Inflammation-related plasma and CSF biomarkers for multiple sclerosis

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Effective biomarkers for multiple sclerosis diagnosis, assessment of prognosis, and treatment responses, in particular those measurable in blood, are largely lacking. We have investigated a broad set of protein biomarkers in cerebrospinal fluid (CSF) and plasma using a highly sensitive proteomic immunoassay. Cases from two independent cohorts were compared with healthy controls and patients with other neurological diseases. We identified and replicated 10 cerebrospinal fluid proteins including IL-12B, CD5, MIP-1a, and CXCL9 which had a combined diagnostic efficacy similar to immunoglobulin G (IgG) index and neurofilament light chain (area under the curve [AUC] = 0.95). Two plasma proteins, OSM and HGF, were also associated with multiple sclerosis in comparison to healthy controls. Sensitivity and specificity of combined CSF and plasma markers for multiple sclerosis were 85.7% and 73.5%, respectively. In the discovery cohort, eotaxin-1 (CCL11) was associated with disease duration particularly in patients who had secondary progressive disease ($P_{CSF} < 4 \times 10^{-5}$, $P_{plasma} < 4 \times 10^{-5}$), and plasma CCL20 was associated with disease severity ($P = 4 \times 10^{-5}$), although both require further validation. Treatment with natalizumab and fingolimod showed different compartmental changes in protein levels of CSF and peripheral blood, respectively, including many disease-associated markers (e.g., IL12B, CD5) showing potential application for both diagnosing disease and monitoring treatment efficacy. We report a number of multiple sclerosis biomarkers in CSF and plasma for early disease detection and potential indicators for disease activity. Of particular importance is the set of markers discovered in blood, where validated biomarkers are lacking.

multiple sclerosis | cerebrospinal fluid | biomarkers | proteomics | proximity extension assay

Multiple sclerosis is an inflammatory disease of the central nervous system (CNS) causing damage to myelin along with neurons and axons and ultimately resulting in neurodegeneration. Together with the clinical presentation, the spatial and temporal occurrence of inflammatory lesions shown by magnetic resonance imaging (MRI) is the primary means for diagnosis (1). However, specificity remains an issue with a proportion of patients not fulfilling the established diagnostic criteria delaying proper disease management and treatment (2, 3). Given the acknowledged benefits of early treatment, there is a need for more accurate biomarkers that would allow rapid identification of disease processes and differentiation against other neurological diseases.

Arguably, the difficulty in identifying multiple sclerosis-associated blood biomarkers is likely due in part to sensitivity. Multiple sclerosis is believed to be driven by systemic immune activation of autoimmunity against CNS components (4, 5), where encephalitogenic cells accumulate in the target organ (6, 7). Due to the proximity to the CNS, cerebrospinal fluid (CSF) has been the primary object of biomarker exploration (8, 9). Immunoglobulin G (IgG) index and detection of oligoclonal bands are currently used to support diagnosis (10). In addition, there is a restricted set of CSF markers, including chemokine (C-X-C motif) ligand 13 (CXCL13), matrix metallopeptidase-9 (MMP-9), and osteopontin (OPN), which have been associated with inflammation, along with neurofilament light chains (NfL), a measure of neuron/axon damage (10–13). However, the systemic immune compartment is to some degree also activated as shown by an increased number of cells expressing proinflammatory cytokines like IFN-γ in blood (14). Despite this "low-grade" peripheral inflammation, no reproducible plasma

Significance

Molecular biomarkers for multiple sclerosis have so far mainly been limited to measures in cerebrospinal fluid (CSF). Here, we identified additional biomarkers for multiple sclerosis, 2 in plasma as well as 10 in CSF. Furthermore, we identified 2 biomarkers: eotaxin-1 (CCL11), associated with disease duration and progression in both CSF and plasma, and plasma CCL20 which showed association with disease severity. However, these findings will require further validation. The capability of measuring biomarkers for multiple sclerosis may assist in the monitoring of patients during routine clinical care such as assessing treatment response but may also allow researchers to more accurately characterize pathological processes of inflammation and neurodegeneration in both the CNS and periphery of patients with multiple sclerosis.

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Data deposition: The data that support the findings of this paper are available at the Swedish National Dataservice database, https://snd.gu.se/en (DOI: 10.5878/p6dc-8149 [discovery cohort], DOI: 10.5878/ta78-gb12 [replication cohort], DOI: 10.5878/2ver-wy19 [treatment cohorts]).

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biomarker has been consistently reported for multiple sclerosis. As increasingly sensitive technological platforms are being developed, the feasibility of identifying soluble biomarkers in blood has improved as supported by the role of NfL in sera/plasma for assessing disease activity and treatment responses (15, 16).

Persons with the relapsing–remitting subtype of multiple sclerosis display stronger inflammatory features in the CSF compared to progressive forms (10). Since therapies are mainly exerting a dampening effect on systemic immunity, this may be one explanation of why therapeutic effects are poor in progressive disease. However, more precise biomarker profiling may be useful in predicting treatment response, identifying progressive patients who are more likely to respond to treatment as well as relapsing–remitting patients with inadequate responses, including prediction of early conversion from relapsing–remitting to progressive disease.

We here report on a proteomic investigation using the proximity extension assay (PEA) (17) with the purpose of 1) determining protein biomarkers in CSF and blood associated with disease development; 2) examining differences between the proteomic profiles of relapsing–remitting and progressive disease; 3) determining biomarkers for evaluating clinical characteristics and disease severity; 4) comparing diagnostic efficacy of biomarker combinations; and 5) monitoring alterations in protein profiles following disease-modifying drugs, natalizumab (18) (Tysabri) and fingolimod (19) (Gilenya).

