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Association of Levels of CSF Osteopontin With Cortical Atrophy and Disability in Early Multiple Sclerosis

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Abstract

Background and Objectives

To evaluate CSF inflammatory markers with accumulation of cortical damage as well as disease activity in patients with early relapsing-remitting MS (RRMS).

Methods

CSF levels of osteopontin (OPN) and 66 inflammatory markers were assessed using an immune-assay multiplex technique in 107 patients with RRMS (82 F/25 M, mean age 35.7 ± 11.8 years). All patients underwent regular clinical assessment and yearly 3T MRI scans for 2 years while 39 patients had a 4-year follow-up. White matter lesion number and volume, cortical lesions (CLs) and volume, and global cortical thickness (CTh) were evaluated together with the 'no evidence of disease activity' (NEDA-3) status, defined by no relapses, no disability worsening, and no MRI activity, including CLs.

Results

The random forest algorithm selected OPN, CXCL13, TWEAK, TNF, IL19, sCD30, sTNFR1, IL35, IL16, and sCD163 as significantly associated with changes in global CTh. OPN and CXCL13 were most related to accumulation of atrophy after 2 and 4 years. In a multivariate linear regression model on CSF markers, OPN ($p < 0.001$), CXCL13 ($p = 0.001$), and sTNFR1 ($p = 0.024$) were increased in those patients with accumulating atrophy (adjusted R-squared 0.615). The 10 markers were added in a model that included all clinical, demographic, and MRI variables: OPN ($p = 0.002$) and IL19 ($p = 0.022$) levels were confirmed to be significantly increased in patients developing more CTh change over the follow-up (adjusted R-squared 0.619). CXCL13 and OPN also revealed the best association with NEDA-3 after 2 years, with OPN significantly linked to disability accumulation (OR 2.468 [1.46–5.034], $p = 0.004$) at the multivariate logistic regression model.

Discussion

These data confirm and expand our knowledge on the prognostic role of the CSF inflammatory profile in predicting changes in cortical pathology and disease activity in early MS. The data emphasize a crucial role of OPN.

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Glossary

CDW = confirmed disability worsening; **CLs** = cortical lesion; **EDSS** = Expanded Disability Status Scale; **FDR** = False Discovery Rate; **FLAIR** = fluid attenuated inversion recovery; **GM** = gray matter; **LRT** = likelihood ratio tests; **LST** = lesion segmentation tool; **MS** = multiple sclerosis; **OPN** = osteopontin; **PIRA** = progression independent from relapsing activity; **PPI** = protein-protein interaction; **TFE** = turbo field echo; **WM** = white matter.

Introduction

Multiple sclerosis (MS) is the most common immune-mediated disorder of the CNS. The disease course is usually characterized by an initial relapsing-remitting phase (RRMS), defined by new neurologic symptoms and subsequent disability.[1] Later in the disease course, most patients develop a progressive accumulation of disability, mostly independent of relapses (secondary progressive MS, SPMS).[1,2]

Focal and diffuse white matter (WM) and gray matter (GM) damage characterize MS pathology. GM damage occurs from earlier disease stages and represents a negative prognostic factor for MS-related disability, associated with compartmentalized meningeal and perivascular inflammations[3,4] that persist in both RRMS and SPMS, associating a severe disease course.[5]

Nevertheless, even if focal GM damage represents a surrogate marker of long-term disability, its pathogenesis continues to be debated. Currently, the main investigated pathologic mechanisms are as follows: (1) persistent inflammation in the adjacent meningeal sulci and subsequent subpial demyelination; (2) focal and diffuse intracortical demyelination; (3) neuroaxonal degeneration associated with persistent WM inflammatory activity.[6,7]

Notably, disease activity in the first years after diagnosing MS and early cortical atrophy accumulation actively influence long-term prognosis.[8,9] These observations catalyzed a search for inflammatory markers associated with an early severe course, allowing for developing a tailored and personalized therapeutic approach.[10] Such research might illuminate new aspects of MS physiopathology, including how GM damage accumulates.

In line with this aim, CSF biomarkers remain an easily accessible and reasonable surrogate of intrathecal inflammatory processes.[11] CSF inflammatory markers, particularly related to B-cell recruitment, have been previously associated with meningeal inflammation and related GM lesion load, disease activity, and accumulation of cortical atrophy in early MS.[4,12] Similarly, molecules implicated in chronic microglial activation, such as CHIT-1L3 and sCD163, have been suggested as good surrogate markers of chronic microglial activity at the edge of demyelinating lesions.[13-16]

Notably, osteopontin (OPN) emerged as one of the major drivers of lymphocyte recruitment and activation in the CNS, being also related to monocyte/microglia activation.[17,18] We, therefore, aimed to evaluate an extensive panel of inflammatory molecules, including OPN, in a cohort of patients with RRMS at the time of diagnosis to explore their association with both early GM damage accumulation, including cortical lesions (CLs) and atrophy, and disease activity. We analyzed their potential value in predicting MRI and clinical outcomes, with the aim to provide surrogate markers of compartmentalized inflammatory processes in early MS.

