

Urokinase, CX3CL1, CCL2, TRAIL and IL-18 induced by interferon- β treatment

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Abstract

Objective: To identify serum proteins associated with MS and affected by interferon beta treatment.

Methods: Plasma samples from 29 untreated relapsing-remitting MS patients and 15 healthy controls were investigated with a multiplexed panel containing 92 proteins related to inflammation. Follow-up samples were available from 13 patients at 1 and 3 months after initiation of treatment with interferon beta-1a.

Results: Ten proteins were differentially expressed in MS patients. Five of these were altered by treatment with IFN- β 1a: uPA, CX3CL1, CCL2, TRAIL and IL18.

Conclusion: CCL2 and TRAIL were confirmed to be modulated with interferon beta treatment in MS. As novel findings, we now report that uPA and CX3CL1 were differentially expressed in MS and increased after IFN-beta-1a treatment. Conflicting results have been reported on how interferon beta affects IL-18.

KEYWORDS

interferon beta, multiple sclerosis, TRAIL, urokinase

1 | INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system. The first disease modifying drug that was approved for MS is interferon beta (IFN- β), which has been a mainstay in treatment of MS for more than twenty years. Although the precise mechanisms of IFN- β remain uncertain, several modes of action have been proposed, including inhibition of T-cell activation and proliferation; inhibition of leucocyte migration; and cytokine modulation.¹ In this study, we investigated the effect of IFN- β 1a on a multiplexed panel of 92 proteins associated with inflammation.

2 | MATERIAL AND METHODS

2.1 | Ethics approval

Written informed consent was provided by all of the participants, and the study was approved by the Regional Ethical Board of Uppsala (Dnr 2012/081 and 2013/219).

2.2 | Subjects

The study cohort consisted of 44 subjects: 29 newly diagnosed and untreated relapsing-remitting MS (RRMS) patients and 15 age- and

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gender-matched healthy controls (HC) all of whom had submitted blood samples. From 13 of the MS patients, follow-up samples were available, from 1 and 3 months after treatment with IFN- β 1a (tiw) was initiated. Patients were diagnosed according to the 2010 revision of the McDonald criteria. The median age of the newly diagnosed MS patients was 28 (range: 21–47), 21 females and 8 males. The median age of HCs was 30 (range: 21–50), 11 females and 4 males.

2.3 | Proximity extension assay

Serum samples from all subjects were analysed with a proximity extension assay (PEA) to quantify 92 proteins included in the ProSeek Inflammation Panel (Olink Proteomics, Uppsala, Sweden). Seventy-three of the analysed proteins were detected in >75% of the samples and were considered for statistical analysis. A detailed description of the PEA is available at the service provider's homepage.²

2.4 | Statistical analysis

Analyses were performed in R v.3.5.1. Principal component analysis (PCA) was used to analyse the variation in the dataset. Parametric tests were used if a population was normally distributed (D'Agostino's test) and non-parametric tests if not. Student's t-test or Mann-Whitney's test was used to compare MS patients with HC where $p < 0.05$ was considered significant. Repeated measures ANOVA or Friedman's test was used to evaluate treatment-related changes in selected proteins, and the results were corrected for multiple testing using false discovery rate (FDR) where $q < 0.05$ was considered significant.

3 | RESULTS

An initial comparison between MS patients and HCs was made, which identified ten proteins significantly ($p < 0.05$) altered in MS patients. These proteins were further compared in RRMS patients treated with IFN- β 1a at three different time points (0, 1, 3 months). After correction for multiple testing, five proteins with at least one

significant difference between the three different timepoints were identified. These were urokinase (uPA), CX3CL1 (fractalkine), CCL2 (MCP-1), TRAIL and IL18 ($q < 0.05$, Table 1, Figure 1).

4 | DISCUSSION

IFN- β and IFN- α belong to the type I interferon family, which is crucial to viral defence and exert broad effects on both the innate and the adaptive immune response by inducing the transcription of interferon-stimulated genes (ISGs).³ IFN- β therapies were the first major therapeutic class of drugs developed for use in MS and have been shown to reduce annual relapse rates and the accumulation of lesions, but the mechanisms of action are still not entirely known.⁴ Studies on the effects on IFN- β on peripheral blood cells have reported on increased production of anti-inflammatory cytokines as well as a decreased production of proinflammatory cytokines, inhibition of T-cell proliferation, signs of a shift from a T helper 1 (Th1) to Th2 response, increased function of T regulatory cells and decreased expression of integrins on T cells.^{1,5-7} Analyses of proteins associated with inflammation in cerebrospinal fluid (CSF), plasma or serum, can be used in order to find useful biomarkers⁸ or to better understand the mechanisms of action.