Results

A Set of CSF Biomarkers Capable of Early and Differential Diagnosis of Multiple Sclerosis. We have investigated the levels of inflammatory protein levels in plasma and CSF in a discovery cohort, consisting of samples from 136 patients with multiple sclerosis and 49 healthy controls sampled at Karolinska University Hospital in Stockholm, and a replication cohort, consisting of samples from 95 patients with multiple sclerosis and 47 healthy controls sampled at Sahlgrenska University Hospital in Gothenburg (Table 1) (20, 21). In the discovery cohort, 11 CSF proteins were associated with multiple sclerosis in comparison to healthy controls ($P < 5 \times 10^{-5}$), of which 10 were successfully validated in the replication cohort ($P < 0.05$) (Fig. 1 and *SI Appendix*, Fig. S1). All markers except for IL-7 and FGF-19 were increased in cases compared to controls. Several markers including IL-12B, CD5, and CXCL9 were already up-regulated in patients during early stages of disease before definite diagnosis ($P < 5 \times 10^{-5}$) and thus were potentially of value for early screening of multiple sclerosis (Fig. 1).

Trace markers with low call rates including IL-7 and CD6 showed suggestive association in the discovery cohort when examining detectable presence with the concentration of CSF IL-7 being lower in cases than in controls ($P = 2 \times 10^{-6}$; *SI Appendix*, Fig. S5). In addition, CSF CD6 was detectable in 22.2% of relapsing–remitting cases, but in none of the healthy controls ($P < 0.002$; *SI Appendix*, Table S4). Detectable CSF CD6 was also associated with higher CSF IL-12B ($P = 1.7 \times 10^{-5}$) and shorter duration of disease ($P = 8.3 \times 10^{-5}$) among relapsing–remitting cases (*SI Appendix*, Table S5).

As a result of minor improvements to the assay kits that occurred between the analyses of the two cohorts, two additional CSF proteins, TNF and IFN-γ, which were not measurable in the discovery cohort (i.e., call rate <20%), along with the newly added CD8a, which replaced BDNF, were associated with multiple sclerosis in comparison to both controls and symptomatic controls, i.e., clinically suspected cases of multiple sclerosis not fulfilling the diagnostic criteria of multiple sclerosis or clinically isolated syndrome (1) (*SI Appendix*, Fig. S2). However, the specificity of these three markers for multiple sclerosis will require further validation.

The correlation between the associated CSF proteins was similar in the discovery and replication cohorts (Fig. 2*A*).

Table 1. Summary characteristics of study cohorts

	Discovery (Stockholm)			Replication (Gothenburg)			
Variable	MS	HC	OND	MS	SC	HC	OND
N	136	49	35	95	86	47	27
N, CSF:plasma	130:111	47:46	31:28	87:94	84:85	43:47	27:26
Age, mean ± SD	39.7 ± 11.4	29.6 ± 7.1	45.7 ± 13.2	34.3 ± 9.2	34.8 ± 9.2	26.9 ± 6.5	34.4 ± 10.5
Male:female ratio	1:2.3	1:1.1	1:2.9	1:3.5	1:4.4	1:0.8	1:2.9
Disease duration							
Onset, mean ± SD	6.5 ± 7.5		7.1 ± 10.3	2.1 ± 3.8	0.3 ± 0.7	—	0.4 ± 0.9
Diagnosis, mean ± SD	2.9 ± 5.5		3.7 ± 5.5	—	—	—	—
Disease severity							
EDSS							
Mean ± SD	2.6 ± 2.0	—	—	1.6 ± 1.0	0.9 ± 1.0	—	—
Median	2	—	—	2	1	—	—
MSSS							
Mean ± SD	4.2 ± 2.6	—	—	4.1 ± 2.6	2.9 ± 2.6	—	—
Median	4.1	—	—	3.9	2.4	—	—
ARMSS							
Mean ± SD	4.4 ± 2.5	—	—	3.9 ± 2.3	2.6 ± 2.3	—	—
Median	4.1	—	—	3.7	1.2	—	—
MRI lesion							
0 to 8 lesions, *n* [*n*%]	47 [34.6%]	0 [0%]	10 [45.5%]	—	—	—	—
≥ 9 lesions, *n* [*n*%]	89 [65.4%]	0 [0%]	4 [18.2%]	—	—	—	—

Shown are descriptive statistics for two Swedish multiple sclerosis (MS) studies including disease duration, number of MRI lesions, and measures of disease severity composed of the expanded disability status score (EDSS) (22), the multiple sclerosis severity score (MSSS) (23), and the age-related multiple sclerosis severity (ARMSS) score (24). Samples were compared with healthy controls (HC), other neurological diseases (OND), and symptomatic controls (SC) who initially were suspected MS cases. Data values are mean and SD, median, or count (*n*) and percentage [*n*%].