Methods

Patient Cohort

A total of 107 consecutive treatment-naive patients with RRMS were enrolled from January 2018 at the time of diagnosis at the MS Center of Verona University Hospital (Italy) and followed up for at least 2 years. A subgroup of 39 patients completed a 4-year follow-up. All patients had a confirmed MS diagnosis according to the most recent diagnostic criteria.[1] They underwent regular neurologic evaluation with the Expanded Disability Status Scale (EDSS)[19] every 3 months in the first year and then every 6 months, with additional visits in case of relapses. A relapse was defined as a worsening of neurologic impairment or appearance of a new symptom or abnormality attributable to MS, lasting at least 24 hours and preceded by the stability of at least 1 month.[20]

At the time of diagnosis, all patients started a first-line disease-modifying therapy (dimethyl fumarate or teriflunomide) and were scheduled to undergo brain 3T MRI after 3 (re-baseline), 12, 24, and, when possible, 48 months after diagnosis and treatment initiation. Intermediate MRI results were taken into account for the analysis. A lumbar puncture with CSF collection and analysis were performed at the time of diagnosis.

The combined three-domain status of "no evidence of disease activity" (NEDA-3) was defined by no evidence of relapses, MRI activity (new or enlarged white matter (WM), T2 lesions, gadolinium-enhancing (Gd+) lesions), and 6-month confirmed disability worsening (CDW), defined as an increase of ≥1 point in EDSS.[21] The appearance of CLs was included in the definition of NEDA-3 that we adopted in this study. Occurrence of progression independent from relapsing activity (PIRA events) was recorded; the PIRA event was defined as a 6-month CDW event, without previous relapses or an onset more than 90 days after the start date of the last

relapse, without occurrence of relapse within 30 days before or after the EDSS confirmation.[22]

CSF Analysis

CSF samples were obtained at diagnosis, at least 1 month after the last relapse and within 1 week of the MRI, according to Consensus Guidelines for CSF and Blood Biobanking.[23] After centrifugation, the supernatant was stored separately at −80°C until use. Analysis of CSF levels of 67 inflammatory mediators was performed using a combination of immune-assay multiplex techniques based on the Luminex technology (40-Plex and 37-Plex, Bio-Plex X200 System equipped with a magnetic workstation; BioRad, Hercules, CA), as previously described.[4] All samples were duplicated in the same experiment and 2 consecutive experiments to verify the results' reproducibility and consistency. The CSF level of each protein detected during the analysis was normalized to the total protein concentration of each CSF sample (measured by the Bradford protocol). The levels of neurofilament protein light chain (Nf-L) in the CSF samples were measured using a Human Nf-L ELISA kit (MyBioSource, San Diego, CA) according to previously optimized procedures.[4] The quantification of Nf-L was performed using a multiwell plate reader at a wavelength of 450 nm (Biorad, Italy). CSF/serum albumin ratio, immunoglobulin G index, and presence/absence of oligoclonal bands were evaluated.

MRI Protocol and Analysis

All patients underwent 3T MRI using a Philips Achieva scanner at the Neuroradiology Unit of the University Hospital of Verona at least 1 month from the last relapse. The following sequences were acquired:

1. 3D T1-weighted turbo field echo (TFE) (repetition time (TR)/echo time (TE) = 8.4/3.7 ms, voxel size of 1 × 1 × 1 mm^3), with the acquisition time of 5:51 minutes
2. 3D double inversion recovery (DIR, TR/TE = 5,500/275 ms, inversion time (TI) TI1/TI2 = 450 ms/2,550 ms, voxel size of 1 × 1 × 1 mm^3); turbo spin echo (TSE) readout with an optimal variable flip angle scheme, the number of excitations of 3, and acquisition time of 10:49 minutes;
3. 3D fluid-attenuated inversion recovery (FLAIR) (TR/TE = 8,000/288 ms, TI = 2,356 ms, voxel size of 1 × 1 × 1 mm^3); the same TSE readout as the DIR sequence, with the number of excitations of 1 and the acquisition time of 4:48 minutes;
4. 3D T1-weighted TFE postcontrast sequence with the same parameters of the precontrast sequence (TR/TE = 8.4/3.7 ms, voxel size of 1 × 1 × 1 mm^3, the acquisition time of 5:51 minutes).

White Matter Lesion Detection and Lesion Load Assessment

The lesion segmentation tool (LST), based on SPM12 software,[24] was applied to FLAIR images to automatically identify and segment WM lesions and to obtain a T2 hyperintense WM lesion volume (WMLv) at baseline.

CL Number and Volume

The number of CLs was assessed on DIR images following the recent recommendations for CL scoring in patients with MS.[25] Such number included both intracortical and mixed (WM/GM) lesions while subpial were not counted because of technical difficulties. The total CL volume (CLv) was calculated using a semiautomatic thresholding technique based on a fuzzy C-mean algorithm included in MIPAV software.

Cortical Thickness Evaluation

Global measurements of the cortical thickness (CTh) were obtained by applying FreeSurfer,[26] release v7.1.0, on a lesion-filled T1-weighted structural volumetric image obtained using LST. The mean of the left and right hemispheres for each ROI of the FreeSurfer parcellation was considered for the analysis.

Statistical Analysis

Differences among groups (patients with and without disease activity) were initially assessed with the Mann-Whitney and chi-square/Fisher exact tests when appropriate. Correlation between protein values and atrophy rates was initially assessed with the Spearman rank test.

The random forest (RF) approach was used to obtain CSF markers associated with CTh change and NEDA-3 outcome using the minimal depth (MD) and the total number of trees (times a root).

The minimal depth variable in a tree equals the depth of the node which splits on that variable and is the closest to the tree's root; the lower the MD, the higher the variable predictive accuracy. The times a root measure corresponds to the total number of trees in which the variable is used for splitting the root node; a higher times a root measure reflects higher prediction power of the variables.

