Peripheral blood age-sensitive immune markers in multiple sclerosis: relation to sex, cytomegalovirus status, and treatment

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Summary

Background Immunosenescence is accelerated by chronic infectious and autoimmune diseases and could contribute to the pathobiology of multiple sclerosis (MS). How MS and disease-modifying therapies (DMTs) impact age-sensitive immune biomarkers is only partially understood.

Methods We analyzed 771 serum samples from 147 healthy controls and 289 people with MS (PwMS) by multiplex immunoassays. We determined cytomegalovirus (CMV) serostatus and collected retrospective clinical information. We performed unsupervised and multivariable analyses.

Findings Unsupervised analyses revealed that MS immune profile was characterized by low relative levels of anti-inflammatory/neuroprotective factors IL-4, IL-10, TNF, and β-NGF but high levels of growth factors EGF and bFGF. Serum levels of IL-4, β-NGF, IL-27, BDNF, and leptin were significantly influenced by sex and/or CMV status. IL-4 and β-NGF levels were lower in untreated PwMS compared to controls, while EGF and bFGF levels were influenced by age and markedly elevated in PwMS in multivariable analysis. Samples from treated PwMS, but not untreated PwMS, showed lower levels of BDNF and TNF than controls. Initiation of high efficacy DMTs, but not low efficacy DMTs, was associated with reduced levels of bFGF and EGF. Samples associated with distinct DMTs exhibited specific profiles for age-sensitive immune markers. Finally, lower levels of IL-6, TNF, IL-10, and β-NGF were observed at baseline in PwMS who subsequently experienced clinical failure after DMTs initiation.

Interpretation Age, sex, CMV status, and specific DMTs significantly influence levels of age-sensitive immune biomarkers associated with MS and must be considered when investigating inflammation-related biomarkers.

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Keywords: Immunosenescence; Aging; Autoimmunity; Neurological disease; Central nervous system

Introduction

Multiple sclerosis (MS) is the most frequent immune-mediated demyelinating disease of the central nervous system (CNS). For the majority of people with MS (PwMS), the onset occurs in young adulthood, and the early phase of the disease is characterized by acute/subacute clinical relapses attributed to the formation of new inflammatory CNS lesions.[1] In young PwMS, relapses can significantly contribute to disability accumulation, although partial or complete recovery after

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Research in context

Evidence before this study

In people with MS (PwMS), aging is associated with a greater risk of progression independent of relapse activity (PIRA). In parallel, response to disease-modifying therapies (DMTs) declines, but the occurrence of infectious and neoplastic complications increases in older PwMS. Biological aging of the immune system, called immunosenescence, is associated with a state of low-grade chronic inflammation and a higher susceptibility to infections and cancers. Multiple environmental and genetic factors modulate age-related biological processes. Previous exposure to cytomegalovirus (CMV), a common lifelong latent viral infection, promotes the senescence of immune cells. Moreover, the sex assigned at birth influences immune activation and gene expression through sex chromosomes and hormones. Notably, autoimmune diseases can also accelerate immunosenescence. As such, shorter telomere length, a hallmark of immunosenescence, was reported in peripheral blood immune cells from PwMS compared to healthy controls (HC) and was associated with disability. We searched Google Scholar and Pubmed for studies investigating the association between immunosenescence or age-sensitive immune markers and MS. Most publications are reviews. Few studies have assessed the impact of MS on immunosenescence markers, and none have explored the influence of age, sex, CMV serostatus, and specific DMTs on peripheral blood age-sensitive immune markers.

Added value of this study

We performed unsupervised and multivariable analysis on multiplex immunoassay and clinical data on a large number (771) of serum samples from 289 PwMS and 147 HC. We identified an immune profile associated with MS and demonstrated that different age-sensitive immune markers are uniquely affected by sex, CMV status, and age. Importantly, the association between age and levels of a subset of immune markers was distinctly altered in MS compared to HC. Moreover, treatment with DMTs reversed the MS-associated trend for some markers but seemed to exacerbate alterations for others. Notably, different DMTs showed distinct impact on age-sensitive immune markers. Finally, higher levels of anti-inflammatory/protective immune markers at baseline, a profile reminiscent of HC samples, were measured in samples from PwMS who subsequently remained free of clinical relapse or significant EDSS increase after starting DMTs.

Implications of all the available evidence

The findings in this study stress the necessity of considering age, sex, and CMV status when investigating peripheral immune biomarkers associated with MS status and treatment response. Moreover, PwMS exhibiting a peripheral immune profile similar to HC before treatment initiation could have a better prognosis while on DMT.

relapses is frequent.[2] Female biological sex triples the risk of developing MS but is associated with a better long-term prognosis.[2] Approved disease-modifying therapies (DMTs), which target various immune cells and responses, significantly decrease the number of relapses in young PwMS.[3] Unfortunately, in a large proportion of older PwMS, overt progression of neurological disability independent of relapse activity (PIRA) is observed over time in relation to slowly expanding CNS lesions and atrophy, while recovery from relapses becomes more limited.[1,2] Aging is indeed the main risk factor for the progression of disability in PwMS.[2,4] In addition, DMT efficacy decreases[5] while DMT-related infectious and neoplastic risks increase with aging.[6] These observations suggest that biological aging of the immune system or immunosenescence contributes to the neurological decline and the higher frequency of treatment failure and treatment-associated complications observed in numerous older PwMS.[6]

Immunosenescence results from the accumulation of alterations, including DNA damage, epigenetic modifications, and mitochondrial dysfunction, and is associated with defective biological functions of immune cells.[7] Multiple cell types globally contribute to chronic inflammation in aging (coined inflammaging) and progressive fibrosis via the production of the senescence-associated secretory phenotype (SASP), which includes a variety of cytokines, chemokines, and growth factors.[7] As observed during SARS-CoV-2 infection and vaccination,[8] aging leads to a reduced capacity to mount a robust immune response coupled with a state of chronic low-grade inflammation and predisposition to autoimmunity.[7]

Multiple genetic and environmental factors can influence immunosenescence. The sex assigned at birth modifies age-associated hormonal and epigenetic modifications, contributing to alterations in immune responses.[9,10] Cytomegalovirus (CMV), a herpesvirus establishing lifelong persistence in humans, has been implicated in shaping immunosenescence.[11] CMV infection is asymptomatic in most immunocompetent people; previous exposure is determined by an antibody serology test, with CMV seroprevalence varying between 40 and 90% of the population across countries.[11,12] CMV can enhance inflammaging (SASP production) via the activation of inflammation-associated pathways such as NF-κB.[11] Moreover, repeated antigen encounters due to chronic viral infections such as CMV or autoimmune diseases can accelerate biological aging. Indeed, such patients exhibit shorter leukocyte telomere length than

age-matched controls,[13] a hallmark of immunosenescence.[14] Compared to non-MS controls, shorter leukocyte telomere length is observed in PwMS and is associated with disability status and progression.[15] Few studies have addressed immunosenescence in MS. A reduction of naïve T cells but an expansion of pro-inflammatory cytotoxic activated T cells[16,17] and pro-inflammatory age-associated B cells have been reported in PwMS compared to controls.[18] The impact of sex, CMV status, and use of DMTs on age-associated biological processes in MS, however, remains only partially understood.

In this study, we assessed the levels of several age-sensitive soluble markers in 771 serum samples from healthy controls (HC) and PwMS to explore the interplay between sex, CMV exposure, MS, DMT, and immunosenescence. Using unsupervised and multivariable analyses, we investigated the effect of age, sex, and CMV exposure on the levels of different immune markers and their association with MS status, treatment status, and treatment response. Our study outlines the necessity to consider age, sex, and CMV status when investigating inflammation-related biomarkers in MS to refine prognosis and inform therapeutic decisions.

Methods

Ethics

Written informed consent was obtained from PwMS and HC in accordance with the local ethical committee; studies were approved by the Centre Hospitalier de l'Université de Montréal (CHUM) ethical board (BH 07.001 and 17.326).

Participants

All participants were recruited from the CHUM MS Clinic (PwMS) and among the CHUM personnel or people accompanying PwMS (HC). Participants with active infectious or autoimmune diseases (other than MS) were excluded from this study. Biosampling was not performed during pregnancy. Participants exposed to immunosuppressors with long-term effects before the first blood draw, such as cladribine, alemtuzumab, or anti-CD20 therapies, or exposed at any time to cyclophosphamide, mitoxantrone, methotrexate, mycophenolate mofetil or azathioprine, or exposed to steroids within the last five weeks or actively receiving immunosuppressive drugs for other conditions, were also excluded from this study. MS diagnosis according to the revised 2017 McDonald criteria[19] was confirmed at the time of blood collection by the treating MS specialist (certified neurologists). EDSS assessments are routinely performed at each clinical visit by neurologists certified for EDSS rating (http://www.neurostatus.net/) and available in the clinical chart (EDSS on the day of biosampling).

Clinical data

All charts were reviewed by a certified MS neurologist to retrieve relevant clinical data (CL). PwMS were considered untreated at the time of sampling if they had not received oral or monoclonal antibody (mAb) DMT for at least six months or injectable DMTs for at least four months before blood collection. Treated PwMS had received the same DMT for at least six months at the time of first blood collection and for at least four months when starting or switching DMT before the following sample. PwMS biosamples not fulfilling the criteria for untreated or treated categories were not included. The DMTs included beta-interferon (IFN), glatiramer acetate (GA), dimethyl fumarate (DMF), fingolimod (FTY), natalizumab (NTZ), teriflunomide (TF), and when started after the first biosample cladribine (CLD) and ocrelizumab (OCV). GA, IFN, and TF were considered low-efficacy therapies; DMF and FTY were considered moderate-efficacy therapies; NTZ and OCV were considered high-efficacy therapies; CLD was not included in treated MS sub-analyses due to low numbers (n = 2). Age, sex, CMV status, presence of vascular comorbidity (hypertension, hypercholesterolemia, diabetes, heart disease, peripheral vascular disease),[20] DMT type, and EDSS at the time of biosample collection are described in Table 1.