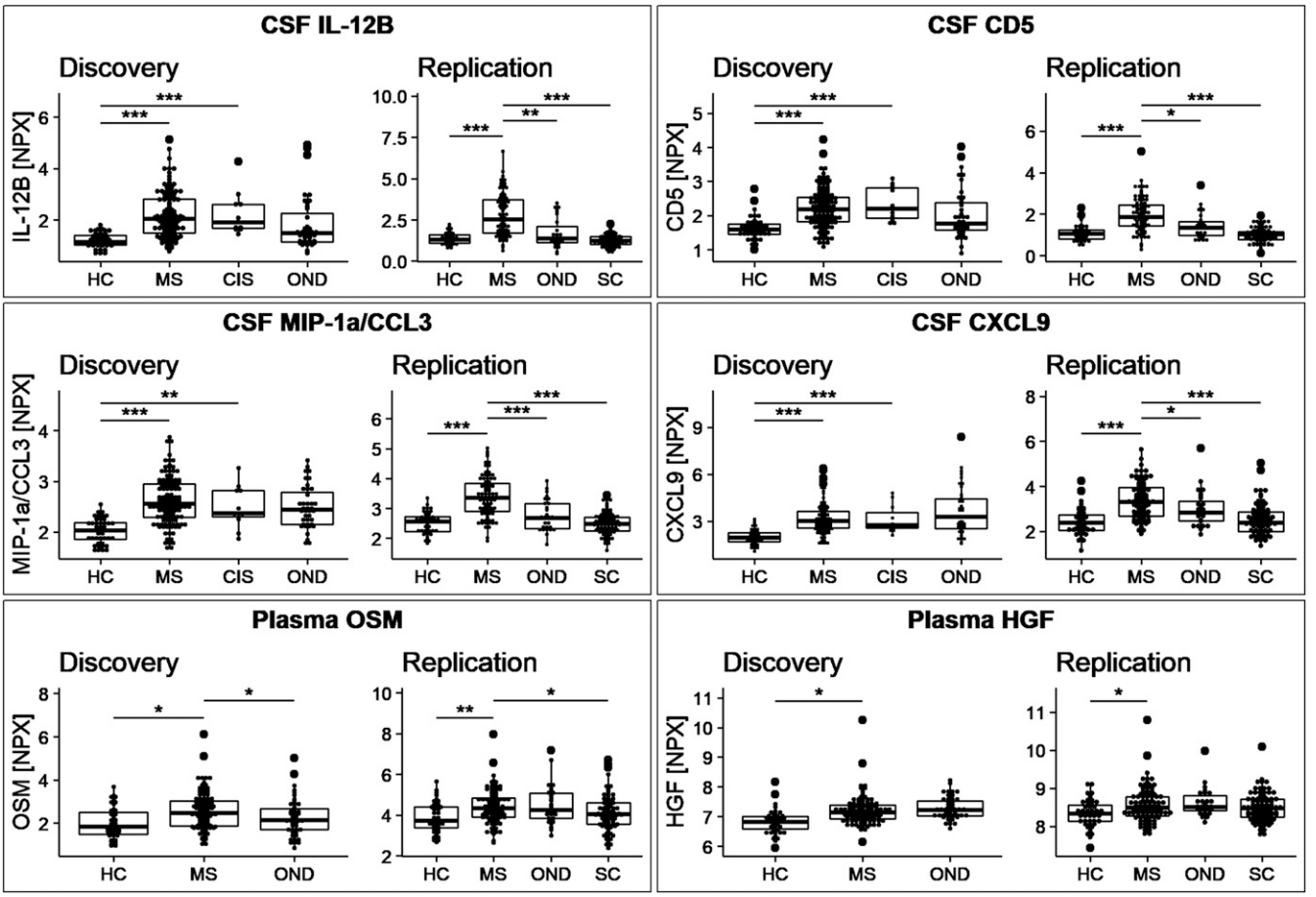


Fig. 1. Differential protein levels in both cerebrospinal fluid and plasma among multiple sclerosis cases and controls. The distributions of the top four CSF and top two plasma disease-associated protein measures are shown stratified by cohort (discovery/replication) and disease status: healthy controls (HC), multiple sclerosis (MS), clinically isolated syndrome (CIS, $n = 11$), symptomatic controls (SC) who initially were suspected MS cases, and other neurological diseases (OND). Significance levels: $*P < 0.05$, $**P \leq 0.001$, and $***P \leq 5 \times 10^{-5}$. Additional associated markers are provided in *SI Appendix*, Fig. S1. See Table 1 for additional information.

Oncostatin M and Hepatocyte Growth Factor Are Potential Plasma Biomarkers for Multiple Sclerosis. After correcting for variation in sample handling (*SI Appendix*, Fig. S4), five of the top proteins associated with multiple sclerosis when compared to healthy controls were selected in the discovery cohort of which two, oncostatin M (OSM) ($P_{dis} = 0.005$, $P_{rep} = 2 \times 10^{-4}$) and hepatocyte growth factor (HGF) ($P_{dis} = 0.01$, $P_{rep} = 0.009$), were then successfully validated in the replication cohort (Fig. 1). Plasma FGF-21 was also associated in both cohorts; however, the direction of association was inconsistent. OSM was not detectable in CSF, and although CSF HGF was higher among multiple sclerosis cases compared to controls, it was not significant after correcting for sex and age at sampling ($P_{dis} = 0.634$, $P_{rep} = 0.27$). Similar to CSF markers, the correlation between plasma OSM and HGF was similar in the discovery and replication cohorts (Fig. 2*A*).

Clinical Course: Cystatin-D (CST5) and Eotaxin-1 (CCL11) Associated with Relapse and Disease Course. In the discovery cohort, samples taken during relapses had a higher level of cystatin-D (CST5) in CSF relative to those sampled during remissions ($P = 8 \times 10^{-5}$, $P < 5 \times 10^{-5}$ with first-line treatment; *SI Appendix*, Fig. S8). In comparison to those with a relapsing–remitting disease course, secondary progressive patients showed a suggestive increase in CCL11 in both CSF and plasma ($P_{CSF} = 0.04$ and $P_{plasma} = 0.01$; *SI Appendix*, Figs. S7 and S12). CCL11 was also the primary protein correlated with duration of disease with an estimated 1.1% and 1.9% increase per year for CSF and plasma, respectively ($P_{CSF} = 3.5 \times 10^{-5}$, $P_{plasma} = 3.11 \times 10^{-5}$; *SI Appendix*, Tables S10 and S11), suggesting a potential overall biomarker for monitoring disease course. However, as the replication cohort contains only early relapsing–remitting disease, future studies will be required to validate its efficacy.

Clinical Characteristics: Plasma and CSF Measures Associated with Multiple Sclerosis. The IgG index was associated with several disease-associated proteins in CSF only (Fig. 2*B*), and, to a lesser extent, similar associations were observed with CSF mononuclear cell count and the number of T2 lesions. In contrast, plasma proteins showed no association to any of these measures and may, therefore, constitute an independent measure of disease activity. Similar analyses with measures of disability and disease severity showed plasma CCL20 was associated with an exponential increase of multiple sclerosis severity score (MSSS), as shown in *SI Appendix*, Fig. S9 ($P_{adj} = 3.8 \times 10^{-5}$). However, this association could not be validated in the replication cohort which may in part be due to the higher proportion of early disease cases.