To better investigate the CSF proteins significantly associated with the sample traits in the previous analyses, we used STRING[27] to quantify (1) module connectivity and (2) enriched biological function. Module connectivity was evaluated using the protein-protein interaction (PPI) enrichment p value. A low PPI p value indicates that the nodes (proteins) are not random and that the observed number of edges (the interaction between proteins) is significant. Strength and false discovery rate (FDR) measures were used to evaluate how large and significant the enrichment effect is for each biological process detected by the pathway analysis.

Multivariable linear regression models were applied to assess the value of clinical, demographic, MRI, and CSF markers collected at the time of diagnosis in predicting CTh changes after 2 and 4 years. Likelihood-ratio tests (LRTs) were used to compare the goodness-of-fit hierarchical models, showing whether adding the CSF variables makes the model significantly more accurate. Multivariable logistic regression models were applied to assess

the value of clinical, demographic, MRI, and CSF markers in predicting disease activity (including CDW, new relapses, and new lesions) after 2 years. A p value <0.05 was considered statistically significant. Statistical analysis was performed by means of R Studio 3.5.3 version and GraphPad Prism 9.

Standard Protocol Approvals, Registrations, and Patient Consents

The local ethics committee of the University of Verona approved the study, and written informed consent was obtained from all the patients.

Data Availability

Anonymized data used in the analyses presented in this report are available on request from qualified investigators.

Results

Study Cohort

Detailed demographic, clinical, and MRI characteristics of patients with MS at the diagnosis and after 2 years of follow-up are listed in Table 1.

After 2 years, 48% (51/107) of patients remained free from disease activity (Table 1). Twenty-nine patients experienced a relapse while the occurrence of new or enlarging T2 lesions, new CLs, or Gd-enhancing lesions was evident in 49 patients; CDW occurred in 32 while PIRA was evident in 5 patients. No severe adverse drug reactions leading to therapy discontinuation were reported during the follow-up.

Patients with disease activity were characterized by a higher EDSS score at the time of diagnosis ($p = 0.002$) and increased CL load (CLn, $p = 0.001$; CLv, $p = 0.002$; Table 1). Characteristics of patients who reached the 4-year follow-up are listed in eTable 1.

Osteopontin and CXCL13 Are Associated With Accumulating Cortical Atrophy

Patients with higher cortical thinning rates in the first 2 years of follow-up showed, among others, increased levels of both osteopontin ($r = -0.374$, $p < 0.001$) and CXCL13 ($r = -0.447$, $p < 0.001$) at the time of diagnosis (additional data are listed in eTable 2). CSF Nf-L levels (mean 2 ng/mL ± 1.48) correlated with accumulating cortical thinning ($r = -0.266$, $p = 0.027$). No differences in accumulating cortical atrophy have been detected according to the treatment administered (mean annualized change of −0.59% ± 0.36 in patients treated with DMF vs −0.56% ± 0.41 in patients treated with teriflunomide, $p = 0.241$).

The random forest selected OPN, CXCL13, TWEAK, TNF, IL19, sCD30, sTNFR1, IL35, IL16, and sCD163 as the most

Table 1 Baseline Demographic, Clinical, and MRI Characteristics of the Whole Population and According to Disease Activity at the 2-Year Follow-Up

	Total MS (n = 107)	2y EDA (n = 56)	2y NEDA (n = 51)	*p* Value
Age, y	35.7 ± 11.8	35.5 ± 11.7	38.4 ± 11.7	**0.021**
Female sex, no. (%)	82 (76.6)	45 (80.4)	37 (72.6)	0.369
EDSS score, median (range)	2 (0–5)	2 (0–5)	1.5 (0–4)	0.168
WMLN, mean ± SD	9 ± 5.1	9.4 ± 4.5	8.5 ± 3.9	0.347
WMLV, mean ± SD	1,015.8 ± 957.1	1,217.6 ± 1,199.1	794.2 ± 514.5	0.121
Spinal cord lesion number	0.6 ± 1.1	0.7 ± 1.2	0.5 ± 1.1	0.418
Gd+ lesions, mean ± SD	0.2 ± 0.5	0.2 ± 0.6	0.1 ± 0.5	0.459
CLn, mean ± SD	4 ± 4.6	5.6 ± 5.1	2.3 ± 3.3	**0.001**
CLv, mean ± SD	353.4 ± 437.0	483.6 ± 492.5	207.5 ± 309.6	**0.002**
Global CTh, mm^3	2.5 ± 0.3	2.4 ± 0.3	2.5 ± 0.2	0.757
Annual CTh change (%), mean ± SD	−0.58 ± 0.38	−0.71 ± 0.46	−0.44 ± 0.19	0.194
Albumin CSF/serum	5.2 ± 1.8	5.2 ± 1.7	5.2 ± 1.8	0.920
IgG Index	0.63 ± 0.27	0.59 ± 0.14	0.69 ± 0.40	0.566
CSF OCBs (yes/no)	81/26	43/13	38/13	0.824

Abbreviations: CLn = cortical lesion number; CLv = cortical lesion volume; CTh = cortical thickness; EDA = evidence of disease activity; EDSS = Expanded Disability Status Scale; Gd+ lesions = gadolinium-enhancing lesions; IgG = immunoglobulin G; NEDA = no evidence of disease activity; OCBs = oligoclonal bands; WMLn = white matter lesion number.
p values for each comparison between EDA and NEDA groups after 2 years are reported. A p value <0.05 was considered significant.
Patients with disease activity after two years were characterized by older age and higher gray matter damage, reflected by the increased cortical lesion number and volume.

important variables associated with accumulating brain atrophy. Osteopontin and CXCL13 showed the best performance (Figure 1A).

