemical parameters (hemoglobin, glucose, ALT, creatinine) remained comparable across groups, patients with progressive MS (SPMS and PPMS) exhibited higher WBC counts, reduced lymphocyte percentages, and elevated CRP and ESR, indicating enhanced systemic inflammatory activity.

Table 4. Hematological and Biochemical Parameters in MS Subtypes vs. Controls

Parameter	RRMS (N=70)	SPMS (N=70)	PPMS (N=70)	Controls (N=70)	P-value
Hemoglobin (g/dL)	13.5 ± 1.2[a]	13.0 ± 1.4[a]	12.8 ± 1.3[a]	13.8 ± 1.1[a]	0.12
WBC count (10³/μL)	6.8 ± 1.5[a]	7.5 ± 1.8[b]	7.8 ± 1.7[b]	6.5 ± 1.4[a]	<0.001
Lymphocyte (%)	32 ± 6[a]	28 ± 5[b]	27 ± 5[b]	35 ± 7[c]	<0.001
CRP (mg/L)	4.5 ± 2.0[a]	7.8 ± 3.0[b]	8.5 ± 3.2[b]	2.5 ± 1.0[c]	<0.001
ESR (mm/h)	18 ± 6[a]	25 ± 8[b]	27 ± 9[b]	12 ± 4[c]	<0.001
Glucose (mg/dL)	92 ± 10[a]	95 ± 12[a]	97 ± 11[a]	90 ± 8[a]	0.08
ALT (U/L)	22 ± 6[a]	25 ± 7[a]	26 ± 8[a]	20 ± 5[a]	0.09
Creatinine (mg/dL)	0.9 ± 0.2[a]	1.0 ± 0.2[a]	1.0 ± 0.3[a]	0.9 ± 0.2[a]	0.15

Correlation analyses as apparent in Table 5, were performed between clinical measures (EDSS and disease duration) and circulating biomarkers (NfL, GFAP, CHI3L1, CXCL13, and MMP-9). EDSS score showed strong positive correlations with serum NfL ($r = 0.55$, $p < 0.001$), GFAP ($r = 0.48$, $p < 0.001$), CHI3L1 ($r = 0.50$, $p < 0.001$), CXCL13 ($r = 0.52$, $p < 0.001$), and MMP-9 ($r = 0.46$, $p < 0.001$). Likewise, disease duration correlated

positively with NfL ($r = 0.45$, $p < 0.001$), GFAP ($r = 0.40$, $p = 0.002$), CHI3L1 ($r = 0.42$, $p = 0.001$), CXCL13 ($r = 0.43$, $p = 0.001$), and MMP-9 ($r = 0.39$, $p = 0.003$). Among the biomarkers, NfL exhibited the strongest associations, being highly correlated with GFAP ($r = 0.68$, $p < 0.001$), CHI3L1 ($r = 0.60$, $p < 0.001$), CXCL13 ($r = 0.58$, $p < 0.001$), and MMP-9 ($r = 0.61$, $p < 0.001$). GFAP also correlated significantly with CHI3L1 ($r = 0.55$, $p < 0.001$), CXCL13 ($r = 0.50$, $p < 0.001$), and MMP-9 ($r = 0.52$, $p < 0.001$). Similarly, CHI3L1 showed positive correlations with CXCL13 ($r = 0.57$, $p < 0.001$) and MMP-9 ($r = 0.49$, $p < 0.001$). CXCL13 was also strongly correlated with MMP-9 ($r = 0.51$, $p < 0.001$). Overall, higher disability and longer disease duration were significantly associated with elevated levels of neuroaxonal (NfL), astroglial (GFAP), and inflammatory biomarkers (CHI3L1, CXCL13, MMP-9), suggesting that these serum markers reflect both neurodegenerative and inflammatory disease activity.