Efficacy of Multiple Biomarker Classification for Multiple Sclerosis. Receiver operating characteristic (ROC) curves for the top

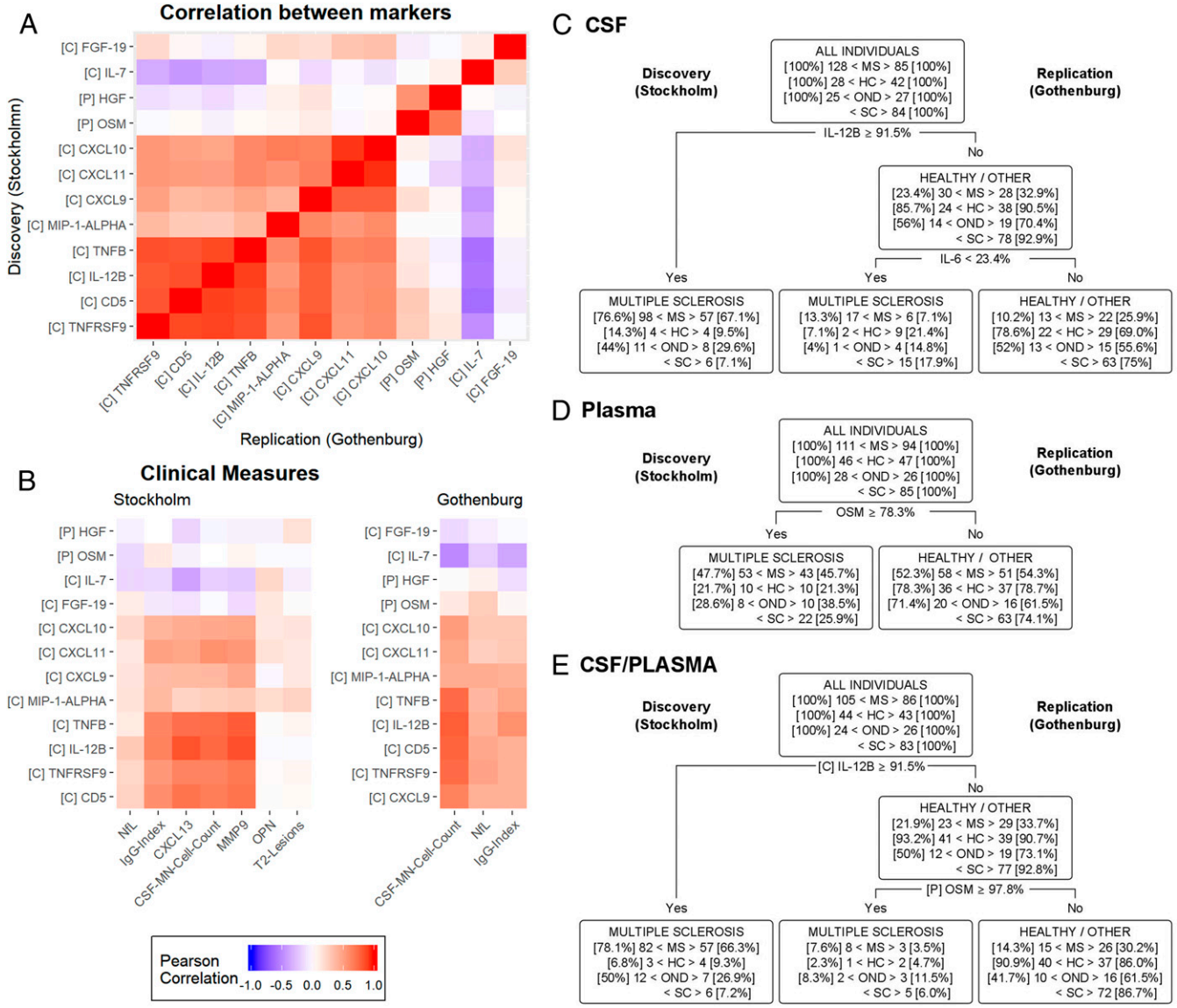
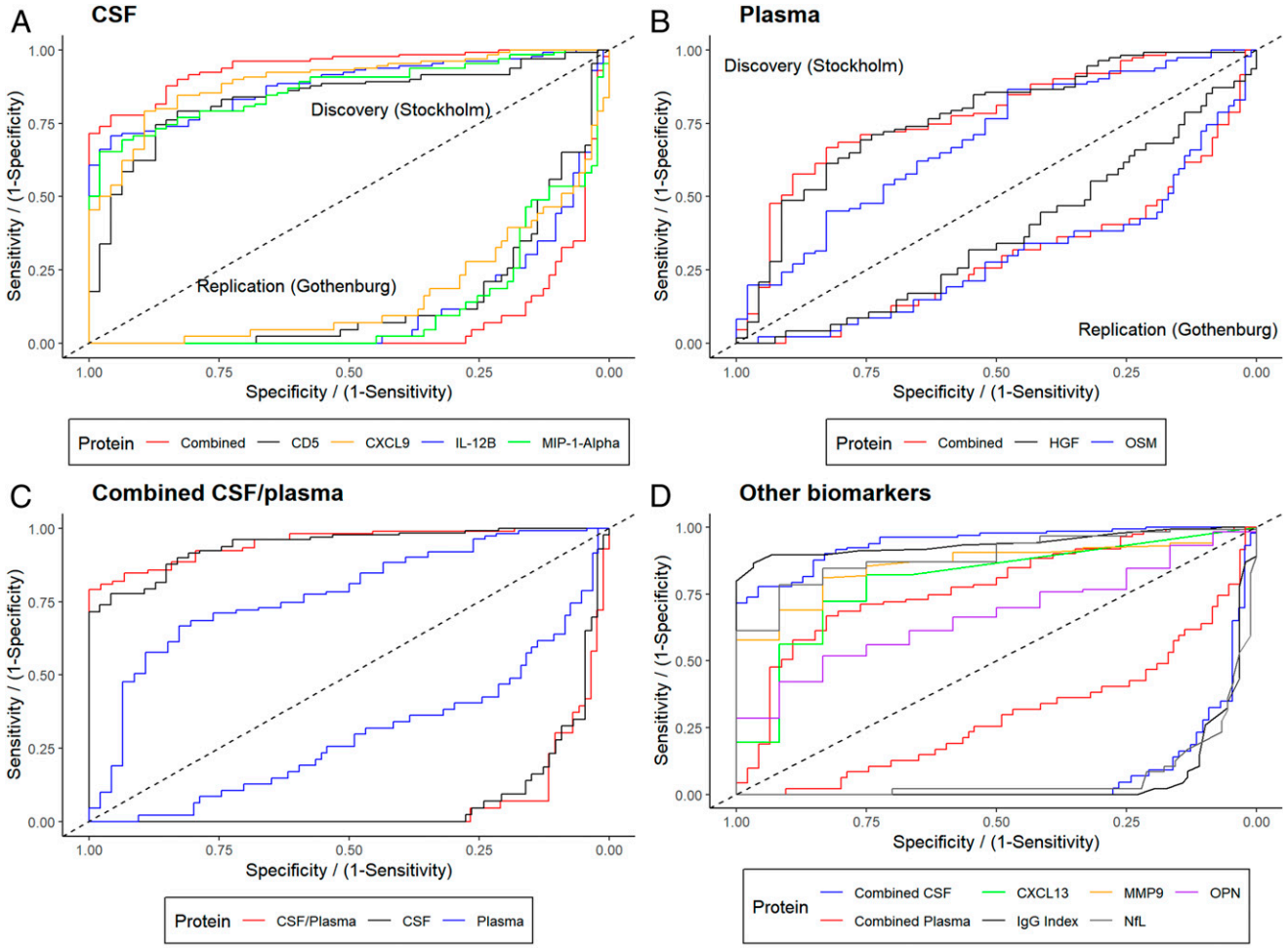


