Biological reflections in body fluids of multiple sclerosis progression and multiple sclerosis-related fatigue

Malekzadeh, A.

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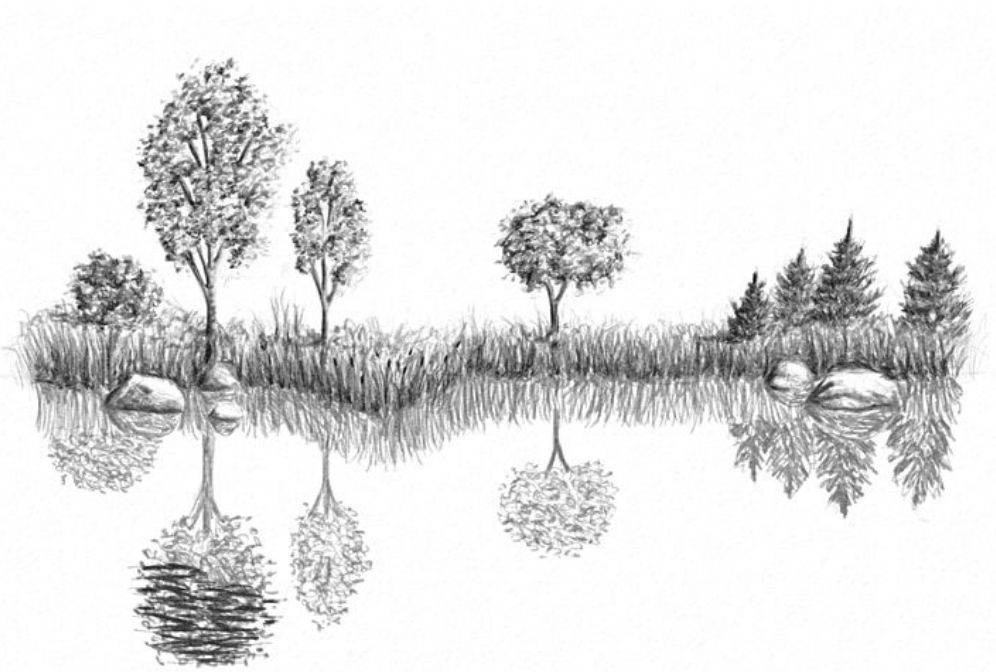
Part 1. Chapter 3.

Plasma proteome in multiple sclerosis disease progression



Arjan Malekzadeh, Cyra Leurs, Wessel van Wieringen, Martijn D Steenwijk, Menno M Schoonheim, Michael Amann, Yvonne Naegelin, Jens Kuhle, Joep Killestein, Charlotte E Teunissen

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Abstract

Background

The pathophysiology of multiple sclerosis disease progression remains undetermined. The aim of this study was to identify differences in plasma proteome during different stages of MS disease progression

Methods

We used a multiplex aptamer proteomics platform (Somalogic) for sensitive detection of 1129 proteins in plasma. MS patients were selected and categorized based on baseline and 4-year follow-up EDSS (delta EDSS) scores; relapse-onset (RO) slow progression (n=31), RO with rapid progression (n=29), primary progressive (n=30), and healthy controls (n=20). The relation of baseline plasma protein levels with delta EDSS and different MRI progression parameters were assessed using linear regression models.

Results

Regression analyses of plasma proteins with delta EDSS showed six significant associations. Strong associations were found for the proteins LGLAS8 ($p= 7.64 \times 10^{-5}$, q= 0.06), CCL3 (p= 0.0001, Q= 0.06) and RGMA (p= 0.0005, q= 0.09). In addition, associations of plasma proteins were found with percentage brain volume for C3 ($p= 2{,}08 \times 10^{-9}$, $q= 1{,}70 \times 10^{-6}$), FGF9 ($p= 3{,}42 \times 10^{-9}$, $q= 1{,}70 \times 10^{-6}$) and EHMT2 (p= 0.0007, q= 0.01) . Most of the significant markers were associated with cell-cell and cell-extracellular matrix adhesion, immune system communication, immune system activation and complement pathways.

Conclusions

Our results revealed 8 novel biomarkers related to clinical and radiological progression in MS. These results indicate that changes in immune system, complement pathway and ECM remodeling proteins contribute to MS progression and may therefore be further explored for use in prognosis of MS.

1. Introduction

Multiple sclerosis (MS) is a chronic degenerative disease of the central nervous system (CNS) that primarily affects young adults. MS is the leading neuropathological, non-traumatic, cause of disability in young adults [1]. The current hypothesis of MS etiology is that MS is an autoimmune disease caused by an interplay between genetic susceptibility of multiple immune system associated loci and environmental factors [2],[3]. Approximately 85% of MS patients experience a relapse-remitting (RRMS) disease course from onset [3]. RRMS is characterized by temporary episodes of neurological malfunctioning, with the presence of focal CNS inflammation and demyelination [3]. Remission occurs upon a relapse with full or partial recovery. The relapse and remission disease course varies between individuals and within 2 decades approximately 60% of the RRMS patients develop a secondary disease (SPMS) course [4]–[7]. SPMS is characterized by steady neurological decline and neurological atrophy, without apparent inflammatory lesions [3],[8]. Approximately 10-15% of MS patients suffer from primary progressive (PPMS) symptoms, characterized by accumulation of neurological deficits from onset without apparent supra-attacks [3],[8].

Despite advances in the MRI field and improvements in clinical assessments for MS diagnostics, the unpredictable nature of lesion formation and MS progression make prognosis and life-planning choices challenging for both the clinician and the patient. Moreover, even within the same MS subtype, different disease progression rates are observed [9]. Some RRMS patients show a more benign disease course, with little to no progression, while others accumulate disability rapidly and show an aggressive disease course [9]. Laboratory quantified blood-based biomarkers would be a relevant tool to aid the clinician in defining prognosis of MS and help with the selection of different therapy regimens. Moreover, biomarkers could help in informing the patient regarding clinical course and aid with life-planning choices [10].