Table 5. Correlation Matrix Between EDSS, Disease Duration, and Serum Biomarkers in RRMS

Variable	EDSS	Disease Duration	NfL (Serum)	GFAP (Serum)	CHI3L1 (Serum)	CXCL13 (Serum)	MMP-9 (Serum)
EDSS	–	r = 0.62, p <0.001	r = 0.55, p <0.001	r = 0.48, p <0.001	r = 0.50, p <0.001	r = 0.52, p <0.001	r = 0.46, p <0.001
Disease Duration	r = 0.62, p <0.001	–	r = 0.45, p <0.001	r = 0.40, p =0.002	r = 0.42, p =0.001	r = 0.43, p =0.001	r = 0.39, p =0.003
NfL (Serum)	r = 0.55, p <0.001	r = 0.45, p <0.001	–	r = 0.68, p <0.001	r = 0.60, p <0.001	r = 0.58, p <0.001	r = 0.61, p <0.001
GFAP (Serum)	r = 0.48, p <0.001	r = 0.40, p =0.002	r = 0.68, p <0.001	–	r = 0.55, p <0.001	r = 0.50, p <0.001	r = 0.52, p <0.001
CHI3L1 (Serum)	r = 0.50, p <0.001	r = 0.42, p =0.001	r = 0.60, p <0.001	r = 0.55, p <0.001	–	r = 0.57, p <0.001	r = 0.49, p <0.001
CXCL13 (Serum)	r = 0.52, p <0.001	r = 0.43, p =0.001	r = 0.58, p <0.001	r = 0.50, p <0.001	r = 0.57, p <0.001	–	r = 0.51, p <0.001
MMP-9 (Serum)	r = 0.46, p <0.001	r = 0.39, p =0.003	r = 0.61, p <0.001	r = 0.52, p <0.001	r = 0.49, p <0.001	r = 0.51, p <0.001	–

Table 6. shown EDSS was strongly correlated with disease duration ($r = 0.70$, $p < 0.001$) and showed significant positive correlations with NfL ($r = 0.60$, $p < 0.001$), GFAP ($r = 0.55$, $p < 0.001$), CHI3L1 ($r = 0.57$, $p < 0.001$), CXCL13 ($r = 0.59$, $p < 0.001$), and MMP-9 ($r = 0.52$, $p < 0.001$). Similarly, disease duration correlated positively with NfL ($r = 0.50$, $p < 0.001$), GFAP ($r = 0.48$, $p < 0.001$), CHI3L1 ($r = 0.50$, $p < 0.001$), CXCL13 ($r = 0.51$, $p < 0.001$), and MMP-9 ($r = 0.45$, $p = 0.002$). Among biomarkers, NfL demonstrated the strongest inter-marker correlation, being highly associated with GFAP ($r = 0.72$, $p < 0.001$), CHI3L1 ($r = 0.65$, $p < 0.001$), CXCL13 ($r = 0.63$, $p < 0.001$), and MMP-9 ($r = 0.60$, $p < 0.001$). GFAP also correlated significantly with CHI3L1 ($r = 0.58$, $p < 0.001$), CXCL13 ($r = 0.55$, $p < 0.001$), and MMP-9 ($r = 0.53$, $p < 0.001$). Likewise, CHI3L1 was positively associated with CXCL13 ($r = 0.60$, $p < 0.001$) and MMP-9 ($r = 0.55$, $p < 0.001$), while CXCL13 correlated with MMP-9 ($r = 0.56$, $p < 0.001$). Overall, higher disability and longer disease duration were consistently associated with elevated levels of neuroaxonal injury (NfL), astroglial activation (GFAP), and inflammatory biomarkers (CHI3L1, CXCL13, MMP-9), underscoring their potential as markers of disease progression and severity.

Table 6. Correlation Between EDSS, Disease Duration, and Serum Biomarkers in the SPMS group

Variable	EDSS	Disease Duration	NfL (Serum)	GFAP (Serum)	CHI3L1 (Serum)	CXCL13 (Serum)	MMP-9 (Serum)
EDSS	–	r = 0.70, p <0.001	r = 0.60, p <0.001	r = 0.55, p <0.001	r = 0.57, p <0.001	r = 0.59, p <0.001	r = 0.52, p <0.001
Disease Duration	r = 0.70, p <0.001	–	r = 0.50, p <0.001	r = 0.48, p <0.001	r = 0.50, p <0.001	r = 0.51, p <0.001	r = 0.45, p =0.002
NfL (Serum)	r = 0.60, p <0.001	r = 0.50, p <0.001	–	r = 0.72, p <0.001	r = 0.65, p <0.001	r = 0.63, p <0.001	r = 0.60, p <0.001
GFAP (Serum)	r = 0.55, p <0.001	r = 0.48, p <0.001	r = 0.72, p <0.001	–	r = 0.58, p <0.001	r = 0.55, p <0.001	r = 0.53, p <0.001
CHI3L1 (Serum)	r = 0.57, p <0.001	r = 0.50, p <0.001	r = 0.65, p <0.001	r = 0.58, p <0.001	–	r = 0.60, p <0.001	r = 0.55, p <0.001
CXCL13 (Serum)	r = 0.59, p <0.001	r = 0.51, p <0.001	r = 0.63, p <0.001	r = 0.55, p <0.001	r = 0.60, p <0.001	–	r = 0.56, p <0.001
MMP-9 (Serum)	r = 0.52, p <0.001	r = 0.45, p =0.002	r = 0.60, p <0.001	r = 0.53, p <0.001	r = 0.55, p <0.001	r = 0.56, p <0.001	–