Fig. 2. Relationship between protein levels and the classification of multiple sclerosis using combined biomarkers in cerebrospinal fluid and plasma. Heat map shows correlation and clustering between disease-associated protein levels among multiple sclerosis cases (*A*) along with correlation to clinical measures and previously established biomarkers (*B*). Measures of CXCL11, MMP-9, OPN, and NfL were normalized using a log base-2 transformation. Decision trees illustrating the suggested biomarkers and classification method for determining multiple sclerosis are shown for CSF (*C*), plasma (*D*), and a combination of both (*E*). Nodes are labeled by the suggested classification and the number and percentage of resulting cases and controls in each node. Statistical details are listed for both discovery (Stockholm, *Left*) and replication (Gothenburg, *Right*) cohorts.

biomarkers in the classification of multiple sclerosis and healthy controls are shown in Fig. 3. The area under the curve was similar for different biomarkers in the discovery and replication cohorts (Fig. 3 *A* and *B*). Although IgG index remains the single best diagnostic tool for multiple sclerosis, IL-12B showed comparable predictivity to CSF NfL with slightly worse differentiation against healthy controls but higher differentiation against other neurological diseases. A combination of the top four CSF biomarkers showed similar discrimination between multiple sclerosis and healthy controls as IgG Index with slightly better differentiation against other neurological diseases (Fig. 3 *D* and *F*).

A decision tree was used to determine the efficacy of disease classification using an optimal combination of protein measures (Fig. 2 *C*–*E*). Analysis with CSF showed higher efficacy with a combined sensitivity/specificity ratio of 89.8/66.0% compared to 47.7/75.7% in plasma. Again, with decision trees, a combination of plasma and CSF showed only a minor improvement in disease classification with a predicted sensitivity and specificity of 85.7% and 73.5%, respectively (Fig. 2*E*).

Biomarkers for Measuring Effect in Immunomodulatory Treatment. Changes in protein level following natalizumab and fingolimod treatment are shown in Fig. 4 (25). As expected by the peripheral compartmentalization of immune cells (18), natalizumab treatment was associated with a decrease in inflammatory cytokines in CSF and a minor increase in plasma. On the other hand, fingolimod treatment resulted in a decrease in peripheral inflammation. In reference, there was no notable difference between patients treated with IFN-beta and untreated cases. Several of the multiple sclerosis-associated proteins (e.g., CD5, TNFSF9,



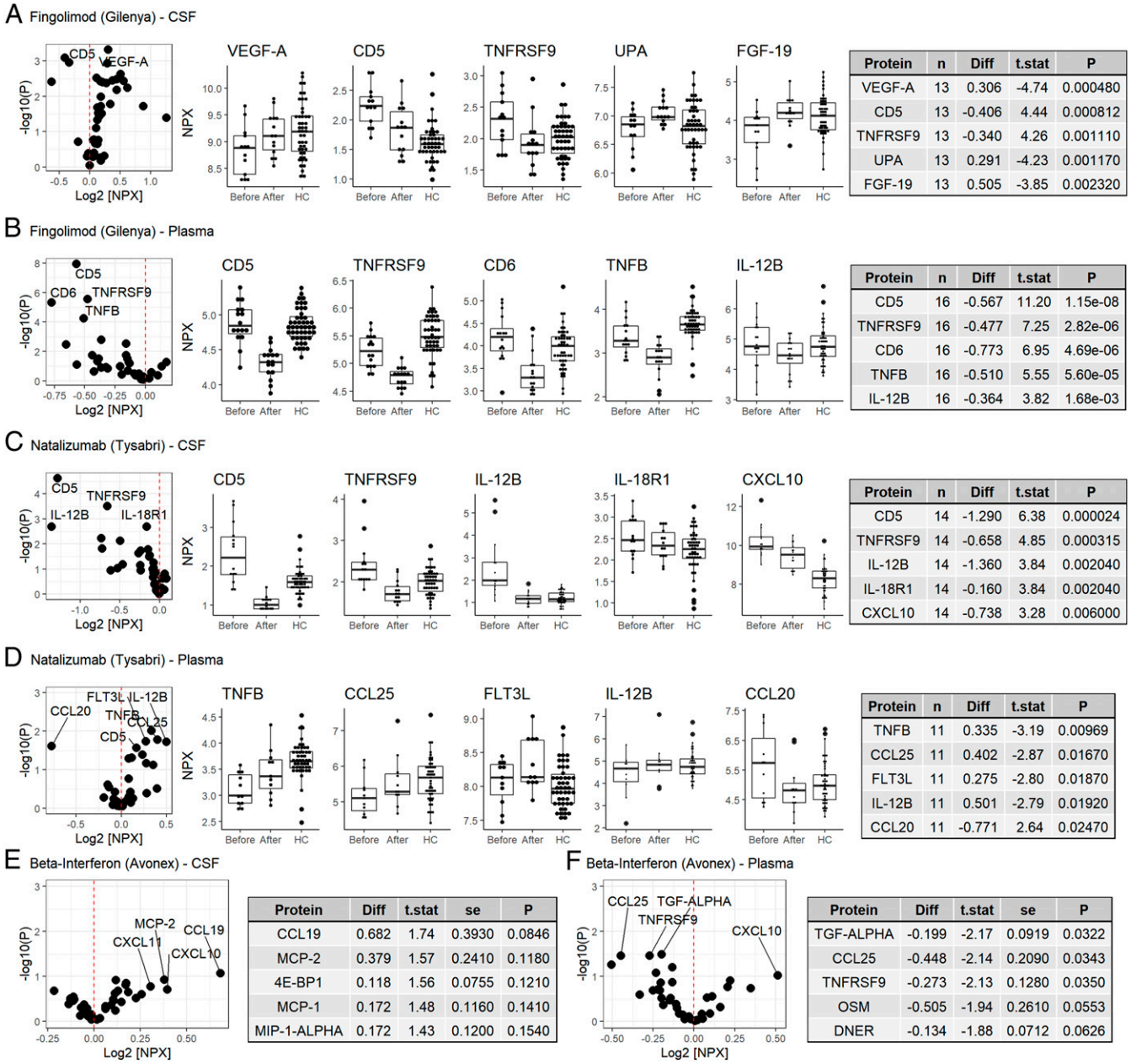
E Discovery (Stockholm)