Notably, CXCL13 was strictly linked to focal cortical damage, associating with both CLs at the diagnosis ($r = 0.716$, $p < 0.001$) and new CLs ($r = 0.611$; $p < 0.001$), while OPN resulted moderately increased in those patients with a higher number of CLs at the time of diagnosis ($r = 0.191$, $p = 0.049$). No correlation between CXCL13 and OPN levels occurred ($r = 0.163$, $p = 0.136$). Patients with both high levels of OPN and CXCL13 had significantly higher atrophy rates, suggesting different, complementary effects on cortical damage accumulation (Figure 2).

OPN was selected as the best marker of cortical atrophy accumulation ($r = -0.691$, $p < 0.001$) in the subgroup of patients who underwent a 4-year follow-up with yearly MRI scans. The other markers significantly increased in those patients with higher atrophy rates after 4 years were CXCL13, IL22, IL19, IL8, IFNalfa2, CCL22, osteocalcin, IL35, and MIF (Figure 1B).

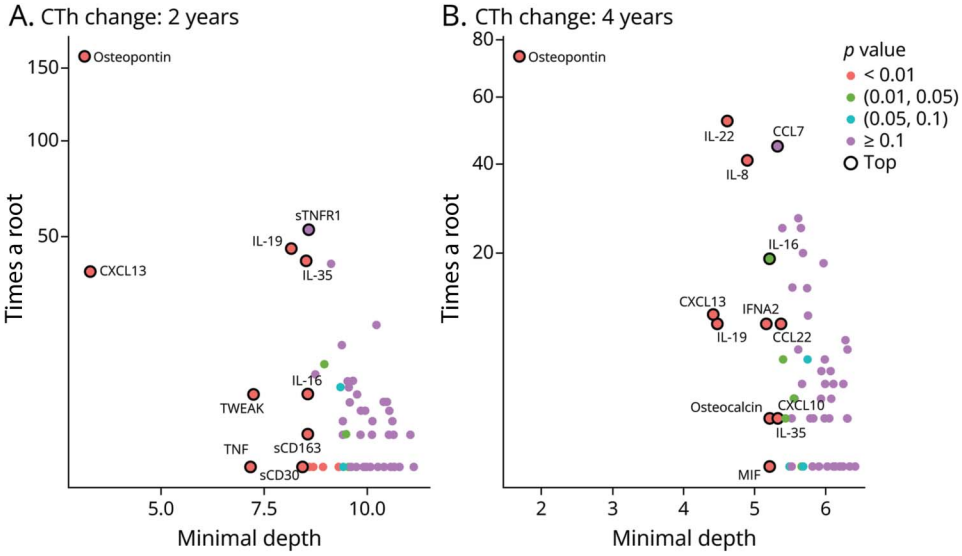
Pathway Analysis

Pathway analysis confirmed a nonrandom interaction among CSF candidate markers (protein-protein interaction p value <0.001, Figure 3). Biological processes that particularly emerged associated with the selected cytokine profiles were the regulation and establishment of the endothelial barrier, positive regulation of a chronic inflammatory response, and activation of the TNF-mediated signaling pathway (Table 2).

