Journal of the Neurological Sciences 396 (2019) 36–41

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Plasma levels of soluble NCAM in multiple sclerosis

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ARTICLE INFO

Keywords:
Multiple sclerosis
MRI
Adhesion molecules
NCAM
VCAM-1
ICAM-1

ABSTRACT

In multiple sclerosis (MS), several adhesion molecules are involved within the central nervous system in inflammatory and neurodegenerative processes that are associated to progressive disability and increasing brain atrophy. The neural cell adhesion molecule (NCAM) has been suggested to participate in the reparative mechanisms and in the remyelination processes, key issues in MS pathology. We aimed at investigating plasma levels of the seldom investigated soluble (s)NCAM, and as comparison those of intercellular adhesion molecule-1 (sICAM-1) and vascular adhesion molecule-1 (sVCAM-1), and their association with clinical and MRI measures of lesion volumes and of global and regional atrophy. The cross-sectional study was conducted in 85 relapsing-remitting (*RR*)-MS, 53 progressive (P)-MS patients, and 42 healthy individuals (HI).

Correlation of MRI measures with plasma levels of these adhesion molecules were not observed.

In the MS and HI groups, sNCAM levels were significantly and positively associated with sVCAM-1 levels. Differently, the correlation between sICAM-1 and sVCAM-1 was observed only in MS patients. sNCAM and sVCAM-1 levels were higher in P-MS compared to HI ($P = 0.05$ and $P = 0.028$ respectively). The sVCAM-1 levels differed ($P < 0.001$) among DMTs groups and HI.

The association of sNCAM plasma levels with MS disease, as well as differences in sVCAM-1 levels in patients receiving different DMTs, deserve further investigation.

1. Introduction

In multiple sclerosis (MS), several adhesion molecules are suggested to be involved in inflammatory processes promoting neurodegeneration within the central nervous system (CNS), that are associated with progressive disability and increasing brain atrophy [1,2].

The members of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), through binding to integrins LFA-1 and VLA-4 respectively, are critical in leucocyte-endothelia interaction, promoting the immuno-inflammatory response in MS [3,4]. The soluble forms sICAM-1 and sVCAM-1 are considered to be markers of blood-brain barrier (BBB) disruption [5] and might regulate functions of the corresponding cell-bound forms [6–8]. A large number of studies investigated plasma or serum levels of sICAM-1 and sVCAM-1 in MS providing often conflicting data and highlighting heterogeneity in "immunological/adhesion pattern" among MS clinical phenotypes [9–13].

The neural cell adhesion molecule (NCAM, also known as CD56), another member of the immunoglobulin superfamily, is involved in cell migration, axonal growth and fasciculation, organization and modulation of synapses (reviewed in [14]). Its possible involvement in the reparative mechanisms and in the remyelination processes, key issues in MS [15], has been suggested [16]. Shedding of sNCAM molecules from cell membrane of neural and glial cells might have a role in brain

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https://doi.org/10.1016/j.jns.2018.10.023
Received 22 June 2018; Received in revised form 24 September 2018; Accepted 26 October 2018
Available online 28 October 2018
0022-510X/

plasticity, as it differentially alters neurite branching in a cell-type dependent manner [17,18].

The large majority of the studies have investigated sNCAM levels in the cerebrospinal fluid (CSF), which appeared to be lower in MS patients CSF compared to controls and to decrease in a step-wise manner through the progression of MS disease (reviewed in [14]). Differently, only one study investigated sNCAM in serum of MS patients [19].

Neuroimaging investigation of MS–adhesion molecule associations received considerable attention over the years, mainly evaluating the presence/absence of brain lesions, or T2 lesion volumes [13,20–22] by magnetic resonance imaging (MRI). Brain atrophy assessment has become important for the evaluation of neurodegeneration and MS disease progression. The whole brain volume (BV), the cortical volume (CV) or lateral ventricular volume (LVV), reflect regional axonal loss as well as demyelination in white and gray matter tissue structures [23,24]. Moreover, atrophy of the deep gray matter (DGM) and particularly of the thalamus, which has a prominent role in integrating signals of complex cognitive and motor functions, is associated to physical and cognitive disability in MS [25].

Up to now, the relationship between brain atrophy parameters and levels in plasma of soluble forms of adhesion molecules has not been explored in MS.