Different biological molecules (e.g. DNA, RNA and proteins) and body fluids have been explored for MS biomarker discovery [10]. A vast amount of

knowledge has been gained at the genetic susceptibility level with genome-wide association studies regarding the risk of MS, rather than the disease onset and progression rate. Functional units within and outside the cells, such as proteins and RNAs, are more likely to be involved in disease mechanisms and can reflect the ongoing pathology as a biomarker. Therefore, the application of proteomics for biomarker research, could give insight into ongoing disease mechanisms and offer prognostic biomarkers. Until now, most of the focus of MS protein biomarker discovery was on cerebrospinal fluid (CSF) proteomics, often using liquid chromatography-mass spectrometry (LC-MS) [10]. Using LC-MS for biomarker discovery for blood biomarker research has proven to be challenging due to pre-analytical and analytical factors, including the interference of abundant proteins e.g. albumin, low sensitivity and low dynamic range within blood [11]. A novel proteomics technology is the SOMAscan, which is based on aptamers that are able to bind conformational protein epitopes with high specificity and sensitivity [12]. This array is able to detect >1000 different proteins with a wide dynamic range (>8 logs of concentration difference) in blood. Since MS is considered to be an auto-immune pathology with a strong involvement of the peripheral immune-system, we hypothesized that assessment of blood proteomics for MS would aid in gaining insight into the proteins and pathways involved in MS progression.

By applying the aptamer proteomics approach in plasma we aimed to identify; 1) disease progression biomarkers that discriminate the different stages of relapse-onset MS, 2) biological pathways that offer insight into disease progression rates of relapse-onset (RO) and primary progressive MS subtype, 3) biomarkers in plasma associated with RRMS and PPMS in comparison with healthy controls

2. Materials and Methods

2.1. Study design and participants

MS patients and healthy controls were included (n= 100) from the GeneMSA multicentre cohort (Amsterdam University Medical Centers (AUMC) and University Hospital Basel) [13]. From the AUMC Presto cohort PPMS (n= 10) patients were included [14],[15] (Table 1). Patients were selected and categorized based on differences in baseline and 4 years follow-up Kurtzke expanded disability status scale (EDSS) scores [16]. Baseline EDSS was defined as the EDSS measured at first blood collection [13]–[15]. Participants who received glucocorticosteroids within one month prior to study entry were excluded. Disease modifying therapies (DMT) for MS were permitted (Table 1) [13]. Three different MS groups were defined: 1) relapse-onset (RO) slow progressors (n= 31, with maximum increase of < 1 point on EDSS in 4 years), RO- rapid progressors (n= 29, with a minimum increase of 1 point EDSS) and primary progressive (PP) (n=30). Finally, age and gender matched healthy controls (n=20) were included (Table 1). Healthy controls consisted of participants with North-Europe ancestry without any familial history or current MS diagnosis. Study protocols of each cohorts were approved by the local institutional ethics committee and participants signed informed consent [13].

2.2. Plasma Samples

EDTA plasma was collected in BD Vacutainer EDTA tubes (BD, New Jersey, USA). Plasma tubes were processed within 2 hours upon collection and were centrifuged at 1800x g for 10 minutes at room temperature. EDTA plasma supernatant was aliquoted in polypropylene tubes (Sarstedt, Germany) and stored at −80°C.

2.3. MRI imaging

The MRI imaging parameters T1-hypo-intense lesion volume, T2-hyper-intense lesion volume (T2-lesion volume) and percentage brain volume change (PBVC) were used as outcome parameters based on the similarities in imaging protocols between centers according to the GeneMSA protocol as previously published [13],[17]. Briefly, participants underwent MRI scanning

upon entry, using 1.5 Tesla MR scanners, using common MR sequences and protocols [13]. T1-hypo-intense lesions and T2-lesions were manually outlined. Follow-up T1-hypo-intense lesion volume and T2-lesion volume after one and two years were calculated in millilitres (ml). We obtained follow-up data of 74 MS patients for both T1-hypo-intense lesion volume and T2-lesion volume. T1-hypo-intense lesions and T2-lesions were centrally measured using the AMIRA software [13]. Delta T1-hypo-intense lesion volume and delta T2-lesion volume were used for statistical analyses by subtraction of T1-hypo-intense lesion and T2-lesion follow-up values by T1-hypo-intense lesion and T2-lesion baseline values [17]. Volumetric analyses for all patients were performed in Amsterdam [17]. We had access to PBVC data of 62 MS patients with 2 years' follow-up from the GeneMSA cohort. Brain volume changes were determined between these two time-points using SIENA [13],[18]. Lastly, because of differences in MRI imaging protocols between the GeneMSA and presto cohorts, we excluded the 10 PPMS patients derived from the presto cohort from MRI parameters regression analyses.

2.4. Somalogic aptamer assay

Proteins were measured using the aptamer based proteomics Somascan v3 measuring 1129 different proteins (Somascan, Somalogic Inc, Boulder, Co) [12]. Aptamers are single stranded DNA molecules that are modified to mimic protein side chains, which allows selective binding to protein targets of interest. Moreover, theses DNA aptamers are able to pair with DNA probes, allowing quantification using a DNA microarray. Therefore, the measurement output is in relative fluorescence units (RFU). The reported intra and inter-run coefficient of variation (CV%) in blood are <5% [12]. Since we analysed the samples in 3 different batches, we included internal controls to control for potential batch variation. In addition, two levels of quality controls were performed; 1) to avoid relative signal variation between the samples at the microarray level, a set of aptamers with the lowest overall signal variation was used for normalization within plates, 2) the median RFU for a set of aptamer used for normalization is calculated from all the samples and a scaling factor for each individual sample is calculated to control for the technical variability.

2.5. Gene enrichment analyses

Explorative enrichment of molecular and biological processes was performed with String database (version 10.5). We included plasma proteins that significantly (p<0.05) associated with delta EDSS (for delta EDSS also q <0.15), delta T1-hypo-intense lesion volume, delta T2-lesion volume and PBVC. The background of enrichment analyses was set on whole human genome.

2.6. Data and statistical analyses

Baseline group comparisons with regards to different MS subtypes and disease-related scales were analysed using Krusikal Wallis. Correlation between the different MRI parameters were assessed using Spearman's rho.

Analysis of 1129 different proteins was performed and the relation of potential biomarkers with clinical progression were determined by regression analysis with; baseline EDSS subtracted from follow-up EDSS (delta EDSS), delta T1-hypo-intense lesion volume, delta T2-lesion volume and 2 years PBVC. Statistics were performed on the R statistical computation platform using Limma-package [19],[20]. The effect of biomarker expression levels (natural log-transformed) on the independent variables was assessed using the linear regression model, taking possible confounders (batch variation, DMT and age) into account. Significance of the estimated effect was determined using a moderated t-test (implemented in Limma), employing empirical Bayes estimation of the variance. The multiplicity problem (multiple proteins testing) was addressed by application of the Benjamini-Hochberg procedure to the raw p-values to control the FDR (q value, False Discovery Rate).