Predicting Cortical Thinning

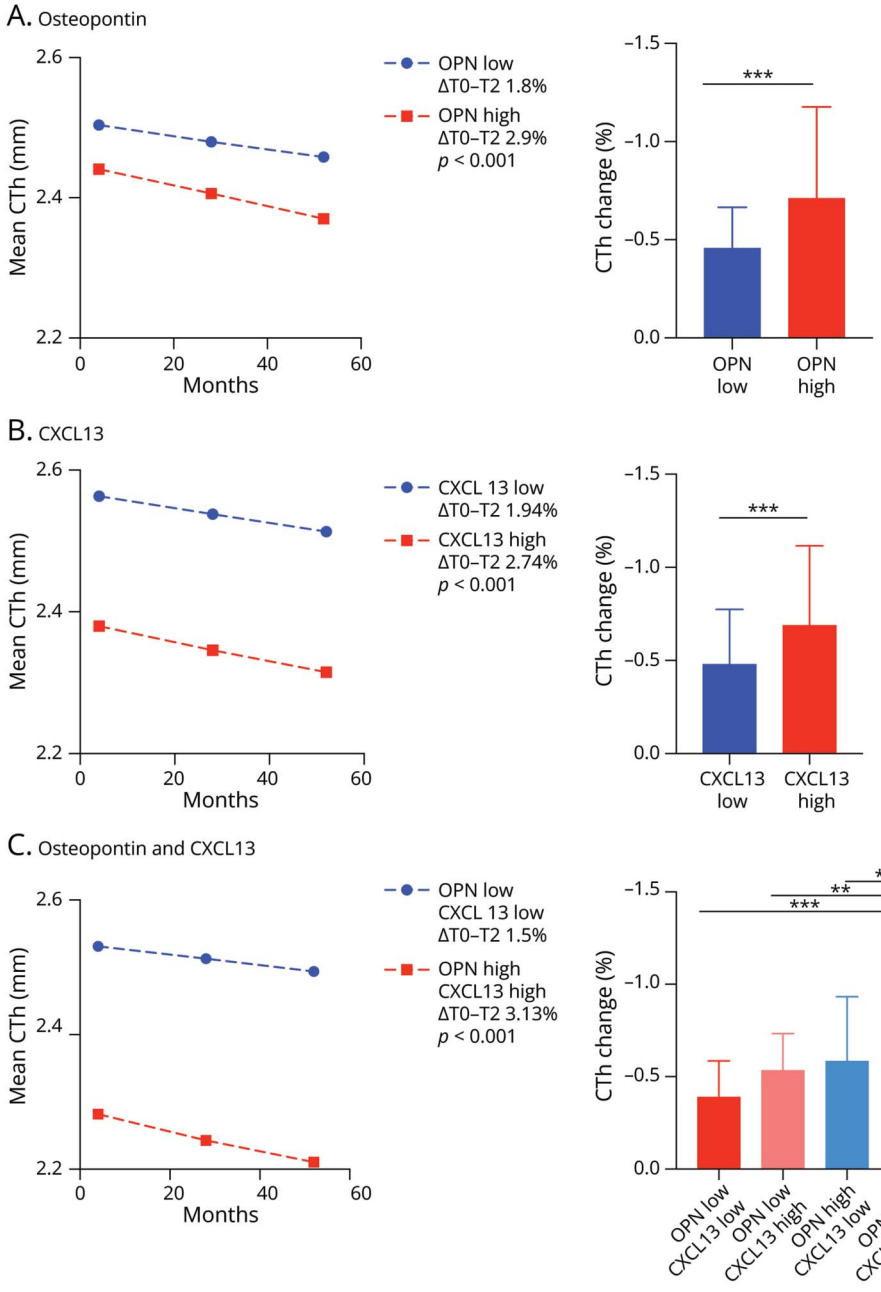
1. When including the molecules that emerged from the random forest approach in a multivariate linear regression model, OPN ($\beta = -1.7*10^{-8}$, $p < 0.001$), CXCL13 ($\beta = -3.5*10^{-5}$, $p = 0.001$), and sTNFR1 ($\beta = -2.6*10^{-7}$, $p = 0.024$) were confirmed to be independent predictors of accumulating atrophy (adjusted R-squared = 0.615).
2. The multiple regression analysis including age, sex, EDSS, WMLn, WMLv, number of spinal cord lesions, and gadolinium-enhancing lesions, but also CLn and CLv, provided evidence of a correlation between WMLn ($\beta = 3.2*10^{-4}$, $p = 0.015$) and WMLv ($\beta = -1.7*10^{-6}$, $p = 0.011$) with accumulating cortical atrophy (adjusted R-squared 0.19). Notably, after the exclusion of CLn and CLv from the model, the adjusted R-squared value was 0.07, with WMLn ($\beta = 3.2*10^{-4}$, $p = 0.033$), WMLv ($\beta = -2.4*10^{-6}$, $p = 0.006$), and the presence of spinal cord lesions ($\beta = -7.2*10^{-4}$, $p = 0.046$) best associated with accumulating atrophy.
3. The 10 selected CSF markers were then added to a single model that included all the abovementioned clinical, demographic, and MRI variables. OPN levels ($\beta = -1.8*10^{-8}$, $p = <0.001$) were confirmed significantly increased in patients with more cortical thinning over the follow-up (adjusted R-squared 0.619). Notably, after the exclusion of measures of cortical damage (CLn and CLv), along with OPN ($\beta = -1.8*10^{-8}$, $p < 0.001$), molecules associated with focal cortical damage such as CXCL13 ($\beta = -3.8*10^{-5}$, $p = 0.001$) and sTNFR1 ($\beta = -2.7*10^{-7}$, $p = 0.022$) provided additional value when compared with the analysis based on demographic, clinical, and radiologic measures most adopted in clinical practice (adjusted R-squared 0.600).

Figure 1 Random Forest Approach: OPN and CXCL13 Best Associated With Cortical Thickness Change After 2 and 4 Years of Follow-Up



(A) Multiway importance plot: OPN and CXCL13 were selected as the most important variables associated with the cortical thickness change after two years of follow-up (panel on the left). (B) OPN was selected as the most important variable after 4 years. CCL22 = chemokine (C-C motif) ligand 22; CCL7 = chemokine (C-C motif) ligand 7; CXCL10 = chemokine (C-X-C motif) ligand 10; CXCL13 = chemokine (C-X-C motif) ligand 13 or B lymphocyte chemoattractant; IFNA2 = interferon alfa2; IL16 = interleukin-16; IL19 = interleukin-19; IL22 = interleukin-22; IL-35 = interleukin-35; IL8 = interleukin-8; MIF = macrophage migration inhibitory factor; OPN = osteopontin; sCD163 = soluble Cluster of Differentiation 163; sCD30 = soluble Cluster of Differentiation 30; sTNFR1 = soluble TNFRSF1A or TNF receptor superfamily member 1A; TNF = tumor necrosis factor; TWEAK = TNFSF12 or TNF superfamily member.

Figure 2 Cortical Atrophy Rates According to Osteopontin and CXCL13 Levels



(A and B) Patients with high levels of osteopontin and CXCL13 showed significantly increased rates of cortical thinning after both 2 and 4 years. (C) Having high levels of both molecules led to higher atrophy rates. High/low was defined according to median values. CTh = cortical thickness. ΔT0-T2: cortical thickness change between baseline and the end of 4 years of follow-up (T2).