Relapses, active disease, and significant EDSS progression were defined according to Lublin and colleagues,[21] and as used by Disanto and colleagues.[22] DMT response was categorized as no sign of failure vs. failure based on clinical visits and EDSS scores following DMT initiation, and MRI data when available. DMT response was categorized as 'no sign of failure' if no clinical relapse and no significant EDSS increase were observed, nor new or enlarging lesions on MRI when available in the next 6–36 months. DMT response was categorized as 'failure' based on ≥1 clinical relapse and/or significant EDSS increase in the next 6–36 months. Significant EDSS increase was defined as an increase of ≥1.5 from a previous EDSS score of 0–1.0, of ≥1.0 from a previous score between 1.0 and 5.5, and of ≥0.5 from a previous score above 5.5. Clinical treatment failure was confirmed by documentation of new or enlarging lesions on MRI for all but two PwMS who did not have an MRI available. Suboptimal response (mild symptoms not typical of a relapse, EDSS increase below the cut-off for treatment failure, or 1–2 new/enlarging lesions on MRI when available in the absence of clinical relapse or significant EDSS progression) was not included in the final sub-analysis presented in Fig. 4c.

Serum

Blood from HC and PwMS was collected between 2011 and 2023 (75% for both HC and PwMS collected between 2016 and 2020) in silica-coated tubes

	MS		HC	
	Female	Male	Female	Male
Characteristics of participants				
Number of participants	207	82	88	59
Samples per participant				
1	19 (9.2%)	7 (8.5%)	83 (94.3%)	56 (94.9%)
2	147 (71.0%)	58 (70.7%)	5 (5.7%)	3 (5.1%)
3	37 (17.9%)	15 (18.3%)	0 (0%)	0 (0%)
4	4 (1.9%)	2 (2.4%)	0 (0%)	0 (0%)
Any vascular comorbidity at baseline	28 (13.5%)	22 (26.8%)	n.a.	n.a.
Number of samples	440	176	93	62
Characteristics at sampling				
Age (years)				
Mean (SD)	44.7 (10.6)	47.2 (11.0)	42.1 (14.2)	42.6 (15.6)
Median [Min, Max]	43 [18, 75]	48 [19, 69]	42 [19, 69]	38 [22, 74]
Disease duration (years)			n.a.	n.a.
Mean (SD)	15.1 (10.6)	15.4 (10.8)		
Median [Min, Max]	14 [0, 51]	13 [0, 49]		
Missing information	8 (1.8%)	0 (0%)		
CMV IgG				
Negative	304 (69.1%)	116 (65.9%)	56 (60.2%)	32 (51.6%)
Positive	135 (30.7%)	59 (33.5%)	36 (38.7%)	28 (45.2%)
Undetermined	1 (0.2%)	1 (0.6%)	1 (1.1%)	2 (3.2%)
Smoking status			n.a.	n.a.
Non-smoker	376 (85.4%)	147 (83.5%)		
Active smoker	64 (14.5%)	29 (16.5%)		
Vascular comorbidity			n.a.	n.a.
0	373 (84.7%)	124 (70.5%)		
1	45 (10.2%)	26 (14.7%)		
2	12 (2.7%)	20 (11.4%)		
3	6 (1.4%)	5 (2.8%)		
4–5	3 (0.7%)	1 (0.6%)		
Missing information	1 (0.2%)	0 (0%)		
Disease course				
RRMS	394 (89.5%)	127 (72.2%)		
SPMS	41 (9.3%)	38 (21.6%)		
PPMS	5 (1.1%)	11 (6.3%)		
EDSS			n.a.	n.a.
0–0.5	164 (37.3%)	44 (25.0%)		
1–1.5	126 (28.6%)	31 (17.6%)		
2–2.5	78 (17.7%)	32 (18.2%)		
3–3.5	29 (6.6%)	19 (10.8%)		
4–5	17 (3.9%)	23 (13.1%)		
≥5.5	26 (5.9%)	27 (15.3%)		
Untreated	141 (32.0%)	64 (36.4%)	n.a.	n.a.
DMT-treated				
GA	58 (13.2%)	15 (8.5%)		
IFN	58 (13.2%)	11 (6.3%)		
TF	69 (15.7%)	30 (17.0%)		
DMF	28 (6.4%)	14 (8.0%)		
FTY	50 (11.4%)	34 (19.3%)		
NTZ	29 (6.6%)	5 (2.8%)		
OCV	5 (1.1%)	3 (1.7%)		
CLD	2 (0.5%)	0 (0%)		

Abbreviations: MS: multiple sclerosis; HC: healthy control; n.a: not available; EDSS: Expanded Disability Status Scale; DMT: disease-modifying therapy; GA: glatiramer acetate; IFN: interferon beta; TF: teriflunomide; DMF: dimethyl fumarate; FTY: fingolimod; NTZ: natalizumab; OCV: ocrelizumab; CLD: cladribine.

Table 1: **Characteristics of participants and biosamples.**

(Greiner bio-one, Monroe, USA), clotted for 30 min before centrifugation at 1800 g for 10 min. Serum was aliquoted and stored at –80 °C until needed.

Analyte detection

Between 2019 and 2023, serum samples were assessed for the levels of interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-18, IL-27, beta-nerve growth factor (β-NGF), leptin, and tumor necrosis factor (TNF) using a U-PLEX multiplex assay from MesoScale Discovery (Rockville, MD, USA). Epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) were determined using R-PLEX singleplex assays (MesoScale Discovery). Mature brain derived growth factor (BDNF) was measured using Mature BDNF Rapid™ test from Biosensis (distributed by Cedarlane, Oakville, ON, Canada). All serum samples were run in duplicate following the manufacturer's instructions; serum samples were added neat for most assays and diluted 1/125 for BDNF and 1/20 for EGF assays. Different time points for a given donor were run on the same plate, and samples from HC and PwMS were included on each plate. IL-2 results were excluded as no detectable levels were measured in most HC and MS samples. Serum samples from two HC (one female and one male) were added to each plate to monitor inter-assay variability. The coefficient of variation for the serum control amongst all plates (18–21 plates per analyte) was <23% for IL-4, IL-6, IL-10, IL-18, IL-27, β-NGF, leptin, and TNF. The coefficient of variation was 37% for BDNF, 31% for EGF, and 29% for bFGF. Plates showing variations outside these ranges were excluded from our analysis (0–3 plates per type of immunoassay). The standard curve range for each analyte included in the analyses is presented in Supplementary Table S1.

CMV status

The CHUM diagnostic lab assessed the CMV IgG titers following the standard diagnostic lab procedures. Values ≥6.0 UA/mL were considered positive (clinical cut-off). When serum from the first time point was positive, later time points from the same participant were assumed to remain positive. When serum from the first time point was negative, later time points were tested to determine if CMV status had changed (seroconversion).

Clustering analysis

All samples with complete datasets (results available for all analytes, n = 648) were included in the clustering analysis. A correlation matrix based on the measurements of analytes was generated, and clusters were formed on the correlation matrix by using hierarchical clustering (built with the pheatmap R package version 1.0.12). The number of clusters was chosen based on the elbow method and visual inspection of the correlation matrix. Clusters are then described by showing the frequency of clinical features across clusters (sex, age, MS or HC status) and by showing the distribution of analytes (cytokines and trophic/growth factors) measurements across clusters.

Statistics

For univariable statistical analyses, as indicated in tables and figure legend, paired or unpaired two-tailed t-test or Mann–Whitney U test (two groups), and ANOVA or Kruskal–Wallis followed by multiple comparison Tukey's or Dunn's post-test (three groups or more) were used according to distribution. Nominal p-values for ANOVA or Kruskal–Wallis were adjusted for multiple testing using the method of Benjamin and Hochberg to control the false discovery rate (FDR) at a threshold we set at 5% (FDR adjusted p value below 0.05).

The relationships between age and biomarker levels were assessed using linear regression analyses, in univariable followed by multivariable models. When biomarker values did not follow a normal distribution, the optimal transformation was used. Non-transformed data were used for BDNF and EGF; log scale values were used for IL-4 and bFGF; square root was used for IL-18 and β-NGF; root of 3 for IL-27, TNF, and leptin; root of 4 was used for IL-6 and IL-10. The results obtained from these analyses were the beta coefficient, which indicated the transformed value of biomarker level change with each increasing year of age, and the 95% confidence intervals (95% CI). To visualize the impact of CMV serostatus/sex/donor (HC/MS) and treatment status on the relation between age and immune marker levels, we graphed the predicted biomarker levels by age, stratified by CMV serostatus/sex/donor (HC/MS) and treatment status.

When analyzing longitudinal samples, linear/logistic regression models with generalized estimating equations (GEE) were used to correct for the clustering of samples within individuals. Multivariable models were adjusted for variables identified as potential confounders (age, sex, CMV serology). For analysis restricted to samples from treated or untreated MS, we assessed smoking as a potential confounder. Introducing smoking as a co-variable did not affect beta values or odds ratios and was therefore not considered in the final multivariable models. To explore the relation of analyte level change and EDSS increase between two time points, a logistic regression using significant EDSS increase as defined above (yes/no) as a binary outcome was performed, adjusting for sampling interval (days) in addition to age, sex, and CMV status.