Biomarker	AUC [HC]	95% CI	AUC [OND]	95% CI
IL-12B	0.88	[0.83,0.93]	0.62	[0.5,0.74]
CD5	0.84	[0.77,0.9]	0.59	[0.46,0.72]
MIP-1-Alpha	0.87	[0.82,0.92]	0.53	[0.42,0.64]
CXCL9	0.89	[0.85,0.94]	0.6	[0.47,0.73]
OSM	0.69	[0.6,0.78]	0.54	[0.42,0.66]
HGF	0.77	[0.69,0.85]	0.69	[0.58,0.81]
IgG Index	0.94	[0.91,0.97]	0.8	[0.71,0.89]
CXCL13	0.8	[0.67,0.93]	0.72	[0.62,0.81]
MMP9	0.86	[0.78,0.94]	0.71	[0.59,0.83]
OPN	0.67	[0.54,0.8]	0.57	[0.45,0.68]
NfL	0.89	[0.82,0.96]	0.56	[0.44,0.69]
Combined CSF	0.94	[0.91,0.97]	0.8	[0.72,0.88]
Combined Plasma	0.77	[0.69,0.86]	0.72	[0.61,0.84]
Both CSF/Plasma	0.95	[0.92,0.98]	0.82	[0.74,0.9]

F Replication (Gothenburg)

Biomarker	AUC [HC]	95% CI	AUC [OND]	95% CI
IL-12B	0.87	[0.82,0.93]	0.78	[0.68,0.88]
CD5	0.85	[0.78,0.92]	0.71	[0.6,0.82]
MIP-1-Alpha	0.87	[0.82,0.93]	0.77	[0.68,0.87]
CXCL9	0.83	[0.76,0.91]	0.64	[0.53,0.76]
OSM	0.71	[0.61,0.8]	0.51	[0.37,0.64]
HGF	0.63	[0.54,0.73]	0.57	[0.46,0.68]
IgG Index	0.94	[0.91,0.98]	0.87	[0.79,0.94]
CXCL13	NA	NA	NA	NA
MMP9	NA	NA	NA	NA
OPN	NA	NA	NA	NA
NfL	0.93	[0.89,0.98]	0.73	[0.61,0.85]
Combined CSF	0.93	[0.88,0.97]	0.81	[0.72,0.9]
Combined Plasma	0.71	[0.61,0.8]	0.57	[0.46,0.68]
Both CSF/Plasma	0.94	[0.9,0.98]	0.82	[0.73,0.91]

Fig. 3. Efficacy of multiple sclerosis biomarkers in differentiating healthy and other neurological disease controls. ROC curves examining the predictive performance of both CSF (*A*) and plasma (*B*) biomarkers for distinguishing multiple sclerosis in reference to healthy controls are shown for both the discovery (Stockholm, *Top Left*) and replication (Gothenburg, mirrored *Bottom Right*) cohort. Combined predictabilities of the top four CSF and top two plasma proteins (cCSF/cPlasma) are shown in *C* along with IgG index, CXCL13, MMP9, OPN, and NfL in *D*. (*E* and *F*) Area under the curve (AUC) and corresponding 95% CI for each measure and combination of measures (healthy controls [HC]) are shown along with similar comparisons of multiple sclerosis against other neurological diseases (OND).



Protein	n	Diff	t.stat	P
VEGF-A	13	0.306	-4.74	0.000480
CD5	13	-0.406	4.44	0.000812
TNFRSF9	13	-0.340	4.26	0.001110
UPA	13	0.291	-4.23	0.001170
FGF-19	13	0.505	-3.85	0.002320

Protein	n	Diff	t.stat	P
CD5	16	-0.567	11.20	1.15e-08
TNFRSF9	16	-0.477	7.25	2.82e-06
CD6	16	-0.773	6.95	4.69e-06
TNFB	16	-0.510	5.55	5.60e-05
IL-12B	16	-0.364	3.82	1.68e-03

Protein	n	Diff	t.stat	P
CD5	14	-1.290	6.38	0.000024
TNFRSF9	14	-0.658	4.85	0.000315
IL-12B	14	-1.360	3.84	0.002040
IL-18R1	14	-0.160	3.84	0.002040
CXCL10	14	-0.738	3.28	0.006000

Protein	n	Diff	t.stat	P
TNFB	11	0.335	-3.19	0.00969
CCL25	11	0.402	-2.87	0.01670
FLT3L	11	0.275	-2.80	0.01870
IL-12B	11	0.501	-2.79	0.01920
CCL20	11	-0.771	2.64	0.02470