Graphical representation of data was performed with SPSS 23.0, by depicting unstandardized predicted values on the y-axis with the different progression groups as fixed factors and DMT, different batch runs and age as covariates. A significant threshold of p<0.001 and or q<0.2 was set. Finally, to assess the overlap of the different proteins that were significantly associated with all outcome parameters, delta EDSS, delta T1-hypo-intense lesion, delta T2-lesion and PBVC, we set an explorative threshold (p<0.05 and q <0.1) and an online Venn diagram tool was used (Venny 2.1) [21])

3. Results

3.1. Cohort characteristics

A total of 90 MS patients and 20 healthy controls were included in this study (Table 1). A significant higher baseline EDSS (Kruskal-Wallis $\chi^2(2)= 33.8$, $p< 0.0001$) and age (Kruskal-Wallis $\chi^2(2)= 17.8$, $p< 0.0001$) was observed for the primary progressive (PP) group. No significant differences between the groups were observed for disease duration and gender (Table 1). At baseline, significant higher T2-lesion volume (Kruskal-Wallis $\chi^2(2)= 7.19$, $p= 0.027$) was observed for the RO-rapid group compared to RO-slow and PP groups (Table 1).

In the total MS group, we found a significant correlation between baseline T1-hypo-intense lesion volume and T2-lesion volume, (Spearman's rho r= 0.86, p< 0.0001). In addition, a significant correlation between delta T1-hypo-intense lesion volume and delta T2-lesion volume was observed (Spearman's rho r= 0.37, $p= 0.001$). Also, a significant association between delta EDSS and delta T1-hypo-intense lesion volume was observed (Spearman's rho r= 0.24, $p= 0.039$). Percentage brain volume change (PBVC) over 2 years showed significant correlations with delta EDSS (Spearman's rho r= -0.39, $p= 0.002$), baseline T1-hypo-intense lesion volume (Spearman's rho r= -0.36, $p= 0.004$) and baseline T2-lesion volume (Spearman's rho r= -0.36, $p= 0.004$).

Table 1. Cohort demographics

	RO-slow (n=31)	RO-rapid (n=29)	PP (n=30)	HC (n=20)
Age mean (SD)	38.8 (9.6)	41.0 (8.15)	48.7 (8.15)	45.3 (5.2)
Sexe (f/m)	F= 17 M= 14	F= 17 M= 12	F= 12 M= 18	F= 10 M= 10
Disease duration (SD)	6.5. (4.4)	9.5 (6.5)	7.0 (5.2)	
EDSS baseline mean (SD)	2.3 (1.1)	2.4 (1.4)	4.0 (0.9)	
EDSS 4-year follow-up mean (SD)	1.3 (1.4)	4.6 (1.5)	5.1 (1.35)	
Δ EDSS mean (SD)	-1.0 (0.9)	2.2 (1.0)	1.3 (1.0)	
T1 hypo-intense lesion volume baseline, mean (SD) *	913.8 (1533.6)	2690.7 (5054.9)	810.8 (1473.7)	
T1 hypo-intense lesion volume 2-year follow-up, mean (Sd) *	890.4 (1546.4)	3041.0 (5094.1)	935.8 (1637.0)	
T2 hyper-intense lesion volume baseline, mean (SD)*	3234.3 (5598.6)	5939.1 (6460.9)	4212.3 (7111.0)	
T2 hyper-intense lesion volume 2-year follow-up, mean (SD)*	3531.4 (6625.3)	6958.3 (6995.7)	4428.3 (7258.8)	
percentage brain volume change 2 years, mean (SD)*	-0.016 (0.038)	-0.049 (0.045)	-0.017 (0.064)	
Disease modifying therapy**	n= 8	n= 12	n= 1	

*The averages of MRI parameters for the primary progressive shown are derived from PP patients of the GeneMSA cohort (n=10).** DMT used upon inclusion consisted of; Beta-Interferon users (n= 19), 1 person from the RO-rapid group was on azathioprine, and PP DMT user was on Methotrexate.

3.2. plasma proteome in relation to EDSS progression

Moderated regression analysis was performed to discover the association of baseline plasma proteins with different EDSS progression rates during follow-up. Six markers were significantly associated with delta EDSS ($p< 0.001$ and $q< 0.2$, Table 2); LGALS8 (Lectin, Galactoside-Binding, Soluble 8), CDH1 (E-Cadherin), CCL3 (Macrophage Inflammatory Protein 1-Alpha), CHEK1 (Checkpoint Kinase 1), TNFRSF13B (TNF Receptor Superfamily Member 13B), and RGMA (Repulsive Guidance Molecule Family Member A) (Figure 1). Plasma levels of LGALS8, CCL3, CDH1 and RGMA were negatively associated with change in EDSS. In contrast, TNFRSF13B and CHEK1 plasma levels were positively associated with change in EDSS (Figure 1).

Lastly, to assess the potential different subtype disease mechanisms, we performed moderated regression analysis on baseline proteins and delta EDSS for each specific MS group. For both RO-slow and rapid group CCL3 and SIGLEC9 were statistically significant, however did not survive the multiple testing cut-off ($q>0.2$). Interestingly, for the PP group; SIGLEC9 ($\beta= -0.13$, $p= 0.001$, $q= 0.12$), PLA2G2A (Phospholipase A2 Group IIA) ($\beta= -0,20$, $p= 0.0009$, $q= 0.12$) and RGMA ($\beta= -0,18$, $p= 0.002$, $q= 0.12$) were significant.