These analyses were conducted on the R statistical software[23] on the RStudio integrated development environment (IDE) with the packages tidyverse (data manipulations),[24] geepack[25] and geeasy (GEE analyses), olsrr (biomarker level distribution normality testing),[26] and broom (regression analysis results display).[27]

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Results
Cohort characteristics
A total of 147 HC and 289 PwMS, having contributed 155 and 616 samples respectively, were included in this study. Most HC contributed only one sample, whereas most PwMS had at least two samples (Table 1). The age range of participants was similar in HC and MS samples; the mean and median age at sampling were slightly higher in MS samples, partly due to 91% of MS donors providing at least a second sample one to four years after baseline. Female PwMS were slightly younger than males but exhibited similar disease duration at time of sampling. As expected, the majority of MS samples (440 samples, 71.4%) were collected from females. Notably, the prevalence of positive CMV serology, a common lifelong infection associated with the senescence of immune cells,[13] was lower in MS samples (30.7 and 33.5% for females and males) compared to HC samples (38.7 and 45.2% for females and males) (Table 1). Only four treated PwMS (two females and two males) CMV seronegative at the first time point, converted to CMV seropositive at subsequent time points (2–3 years later). PwMS samples were collected while 14.5% of females and 16.5% of males were active smokers, and 15.3% of females and 29.5% of males were diagnosed with at least one vascular comorbidity (this information was not collected for HC due to ethical considerations). SPMS and PPMS courses were more frequent in males, as was the proportion of EDSS scores ≥3.0.[2] Based on the recent large consensus that the clinical MS course represents a continuum rather than defined disease forms, we did not separate samples according to disease course (relapsing-remitting vs. progressive) for analyses.[1] One-third of the samples (n = 141 female and n = 64 male samples) were from untreated PwMS. The proportions of samples from treated PwMS according to the different DMTs currently used are shown in Table 1.

Age, sex, and MS status are associated with clustering based on unsupervised analysis of soluble immune markers
A short description of each analyte and its role in physiology, MS, and aging are presented in Supplementary Table S1. The mean, median, and range values for the measured soluble markers in samples from HC and MS are shown in Supplementary Table S2. Serum samples from PwMS exhibited lower levels of IL-4, β-NGF, TNF, and BDNF than HC (Supplementary Table S2). In contrast, the levels of EGF and bFGF were significantly higher in PwMS than in HC samples. To evaluate the influence of MS and DMTs on serum markers, we compared absolute levels of the different analytes between HC, untreated MS (untx MS), and DMT-treated MS (tx MS). Four different 'patterns' were observed (Fig. 1). The first showed altered levels in MS compared to HC regardless of treatment status; EGF and bFGF were higher, whereas IL-4 was lower, in untreated and treated MS vs. HC, while β-NGF was lower in both groups but reached significance only in untreated MS. The second was characterized by lower levels in treated MS compared to HC, as observed for BDNF and, to a lower extent, TNF. The third pattern showed lower levels in untreated MS compared to treated MS, as observed for IL-18 and leptin. Finally, the fourth was characterized by similar levels in HC, untreated, and treated MS, and was seen for IL-6, IL-10, and IL-27.

To investigate whether clinical factors such as age, sex, and disease status (MS versus HC) contribute to the observed heterogeneity and variance in the levels of eleven measured immune markers, we applied clustering analysis to the sample data. The dendrogram illustrates the hierarchical clustering of samples from HC and PwMS (Fig. 2) into eight clusters. Clusters 7 and 8 contained a high proportion of samples from PwMS, while cluster 4 also had a notable representation of PwMS samples. In contrast, samples from HC were predominant in clusters 1–3 (Fig. 2b). Notably, samples in cluster 4 largely came from older (≥50 years old) MS and HC donors, whereas clusters 7 and 8 contained relatively more samples from younger (<50 years old) MS donors. Moreover, clusters 5 and 6 contained a higher proportion of male samples, while cluster 4 predominantly consisted of female samples (Fig. 2c).

We also assessed the overall distribution of samples from HC, untreated PwMS and treated PwMS into the different clusters (Fig. 2d). We observed that cluster 1 contained the largest number of samples from HC (31% of HC samples) but not from MS. This cluster was characterized by high relative levels of cytokines considered anti-inflammatory (e.g., IL-4, IL-10) or protective (TNF) in MS or neurotrophic factor (β-NGF). Cluster 1 also had low levels of growth factors associated with SASP: EGF and bFGF (Fig. 2e). Similarly, cluster 2 was composed of a high proportion of samples from HC (23%) vs. untreated PwMS (13.2%) or treated PwMS (16.8%) and showed high relative levels of TNF and β-NGF, IL-27, and leptin. Cluster 3, also associated with HC, was characterized by elevated levels of IL-4 and BDNF, two factors generally considered beneficial in MS (Fig. 2e). Clusters 7 and 8 contained the largest proportions of samples from untreated (cluster 7: 13.2% and cluster 8: 22.9%) and treated MS (cluster 7: 22.5% and cluster 8: 22.2%) but represented only a small

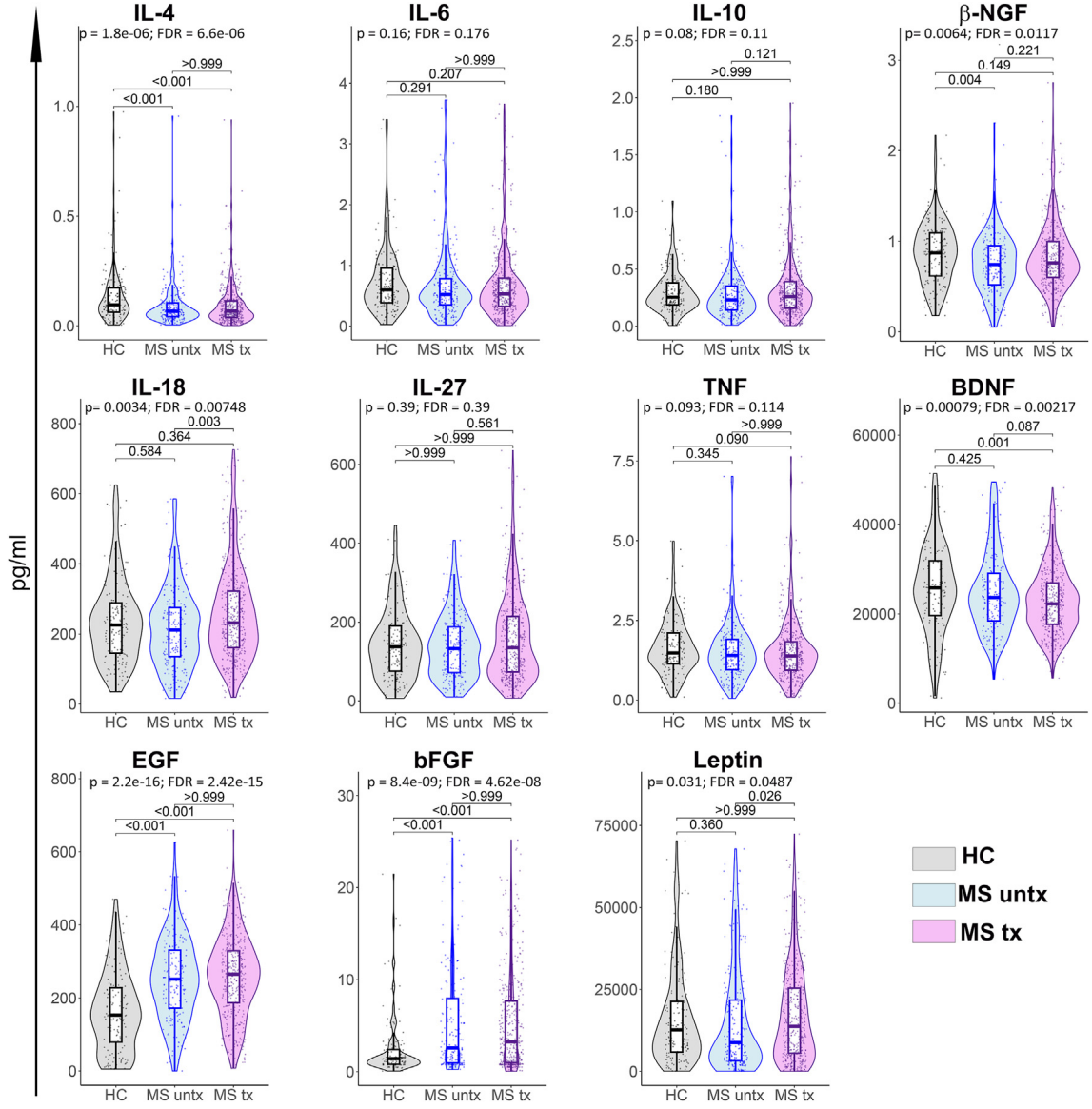

***Fig. 1:* Comparison of analyte levels in serum samples from HC, untreated, and treated MS**. Violin plots of serum analyte levels in healthy controls (HC, gray), untreated MS (MS untx, blue), and treated MS (MS tx purple) samples. Each dot represents one sample. The median and p values for the Kruskal–Wallis (IL-4, IL-6, IL-10, β-NGF, IL-18, IL-27, TNF-α, bFGF, leptin) or ANOVA (BDNF, EGF) tests comparing the three groups are shown along with the false discovery rate (FDR)-adjusted p-value.

proportion of HC samples (cluster 7: 5.3% and cluster 8: 3.5%). Both clusters had the highest relative levels of growth factors EGF and bFGF. Cluster 8 was characterized by low relative levels of IL-4, IL-6, IL-10, TNF, and β-NGF (Fig. 2e). Finally, cluster 6, which included a high proportion of samples from young male HC individuals, was associated with higher levels of BDNF (Fig. 2d and e).