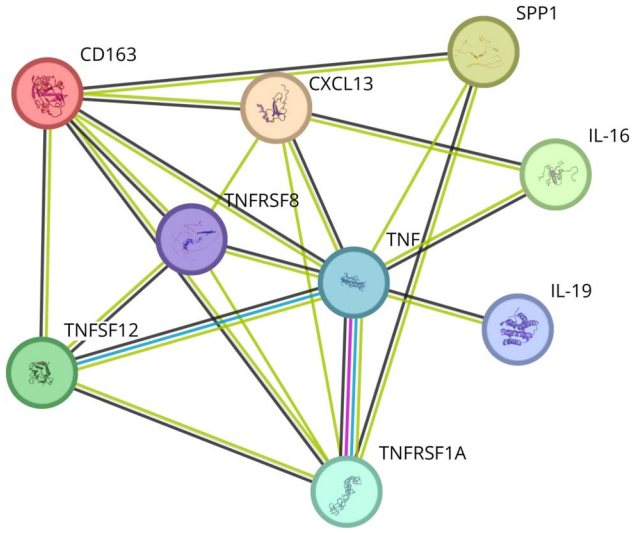
The LRT approach confirmed that these latter models (iii) inclusive of CSF variables were significantly more accurate than the ones with only clinical, demographic, and MRI variables at baseline ($p < 0.001$), suggesting an additional prognostic value when testing CSF markers.

Predicting Cortical Atrophy After 4 Years

A similar approach was adopted to further evaluate and confirm the association between molecules selected by the random forest (OPN, CXCL13, IL22, IL19, IL8, IFNalfa2, CCL22, osteocalcin, IL35, and MIF); clinical, demographic, and MRI variables; and 4 years of cortical thinning:

1. A multivariate linear regression model, among the 10 cytokines selected by the RF approach, confirmed that OPN ($\beta = -2.7*10^{-8}$, $p < 0.001$), CXCL13 ($\beta = -3.1*10^{-6}$, $p < 0.001$), and IL35 ($\beta = 4.2*10^{-6}$, $p = 0.030$) as highly expressed in patients with accumulating atrophy (adjusted R-squared 0.773).
2. A model with clinical, demographic, and MRI variables selected WMLn ($\beta = 7.7*10^{-4}$, $p = 0.027$) and WMLv ($\beta = -4.6*10^{-6}$, $p = 0.018$) as best associated with annual atrophy rates (adjusted R-squared 0.348).
3. A comprehensive model that included all CSF variables had improved accuracy (adjusted R-squared

Figure 3 Pathway Analysis of CSF Markers Identified as Associated With Accumulating Cortical Atrophy



Protein-protein associations between molecules detected at the RF approach (protein-protein enrichment p value = 7.93e-13). Table II lists the specific functions related to the pathway. CD163 = Cluster of Differentiation 163; CXCL13 = chemokine (C-X-C motif) ligand 13 or B lymphocyte chemoattractant; IL-16 = interleukin-16; IL-19 = interleukin-19; IL-35 = interleukin-35; SPP1 = secreted phosphoprotein 1 or osteopontin; TNF = tumor necrosis factor; TNFRSF1A = TNF receptor superfamily member 1A or TNFR1; TNFRSF8 = TNF receptor superfamily member 8 or Cluster of Differentiation 30; TNFSF12 = TNF superfamily member 12 or TWEAK.

0.818), with OPN being the most significant variable ($\beta = -2.7^{*}10^{-8}$, $p < 0.001$), further suggesting the additional value of testing CSF variables. The LTR approach confirmed the higher accuracy of the latter model ($p < 0.001$).

Predicting Disease Activity and Disability Accumulation

Patients with disease activity had higher cortical atrophy accumulation rates (mean annual reduction of −0.71 ± 0.46 vs −0.44 ± 0.19 mm) and increased levels of several proinflammatory molecules (additional data are listed in eTable 3). CSF Nf-L levels were slightly increased in those patients with disease activity (2.27 ng/mL ± 1.65) when compared with the NEDA group (1.70 ng/mL ± 1.22, $p = 0.225$).

The random forest approach was applied to evaluate molecules best associated with disease activity. When considering the minimal depth and times a root measures, CXCL13 and OPN were revealed as best associated, along with TNF, IFN gamma, IL8, CXCL5, CCL1, IL4, and IL6 (eFigure 1). A logistic regression model confirmed CXCL13 levels as significantly increased (OR 1.18 [1.01–1.4], $p = 0.040$) in those patients with MS activity.

Confirmed Disability Worsening

Notably, among markers of disease activity, OPN emerged as the best molecule associated with CDW over the follow-up (OR 1.85 [1.11–3.39], $p = 0.028$, Figure 4).

Accordingly, with the hypothesis that higher atrophy accumulation is associated with disability accumulation in early MS, patients with CDW developed more cortical thinning (mean annual percentage change of −0.85% ± 0.01 vs −0.47% ± 0.21, $p < 0.001$).