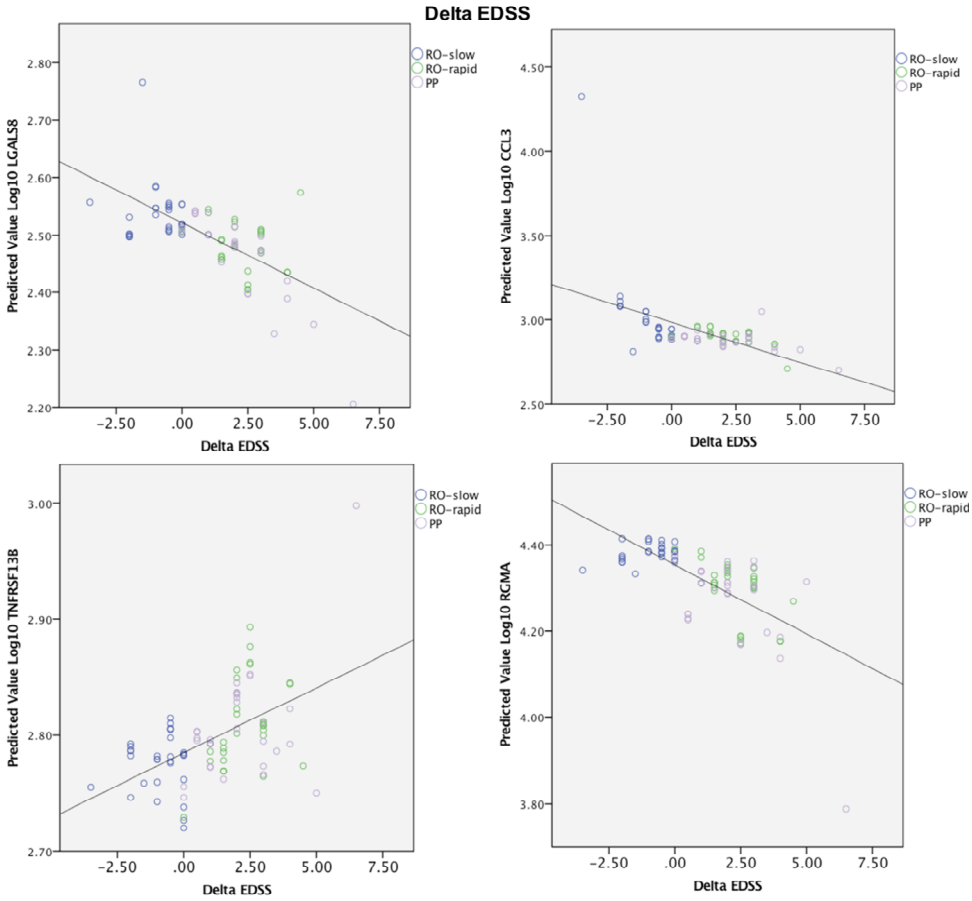

Figure 1. Scatter plots of several significant markers associated with delta EDSS. On the y-axis corrected Log10 levels of significant markers are shown, and on the x-axis delta EDSS

3.3. Plasma proteome and MRI parameters

Next, moderated regression analyses were performed to determine the association of baseline plasma proteome in relation to several MRI progression parameters during follow-up, including delta (the difference of baseline and 2 years' follow-up values) T1-hypo-intense lesion volume, delta T2-lesion volume and percentage brain volume changes (PBVC, 2 years' follow-up) (Table 2).

Both ACE2 (Angiotensin I Converting Enzyme 2) and PIK3CA/PIK3R1 (Phosphoinositide-3-Kinase, Catalytic, Alpha complex) were significantly positively associated with increasing T2-lesion volume (Figure 3 and Table 3).

For delta T1-hypo-intense lesion volume no proteins survived the multiple testing ($q<0.2$), however PIK3CA/PIK3R1 also showed a positive association with increasing T1-hypo-intense lesion volume at an explorative level ($p<0.001$) (Figure 2 and Table 2).

Eight plasma proteins were significantly associated with PBVC; C3 (Complement C3 fragment a and d), FGF9 (Fibroblast Growth Factor 9), MATK (Megakaryocyte-Associated Tyrosine Kinase), LTBR (Lymphotoxin Beta Receptor), ESR1 (Estrogen Receptor 1), CTSE (Cathepsin E) and EHMT2 (Euchromatic Histone Lysine Methyltransferase 2). Plasma levels of C3 (both fragment a and d), ESR1, MATK and CTSE were positively associated with decreasing annualized PBVC scores (2 years), whereas decreasing annualized PBVC scores were negatively associated with LTBR, FGF9 and EHMT2 plasma levels (Figure 3).

Lastly, to determine whether MRI parameters were differentially associated with plasma proteome in different MS groups, we performed moderated regression analysis for the different subtypes. Interestingly, specific significant associations were observed for the RO-slow group for delta T2-lesion volume with; HBEGF (Heparin-binding EGF like growth factor) ((β= 0,0005, p= 9.5 x 10^{-8} , q= 0.009) and IL3RA (Interleuking 3 receptor alpha) β= 0,0006, p= 3.1 x 10^{-8} , q= 0.015). Both RO-rapid and PPMS groups showed significant associations for both delta T1-hypo-intense volume and delta T2-lesion volume with PIK3CA/PIK3R, however it did not survive the multiple testing cut-off.

Table 2. Overview of significant markers for regression analyses on delta EDSS and different MRI parameters

peptide	Uniprot	Beta	*p* value	q value
Delta EDSS				
LGALS8	O00214	-0.05	7.64×10^{-5}	0.06
CCL3	P10147	-0.12	0.0001	0.06
CHEK1	O14757	0.040	0.0002	0.06
TNFRSF13B	O14836	0.031	0.0003	0.07
CDH1	P12830	-0.04	0.0004	0.09
RGMA	Q96B86	-0.07	0.0005	0.09
Delta T1 Hypo-intense lesion volume				
PIK3CA/PIK3R1 complex	P42336/P27986	0.00016	0.0009	0.88
Delta T2 Hyper-intense lesion volume				
ACE2	Q9BYF1	9.76×10^{-5}	0.0001	0.16
PIK3CA/PIK3R1 complex	P42336/P27986	8.17×10^{-5}	0.0003	0.16
PBVC 2 years				
C3 (C3 fragment d)	P01024	-2.65	2.08×10^{-9}	1.70×10^{-6}
FGF9	P31371	1.22	3.42×10^{-9}	1.70×10^{-6}
C3 (C3a anaphylatoxin)	P01024	-1.59	$2{,}20 \times 10^{-7}$	7.27×10^{-5}
MATK	P42679	2.46	3.85×10^{-5}	0.009
LTBR	P36941	2.00	$6{,}06 \times 10^{-5}$	0.01
ESR1	P03372	1.88	6.48×10^{-5}	0.01
CTSE	P14091	3.20	0.0001	0.01
EHMT2	Q96KQ7	0.86	0.0007	0.01