To exclude the effect of DMTs on the measured immune profiles, we performed a separate clustering analysis only with samples from HC and untreated PwMS. Running unsupervised analysis on this subset of samples (from HC and untreated PwMS), we observed similar associations of MS status, age, and sex with data-driven clustering based on serum levels of soluble immune markers. Indeed, clusters exhibiting the highest EGF and bFGF levels (cluster G, Supplementary Figure S1b–d) included the highest proportion of MS samples. In contrast, the cluster presenting high relative levels of IL-4, TNF, and β-NGF (cluster C, Supplementary Figure S1b and d) included a greater proportion of HC than MS samples. Overall, these

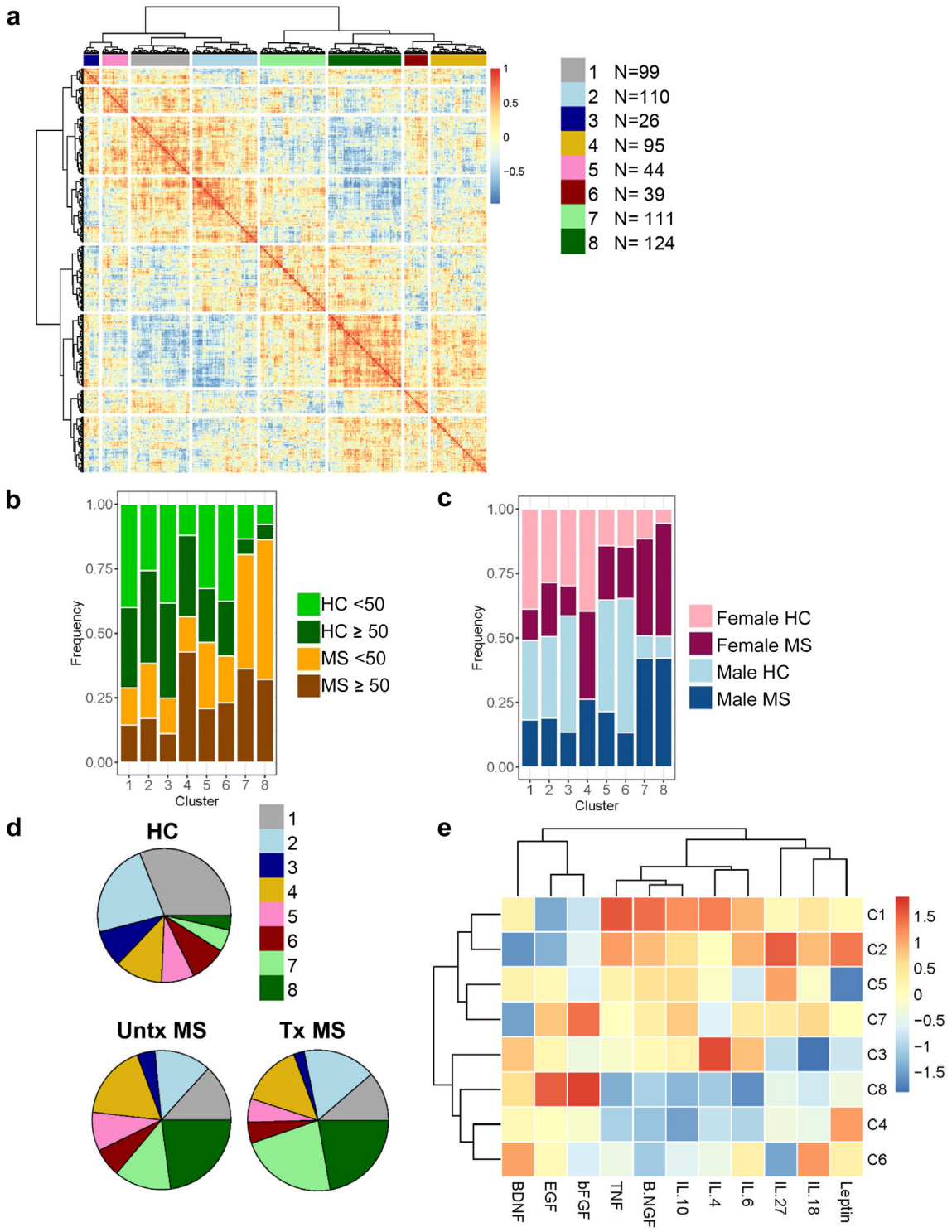

***Fig. 2:* Data-driven clustering of HC and MS samples based on serum marker levels**. Unsupervised clustering of serum samples based on immune marker levels was performed using the 648 samples for which all analyte levels were available. a) Representation of hierarchal clustering; clusters 1–8. The number of samples in each cluster is indicated. b) Frequency of HC (green) and MS (brown) samples from <50 years old (light tone) or ≥50 years old. (dark tone) donors in each cluster. c) Frequency of female (pink) and male (blue) from HC (light tone) and MS (dark tone) samples in each cluster. d) Distribution of samples from HC, untreated MS (Untx MS), and treated MS (Tx MS) by cluster. e) Heatmap showing relative levels of serum markers for the different clusters (data-driven analysis). Color scale legends the z-score, across the clusters, of each analyte median level; analytes are indicated on the x-axis and clusters on the y-axis.

results suggest that age, sex as well as MS status and treatment shape the heterogeneity in immune marker levels in serum.

CMV serostatus and sex influence the relationship between age and levels of soluble markers

CMV infection is associated with T cell senescence and could influence the relation between age and levels of immune markers.[11] The prevalence of previous CMV exposure was lower in MS than in HC participants (Table 1). We observed an inverse linear association with age in CMV-negative but not CMV-positive individuals for IL-10, IL-18, and EGF levels (Supplementary Figure S2a). In contrast, leptin levels were positively associated with age in CMV-negative individuals only. Moreover, predicted levels of BDNF were lower at all ages in samples from CMV-positive compared to CMV-negative individuals, although confidence intervals overlapped.

As sex assigned at birth also influences the aging process, we similarly graphed the predicted levels according to age for CMV-negative HC individuals, stratified by sex (Supplementary Figure S2b). Predicted levels of β-NGF were inversely related with age in females but an opposite trend was observed in males, leading to marked differences in predicted levels in older individuals. IL-10 and EGF showed a similar statistically significant negative slope with age in both males and females (Supplementary Figure S2b). Leptin showed similarly increasing levels over the ages in both sexes, but females exhibited higher levels than males, as shown by others,[28] with more marked sex differences at younger ages. Other analytes did not show a significant linear association with age in either male or female samples. Taken together, these data support the impact of sex and CMV serostatus on levels of age-sensitive immune markers.

MS and DMTs modify the relation between age and levels of immune markers

We next investigated the impact of MS and its treatment on levels of immune markers according to age. Taking CMV negative female donors as reference, we observed that across virtually all ages, the predicted levels of EGF and bFGF were higher among MS donors, irrespective of treatment, than HC (Fig. 3). EGF levels significantly decreased with age among untreated PwMS, not significantly in other groups. We found that β-NGF and TNF showed reverse trends in untreated vs. treated MS, with higher levels of β-NGF but lower levels of TNF in older treated PwMS compared to older HC and older untreated PwMS (Fig. 3). Predicted leptin levels increased with age similarly in treated and untreated PwMS, and IL-18 levels decreased with age in treated but not in untreated PwMS or HC (Fig. 3). IL-10 decreased with age in all groups but only reached significance in treated PwMS. Finally, although not reaching significance, predicted IL-4 levels declined with age in HC but not in PwMS in whom levels tended to be lower than HC at all ages. Our results suggest that MS per se and DMT treatment are associated with altered levels of age-sensitive immune markers and can modify the relation of these markers with age (Fig. 3).

Levels of several age-sensitive immune markers associated with sex and CMV exposure are altered by MS and by DMT compared to HC in multivariable analysis

Performing multivariable analysis adjusted for age and other relevant factors, we found that the levels of β-NGF, IL-4, IL-18, IL-27, and TNF (all higher in males) and leptin (lower in males) were significantly influenced by sex (Table 2). Moreover, CMV seropositivity was associated with reduced levels of β-NGF, TNF, and BDNF (Table 2). In line with unsupervised analyses (Fig. 1), we found that IL-4 was significantly lower whereas EGF and bFGF were higher in both untreated MS and treated MS than HC after adjusting for age, sex, and CMV exposure (Table 2). Moreover, β-NGF and leptin levels were significantly lower in untreated PwMS but not in treated PwMS compared to HC after adjusting for age, sex, and CMV status. Finally, while showing similar trends in untreated MS, we found that TNF and BDNF were significantly lower in treated PwMS than HC after adjusting for age, sex, and CMV exposure (Table 2). These results support an association of untreated MS or DMT-treated MS with lower levels of age-sensitive, CMV- and/or sex-influenced factors associated with neuroprotection in MS (IL-4, β-NGF, BDNF, TNF) but intriguingly with higher levels of growth factors EGF and bFGF.