In this study, we have used a highly sensitive and specific multiplex PEA assay to analyse 92 proteins in serum from patients newly diagnosed with MS before and three months after initiation of IFN- β treatment, in order to broadly investigate the treatment effects on the immune system. The levels of five proteins were both significantly altered in MS patients as compared to controls and subsequently also altered by IFN- β treatment: urokinase-type plasminogen activator (uPA), fractalkine/CX3CL1, MCP-1/CCL2, TNF-related apoptosis-inducing ligand (TRAIL) and interleukin (IL)-18. These five proteins were all decreased in sera from MS patients as compared to healthy controls and increased again after IFN- β treatment.

Urokinase-type plasminogen activator (urokinase, uPA) is a serine protease and a key component of thrombolysis and extracellular matrix degradation and is predominantly expressed by neutrophils, monocytes, macrophages and activated T cells. It is involved in vascular disease and cancer progression, but has also been described to

TABLE 1 (A) Student's t-tests/Mann-Whitney's tests between newly diagnosed RRMS patients ($n = 29$; 21 female, 8 male) and HC ($n = 15$; 11 female, 4 male) were performed, which identified 10 proteins of interest. B) Repeated Measures ANOVA between three different time points (0, 1, 3 months) among MS patients treated with IFN- β -1a ($n = 13$; 10 female, 3 male), 5 of these were chosen due to their FDR corrected significance ($q < 0.05$).

	A) Initial comparison of newly diagnosed RRMS patients and healthy controls. <i>P</i> -values for Student's t-test/Mann-Whitney's test (#)	B) Comparison between three time points in IFN- β -1a-treated patients. FDR corrected <i>q</i> -values for repeated measures ANOVA, (*)
1. uPA	0.045	0.034
2. CCL2	0.046	0.0081
3. TRAIL	0.014	0.0022
4. IL-18	0.0035	<0.0001
5. CX3CL1	0.024	<0.0001