Table 3. Overview of significant markers for different group comparisons

peptide	Beta	Uniprot	*p* value	q value
RO-rapid x RO-slow				
SIGLEC9	-1.78	Q9Y336	0.00057	0.56
PP x RO-all				
CDH1	-0.19	P12830	0.00027	0.16
KLK4	0.43	Q9Y5K2	0.00033	0.16
FN1 (Fragment 4)	-0.20	P02751	0.00089	0.19
C3 (inactivated C3b)	0.65	P01024	0.00094	0.19
FCN1	0.21	O00602	0.00099	0.19
HC x RO-all				
MMP3	1.00	P08254	7.98×10^{-6}	0.007
FN1	-0.27	P02751	0.00042	0.18
FN1(Fragment 3)	-0.25	P02751	0.00055	0.18
HC x PP				
PLA2G2A	0.52	P14555	0.000125	0.11
CDH1	0.21	P12830	0.00021	0.11
LGALS8	0.25	O00214	0.00047	0.15
HC x MS-all				
MMP3	0.83	P08254	6.77×10^{-5}	0.067

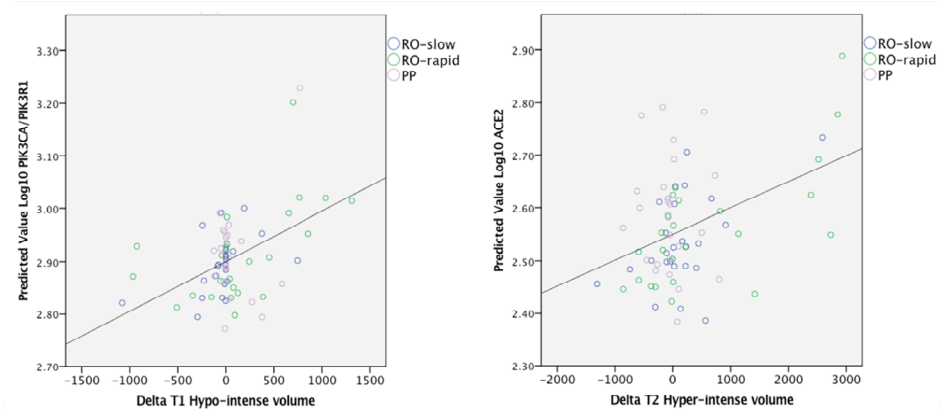

Figure 2. Scatter plots of PIK3CA complex associated with delta T1 hypo-intense and delta T2 hyper-intense volume. On the y-axis corrected Log10 levels of significant markers are shown, and on the x-axis delta delta T1 hypo-intense volume and delta T2 hyper-intense volume.

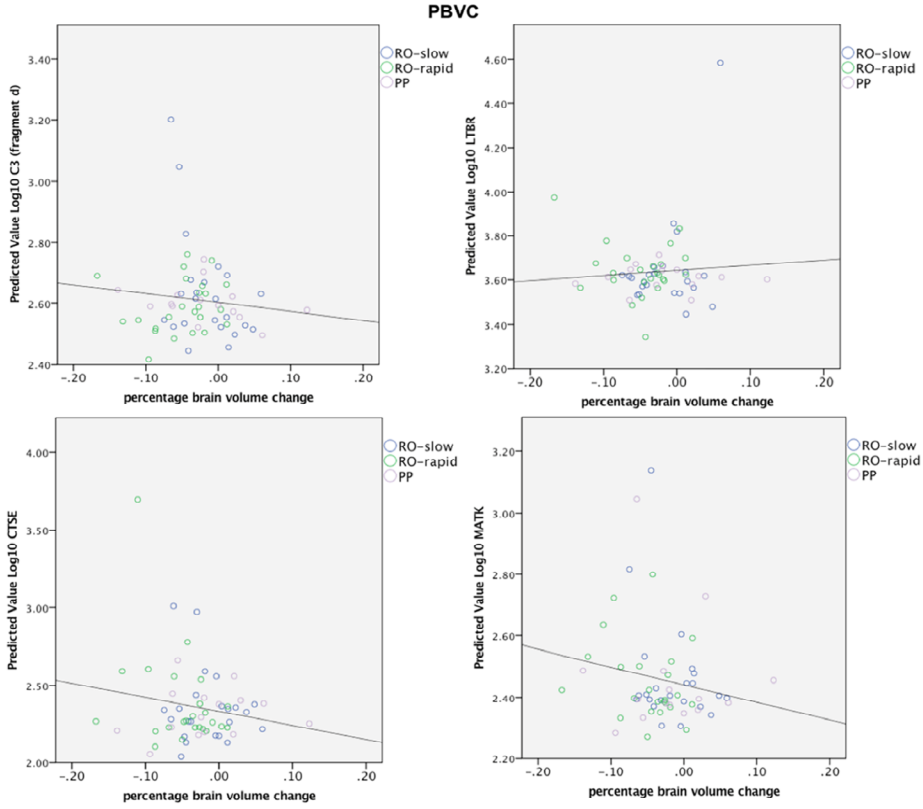

Figure 3. Scatter plots of several significant markers associated with percentage brain volume change (PBVC). On the y-axis corrected Log10 levels of significant markers are shown, and on the x-axis PBVC values for 2 years.

3.4. Overlap analyses of plasma proteins with EDSS and MRI parameters

We exploratively determined the overlap in plasma proteins that were associated with delta EDSS, delta T1-hypo-intense lesion volume, delta T2-lesion volume and PBVC (Figure 4), applying a more stringent threshold of significance ($p<0.05$ and $q<0.1$). A total of 64 proteins were associated with delta EDSS, 34 proteins with delta T1-hypo-intense lesion volume, 46 with delta T2-lesion volume and 28 proteins with PBVC (Figure 4 and Table 4). CHEK1 was related to both delta EDSS and T1-hypo-intense lesion volume and T2-lesion volume. CXCL13 was related to both delta EDSS and T1-hypo-intense lesion volume. MPO (Myeloperoxidase) and CST5 (Cystatin D) were related to both delta EDSS and PBVC. For the relation with delta T1-hypo-intense lesion volume and delta T2-lesion volume, ten proteins were found to be overlapping, including the three earlier mentioned; ACE2, PIK3CA/PIK3R1 complex, and SIGLEC9 (Table 4). INS (insulin) and CTSE were related to both delta T1-hypo-intense lesion volume and PBVC. No proteins were related to delta EDSS and delta T2-lesion volume. Lastly, no markers were overlapping between delta T2-lesion volume and PBVC

Interestingly, similar enrichments patterns were observed for pathway evaluations for all the clinical and MRI parameters, with most of the markers being enriched for pathways; involved with cell to cell and extracellular matrix (ECM) adherence, immune system communication and immune system activation. Further analyses of the immune associated markers showed that a majority of the immune markers were involved with T and B cell homeostasis.