EDSS and disease duration show a modest association with the pattern of immune markers observed in MS

In MS EDSS and disease duration increase with age.[6] We assessed the relation between analyte levels and EDSS at biosampling in untreated and treated MS adjusted for sex, age, and CMV status. No significant association was identified; trends were observed for an inverse relation between TNF levels and EDSS in treated MS (Supplementary Figure S3). IL-27 predicted levels differed between treated and untreated PwMS at EDSS values below 5 but not beyond (Supplementary Figure S3). Using logistic regression to explore the relation of analyte level change and EDSS increase between two time points among PwMS remaining untreated, we did not find significant associations after adjusting for age, sex, CMV status, and sampling interval (data not shown). Assessing specifically the relation between analyte levels and disease duration at biosampling in untreated and treated MS adjusted for sex, age, and CMV status (Supplementary Figure S4), no significant association was found in untreated MS;

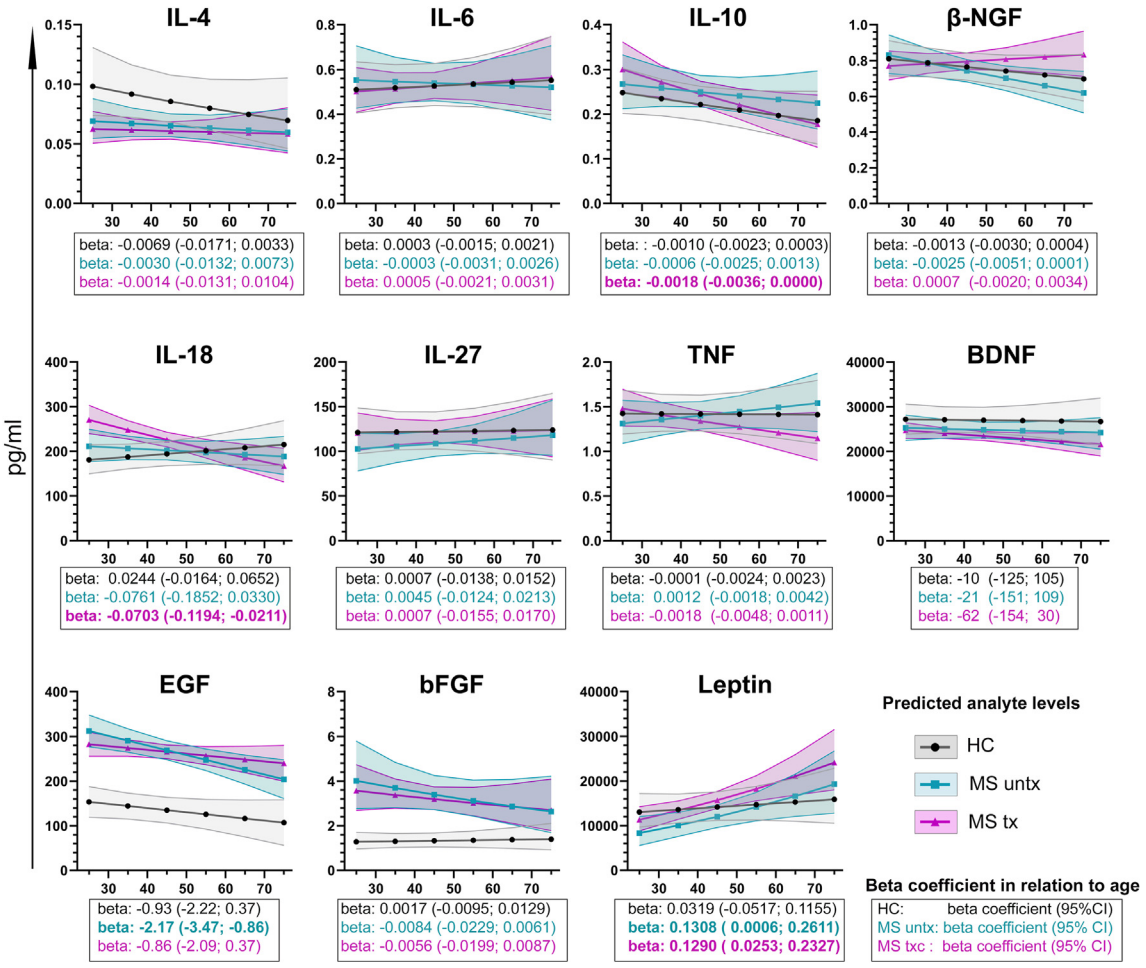

Fig. 3: **Impact of MS status on the relation between age and analyte levels**. Linear regression models for the relation between age and analyte levels were estimated for each group: HC (black), untreated MS patients (aqua), and DMT-treated MS patients (magenta). Generalized estimating equations (GEE) were used to correct for the clustering of samples within individuals. Non-transformed data were used for BDNF and EGF; log scale values were used for IL-4 and bFGF; square root was used for IL-18 and β-NGF; root of 3 for IL-27, TNF, and leptin; root of 4 was used for IL-6 and IL-10. For each analyte, the beta coefficient, indicating the transformed value of biomarker level change (untransformed for BDNF and EGF) with each increasing year of age, and the 95% confidence intervals (95% CI) are provided. 95% CI that do not cross zero are shown in bold. Graphs show predicted analyte levels (line) with range of predicted values based on the 95% CI (colored area) according to age in CMV-negative females for each group.

only IL-27 (increased) and BDNF (decreased) in treated PwMS showed a significant association with disease duration.

Vascular comorbidities but not disease course are associated with a distinct profile of immune markers in older PwMS

Vascular comorbidities exhibit complex reciprocal interactions with aging, immunosenescence, and inflammation. MS is associated with an increased incidence of vascular comorbidities, which are, on the other hand, associated with disability in MS.[20,29] Compared to the absence of such comorbidities, the presence of vascular comorbidities at the time of sampling was associated with older age in both untreated (58.8 ± 9.3 vs. 46.2 ± 11.8 years old) and treated (49.0 ± 8.2 vs. 42.6 ± 9.2 years old) MS, an elevated proportion of males (untreated: 43.2% vs. 28.1%; treated 44.6% vs. 23.4%) and a progressive (SPMS and PPMS) disease course (untreated: 56.9% vs. 21.9%; treated 19.0% vs. 6.2%). To explore the association of vascular comorbidities with age-sensitive immune markers in PwMS, we restricted the analysis to individuals ≥45 years old (Supplementary Table S3). Notably, levels of cytokines associated with metabolic syndrome, atherosclerosis and cardiovascular disease[30–32] were significantly elevated in untreated (IL-6) or treated (IL-18, leptin) PwMS with comorbidity compared to those without, in analyses unadjusted for

age, sex, and CMV status (Supplementary Figure S5). In contrast, the levels of age-sensitive immune markers we had identified as altered in untreated MS or DMT-treated MS compared to HC were not different according to the presence of vascular comorbidities. Finally, no significant differences were observed for levels of age-sensitive immune markers between RRMS and SPMS when comparing individuals ≥45 years old (data not shown). These data suggest that vascular comorbidities in older PwMS are associated with an inflammaging profile similar to those in the general population affected by such comorbidities.

Initiation of treatment is associated with increased levels of β-NGF in MS

To investigate the impact of DMT on age-sensitive immune markers, we compared the levels in consecutive samples (longitudinal analysis) from untreated MS who remained untreated (Untx-untx) vs. those who subsequently started a DMT (Untx-Tx). In this multivariable analysis of consecutive samples from PwMS, we found that starting a DMT was significantly associated with a higher increase in β-NGF levels compared to the pairs of samples from PwMS remaining untreated (Table 3). We performed a similar analysis on paired samples from treated MS. We compared samples obtained from treated PwMS remaining on the same DMT at both biosampling times (stable group: Tx-Tx) versus those switching from one DMT to another between biosampling times (switch group: Tx-Swtx). Only BDNF variation differed between groups; BDNF levels increased in the follow-up sample in the switch but not the stable group (Supplementary Table S4). These results show that DMT initiation and switch can influence levels of neurotrophic factors in PwMS.

Distinct alterations in immune profile according to DMT choice

To assess whether different DMTs are associated with a distinct profile of age-sensitive immune markers, we performed a principal component analysis (PCA) analysis based on analyte levels. We grouped the samples based on current DMT efficacy: low (GA, IFN, and TF), moderate (FTY and DMF), or high efficacy (NTZ and OCV),[3,33] and compared the levels to untreated and HC groups (Fig. 4). We observed that samples from PwMS treated with high-efficacy therapies were closer to HC than those from untreated PwMS or PwMS treated with low or moderate efficacy therapies, especially on the second principal component. Notably, the second principal component (y axis) was associated with variation in EGF and bFGF levels as illustrated (Fig. 4a). We then compared the changes in analyte levels between the paired MS samples obtained at baseline (untreated) and after initiation of treatment with low, moderate or high