In this study, we aimed at investigating associations of soluble plasma levels of key adhesion molecules, the seldom studied sNCAM and as comparison sICAM-1 and sVCAM-1, with clinical and MRI measures of disease severity, in a cohort of MS patients and in healthy individuals (HI).

2. Materials and methods

2.1. Study population

The population of this cross-sectional study included subjects recruited in a case-control study of cardiovascular, environmental and genetic risk factors for disease progression in patients with MS (CEG-MS study; IRB ID: MODCR00000352) [26].

Subjects with the following characteristics were included: having MS according to the revised McDonald criteria [27] or being a healthy individual (HI), having an MRI scan at the 3 T scanner using the standardized MRI protocol, age between 18 and 75 years and physical/neurologic examination within 30 days from the standardized MRI study protocol. The exclusion criteria consisted of presence of relapse and steroid treatment within the 30 days preceding study entry, pre-existing medical conditions known to be associated with brain pathology (e.g., neurodegenerative disorders, cerebrovascular disease, positive history of alcohol abuse, etc.) and pregnancy.

Subjects underwent neurological and MRI examinations and provided blood samples. The collected data included demographic and clinical information. The Expanded Disability Status Scale (EDSS) was assessed in MS patients. The study protocol was approved by the local Institutional Review Board and all participants gave their written informed consent.

2.2. MRI acquisition and image analysis

MRI were obtained on a General Electric (GE) 3 T Signa Excite HD 12.0 scanner (Milwaukee, WI) using an eight-channel head and neck coil. 2D T2/PD-weighted images (WI), fluid-attenuated inversion recovery (FLAIR), spin-echo T1-WI without gadolinium contrast, 3D high resolution T1-WI and susceptibility-weighted imaging (SWI) were acquired. 2D sequences were acquired using a 256 × 192 matrix and 256 × 192mm^2 FOV, resulting in a nominal in-plane resolution of 1x1mm^2, and 48 gap-less 3 mm thick slices were acquired for whole-brain coverage. Sequence-specific parameters were: dual FSE proton density and T2-WI (TE1/TE2/TR = 9 ms/98 ms/5300 ms; echo-train length = 14), 4:31 min long; FLAIR (TE/TI/TR = 120 ms/2100 ms/8500 ms; flip angle = 90°; echo-train length = 24), 4:16 min long; and spin-echo T1-WI (TE/TR = 16 ms/600 ms), 4:07 min long. In addition, a 3D high resolution T1WI fast spoiled gradient echo sequence with a magnetization-prepared inversion recovery pulse was acquired (TE/TI/TR = 2.8 ms/900 ms/5.9 ms, flip angle = 10°), 4:39 min long, with 184 slices of 1 mm thickness, resulting in isotropic resolution.

MRI analysts were blinded to the subject's physical and neurologic condition. T2- and T1 lesion volume (LV) were assessed using a semi-automated edge detection contouring/thresholding technique [28]. Normalized brain volume (NBV) and cortical volume (NCV) were obtained using SIENAX software (version 2.6) [29]. DGM and thalamus volumes were calculated using FIRST, [30] and subsequently normalized using the SIENAX-derived scaling factor. Prior to tissue segmentation, lesion filling was utilized to reduce the impact of T1 hypointensities [31].

2.3. Assays for adhesion molecules

Adhesion molecules were measured in EDTA plasma samples obtained only once at the time of the neurological and MRI examinations. sNCAM, sICAM-1 and sVCAM-1 levels were assayed using Milliplex™ magnetic bead kits (human neurodegenerative disease bead panel 3, HNDG3MAG-36 K, Merck Millipore, Germany). Based on producer's information, this assay recognizes total sNCAM and not a specific isoform. Samples were processed following the manufacturer recommended protocols and read on a MAGPIX instrument equipped with the MILLIPLEX-Analyst Software 5.1 (Merk Millipore) using a five-parameter nonlinear regression formula to compute sample concentrations from the standard curves. Concentrations were expressed as ng/mL. The calculated inter-assay coefficient of variations for sNCAM, sICAM-1 and sVCAM-1 were 4.9%, 5.7% and 7.3% respectively, while intra-assay coefficient of variations were 3.3%, 4.0% and 6.8%. The lower limits of detection for sNCAM, sICAM-1 and sVCAM-1 were 4.81 pg/mL, 6.29 pg/mL and 6.44 pg/mL, respectively. Assays were performed blinded to clinical status.