Table 4. Overview of overlapping significant markers for delta EDSS and different MRI parameters

Protein Name	Δ EDSS	Δ T1-HL	Δ T2-HL	PBVC
CHEK1	p= 0.00017	p= 0.0055	p= 0.022	
CXCL13	p= 0.0031	p= 0.013		
PIK3CA/PIK3R1		p= 0.00089	p= 0.00032	
PLXNC1		p= 0.0029	p= 0.032	
SIGLEC9		p= 0.0095	p= 0.045	
EDA2R		p= 0.012	p= 0.046	
IL20		p= 0.015	p= 0.0039	
ACE2		p= 0.015	p= 0.00019	
CSNK2A1		p= 0.018	p= 0.017	
PTN		p= 0.021	p= 0.046	
CAT		p= 0.047	p= 0.043	
MPO	p= 0.0011			p= 0.023
CST5	p= 0.0012			p= 0.0039
INS	p= 0.0089			p= 0.029
CTSE	p= 0.048			p= 0.00012

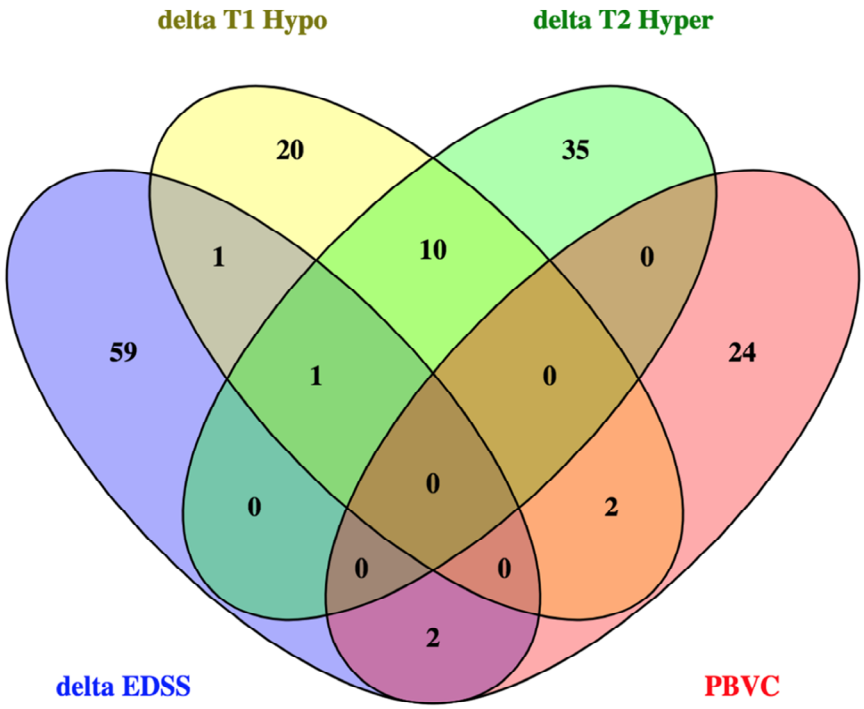

Figure 4. Venn-diagram of overlapping significant plasma proteins ($p < 0.05$) associated with delta EDSS, delta T1 hypo-intense volume, delta T2 hyper-intense volume and percentage brain volume change (PBVC)

3.6. Plasma proteome of different MS groups and healthy controls

There was no significant difference in expression of almost all proteins between RO-rapid and RO-slow patients. Siglec9 (Sialic Acid Binding Ig Like Lectin 9) was significantly downregulated in the RO-rapid group in comparison with the RO-slow group ($p<0.001$, Figure 5 and Table 3), however, the effect did not survive the multiple testing threshold ($q<0.2$). Interestingly, Siglec9 plasma levels for RO-slow were similar to those in HC (Figure 5).

Five markers were differentially expressed between PPMS and pooled RO-patients; CDH1 (E-Cadherin), KLK4 (Kallikrein Related Peptidase 4), FN1 (Fibronectin), FN1 fragment 4, C3 (Complement C3, inactivated C3b) and FCN1 (Ficolin 1). All these markers met both multiple testing significance criteria ($p<0.001$, $q<0.2$). C3, FCN1 and KLK4 were significantly upregulated in PPMS compared to the RO group (Figure 5). Additionally, FN1 was specifically downregulated in the PPMS compared to RO group, whereas CDH1 was downregulated in both the RO-rapid and PP group. Lastly, comparison of all MS patients with HC showed a highly significant decrease of MMP3 ($p= 6.77 \times 10^{-5}$, $q= 0.067$) in MS subtypes compared to HC (Figure 5 & Table 3). Comparing the plasma proteome between RO-all and HC, three markers were differentially expressed; MMP3 (Matrix Metallopeptidase 3), FN1 (Fibronectin 1) and FN1 (fragment 3). Both FN1 fragments were upregulated in the RO group in comparison with HC group. Comparison between PPMS and HC showed three different significant markers; PLA2G2A, CDH1 (E-Cadherin) and LGALS8 (Lectin, Galactoside-Binding, Soluble 8). All the three markers were significantly downregulated in PPMS patients compared to HC (Figure 5).