Protein	Diff	t.stat	se	P
CCL19	0.682	1.74	0.3930	0.0846
MCP-2	0.379	1.57	0.2410	0.1180
4E-BP1	0.118	1.56	0.0755	0.1210
MCP-1	0.172	1.48	0.1160	0.1410
MIP-1-ALPHA	0.172	1.43	0.1200	0.1540

Protein	Diff	t.stat	se	P
TGF-ALPHA	-0.199	-2.17	0.0919	0.0322
CCL25	-0.448	-2.14	0.2090	0.0343
TNFRSF9	-0.273	-2.13	0.1280	0.0350
OSM	-0.505	-1.94	0.2610	0.0553
DNER	-0.134	-1.88	0.0712	0.0626

Fig. 4. Changes in cerebrospinal fluid and plasma protein levels following fingolimod and natalizumab treatments in multiple sclerosis cases. Shown is a summary of the comparison between paired samples before and after treatment with fingolimod (Gilenya, *A* and *B*) and natalizumab (Tysabri, *C* and *D*). Results are illustrated using a volcano plot (*Left*) with the distribution of the top five proteins (based on significance) further depicted as a dot plot along with healthy controls for reference (*Center*). Results from paired *t* tests are listed in the table (*Right*) with additional results in *SI Appendix*, Tables S13 and S14. Furthermore, patients with multiple sclerosis ($n = 18$) treated with IFN beta-1a (Avonex, *E* and *F*) were compared to untreated individuals with multiple sclerosis, adjusting for sex and age at sampling (*SI Appendix*, Table S15).

IL12B) were affected by disease-modifying treatment, showing the potential application for treatment monitoring.

Both CD5 and TNFSF9, which were associated with multiple sclerosis only in CSF, were lowered in both CSF and plasma following fingolimod treatment, in line with an effect on T cell activation in the periphery. Enrichment analysis using our validated biomarkers shows that natalizumab targets disease-associated proteins including many inflammatory cytokines, closing the gap with healthy controls (*SI Appendix*, Fig. S15). However, although there was some overlap, fingolimod primarily targeted different proteins compared to disease-associated proteins. This is further shown in reference to healthy controls as proteins affected by fingolimod particularly in plasma deviated farther away from controls. Surprisingly, plasma proteins affected by fingolimod treatment seemed to be partially enriched for CSF biomarkers, suggesting a potential suppression of CNS immune-related factors before crossing the blood–brain barrier.

Discussion

Our study provides a comprehensive examination of the immune-protein profile of multiple sclerosis showing up-regulation of several inflammatory cytokines with many in agreement with

previous studies (12, 26, 27). The results particularly highlight the importance of T lymphocyte activity in disease pathogenesis (6, 26). For example, several ligands of the chemokine receptor CXCR3 (e.g., CXCL9/10/11) were overexpressed in the CSF. This receptor is primarily expressed on Th1 cells and certain regulatory T cells. Studies have shown increased infiltration of CCR5+ and CXCR3+ T cells along with a higher expression of corresponding ligands MIP-1a and CXCL10 in active lesions (28–30). We here show that MIP-1a and CXCL10 are upregulated in CSF of patients with multiple sclerosis. In summary, these cytokine biomarkers provide an important proxy of T cell activity, providing an independent yet complementary measure to the previously published CXCL13, a chemokine targeting B lymphocytes (10, 12).

Several identified CSF biomarkers have already been implicated as genetic risk factors for multiple sclerosis, for instance, CD6, IL7, and IL12B along with its corresponding subunit IL12A (31). In addition, several of the identified biomarkers are known to interact (e.g., ligand/receptors) with genetic risk variants for multiple sclerosis including IL12RB1 and IL7R. Overlapping evidence between proteomics and genomics further supports the involvement of these proteins in multiple sclerosis.

We have also demonstrated that certain inflammatory aspects of multiple sclerosis can be measured in blood as shown by OSM and HGF, which could open additional ways of improving diagnostic procedures and disease monitoring. However, the role of these proteins in multiple sclerosis pathology is still unclear as both cytokines have wide pleiotropic effects. However, unlike NfL, the lack of an association in CSF suggests peripheral localization and their limited correlation with other inflammatory markers or NfL levels may indicate a measure independent of direct CNS inflammation or neuron damage. Studies have shown that OSM is highly expressed in the infiltrating lymphocytes of multiple sclerosis lesions and its direct immunoregulatory effects on cerebral endothelial cells indicate a potential role in regulating lymphocyte infiltration through the blood–brain barrier (32, 33). As a result, increased OSM is shown to be protective in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) mice, preventing infiltration of lymphocyte into the CNS (34). Similarly, HGF also has neuroprotective effects through neuro-regeneration which has been shown in EAE animal models to limit development of physical disabilities and promote recovery (35, 36). Therefore, high levels of HGF and OSM may be a natural compensatory effect for increased neuronal damage which is a consequence of disease-associated inflammation. It may also explain the minor increase of both OSM and HGF from relapsing–remitting to secondary progressive disease (*SI Appendix*, Fig. S7). However, further investigation will be required to adequately understand the mechanism of these measures which in turn may improve our understanding of its applicability as a diagnostic measure or perhaps its potential as a prognostic measure.

Furthermore, we also identify several potential biomarkers for measuring disease development, course, and severity, representing features for which soluble biomarkers may prove useful for clinical decision making. Cystatin-D (CST5) showed potential as a relapse marker in the discovery cohort and has been shown in an earlier study using the same proximity extension technology to serve as a proxy for neural tissue damage in traumatic brain injury (37). Lack of replication in the Gothenburg cohort could be due to systematic differences in the stage of relapse at sampling or method of discerning a relapsing episode; therefore, it warrants further investigation. The chemokine CCL11 was associated with disease duration and is a potential biomarker for conversion to secondary progressive disease. As an eotaxin, CCL11 is an eosinophil chemokine attractant but also functions as a ligand for the CCR5 receptor of T cells. In neuromyelitis optica, higher concentrations of eosinophils and various eotaxins were found in disease-associated lesions (8, 38, 39). In addition, CCL11 has been found to influence the course of MOG-induced disease in animal models of multiple sclerosis (40). Finally, CCL20 was exponentially associated with disease severity and has been shown to be crucial in the trafficking of pathogenic T cells in experimental models, suggesting that it may serve as a marker for T cell recruitment into the CNS (41, 42).