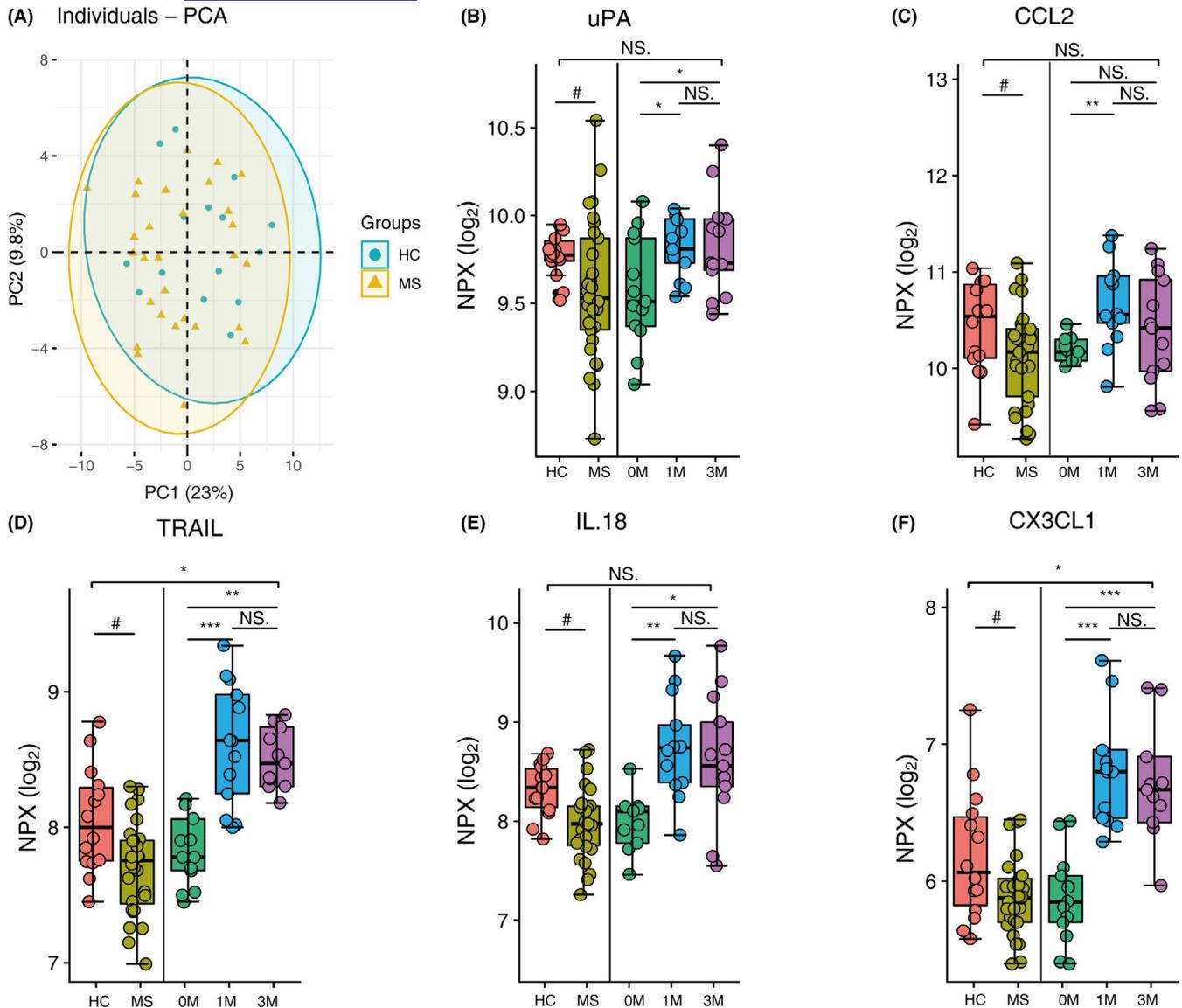


FIGURE 1 Levels of uPA, CX3CL1, CCL2, TRAIL and IL-18 in HC, untreated MS patients and IFN- β -1a treated MS patients. A, A principle component analysis (PCA) was used in order to analyse the spread and variation within and between individuals newly diagnosed with multiple sclerosis (MS) and healthy controls (HC). B-F, To the left, NPX values (\log_2 scaled) and comparisons between MS and HC alongside the three time points for each of the 5 proteins of interest, # ($p < 0.05$), # # ($p < 0.01$). To the right, data from 13 individuals receiving IFN- β -1a treatment who were followed longitudinally. 0 M (0 months or before treatment started), 1 M (1 month after treatment start) and 3 M (3 months after treatment start); * ($q < 0.05$), ** ($q < 0.01$), *** ($q < 0.001$), N.S. (not significant)

be involved in several inflammation-related diseases such as rheumatoid arthritis, systemic lupus erythematosus and allergic asthma. uPA cleaves plasminogen, generating the active protease plasmin which cleaves and activates the extracellular proteolytic enzymes matrix metalloproteases (MMPs). Both plasmin and MMPs degrade many extracellular matrix (ECM) components. MMPs are thought to be an essential step for migration of activated T cells into the CNS by digesting the extracellular matrix underneath the cerebral endothelial cells, and MMP-8 and MMP-9 levels are increased in serum of MS patients, but corrected after 6 months of IFN- β treatment.⁹

Increases in uPA, urokinase receptor (uPAR) and plasminogen activator inhibitor-1 are detected in acute MS lesions.¹⁰ It is also reported that patients with progressive MS have sustained high

levels of circulating uPAR⁺ monocytes. In RRMS patients with a clinical relapse, uPAR⁺ monocytes in blood increase prior to the onset of relapse and uPAR levels correlate with both clinical activity and severity in all groups. After treatment with glatiramer acetate, uPAR levels are significantly lower after two years.¹¹ To our knowledge, soluble uPA has not previously been directly investigated in MS. In the present study, we could demonstrate that patients with RRMS had lower levels of uPA in serum than HC. A possible explanation of this observation is that free uPA is absorbed by uPAR⁺ monocytes. Low levels of uPA may be related to the previously reported increased risk of cardiovascular disease in MS¹² through its action on plasminogen. This increased risk for acute myocardial infarction, stroke and heart failure was highest

within the first year after MS-diagnosis. Interestingly, we could observe that levels of uPA were raised to the levels of HC after three months of IFN- β treatment. This increase could be an effect of reduced inflammation, resulting in a downregulation of uPAR and increased levels of free uPA.

CCL2, TRAIL and IL-18 have previously been shown to be modulated with IFN- β treatment in MS and have been implicated in several inflammatory diseases. Similar to our findings, previous investigators reported that CCL2 was induced by IFN- β treatment,¹³ an increase in the levels of TRAIL¹ and simultaneous increase in the levels of both CCL2 and TRAIL.¹⁴

CCL2 is secreted by various cell types such as fibroblasts, epithelial cells and leucocytes and is considered the principal chemokine involved in the recruitment of monocytes/macrophages and activated lymphocytes. It has recently been suggested that CCL2 also impacts leucocyte behaviour, influence adhesion, polarization, effector molecule secretion, autophagy, killing and survival.¹⁵ CCL2 has been detected in high levels in acute MS lesions, and to some extent also in chronic-active MS lesions,¹⁶ which may be important for the recruitment of inflammatory cells from the circulation. The CCL2 levels in CSF are transiently reduced with disease activity, but studies in serum have provided inconsistent findings.^{17,18}

Our results support a role for CCL2 in the beneficial mechanisms of IFN- β in MS, possibly by altering both the recruitment of leucocytes to the CNS and their behaviour.