Analyte	Variables	Regression results		Predicted analyte levels (pg/ml)[a]
		Beta	95% CI	
IL-4[b]	Sex	**0.2164**	**0.0808; 0.3520**	
	CMV	−0.1274	−0.2593; 0.0045	
	HC	Ref		0.088
	MS untreated	**−0.3199**	**−0.5091; −0.1307**	0.064
	MS treated	**−0.3458**	**−0.5108; −0.1808**	0.062
IL-6[c]	Sex	0.0231	−0.0037; 0.0498	
	CMV	−0.0087	−0.0349; 0.0174	
	HC	Ref		0.587
	MS untreated	−0.0284	−0.0656; 0.0088	0.514
	MS treated	−0.0255	−0.0580; 0.0070	0.522
IL-10[c]	Sex	0.0174	−0.0041; 0.0388	
	CMV	−0.0027	−0.0235; 0.0181	
	HC	Ref		0.247
	MS untreated	−0.0145	−0.0446; 0.0157	0.227
	MS treated	0.0038	−0.0224; 0.0300	0.252
β-NGF[d]	Sex	**0.0429**	**0.0132; 0.0726**	
	CMV	**−0.0313**	**−0.0602; −0.0024**	
	HC	Ref		0.818
	MS untreated	**−0.0621**	**−0.1033; −0.0208**	0.709
	MS treated	−0.0224	−0.0585; 0.0137	0.778
IL-18[d]	Sex	**1.2481**	**0.6289; 1.8672**	
	CMV	0.1219	−0.4804; 0.7242	
	HC	Ref		204.576
	MS untreated	−0.5880	−1.4474; 0.2714	188.101
	MS treated	0.7485	−0.0027; 1.4997	226.548
IL-27[e]	Sex	**0.2513**	**0.0694; 0.4332**	
	CMV	0.0810	−0.0960; 0.2579	
	HC	Ref		115.572
	MS untreated	−0.0114	−0.2643; 0.2414	114.763
	MS treated	0.1443	−0.0766; 0.3651	126.151
TNF[e]	Sex	**0.0472**	**0.0132; 0.0811**	
	CMV	**−0.0582**	**−0.0912; −0.0252**	
	HC	Ref		1.531
	MS untreated	−0.0445	−0.0924; 0.0033	1.360
	MS treated	**−0.0422**	**−0.0837; −0.0007**	1.369
BDNF	Sex	535	−798; 1868	
	CMV	**−2256**	**−3548; −963**	
	HC	Ref		26,495
	MS untreated	−1118	−3665; 828	25,377
	MS treated	**−3136**	**−4821; −1450**	23,359
EGF	Sex	0.79	−16.9; 18.5	
	CMV	−15.4	−32.5; 1.7	
	HC	Ref		163.3
	MS untreated	**98.3**	**73.4; 123.2**	261.6
	MS treated	**96.9**	**75.3; 118.5**	260.2
bFGF[b]	Sex	0.002	−0.176; 0.180	
	CMV	−0.166	−0.339; 0.007	
	HC	Ref		1.619
	MS untreated	**0.672**	**0.425; 0.920**	3.171
	MS treated	**0.660**	**0.444; 0.875**	3.133
Leptin[e]	Sex	**−4.97**	**−5.809; −3.386**	
	CMV	−0.114	−1.293; 1.065	

(Table 2 continues on next page)

Analyte	Variables	Regression results		Predicted analyte levels (pg/ml)[a]
		Beta	95% CI	
(Continued from previous page)				
	HC	Ref		15974
	MS untreated	**−2.305**	**−3.988; −0.622**	11978
	MS treated	−0.459	−1.931; 1.011	15117

Linear regression models, one per analyte, for the relation of MS status and analyte levels, with generalized estimating equations to account for the clustering of samples within individuals, and including age, sex, and CMV serostatus (for sex, reference = female; for CMV, reference = seronegative; for MS status, reference = HC). Model with analyte levels transformed by taking. [a]Predicted analyte levels estimated for a 45 years old CMV-seronegative female. [b]Model with analyte levels transformed by taking natural logarithm. [c]Model with analyte levels transformed by taking the root of 4. [d]Model with analyte levels transformed by taking the square root. [e]Model with analyte levels transformed by taking the root of 3.

Table 2: **Linear regression results for the relation of MS status and analyte levels.**

efficacy DMTs. We observed that starting a low efficacy DMT was associated with a strong trend toward increased β-NGF but decreased IL-4 levels (Supplementary Figure S6). In contrast, only the initiation of high-efficacy therapies was associated with a significant reduction in serum levels of EGF and bFGF.

Analyte	Treatment status	Mean level at baseline	Regression results		
			Beta	95% CI of beta	Predicted level change (pg/ml)[a]
IL-4[b]	Untx-untx	0.076	Reference		−0.0071
	Untx-Tx	0.106	−0.0164	−0.0362; 0.0033	−0.0235
IL-6[c]	Untx-untx	0.610	Reference		−0.0465
	Untx-Tx	0.805	−0.0215	−0.1978; 0.1548	−0.0680
IL-10[c]	Untx-untx	0.265	Reference		−0.052
	Untx-Tx	0.313	0.0539	−0.0488; 0.1565	0.002
β-NGF[d]	Untx-untx	0.716	Reference		−0.013
	Untx-Tx	**0.844**	**0.0943**	**0.0148; 0.1737**	**0.081**
IL-18[d]	Untx-untx	206.9	Reference		−4.8878
	Untx-Tx	235.8	22.409	−7.5860; 52.4032	17.5308
IL-27[e]	Untx-untx	141.387	Reference		3.5902
	Untx-Tx	136.513	11.7349	−6.2899; 29.7598	15.3252
TNF[e]	Untx-untx	1.428	Reference		−0.0256
	Untx-Tx	1.555	−0.0777	−0.2845; 0.1290	−0.1034
BDNF	Untx-untx	25,174	Reference		−2201
	Untx-Tx	24,293	642	−2400; 3683	−1559
EGF	Untx-untx	278.7	Reference		−80.9
	Untx-Tx	303.0	−1.2	−65.0; 62.7	−82.1
bFGF[b]	Untx-untx	6.379	Reference		−2.319
	Untx-Tx	6.882	−0049	−3.0183; 3.0086	−2.323
Leptin[e]	Untx-untx	14,902	Reference		155
	Untx-Tx	14,359	−797	−3939; 2344	−642

Linear regression models, one per analyte, for the relation of treatment status and analyte levels adjusted for age, sex, CMV serostatus, and time interval between samples (days). [a]Predicted analyte levels estimated for a 45 years old CMV-seronegative female. [b]Model with analyte levels transformed by taking natural logarithm. [c]Model with analyte levels transformed by taking the root of 4. [d]Model with analyte levels transformed by taking the square root. [e]Model with analyte levels transformed by taking the root of 3.

Table 3: **Linear regression results for the relation of treatment status change and analyte change in two consecutive samples, among PwMS untreated at baseline.**

Beyond their efficacy profile, DMTs exhibit a wide range of mechanisms of action.[34] Moreover, clinical characteristics influence DMT choice, as illustrated by the higher proportion of female PwMS receiving platform therapies than males. In contrast, FTY was more commonly chosen for male PwMS (Table 1). The clinical data of PwMS according to specific DMT use are shown in Supplementary Table S5. We observed a lower proportion of CMV seropositivity among samples collected from PwMS while on IFN and to a lesser extent TF and DMF. Although these data were not adjusted for age, sex, and CMV status, we found different analyte signatures associated with various DMTs (Fig. 4b). Compared to untreated PwMS, GA-treated samples showed no significant alterations, and samples from DMF-treated PwMS showed few differences except for lower BDNF levels. IFN-treated PwMS showed a distinctive profile with uniquely elevated levels of IL-18, IL-10, and IL-6 but lower BDNF levels compared to untreated MS and/or multiple other DMTs. Samples from TF-treated PwMS showed low levels of IL-10 and TNF compared to untreated MS and most DMT-treated groups. FTY-treated samples exhibited elevated levels of IL-27 and β-NGF compared to untreated MS. Samples from NTZ-treated PwMS showed elevated levels of IL-6, IL-10 and TNF compared to TF, as well as lower BDNF levels and a similar trend for EGF and bFGF compared to untreated PwMS. Although few samples were available in our cohort, in OCV-treated samples, which included three patients with primary progressive disease (identified as red dots), most age-sensitive immune markers except BDNF exhibited a trend of lower levels (Fig. 4b). Taken together, these results suggest that immune alterations associated with DMTs might be due to the clinical characteristics, level of DMT efficacy, and specific biological effects on the immune profile mediated by DMTs in PwMS.

Levels of β-NGF, TNF, IL-10, and IL-6 at baseline predict treatment failure

To investigate whether baseline levels of age-sensitive immune markers could represent biomarkers for DMT response, we assessed the analyte levels in samples from untreated PwMS who subsequently initiated a DMT within the next two years. We compared the analyte levels at the untreated time point between PwMS who subsequently experienced treatment failure 6–36 months post DMT initiation, as defined by a documented clinical relapse or significant EDSS progression (failure group), to PwMS with no evidence of disease activity clinically and on MRI when available (no sign of failure group) (Supplementary Table S6). We found that samples from PwMS with no signs of DMT-treatment failure had significantly higher baseline levels of IL-6, IL-10, β-NGF, and TNF compared to PwMS with subsequent signs of treatment failure (Fig. 4c). Those comparisons remained significant when excluding