2.4. Statistical analysis

SPSS (IBM Corp. Armonk, NY, USA, version 24.0) statistical software was used for all statistical analyses and GraphPad (GraphPad Software, Inc. La Jolla, CA, USA, prism version 6.01) for the figures.

Data were assessed for normality using the Kolmogorov–Smirnov test. The Fisher's exact test was used to compare differences in categorical variables and the Student's *t*-test to compare age.

Differences in brain volumes between total MS and HI groups and between RRMS and PMS were evaluated by ANCOVA, with age and gender as covariates. Spearman's rank correlation was used to assess associations among the adhesion molecules levels and with demographic characteristics, EDSS and disease duration. Differences in adhesion molecules levels between clinical MS subgroups and HI were determined by the Kruskal-Wallis test, followed by Dunn's multiple comparison test. The same statistical tests were used to analyze variations of adhesion molecules levels in the presence of different disease-modifying treatments (DMTs). *P*-values ≤ 0.05 were considered as statistically significant. The associations of MRI measures with adhesion molecules were assessed using linear regression analysis, with the MRI measure of interest as the dependent variable, and age, gender, drug-treatment and the adhesion molecule of interest as the predictor variables. Given the multiple testing involved, a conservative *p*-value ≤ 0.01 was used for significance assessment and a *p*-value ≤ 0.05 was considered a trend.

3. Results

The demographic and clinical characteristics of the study populations are summarized in Table 1 and have been previously reported

Table 1
Demographic and clinical characteristics of the cohorts.

	All MS n = 138	RR-MS n = 85	P-MS *n* = 53	HI *n* = 42
Female, n (%)	100 (72.5)	60 (70.6)	40 (75.5)	31 (73.8)
Age, years	54.3 ± 10.8	50.1 ± 10.7	60.9 ± 7.2	51.0 ± 14.3
Age onset, years	32.9 ± 9.5	32.6 ± 9.1	33.3 ± 10.1	–
Disease duration, years	21.1 ± 10.6	17.0 ± 8.8	27.6 ± 10.0	–
EDSS, median (IQR)	3.5 (2–6)	2 (1.5–3.5)	6 (4–6.5)	–
Annual relapse rate	0.2 (0.4)	0.2 (0.4)	0.1 (0.3)	–
DMT type, number of patients (%)				–
Interferon-beta	45 (32.6)	30 (35.3)	15 (28.3)	
Glatiramer acetate	42 (30.4)	23 (27.1)	19 (35.9)	
Natalizumab	5 (3.6)	4 (4.7)	1 (1.9)	
Other DMT*	19 (13.8)	13 (15.3)	6 (11.3)	
No DMT	27 (19.6)	15 (17.6)	12 (22.6)	

MS: Multiple Sclerosis; RR-MS: Relapsing Remitting Multiple Sclerosis; P-MS: Progressive Multiple Sclerosis; HI: Healthy Individuals; EDSS: Expanded Disability Status Scale; IQR: interquartile range; SD: standard deviation; n: number; DMT: disease-modifying treatment.
Descriptive analysis between MS and HI were performed using Fisher's exact test and Student *t*-test.
* Other DMTs include: 5 fingolimod, 3 teriflunomide, 5 dimethyl-fumarate, 4 intravenous immunoglobulin, 1 mitoxantrone and 1 methotrexate treated patients. All continuous variables (age and disease duration) are reported as mean ± standard deviation. For the ordinal EDSS, the median (interquartile range) is given.

[32]. The patient population (*n* = 138) included 85 relapsing remitting (RR-MS) and 46 secondary-progressive and 7 primary-progressive MS, categorized as progressive (P-MS) group for the purpose of the analyses.

3.1. Adhesion molecules levels in plasma

The adhesion molecules levels in plasma of MS and HI groups are summarized in Fig. 1A.

The levels of sNCAM and sVCAM-1 differed among MS and HI ($p = 0.033$ for both proteins, Kruskal-Wallis test). Higher levels in P-MS compared to HI were detected for sNCAM (median = 302.6, IQR = 276.8–349.3 ng/mL vs. 272.8, IQR = 230.2–331.8 ng/mL; $p = 0.050$) and for sVCAM-1 (median = 1039, IQR = 881.5–1249 ng/mL vs. median = 855.3, IQR = 782.6–1066 ng/mL; $p = 0.028$) by Dunn's multiple comparison test. No significant differences in sICAM-1 plasma levels were observed between the study groups (Fig. 1A).