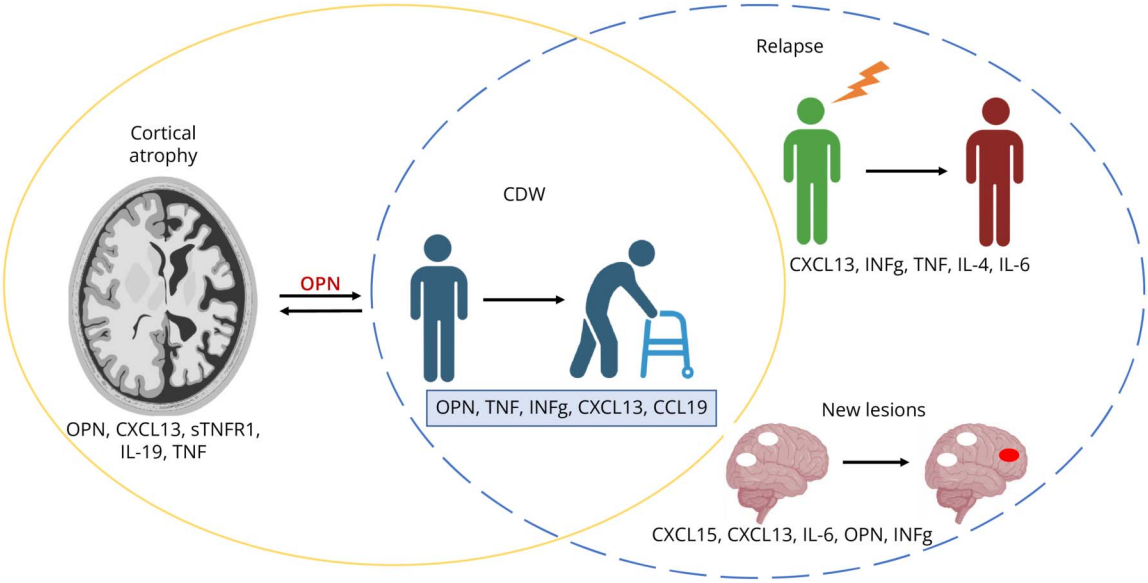
A logistic regression model with baseline demographic, clinical, and MRI variables confirmed OPN (OR 2.468 [1.46–5.034] $p = 0.004$), along with EDSS (OR 2.747 [1.379–6.186] $p = 0.007$), as significantly increased in those patients accumulating more disability. After 2 years, osteopontin was significantly

Table 2 Pathway Analysis

Biological process	Strength	False discovery rate
Cytokine-mediated signaling pathway	1.35	2.71e-05
Tumor necrosis factor–mediated signaling pathway	1.84	0.00071
Cellular response to organic substance	0.87	0.00097
Inflammatory response	1.32	0.0032
Death-inducing signaling complex assembly	2.74	0.0099
Extrinsic apoptotic signaling pathway	1.81	0.0115
Negative regulation of response to external stimulus	1.37	0.0136
Chronic inflammatory response	2.56	0.0146
Positive regulation of ceramide biosynthetic process	2.56	0.0146
Regulation of establishment of endothelial barriers	2.46	0.0182
Positive regulation of inflammatory response	1.66	0.0217
Regulation of response to external stimulus	1.03	0.0229
Positive regulation of response to external stimulus	1.23	0.0265
Regulation of anatomical structure morphogenesis	1.0	0.0275
Regulation of multicellular organismal process	0.67	0.0404
Signal transduction	0.55	0.0464
Molecular function		
Cytokine activity	1.75	8.21e-07
Cellular component		
Extracellular region	0.67	0.0015
Extracellular space	0.74	0.0033

Biological processes that particularly emerged associated with the selected cytokine profile were the regulation and establishment of the endothelial barrier, a positive regulation of a chronic inflammatory response, and the activation of the TNF-mediated signaling pathway.

Figure 4 Cytokines and Chemokines That Are Associated With Cortical Atrophy and Disease Activity



The molecules associated with each outcome are shown. OPN emerged as the best associated with accumulating cortical atrophy and confirmed disability worsening. CCL19 = chemokine (C-C motif) ligand 19; CDW = confirmed disability worsening; CXCL13 = chemokine (C-X-C motif) ligand 13 or B lymphocyte chemoattractant; CXCL15 = chemokine (C-X-C motif) ligand 15; IFNg = interferon gamma; IL-19: interleukin 19; IL-4 = interleukin 4; IL-6 = interleukin 6; OPN = osteopontin; sTNFR1 = soluble TNFRSF1A or TNF receptor superfamily member 1A; TNF = tumor necrosis factor. Created with BioRender.com.

correlated with cortical thinning in the whole CDW group (n = 32, $r = -0.486$, $p = 0.005$) while, in the 5 patients with PIRA, the correlation was high ($r = -0.600$) but without statistical significance ($p = 0.350$).

Results were overall confirmed after 4 years when CDW was evident in 22 of 39 patients, of which 10 showed PIRA events. In particular, CDW after 4 years was associated with accumulating atrophy (mean percentage annual decrease of 0.814% ± 0.005 vs 0.458% ± 0.183 mm^3, $p < 0.001$) and higher baseline OPN levels (114174.48 ± 60991.97 pg/mL vs 53338.52 ± 76842.52, $p < 0.001$). When considering patients who reached the 4-year follow-up, OPN was correlated with accumulating atrophy in the whole CDW group (n = 22, $r = -0.692$, $p = 0.001$), in those patients with PIRA (n = 10, $r = -0.8308$, $p = 0.004$), and in those with CDW without PIRA events (n = 12, $r = -0.661$, $p = 0.022$).

Occurrence of New Relapses and Radiologic Activity

Among inflammatory markers, CXCL13 (OR 1.329 [1.082–1.705], $p = 0.013$) and IFNgamma (OR 1.913 [1.132–3.499], $p = 0.023$) were best associated with relapses (Figure 4) while decreased CXCL5 levels (OR 0.743 [0.560–0.948], $p = 0.038$) and increased CXCL13 (OR 1.197 [1.01–1.453], $p = 0.047$) were confirmed in the regression analysis in those patients experiencing new radiologic activity (Figure 4).