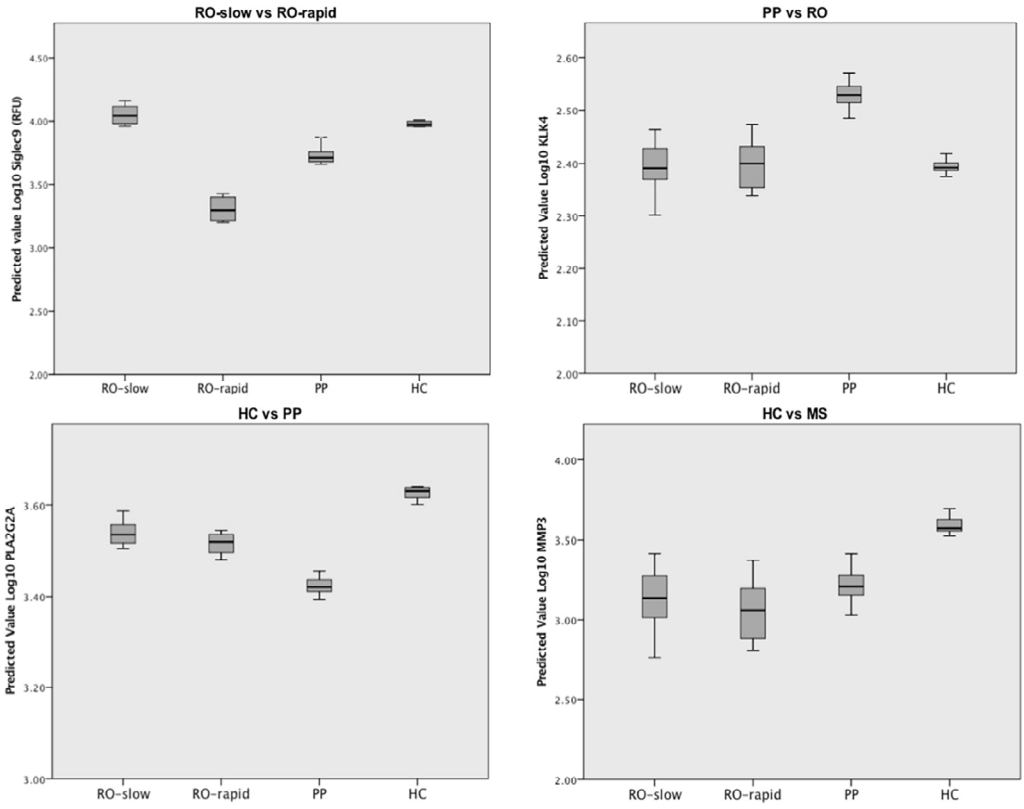

Figure 5. Graphical representation of several significant markers for different group comparisons. On the y-axis corrected Log10 levels of significant markers are shown. The dark lines in the boxplot represent the median, and the whiskers represent the 95% confidence interval.

4. Discussion and conclusion

In this study, we explored the relation of 1129 plasma proteins with multiple sclerosis (MS) disease progression, and for this performed group comparison on three different MS groups. MS patients were grouped in relapse-onset (RO)-slow progression, RO-rapid progression, primary progressive (PP) and finally healthy controls were included. Several interesting plasma proteins were significantly associated with EDSS progression; CDH1, CHEK1, LGALS8, TNFRSF13B, RGMA, and CCL3. Annualized percentage brain volume changes strongly associated with C3, FGF9 and EHMT2. Assessment of markers related with EDSS progression and change in different MRI parameters showed no overlapping markers for all of the parameters. Lastly, explorative analyses on markers associated with EDSS progression and MRI imaging parameters, showed primarily enrichments in biological pathways associated with cell adhesion, immune system communication and immune activation pathways.

Previous MS biomarker studies performed mostly LC-MS blood proteomics [22],[23]. Moreover, few studies have focused on the different progression courses within MS, making comparison with earlier publications challenging [22],[23]. Nonetheless, some of our results coincide with earlier findings from candidate approach and pathology based studies, as we will discuss below.

Decreasing repulsive guidance molecule A (RGMA) levels in blood were significantly associated with EDSS progression. RGMA is involved in axon guidance and neurogenesis and has been shown to be expressed in chronic and active MS lesions [24]. Interestingly, decreased RGMA CSF levels were associated with functional improvements in MS patients [24], showing the opposite direction from our findings. Possibly, the compartmentalization differences of the body fluids could be an explanation for the observed difference. RGMA could be an interesting progression marker, however differences in RGMA expression in CSF and blood in MS should be further explored in future studies.
We also identified several interesting immune system markers, i.e. CCL3, TNFRSF13B and LGALS8, to be significantly associated with EDSS progression. For CCL3 and LGALS8, lower blood levels were related with

EDSS progression. In a recent study, LGALS8 was shown to have immunosuppressive protective role in CNS inflammation, by balancing the Th17 and Th1 cells [25]. Moreover, LGALS8 was shown to be present in CSF of MS patients, and RRMS patients with antibodies against LGALS8 showed EDSS progression and worse disease prognosis [25]. Interestingly, potential imbalance in peripheral Th17 and Th1 cells has also been shown to be associated with reduced gene expression of several different cytokines and chemokines in MS patients, among which CCL3 [26]. It is possible that the observed decrease in both LGALS8 and CCL3 reflect imbalance in T helper cells, which promote a pro-inflammatory self-antigen reaction and MS progression. Lastly, TNFRSF13B is a transmembrane receptor involved in B cell homeostasis [27]. Increased TNFRSF13B CSF levels have been observed in MS patients compared to other neurological controls, whereas no differences in plasma TNFRSF13B levels were observed between MS and controls [27]. Others report IFN-β therapy is able to reduce pathogenic subset of memory B cells, thereby reducing the MS associated humoral response [28]. TNFRSF13B is an interesting marker because of persistent presence of B cell producing antibodies (e.g. IgG and IgM) in early and later stages of MS [29]. It is possible that the increase in soluble TNFRSF13B in MS progression reflects attempts of restoring B cell homeostasis. These three markers in conclusion might therefore reflect in imbalance in T and B cell regulatory process and reflect ongoing MS pathophysiological mechanisms in plasma.

The most prominent overlapping marker that was associated with both delta T1-hypo-intense lesion volume and delta T2-lesion volume, was the PIK3CA/PIK3R1 complex. The PIK family of receptors are involved in the activation of the B and T-cell receptors and regulation of self-antigen recognition [30]. Single nucleotide polymorphisms (SNP) in *PIK3R1* gene are associated with MS susceptibility *HLA* genes and are likely to contribute to MS susceptibility [31]. Increased PIK3R1 complex plasma levels associated with increasing delta T1-hypo-intense lesion volume and delta T2-lesion volume, could indicate a continuous imbalance of immune cell activation and faulty self-recognition mechanisms, contributing to MS disease progression.

In addition, we observed a strong association of C3 and EHMT2 with percentage brain volume change (PBVC), with higher C3 and lower EHMT2 plasma levels related to increasing brain atrophy. Interestingly, the group

comparisons between RO and PP-MS subtypes showed upregulation of plasma proteins in the RO group that are associated with complement pathway; C3, Ficolin 1, and fibronectin, which is an extracellular matrix (ECM) protein. Complement activation is a well-known feature in grey matter lesions, these lesions were surrounded by complement receptor-positive microglia, and known to contribute to irreversible MS progression [32]. Additionally, the presence of C3 together with other complement factors, and Fibronectin aggregates at lesions have been shown to prevent remyelination and shown to be differentially regulated in serum of pre-symptomatic and symptomatic MS patients [22]. We observed a significant increase of C3 plasma levels related to increasing brain atrophy and increased FN1 plasma levels in RO-all compared to healthy controls. In contradiction with our results, lower expression of C3 and FN1 were observed in serum of pre-symptomatic and symptomatic MS patients compared to healthy controls [22]. The average disease duration of the pre-symptomatic and symptomatic MS cohort was less than 2 years, whereas the average disease duration of our cohort was 6 years and higher [22]. It is possible that, with longer disease duration and ongoing brain atrophy, C3 and FN1 plasma levels rise in MS patients.