The association among the adhesion molecules levels was explored

Table 2
Correlations among adhesion molecules levels in Multiple Sclerosis patients and healthy individuals.

		sNCAM	sICAM-1
Multiple sclerosis patients			
sICAM-1	*Rho:*	−0.08	
	p-value:	0.382	
sVCAM-1	*Rho:*	0.26	0.20
	p-value:	0.002	0.021
Healthy individuals			
sICAM-1	*Rho:*	0.21	
	p-value:	0.182	
sVCAM-1	*Rho:*	0.49	0.25
	p-value:	0.001	0.107

sNCAM: soluble neural cell adhesion molecule; sICAM-1: soluble intercellular adhesion molecule; sVCAM-1: soluble vascular cell adhesion molecule 1.
Spearman correlation coefficient and *p*-values are reported.

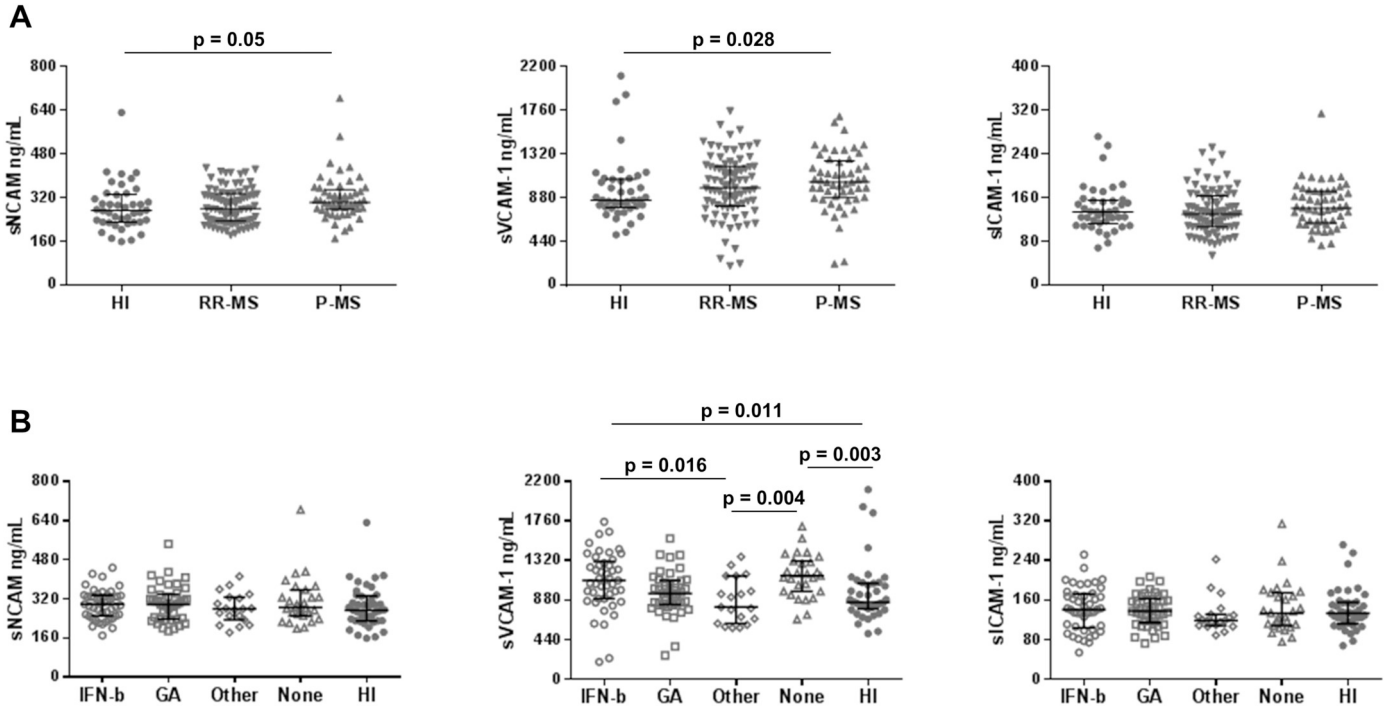

Fig. 1. Adhesion molecules levels in MS patients and in healthy individuals (A) and in relation to MS disease-modifying treatment (B).

Table 3
MRI characteristics of the cohorts.