Table 7. DSS correlated strongly with disease duration ($r = 0.65$, $p < 0.001$) and was positively associated with NfL ($r = 0.58$, $p < 0.001$), GFAP ($r = 0.52$, $p < 0.001$), CHI3L1 ($r = 0.55$, $p < 0.001$), CXCL13 ($r = 0.56$, $p < 0.001$), and MMP-9 ($r = 0.50$, $p < 0.001$). Similarly, disease duration showed significant correlations with NfL ($r = 0.48$, $p < 0.001$), GFAP ($r = 0.45$, $p = 0.001$), CHI3L1 ($r = 0.47$, $p = 0.001$), CXCL13 ($r = 0.48$, $p = 0.001$), and MMP-9 ($r = 0.43$, $p = 0.002$). Among biomarkers, NfL demonstrated the strongest inter-marker correlations, being highly associated with GFAP ($r = 0.70$, $p < 0.001$), CHI3L1 ($r = 0.62$, $p < 0.001$), CXCL13 ($r = 0.60$, $p < 0.001$), and MMP-9 ($r = 0.58$, $p < 0.001$). GFAP also correlated significantly with CHI3L1 ($r = 0.55$, $p < 0.001$), CXCL13 ($r = 0.52$, $p < 0.001$), and MMP-9 ($r = 0.50$, $p < 0.001$). CHI3L1 was associated with CXCL13 ($r = 0.58$, $p < 0.001$) and MMP-9 ($r = 0.53$, $p < 0.001$), while CXCL13 correlated positively with MMP-9 ($r = 0.54$, $p < 0.001$). Overall, higher disability and longer disease duration were consistently associated with increased levels of NfL, GFAP, CHI3L1, CXCL13, and MMP-9, highlighting their interrelated roles as markers of neurodegeneration and inflammation in MS.

Table 7. Correlation Between EDSS, Disease Duration, and Serum Biomarkers in PPMS Group

Variable	EDSS	Disease Duration	NfL (Serum)	GFAP (Serum)	CHI3L1 (Serum)	CXCL13 (Serum)	MMP-9 (Serum)
EDSS	–	r = 0.65, p <0.001	r = 0.58, p <0.001	r = 0.52, p <0.001	r = 0.55, p <0.001	r = 0.56, p <0.001	r = 0.50, p <0.001
Disease Duration	r = 0.65, p <0.001	–	r = 0.48, p <0.001	r = 0.45, p =0.001	r = 0.47, p =0.001	r = 0.48, p =0.001	r = 0.43, p =0.002
NfL (Serum)	r = 0.58, p <0.001	r = 0.48, p <0.001	–	r = 0.70, p <0.001	r = 0.62, p <0.001	r = 0.60, p <0.001	r = 0.58, p <0.001
GFAP (Serum)	r = 0.52, p <0.001	r = 0.45, p =0.001	r = 0.70, p <0.001	–	r = 0.55, p <0.001	r = 0.52, p <0.001	r = 0.50, p <0.001
CHI3L1 (Serum)	r = 0.55, p <0.001	r = 0.47, p =0.001	r = 0.62, p <0.001	r = 0.55, p <0.001	–	r = 0.58, p <0.001	r = 0.53, p <0.001

CXCL13 (Serum)	r = 0.56, p <0.001	r = 0.48, p =0.001	r = 0.60, p <0.001	r = 0.52, p <0.001	r = 0.58, p <0.001	–	r = 0.54, p <0.001
MMP-9 (Serum)	r = 0.50, p <0.001	r = 0.43, p =0.002	r = 0.58, p <0.001	r = 0.50, p <0.001	r = 0.53, p <0.001	r = 0.54, p <0.001	–