Next, with increased brain atrophy we observed lower EHMT2 plasma levels. EHMT2 is a methyltransferase and involved with demethylation of histone H3 at lysine 9. This results in recruitment of other transcription regulators and results in repression of transcription [33],[34]. *In vitro* studies show that EHMT2 promotes neuronal and immature oligodendrocyte differentiation and is required for oligodendrocyte maturation [35]. It is likely that with ongoing MS progression and brain atrophy, C3, FN1 and EHMT2 reflect the pro-inflammatory environment, lack of neuro-glial maturation and remyelination mechanisms and indicative of MS progression.

Lastly, we observed increasing HBEGF plasma levels were significantly associated with increasing T2-lesion volume for the RO-slow group. Astrocytes surrounding MS lesions have an increased expression of HBEGF, which potentially allow trans-endothelial monocyte migration[36]. This could be an indication that plasma HBEGF levels reflect ongoing monocyte migration into the lesions which leads to increased T2-lesion volume. It is possible that different mechanisms of chemo-traction and immune activation might be altered between RO-rapid and slow groups, resulting in different disease progression rates.

Surprisingly, no markers survived the multiple testing for MS subtype analyses of PBVC with plasma proteome. This is most likely due to a lack of power upon group segregations and having less PBVC data points compared to the other MRI parameters.

Finally, lower MMP3 (Matrix Metallopeptidase 3) plasma levels were observed for all MS subtypes in comparison with HC, especially for the RO group compared to HC. MMP3 and other MMP family members have previously been described to be involved in MS [37],[38]. Our results seemingly contrast to earlier reported increases during relapses in MS [38]. Active MMP3 and other MMP family members contribute to BBB breakdown and aid to leukocyte infiltration at focal in chronic and active lesions [39]. Interestingly, MMP3 is able to degrade Fibronectin (higher in RO-MS compared to PP-MS and HC in our study) and E-Cadherin (CDH1 lower in RO-MS compared to PP-MS in our study), which are components of the BBB [40],[41]. The overall upregulation of MMP3 in MS sera and lesions in the previous studies does not necessarily contradict our results [38],[39], since earlier studies did not include healthy controls, it can therefore not be excluded that the overall MMP3 expression in blood of MS patients is lower compared to healthy controls. The overall reduced MMP3 plasma levels in MS patients in comparison to HC could potentially be a physiological attempt to restore balance and restore the BBB and inhibit progression mechanisms in MS patients.

Strengths of the study are the design, including MS patients with different disease courses and progression rates in a relatively large sample size, and a novel proteomics approach that is optimized for plasma analysis. Furthermore, in this study we specifically choose to perform blood proteomics, while most of proteomics in MS have focused on more tissue specific cerebrospinal fluid (CSF) [10]. CSF being adjacent to the brain tissue could give more insight into lesion microenvironment, however CSF sampling is relatively uncomfortable [42]. While blood lacks brain tissue specificity, it does provide a broad overview of different tissues and cell types. This is of importance for MS because of its probable, peripheral auto-immune onset and progression mechanisms. Moreover, we have had access to different lesion and brain volume MRI baseline and follow-up data, which showed significant correlations with different blood markers.

However, this study also has limitations, such as the relative limited selection of markers present on the SOMAscan array, although these markers cover a broad range of pathways and include brain specific proteins, potentially allowing discovery of different pathways involved with MS progression. Another weakness of this study was the high amount of measured proteins relative to the sample size. Therefore, to exclude potential false-positives we performed multiple testing corrections. However, we chose for a relative mild false discovery cut-off, this to minimize losing true-positive markers, while attempting to exclude false-positive markers. Lastly, we did not exclude MS patients who were on disease-modifying therapy (DMT) at baseline. Including participants with a rapid disease progression, that are not on DMT upon inclusion is challenging. Therefore, we performed statistical corrections for DMT usage.

Taken together, to our knowledge, this is the first explorative study that has assessed large numbers of plasma proteins in a substantial amount of MS patients with different rates of disease progression, as defined by both clinical and MRI measures. The differentially regulated biomarkers in relation to MS prognosis were primarily associated with molecular pathways involved with cell-cell adherence, cell-extracellular matrix, adherence, immune system communication and immune system activation. Validation of these markers in an independent cohort with longer follow-up and repeated blood analysis would be needed. The identified markers increase our insight into the prominent mechanisms involved with MS disease progression and potentially yield new prognostic and monitoring blood-based biomarkers for MS.

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Author contributions

Study concept and design were performed by A.M, J.K (J. Killestein) and C.T. Data regarding Amsterdam cohort were provided by; C.L, M.S (M. Steenwijk) and M.S (M. Schoonheim). Data regarding Basel GeneMSA cohort were provided by; Y.N, J.K (J. Kuhle) and M.A. Statistics were performed and controlled by W.W. Drafting significant portions of the manuscript: A.M and C.T. All authors critically reviewed and approved the final manuscript.

Conflict of interests

Prof. Dr. C. Teunissen has functioned in advisory boards of Fujirebio and Roche, received non-financial support in the form of research consumables from ADxNeurosciences and Euroimmun, performed contract research or received grants from Probiodrug, Janssen prevention center, Boehringer, Brainsonline, AxonNeurosciences, EIP farma, Roche.
Prof. Dr. Jens Kuhle served on scientific advisory boards for Novartis Pharmaceuticals, Merck, Biogen, Sanofi Genzyme, Roche and Bayer; has received funding for travel and/or speaker honoraria from Biogen, Sanofi Genzyme, Novartis, Merck Serono, Roche, Teva and the Swiss MS Society; and research support from Bayer, Biogen, Merck, Sanofi Genzyma, Novartis, Roche, ECTRIMS Research Fellowship Programme, University of Basel, Swiss MS Society, Swiss National Research Foundation (320030_160221).

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