TRAIL is a member of the tumour necrosis factor family and coded by a known ISG. It is expressed by most cells and is involved in immunoregulatory processes such as apoptosis, inhibition of T cells and promotion of T regulatory cells.¹⁹⁻²¹ Studies on the experimental autoimmune encephalomyelitis (EAE) mouse model show that blockade of the TRAIL-pathway in mice exacerbated EAE, while treatment with recombinant soluble TRAIL delayed disease onset and reduced the severity of EAE.^{22,23} This did not seem to be related to apoptosis induction in inflammatory cells, but rather to the prevention of autoreactive T-cell activation.^{20,22,23} However, TRAIL has also been reported to mediate apoptosis of human neurons and oligodendrocytes,^{24,25} and high levels have been reported in established MS lesions.^{26,27} While some studies have reported on similar TRAIL levels in MS patients and healthy controls,²⁸ our results are consistent with several studies reporting on lower TRAIL levels in serum in MS patients which increases after IFN- β treatment²⁹⁻³¹ and TRAIL has been identified as a potential response marker for IFN- β in MS.^{32,33} The increase of TRAIL as a response to IFN- β treatment may be important in correcting a defective immunoregulation, where autoimmune lymphocytes do not undergo apoptosis.

IL-18 is a proinflammatory cytokine primarily involved in T helper 1 (Th1) cell and natural killer (NK) cell responses by inducing IFN- γ and also has the ability to induce, that is CCL2.³⁴ It is also, together with IL-1 β , processed by the multiprotein complex called the inflammasome, which is believed to contribute to the inflammatory response in MS.^{35,36} In the present study, the levels of IL-18 in MS patients were initially lower than in HC, but increased twofold after treatment with IFN- β . This finding is in contrast with other studies

who reported increased IL-18 in MS^{37,38} with a decrease after treatment with IFN- β ,³⁹ altogether a mirror image of our results. Another study showed no effect on IL-18 after six months of IFN- β treatment.⁴⁰ The reason for this discrepancy is unclear, but previous treatment and longer duration of disease at the time of sampling may be a part of the explanation. Nevertheless, this raises concern about the validity of the findings relating to IL-18.

CX3CL1 (or fractalkine) is a chemokine and a mediator of several aspects of the immune response that can function as both a proinflammatory chemoattractant and anti-inflammatory neuroprotective agent.⁴¹ It is reported that CX3CL1 is increased in serum from MS patients.⁴² However, the CSF/serum-ratio tended to be lower in MS patients than in healthy controls, which does not support a role for CX3CL1 as a chemokine in the CSF.⁴² In contrast, another study found that CX3CR1⁺CD4⁺ T cells are enriched in blood samples from RRMS patients and even more in CSF.⁴³ None of these studies were performed on patients newly diagnosed with MS, which may affect the results. In our study, CX3CL1 levels in newly diagnosed MS patients were lower to that of HC, but after treatment with IFN- β , CX3CL1 levels increased surpassing the level of HC in a similar fashion to TRAIL. It is possible that the increase in serum and peripheral blood cells noted in studies on MS patients with a longer duration reflects a more chronic inflammation than what is present in the patients in our study.

While IFN- γ is a known inducer of CX3CL1, there are to our knowledge no studies on the effect from IFN- β . Due to its strong ability to attract leucocytes, the increase in CX3CL1 concentration in peripheral blood after IFN- β seen here may reduce the CSF/serum-ratio and thereby switch the gradient for leucocyte migration. Analysis of CSF would be needed to examine that hypothesis.

In conclusion, the results in this study support the notion that IFN- β exerts its actions on several aspects of the immune system in MS, including adhesion and migration of leucocytes to the CNS, immune regulation and degradation of the ECM.

The main limitation of this study is the low number of study subjects in relation to the large number of measured proteins. This was in part mitigated by the two-pronged approach to identify differentially expressed proteins affected by interferon- β treatment. After an initial screening process to identify proteins of interest in MS, the number of investigated proteins was reduced by a factor of seven. Only then did we consider if they were affected by interferon- β and applied correction for multiple testing. With this, we could confirm previous findings on the effects of interferon- β on CCL2 and TRAIL. As novel findings, we report that uPA and CX3CL1 were differentially expressed in MS and increased after IFN-beta-1a treatment. Those results should be confirmed in an independent cohort.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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