Discussion

In this work, we observed that OPN, along with other inflammatory markers such as CXCL13 and more than other molecules related to the TNF superfamily, is associated with accumulating cortical thinning as well as with disease activity and disability accumulation, beginning with the early stages or RRMS.

Accumulating GM damage has been recognized as a major factor in driving MS-associated disability,[5,6,28,29] with extensive GM demyelination characterizing progressive MS but also being suggested as a reliable marker of disability progression occurring independently from disability in the first years of the disease course.[30]

In a previous study, we showed how inflammatory markers of focal GM damage, including the lymphoid chemokines CXCL12 and CXCL13 and TNF, assessed at the time of diagnosis, are associated with disease activity, elevated CL load, and accumulating cortical atrophy.[12] This is congruent with the notion that meningeal inflammatory aggregates are strictly linked to the underlying subpial demyelination.[4,5] Nevertheless, the complex physiopathology of diffuse GM damage, its relationship with meningeal inflammation, focal GM demyelination, and persisting WM smoldering lesions with subsequent retrograde degeneration, suggested us a need to further investigate candidate markers that apparently might reflect focal GM damage, as detected by DIR sequences.

Among all markers, OPN clearly emerged as the most prominent associated with accumulating GM atrophy, notably providing additional value when included in a regression model with demographic and radiologic variables.

OPN represents a member of SIBLING (small integrin-binding ligand, N-linked glycoproteins) family of proteins.

OPN is a binding partner of a4b1 integrin, the main homing molecule for lymphocyte entry to the brain in MS, as well as several extracellular matrix proteins, including fibronectin and vitronectin.[17,18] OPN, by interacting with several binding partners, influences various biological processes, including cell adhesion, coagulation, and upregulation of proinflammatory cytokines from both the TH1 and TH17 pathways in mice and in man.[17,31,32] OPN influences the downregulation of IL10 levels through CD44 ligand[33] and is involved in the induction of autoreactive T cells that persistently produce inflammation in the CNS by protecting from apoptotic death.[17,33,34]

Accordingly, OPN is expressed by the inflamed endothelium in the brains of patients with MS within active plaques but also in the white matter adjacent to plaques, reactive astrocytes, and microglial cells.[17,35] Notably, neurons have been suggested as capable of secreting OPN, a process that could lead to the inhibition of cell lysis, thus protecting the axon from degeneration during autoimmune demyelination.[17,18]

Our results are in line with an initial in vivo report of OPN associated with long-term atrophy in patients with MS.[36] Whether these results reflect chronic inflammatory processes occurring in active plaques in both WM and GM or microstructural changes in NAWM[37] remains to be elucidated.

Of note, these findings suggest a potential role of OPN in mediating progressive chronic inflammation that underlies the course of progressive MS. Accordingly, the progressive EAE course is rarely observed in OPN-deficient mice after immunization with MOG35–55, with OPN−/− mice being totally protected from EAE-related death.[17] Furthermore, in relapsing EAE induced by the PLP139–151 in SJl/j mice, recombinant OPN injection, after remission from a relapse, led to a more progressive disease without return to a state of remission.[34] In line with experimental evidence, we similarly found increased CSF levels of OPN in patients with primary progressive MS at the time of diagnosis,[38] further suggesting a role of OPN in not only relapse-associated inflammatory bouts but also the occurrence of disability progression independent of relapses. Notably, OPN was revealed as the CSF factor most associated with confirmed disability accumulation, in line with the notion that cortical thinning parallels disability accumulation over the disease course,[6] as observed in our patient cohort. However, CSF OPN sources need to be further addressed.

Accordingly with evidence in EAE, a role of OPN in mediating neurodegeneration has been shown by a population of OPN+ microglia bearing the CD11c surface marker that was associated with signs of Alzheimer disease neuropathology. Furthermore, OPN levels correlated with the severity of cognitive deficits.[39]

Along with OPN, we confirmed the role of the lymphoid chemokine CXCL13 as a marker of focal gray matter damage and accumulating GM atrophy,[12] known to be associated with B-cell and T-cell recruitment and organization of meningeal tertiary lymphoid structures in the sulci adjacent to the demyelinated GM.[5,40] Notably, the additive effect of OPN and CXCL13, with the latter clearly associated with focal cortical damage, also emerged from the regression models. This suggests the involvement of these molecules in combined pathologic mechanisms of cortical damage and places them as promising, complementary candidate markers of gray matter damage accrual.

Certainly, measuring CSF markers could limit the translation of these findings into clinical practice. Efforts has now been made for the detection and potential use of plasma or serum markers, with OPN as a candidate molecule being the single serum biomarkers with highest capability to distinguish MS from control cases[41] and associating with cognitive function in RRMS.[42] Similarly, serum CXCL13 levels rapidly decrease in patients with RRMS who are treatment responders, and further studies are needed to validate the use of these markers in larger populations.[43]

In conclusion, our study expands the field of possible markers of cortical atrophy and disability accumulation, emphasizing the importance of CSF assessment at the time of diagnosis and its potential to provide reliable predictors of disability accumulation as well as treatment response.[38]

Along with molecules associated with focal cortical damage that clearly emerge as reflecting chronic meningeal inflammation, we suggest OPN as a reliable marker, needing further validation in a larger and separate cohort. This study is consistent with accumulating both preclinical and ex vivo and in vivo evidence of its role in chronic compartmentalized inflammation. OPN may have varying roles, whether in smoldering chronic WM lesions, in WM or in GM, or in normal-appearing or demyelinated tissue. Clearly, OPN deserves increased attention for its role in the pathogenesis of different forms of MS.

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