This study provides one of the earliest proteomic analyses for multiple sclerosis using the proximity extension technology, resulting in additional biomarkers in both CSF and plasma. The higher assay sensitivity due to amplification through qPCR provides a significant advantage relative to classical immunoassays for measuring low-level inflammatory cytokines. In addition, proper hybridization requiring both dual-antibody binding and correct spatial orientation provides an additional layer of specificity. However, such benefits may result in greater sensitivity to variation from sample handling as the result of protein degradation or cell leakage (43, 44). In this study, all samples were processed on site, minimizing processing time and ensuring consistent handling. However, variability, particularly with the handling of blood/plasma which seems more susceptible to intracellular protein leakage, may require active correction and filtering to prevent artifacts as was necessary for this study. In addition, validation of results with a separate cohort handled independently was a necessity for minimizing the likelihood of false positives.

In summary, we here identify a number of potential biomarkers segregating between multiple sclerosis and both healthy controls and other neurological diseases. In particular, the identification of potential plasma biomarkers is highly encouraging, since this opens the route for additional ways of monitoring disease development and response to therapy (45). However, further studies are needed to validate their capabilities for clinical application.

Materials and Methods

Study Design and Cohort. Cell-free CSF and plasma samples were obtained from two independent Swedish multiple sclerosis cohorts consisting of persons undergoing diagnostic procedures for possible multiple sclerosis in either Stockholm (discovery) or Gothenburg (replication) (12) (Table 1). Potential biomarkers were determined in two stages: 1) an initial identification of potential biomarkers using the discovery cohort followed by 2) validation of those selected markers in a replication cohort.

The discovery cohort consisted of 123 multiple sclerosis cases and 13 cases with "clinically isolated syndrome," i.e., not initially fulfilling diagnosis criteria at the time of sampling but later converting to clinically definite relapsing–remitting disease. This cohort consists of both relapsing–remitting ($n = 98$) and progressive disease ($n_{PPMS} = 10$, $n_{SPMS} = 15$) while the replication cohort consists primarily of cases with a recently diagnosed relapsing–remitting disease ($n = 95$) (46). In addition, the replication cohort includes symptomatic controls (SC), initially clinically suspected cases of multiple sclerosis, often presenting with sensory disturbances but not fulfilling diagnostic criteria of multiple sclerosis or clinically isolated syndrome (1). For comparison, samples were also obtained from healthy controls along with individuals with other neurological diseases (e.g., systemic lupus erythematosus, Sjögren syndrome, neurosarcoidosis, myelitis).

Additionally, paired samples were also taken from a cohort of patients with multiple sclerosis before and after (3 to 6 mo) starting disease-modifying treatment with either natalizumab ($n = 16$) or fingolimod ($n = 16$) (*SI Appendix*, Table S12) (12). As a reference, we also analyzed single time-point samples ($n = 18$) from patients undergoing treatment with IFN beta-1a (Avonex), a previously common first-line treatment.

Cases and controls were processed and handled similarly with samples being taken on site and stored in a −80 °C freezer within 2 h. All multiple sclerosis cases (except for the posttreatment cohort) were taken before treatment or after a washout period of ∼3 wk for first-line treatments and ∼2.5 mo after second-line treatments, although the majority of patients were treatment naive.

Clinical diagnoses of multiple sclerosis were determined by qualified neurologists using the McDonald criteria (47). The study was approved by the Stockholm Regional Ethical Review Board (reference nos. 2009/2107-31/2, 2015/1280-32) and the Gothenburg Regional Ethical Board (reference no. 895-13) with all participants having provided informed and written consent in accordance with the Declaration of Helsinki.

Clinical Characteristics. Data on available clinical and MRI assessments at sampling were obtained from medical records composed of IgG index, presence of oligoclonal bands, CSF mononuclear cell count, and MRI T2 lesion count. Disability was scored by a qualified neurologist using the expanded disability status scale (EDSS) (22). In addition, severity was determined using both the MSSS (23) and the age-related multiple sclerosis severity (ARMSS) score (24).

Proteomic Analysis. Proteins were measured with the Olink INFLAMMATION panel using proximity extension technology, a high-throughput multiplex proteomic immunoassay (17). Details regarding assay protocol and preprocessing are outlined in *SI Appendix, SI Materials and Methods*. In short, the panel includes 92 immune-related proteins, primarily cytokines and chemokines as listed in *SI Appendix*, Table S1. The assay utilizes epitope-specific binding and hybridization of a set of paired oligonucleotide antibody probes, which is subsequently amplified using a quantitative PCR, resulting in log base-2 normalized protein expression (NPX) values. Furthermore, levels of CXCL13, MMP-9, OPN, and NfL were analyzed separately as detailed in our previous study (10).

Statistical Analysis. Protein associations to multiple sclerosis and disease-related characteristics were analyzed using a multivariable linear regression model, adjusting for sex and age at sampling. Analysis of plasma proteins was corrected for variability in sample handling, using plasma axin-1 levels as a reference (*SI Appendix*, Figs. S3 and S4) (43). Paired pre- and posttreatment samples were analyzed using a paired Student's *t* test. Potential disease-associated markers were initially determined from the discovery cohort using a false discovery rate (FDR)-corrected significance (*P*) of $P_{FDR} < 0.05$. For CSF markers, a more conservative Bonferroni-corrected cutoff of $P < 5 \times 10^{-5}$ was used to minimize the likelihood of false positives. Selected markers were then validated, $P < 0.05$, in the replication cohort. All statistical analyses and figures were computed in R-3.2.3. Additional details regarding statistical analyses are outlined in *SI Appendix*.

Data Availability. The data that support the findings of this paper are available at the Swedish National Dataservice database, https://snd.gu.se/en (DOI: 10.5878/p6dc-8149, DOI: 10.5878/ta78-gb12, DOI: 10.5878/2ver-wy19).

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