Molecular biomarkers for multiple sclerosis have so far mainly been limited to measurements 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: eosinophil-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.  

Significance

Molecular biomarkers for multiple sclerosis have so far mainly been limited to measurements 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: eosinophil-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.
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 NIL 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).

### Table 1. Summary characteristics of study cohorts

| Variable                  | Discovery (Stockholm) | Replication (Gothenburg) |
|---------------------------|-----------------------|--------------------------|
|                           | 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 [%].

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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) ($P_{\text{dis}} = 0.005, P_{\text{rep}} = 2 \times 10^{-4}$) and hepatocyte growth factor (HGF) ($P_{\text{dis}} = 0.01, P_{\text{rep}} = 0.009$), 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 ($P_{\text{dis}} = 0.634, P_{\text{rep}} = 0.27$).

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 ($P_{\text{CSF}} = 0.04$ and $P_{\text{plasma}} = 0.01$; 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 ($P_{\text{CSF}} = 3.5 \times 10^{-5}, P_{\text{plasma}} = 3.11 \times 10^{-5}$; 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. 2B), 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 ($P_{\text{adj}} = 3.8 \times 10^{-5}$). 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...
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, 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. 3A 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. 3D and F).

A decision tree was used to determine the efficacy of disease classification using an optimal combination of protein measures (Fig. 2C–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. 2E).

Biomarkers for measuring effect in immunomodulatory treatment.
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).
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 demonstrated that certain inflammatory factors 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 cosmiphils 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 (nrelaps = 10, nprogress = 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, Sjogren syndrome, neuromyelitis optica).

Additionally, paired samples were also taken from a cohort of patients with multiple sclerosis before (6 to 8 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. 2009/2107-3/2, 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, Table 31. 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, MIP-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 FDR < 0.05). For CSF markers, a more conservative Bonferroni-corrected cutoff of P < 5 × 10−7 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/aj7-gb12, DOI: 10.5878/2ver-wy19).

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