The present study showed that both serum and cerebrospinal fluid (CSF) biomarkers were significantly altered in patients with multiple sclerosis, with the most pronounced abnormalities observed in the progressive phenotypes, namely SPMS and PPMS. These findings are consistent with the modern understanding of MS as a disease characterized not only by inflammatory demyelination but also by neuroaxonal injury, astroglia activation, and chronic innate immune dysregulation. Previous reviews have emphasized that the difference between relapsing and progressive MS is not only clinical, but also pathophysiological, reflecting a shift from predominantly acute inflammatory activity toward chronic neurodegenerative and compartmentalized inflammatory processes (12,13). The current findings strongly support this concept by demonstrating a higher biomarker burden in the progressive subtypes. The marked increase in NfL in MS patients, especially in the SPMS and PPMS groups, is in strong agreement with previous studies that identified this marker as one of the most informative indicators of neuroaxonal damage in MS. NfL increases in the setting of active tissue injury, correlates with inflammatory activity and MRI lesion load, and may also reflect cumulative long-term damage. Therefore, the higher NfL levels observed in the progressive forms in this study are in agreement with the view that SPMS and PPMS are characterized by ongoing axonal loss and irreversible tissue injury (14). In addition, the superior performance of CSF NfL compared with serum NfL is expected, since CSF is more directly related to intrathecal pathological processes (15). The findings for GFAP are also consistent with previous studies suggesting that astrocytic activation becomes more prominent as MS advances toward the progressive phase. Systematic reviews and biomarker studies reported that GFAP may be particularly useful in identifying progressive disease, where chronic gliosis and astrocyte-related pathology are more pronounced than in early relapsing disease. According to the current research, elevated levels of serum GFAP and CSF GFAP were observed in SPMS and PPMS when compared to RRMS, consistent with an active component of disease progression (16). Similarly, this finding supports the notion that GFAP may add value to NfL, as it reveals a different biological aspect of MS pathology (17). Relating to CHI3L1, this elevation in the present study agrees with many previous reports characterising this biomarker of glial activation, innate immune activity, and worse clinical outcomes. Previous research has indicated that CHI3L1 is connected to higher inflammatory severity, a switch to clinically definite MS, and a less favorable long-term prognosis. As a result, the heightened presence of CHI3L1 in the SPMS and PPMS groups aligns with these findings, indicating that this biomarker might point toward active tissue remodeling and chronic neuroinflammation rather than acute relapse-derived activity (18). The better discriminatory value of CSF CHI3L1 compared with serum CHI3L1 is also in accordance with prior studies; intrathecal inflammatory markers are more sensitive in CSF (19). The findings for CXCL13 also corroborate with the literature. Research has shown that the recruitment of B-cells and intrathecal immune activation is closely linked with this chemokine. Also, studies show that elevated CSF CXCL13 is associated with greater inflammatory activity and disease severity. In the present study, there was a significant increase of CXCL13 in all MS phenotypes with the highest being in progressive forms of the diseases. It concurs with the notion that B-cell-driven inflammation is not limited to early relapsing disease, but may persist in progressive MS compartmentalized within the CNS (20). Equally, the higher performance of CSF CXCL13, relative to serum CXCL13, is consistent with previous studies emphasizing its greater relevance in the CSF compartment (21). The results obtained in this study about MMP-9 are consistent with previous studies, where an association has been found between this marker and blood–brain–barrier disruption, as well as extravasation of inflammatory cells into the CNS. Previous research indicates that the MMP-9 participate in the MS pathogenesis by allowing the immune cells to enter the brain and the spinal cord. MMP-9 concentrations in both serum and CSF were markedly elevated in patients with secondary progressive multiple sclerosis (SPMS) and primary progressive multiple sclerosis (PPMS) compared with relapsing remitting multiple sclerosis (RRMS) and healthy controls. This finding suggests that tissue remodeling and chronic inflammatory activity continue beyond the relapsing phase of the disease (22). A noteworthy point that emerges from our study is that CSF biomarkers are more effective than serum biomarkers in differentiating disease phenotypes. Results are consistent with established principles of biomarker research. Also, other previous MS studies showed that biomarkers measured in CSF are more closely related to the site of pathology and generally yield higher sensitivity and specificity. While serum biomarkers are more practical and less invasive, the results confirm the continuing utility of CSF, especially in more demanding or more progressive cases where greater biological precision is warranted (23). The positive associations noticed with EDSS, disease duration, and biomarkers (NfL, GFAP,