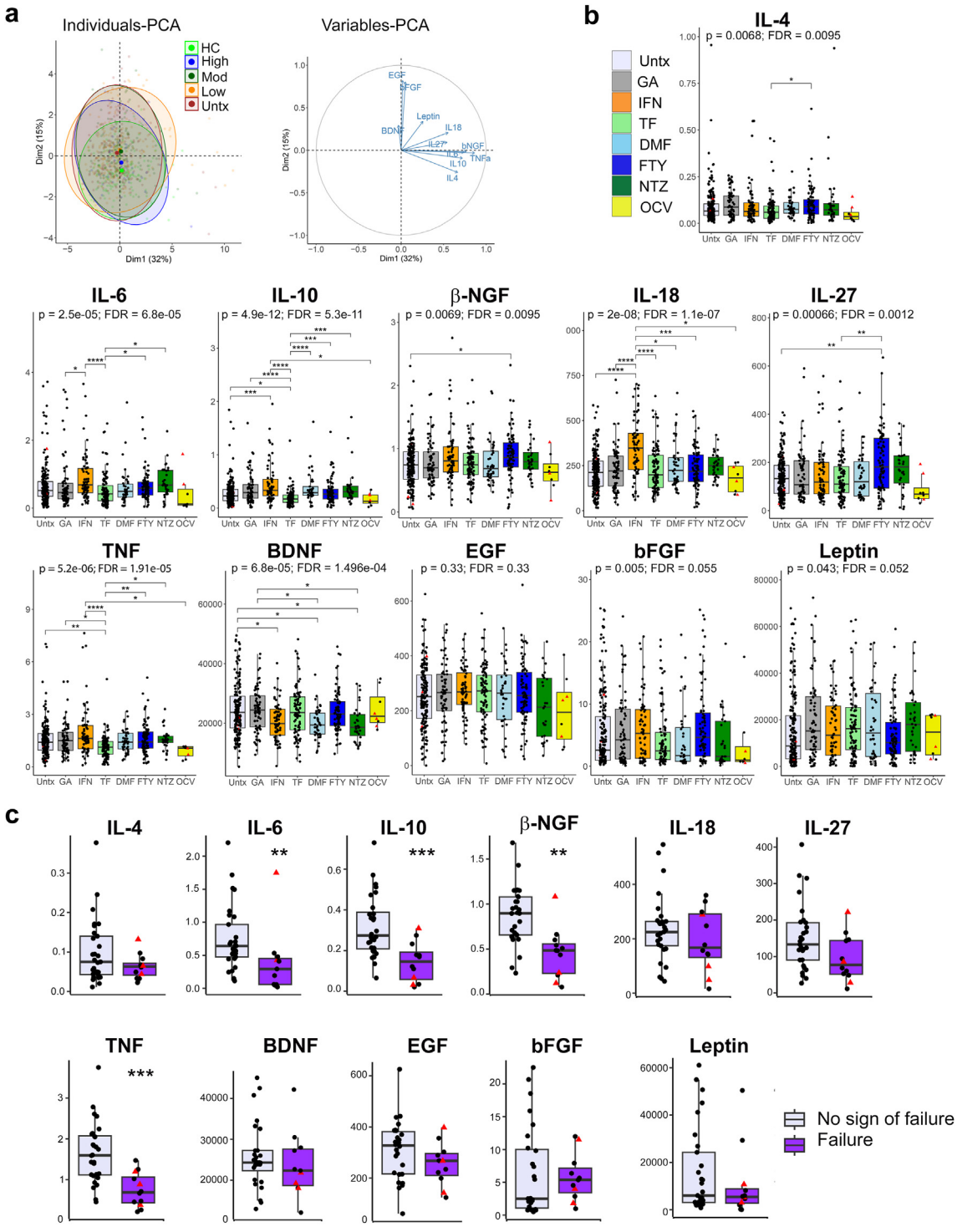

***Fig. 4:* Variations in the immune profile according to DMTs and DMT-response**. a) Principal component analysis (PCA) based on analyte levels according to HC, untreated, low-efficacy, moderate efficacy, and high-efficacy DMT. b) Analyte levels (pg/ml) in samples from untreated, or DMT-treated PwMS. The median and p values for the Kruskal–Wallis (IL-4, IL-6, IL-10, β-NGF, IL-18, IL-27, TNF-α, bFGF, leptin) or ANOVA (BDNF, EGF) tests comparing the three groups are shown along with the false discovery rate (FDR)-adjusted p-value. c) Analyte levels (pg/ml) in samples from untreated PwMS who subsequently started a DMT and showed either no sign or sign of treatment failure in the 6–36 months post-DMT initiation. Each dot represents one sample. Adjusted p values * < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

PwMS starting OCV for active primary progressive disease (other OCV samples were not from DMT naïve PwMS). Notably, the HC-associated cluster 1 identified in our unsupervised analysis (Fig. 2) showed elevated levels of IL-4, IL-6, IL-10, TNF, and β-NGF (Fig. 2c). These results suggest that samples from untreated PwMS exhibiting an immune profile closer to HC were more likely to experience an absence of clinical relapse or progression following DMT initiation than those from PwMS not showing such a 'good prognosis' immune profile.

Discussion

Identifying accessible (e.g., peripheral blood) disease-relevant immune markers associated with clinical course and DMT response in PwMS remains an unmet need. Several publications have produced conflicting results, with substantial heterogeneity between studies frequently observed. Our data showed that levels of multiple age-sensitive immune markers relevant to MS pathobiology are uniquely modified by sex, CMV serostatus, and treatment with specific DMTs in addition to MS per se. Moreover, these factors can alter the association of immune markers with age. These confounding factors have not been systematically considered in multiple immunological studies and could contribute, at least partially, to the inconsistent reproducibility of immune biomarkers in MS.

Our analysis establishes the importance of considering CMV serostatus when comparing levels of immune markers in human samples. This is particularly relevant in MS given the lower CMV-seroprevalence compared to HC, which we and others observed in PwMS.[35,36] CMV seroprevalence varies according to age, ethnicity, and country of residence.[12,37] Therefore, the lack of inclusion of CMV serostatus in analyses likely contributes to some of the discrepancies reported in studies of immune markers in MS worldwide. CMV infection or reactivation can cause transient immunosuppression, impair the development of specific B cell responses, and induce type I cytokine signature.[38] Also, CMV infection might display a protective role in MS through activating NKG2C$^+$ NK cells by a potent CMV peptide variant in susceptible individuals, enabling control (killing) of autoreactive immune cells.[39] Notably, CMV seropositivity in presymptomatic samples has been associated with a reduced risk of MS onset regardless of EBV and HHV6 status, two viruses also in the herpesviridae family.[40] Moreover, CMV seropositivity has been linked with a more benign clinical course in PwMS[41] and MS animal models.[42] Although our study was not designed to compare the prevalence of CMV seropositivity between groups, we found the lowest proportion of CMV$^+$ samples among PwMS treated with IFN and TF and, to a lower extent, DMF compared to untreated PwMS. These DMTs exhibit antiviral properties[43,44] and were associated with lower SARS-CoV-2 lethality.[45] Notably, IFNβ can inhibit CMV replication in vitro.[46] Taken together, these data support a complex relationship between age, MS, CMV status, and DMT treatment on the immune profile of individual PwMS, which we speculate could contribute to the heterogeneity of MS clinical course and DMT response.

The impact of sex is well documented in MS.[47] As previously described, the course of MS was more severe in men.[1,2,4] Of note, vascular comorbidities were more frequent in males.[48] Since these comorbidities are associated with accelerated disability progression, they could contribute to the elevated proportion of males exhibiting high EDSS scores and a progressive MS course.[20] Our study underlines significant sex differences in levels of age-sensitive immune markers. In particular, sex modified the association between age and levels of β-NGF, a neurotrophic factor with anti-inflammatory properties; lower levels were observed in females than males, more markedly at older ages. The CMV status also influenced the β-NGF serum levels. Trends for lower levels of β-NGF in serum from healthy female vs. male donors and older vs. younger donors were previously described.[49] Our multivariable analysis, adjusting for age, sex, and CMV status, showed that samples from untreated PwMS exhibited significantly lower levels of β-NGF than HC. A small study reported higher levels in the serum of PwMS, most of whom were currently on treatment (GA or IFN) and less than 40 years old.[50] We observed elevated β-NGF levels in FTY-treated patients compared to untreated patients. We can speculate that the FTY-mediated lymphopenia might have reduced lymphocyte-mediated consumption of peripheral β-NGF, which could explain higher levels in the peripheral blood. Others documented decreased blood levels of β-NGF in stable PwMS but increased levels post-relapse; notably, lower levels were associated with incomplete recovery.[51] Of note, our data show that baseline levels of β-NGF in PwMS who subsequently exhibited treatment failure were lower than levels measured in stable PwMS. Finally, treatment initiation was associated with a significant increase in β-NGF levels compared to baseline levels after adjustment for sex, age, and CMV serostatus. Further prospective studies will be necessary to validate β-NGF as a prognostic biomarker linked to DMT response. We can speculate that β-NGF levels could guide the selection of older or progressive PwMS who could benefit from DMT initiation, and younger PwMS who could consider initiating a low-efficacy DMT. Reduced β-NGF levels were reported in the peripheral blood of patients with dementia and Parkinson's disease, supporting a general role for β-NGF in CNS health.[52,53]

We found lower IL-4 levels in female donors than males, in line with previous reports,[54] and lower levels of IL-4 in both untreated and treated PwMS compared to HC. This finding is consistent with previous studies[55]

but contradicts others.[56] IL-4 displays pro-regenerative properties in MS animal models,[57] supporting the idea that this cytokine would be associated with neuroprotection or repair in PwMS. Moreover, type 2 cytokine signaling deficiency has recently been described in aging mice and humans, and was associated with accelerated senescence and impaired DNA repair in macrophages.[58] Other factors considered protective/pro-regenerative in MS, namely TNF and BDNF, were lowest in samples from treated PwMS, especially in older individuals. BDNF also significantly decreased with increasing disease duration in treated PwMS. The serum levels of IL-10, an anti-inflammatory cytokine, showed a significant negative correlation with age only in treated MS. Of note, lower levels of TNF and IL-10 at baseline (before treatment initiation) were also associated with subsequent treatment failure in PwMS. Among the DMT-treated samples, only IFN-treated ones showed higher levels of IL-10 when compared to untreated samples. However, these IFN-treated samples were also associated with low BDNF levels. DMTs could induce lymphocyte changes reminiscent of immunosenescence.[59] Although speculative, we propose that the failure of most DMTs to restore protective (e.g., BDNF) and anti-inflammatory cytokines (e.g., IL-4, IL-10) levels and to prevent their age-associated decline could contribute to their lack of beneficial impact on PIRA and on repair processes in aging PwMS.

IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties. Conflicting results have been reported for serum IL-6 levels in MS.[60,61] As previously described in other populations, in our MS cohort levels of IL-6 were significantly higher in untreated PwMS ≥45 years old with vascular comorbidities. In contrast, within a younger subgroup despite similar levels between PwMS and HC, we found significantly lower IL-6 levels at baseline in initially untreated PwMS who subsequently experienced treatment failure compared to PwMS with no sign of treatment failure. Notably, HC-associated clusters (clusters 1–3) exhibited relatively high levels of IL-6 in our unsupervised analysis. The role of IL-6 in MS remains controversial, with case reports of anti-IL-6 monoclonal antibodies associated with both MS onset/relapse and MS quiescence.[62] Our data suggest that higher levels of IL-6 at baseline in younger PwMS might be associated with a better response to DMT, and warrant caution concerning anti-IL-6 treatment in PwMS.

EGF and bFGF are trophic factors secreted by multiple peripheral and CNS sources, including immune cells.[63,64] Both factors can display anti-inflammatory, neuroprotective, remyelinating, and neuroregenerative properties in MS animal models.[64] Compared to controls, EGF levels were reduced in CSF and postmortem brain samples from PwMS[64,65]; such low CNS levels may contribute to the remyelination failure and reduced repair capacity observed in older PwMS.[6,64] In contrast, elevated bFGF expression has been reported in postmortem brain tissue and in CSF, especially during disease relapses, from PwMS.[66,67] In our study, EGF and bFGF serum levels were higher in both untreated and treated MS compared to HC at all ages. Other groups also reported elevated EGF and bFGF serum levels in RRMS compared to controls.[68] We observed a negative relation between EGF levels and age, especially in untreated PwMS. Others observed lower EGF levels in progressive MS compared to RRMS,[69] which could be partially mediated by the relation with age in line with our results. Peripheral blood mononuclear cells (PBMC) from untreated or IFN-treated MS patients produced greater amounts of EGF than counterparts from controls[70]; they could thus contribute to elevated peripheral levels of this trophic factor. We can speculate that increased peripheral levels of growth factors (EGF, bFGF) represent compensatory mechanisms to promote CNS repair that are not sustained over time and in older individuals. Moreover, we found that initiation of high-efficacy DMTs with different mechanisms of action (NTZ and OCV), but not low or moderate DMTs, was associated with a significant reduction in EGF and bFGF levels. While these observations warrant confirmation, this suggests that halting the formation of inflammatory lesions in the CNS rather than impacting the immune response underlies the 'normalization' of EGF levels in PwMS on high-efficacy DMTs. Notably, EGF can induce cellular senescence; activation of the cognate receptor, EGFR, can trigger the secretion of SASP-related factors.[71] Moreover, bFGF exhibits both anti- and pro-senescence activity.[72,73] Further investigations will be necessary to determine whether EGF and bFGF are double-edged swords promoting remyelination/repair while enhancing cellular senescence in the context of MS.

In contrast to age, sex, and CMV status, EDSS score at sampling had a very modest association with the levels of the measured age-sensitive immune markers when adjusting for the aforementioned confounding variables in untreated or treated MS in our cohort. The number of samples collected from PwMS with EDSS over 3.5 were limited, especially for female PwMS, and the EDSS scale itself is a non-linear ordinal scale in contrast to chronological age. We hence may have underestimated the impact of EDSS or mobility on the analyte levels. However, previous studies had similarly found a limited correlation between most cytokine levels and EDSS score in MS.[74] As the EDSS score is driven both by relapse-associated worsening and PIRA[1,2] and is impacted by comorbidities,[20] it reflects the lasting consequences of distinct pathobiological mechanisms at play at different time points before biosampling, which might contribute to the modest correlation with immune markers.

Our study has limitations. Samples were collected at one hospital in Canada, which might limit the

generalization of the findings, including in light of the different CMV seroprevalence across countries.[12] Information regarding the weight, height, and feeding status at the time of sampling was not collected. Therefore, while we observed lower leptin levels in younger but not older untreated PwMS versus HC, it is not possible to draw final conclusions due to the limited metabolic information. Information on vascular comorbidities was collected retrospectively from clinical charts but not assessed by systematic questionnaires or tests. As this information may not have been uniformly documented for PwMS and was unavailable for HC, we did not include comorbidities in multivariable analyses. In this study we identified, in unsupervised, univariable and multivariable analyses, age-sensitive immune markers associated with MS, with specific DMTs and with DMT response, but the possibility to use a combination of such markers as an additional tool to inform clinical management on an individual basis remains to be investigated in a prospective study. Our study specifically included numerous PwMS with disease onset decades ago. Although we assessed the relation between levels of analytes and disease duration in our study, its definition is tributary of the contemporaneous diagnostic criteria which have evolved over time. It is established that MS onset can start years before the first clinical episode[1]; this could have led to an underestimate of the disease duration, that could have resulted in an underestimate of its association with levels of immune markers. Furthermore, samples were collected in a 'real-life' clinical setting, including older PwMS not eligible for DMT initiation or switch, PwMS living far from the hospital, and COVID-related restrictions for follow-ups in 2020–2021. Annual MRIs were not systematically available for all PwMS. The definition of treatment failure was therefore stringent and relied on clinical assessment of relapse and/or significant EDSS progression compared to the previous visit as assessed by an MS specialist. Samples from PwMS showing suboptimal response clinically or radiologically but no overt clinical treatment failure were therefore not considered in the treatment response analysis; some were nevertheless most likely included in the 'no evidence of treatment failure group' when MRI was not performed annually, which could reduce the possibility to identify differences between groups. Finally, our study mainly included samples collected before approval and widespread use of anti-CD20 therapies in Canada. We therefore did not obtain samples from naïve RRMS starting such therapy as first line to assess baseline biomarkers associated with anti-CD20 response in that context, warranting future dedicated studies.

Disease activity might recur after stopping DMTs[75] but the risk to benefit ratio increases in older PwMS,[6] strongly supporting the need to improve our understanding of the contribution of aging to the lower efficacy and the higher risk of serious adverse events from high efficacy therapies. Using unsupervised and multivariable analysis, we found that MS per se and DMT treatment influence the profile of age-sensitive immune markers that could represent potential biomarkers that can guide the selection of DMT for PwMS. Importantly, our data suggest that consideration of CMV status in addition to age and sex is warranted in studies assessing immune markers in PwMS, and that pooling data from samples of untreated and treated PwMS or PwMS on different DMTs can potentially significantly blur or magnify differences in levels of immune markers observed between subgroups. Overall, our analysis paves the way to enhance the personalization of care by assessing immunological profiles in PwMS.

Contributors

H.D. performed unsupervised analysis and interpreted data, and wrote the manuscript.

R.B. conducted experiments and revised the manuscript.

A.D., S.D.C., W.K., M-L.C., C.M., and A.L. conducted experiments.

O.T. performed unsupervised analysis, interpreted data, and revised the manuscript.

J-M.G., P.D., and A.P. were involved in the collection of human samples and clinical characterization of patients.

P.D. and A.P. revised the manuscript.

G.M. provided important scientific and clinical input, and revised the manuscript.

J.Y., C.F., and M-C.R. performed the descriptive and regression analyses, interpreted the data, and revised the manuscript.

C.L. retrieved and reviewed relevant clinical data.

N.A. and C.L. designed the study, analyzed and interpreted the data, wrote the manuscript, and secured funding.

All authors had full access to all the data in the study and approved the final version of the manuscript.

Data sharing statement

The data that support the findings of this study are available from the corresponding authors, [N.A. and C.L.], upon reasonable request.

Declaration of interests

H.D. received a postdoctoral award from the National Multiple Sclerosis Society.

R.B. and M.L.C. received a doctoral award from MS Canada.

P.D. served on editorial boards and has been supported to attend meetings by EMD, Biogen, Novartis, Genzyme, and TEVA Neuroscience. He holds grants from the CIHR and the MS Society of Canada and has received funding for investigator-initiated trials from Biogen, Novartis, and Genzyme.

A.P. holds the Senior Canada Research Chair in Multiple Sclerosis and active patents.

WO2016095046A1, US20110014183A1, and US20100310568A1; has served sporadically on scientific advisory boards or as a speaker for Novartis, Biogen, Sanofi, Bristol Myers Squibb, Actelion, Roche, and EMD-Serono.

G.M. has received an Innovations in Well-Being Award for National MS Society/International Progressive Multiple Sclerosis Alliance (grant #PA-2304-41062) (2024–2025), has participated in Advisory boards for Novartis, Merck/EMD Serono and Genentech-Roche, and educational programs for John Hopkins e-Literature review, Neurology Live, MedEdge, BIogen and Novartis.

C.L. is an FRQS Clinicien–Chercheur Junior 2 Scholar; has served sporadically on scientific advisory boards or as a speaker for FIND Therapeutics, Amgen, Novartis, Biogen, Sanofi, Bristol Myers Squibb, Actelion, Roche, and EMD Inc. Mississauga an affiliate of Merck KGaA.

All other authors report no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105559.

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