CHI3L1, CXCL13, and MMP-9) are consistent with previous studies. According to multiple reports, it was found that the axonal loss, glial activation and chronic inflammation evidence were greater when the disability scores and disease duration were higher. The current study showed that these associations occurred in all MS types and they were strongest for the progressive groups, consistent with the clinical notion that disability accumulation in progressive MS reflects a combined effect of neurodegeneration and ongoing inflammation (24). Cathepsin S findings appear biologically plausible and are largely in line with preceding mechanistic studies, however, the relevant MS biomarker literature is scarce. Cathepsin S is the pivotal enzyme involved in the MHC class II processing of the invariant chain, an essential antigen presentation component, making it enrich in autoimmune disease. Furthermore, studies suggest that Cathepsin S may cause microglial activation and chronic inflammation in the CNS. Thus, the levels of this protein observed in SPMS and PPMS in the present study support the literature suggesting they promote progression by implicating antigen presentation and persistent immune activation (25). Unlike the findings concerning Cathepsin D, those surrounding Cathepsin H ought to be viewed with greater caution. Information on Cathepsin H specifically for MS is limited. The representation of d-peptidase in progressive types is not at all contrary to literature on other regulatory and immunomodulatory proteases being elevated in disease in progressive forms, as it belongs to the lysosomal protease family involved in immune regulation and tissue remodeling. As such, this finding can be interpreted as exploratory but promising and should be assessed in further study (26). Findings regarding the NPSs also agree with the increasing attention given to the role of neutrophils as hitherto underestimated contributors to the pathogenesis of MS. In recent retrospective examinations, neutrophils were suggested to participate in injuring tissues via degranulation, formation of extracellular traps, oxidative stress, as well as release of proteases, aggravating blood-brain barrier dysfunction in addition to enhancing inflammation. In other words, PSPMS and SPMS patients have persistently elevated Neurologic Severity Index (NP-wide) scores at the same level due to chronic inflammation in lesions. An influx of CRP, ESR, and WBC amid the progressive groups suggests a more vigorous peripheral innate inflammation component at play in severe disease (27). As for AAP-1, the present study suggests a possible association with progressive MS, but the other study appears insufficient for a strong direct comparison. Therefore, it is more accurate to describe this finding as a novel and biologically interesting observation that is compatible with the general role of protease activation pathways in chronic inflammation, while still requiring independent validation in future investigations (28). Overall, the findings of this study imply that the process of MS (Multiple Sclerosis) progression involves a number of mediators acting simultaneously including neuroaxonal degeneration, astroglial activation, immune-cell recruitment, and proteases that injure their tissue. The current model of MS pathogenesis does not view MS solely as a demyelinating disorder mediated by lymphocytes but rather a more complex affair involving the interplay of adaptive immunity, often against self, innate immunity, glia, and inflammation in the CNS that is chronic and compartmentalized (29). The finding that the levels of NfL, GFAP, CHI3L1, CXCL13, MMP-9, cathepsins and NSP-related markers were elevated in this study strongly support this integrated pathological model. The current results are compatible with the view that combined biomarker panels are more informative than single biomarkers. Although NfL serves as a robust indicator of neuroaxonal injury, combining it with GFAP, CHI3L1, CXCL13, and MMP-9 provides insight into astroglia activation and blood–brain barrier dysfunction. Including protease-associated signals like Cathepsin S and NSPs might increase damage-related complexity. Consequently, this study aligns with the widely accepted view of better utilizing their combination by almost all scientists, as it will improve the biological characterization of MS most notably RRMS, SPMS, and PPMS (30).

4. CONCLUSION

This study demonstrates that cathepsin S, cathepsin H, and other neuroinflammatory biomarkers play a significant role in the pathophysiology and clinical characterization of Multiple Sclerosis. Cerebrospinal fluid (CSF) biomarkers consistently showed superior diagnostic accuracy compared to serum counterparts across all MS subtypes, highlighting their closer association with central nervous system pathology. Among the evaluated markers, neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) exhibited the highest discriminative performance, particularly in progressive forms of the disease. The findings support the potential utility of cathepsin S and cathepsin H as complementary biomarkers for disease monitoring and stratification. While CSF-based assays provide greater sensitivity and specificity, serum biomarkers remain a practical and less invasive alternative for routine clinical use, especially when advanced detection technologies are available. Overall, integrating multiple biomarkers may enhance diagnostic precision and improve the assessment of disease activity and progression in MS patients. Further longitudinal studies are warranted to validate these findings and to establish standardized biomarker panels for clinical application.

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