	All MS	RR-MS	P-MS	HI	MS vs. HI *p*-value	RR-MS vs. P-MS *p*-value
T2-LV, ml	15.8 (19.0)	11.8 (15.9)	22.2 (21.9)	0.2 (0.6)	< 0.001	0.016
T1-LV, ml	2.9 (6.2)	2.0 (4.6)	4.4 (8.1)	0.0 (0.0)	0.005	0.075
NBV, ml	1438 (92.1)	1469 (82.4)	1387 (85.2)	1528 (97.9)	< 0.001	0.001
NCV, ml	591 (48.6)	606 (44.8)	567 (44.8)	630 (53.3)	< 0.001	0.028
LVV, ml	55.1 (27.0)	50.7 (25.2)	62.3 (28.5)	32.2 (14.5)	< 0.001	0.229
DGM volume, ml	53.6 (7.1)	55.5 (6.5)	50.4 (6.9)	60.5 (46.4)	< 0.001	0.007
Thalamus volume, ml	17.7 (2.5)	18.4 (2.3)	16.5 (2.4)	20.3 (1.9)	< 0.001	0.008

MS: Multiple Sclerosis; RR-MS: Relapsing Remitting Multiple Sclerosis; P-MS: Progressive Multiple Sclerosis; HI: Healthy Individuals; LV: lesion volume; NBV: normalized brain volume; NCV: normalized cortical volume; LVV: lateral ventricular volume; DGM: deep gray matter.
Lesion and brain volumes are expressed in milliliters and reported as mean values (standard deviation).
P-values derived by ANCOVA with age and gender as covariates, are reported.

in the MS and HI groups (Table 2). In the MS and HI groups, sVCAM-1 levels were significantly and positively associated with sNCAM levels ($r = 0.26$, $p = 0.002$ in MS and $r = 0.49$, $p = 0.001$ in HI). Additionally in MS, trend for positive association between sICAM-1 and sVCAM-1 ($r = 0.20$, $p = 0.021$) was observed.

3.2. Association of clinical and MRI features with adhesion molecules levels

Soluble adhesion molecule levels were not found to be associated with EDSS and disease duration.

Significant differences for 5 out of 7 MRI parameters were observed between RR-MS and P-MS groups, and in particular for NBV ($P = 0.001$), DGM volume ($P = 0.007$) and thalamus volume ($P = 0.008$). As expected, all brain MRI measures were significantly different between total MS and HI (Table 3).

We investigated whether adhesion molecule levels could be associated with MRI measures in MS patients by regression analyses adjusting for age, gender and type of DMTs. None of the adhesion molecules investigated was associated with the MRI measures either in the whole MS and HI populations (Supplementary Tables 1 and 2 respectively) or in the P-MS group (data not shown).

3.3. Adhesion molecules levels in patients grouped by disease-modifying treatments

As the plasma levels of adhesion molecules can be influenced by DMTs for level analysis (Fig. 1B), those were categorized into four groups: IFN-beta, GA, Other and None. Since very few patients (Table 1) had been treated with natalizumab, they were not included in the analysis.

The levels of sVCAM-1 differed ($p < 0.001$, Kruskal-Wallis test) among the DMT groups and HI sVCAM-1 levels were higher in MS patients treated with IFN-b (median = 1098, IQR = 895.2–1311 ng/mL) compared to Other DMTs (median = 802.4, IQR = 619.6–1148 ng/mL; $p = 0.016$) and to HI (median = 855.3, IQR = 782.6–1066 ng/mL, $p = 0.011$). Additionally, sVCAM-1 levels were higher in MS with None treatment (median = 1149 ng/mL, IQR = 975.2–1314) compared to HI (median 855.3, IQR = 782.6–1066 ng/mL; $p = 0.003$) and to Other treatment (median = 802.4, IQR = 619.6–1148 ng/mL; $p = 0.004$). Neither sNCAM or sICAM1 levels showed significant differences among patients receiving different DMTs.

4. Discussion

Adhesion molecules are suggested to take part in the different processes that lead to the development of lesions and neurodegeneration in MS. Based on the functional importance in MS pathogenesis, we investigated the levels of sNCAM, and in comparison, those of sICAM-1 and sVCAM-1, in a large cohort of subjects, in which multiple clinical and MRI measures of disease severity were assessed. Levels were evaluated in plasma by a multiplex assay, which favors detection of associations by decreasing experimental variations.

Highly significant correlations between plasma levels of sNCAM and sVCAM-1 were detected both in patients and HI. The noticeable values of the correlation coefficients for these molecules, which are produced by different genes and might be expressed by different cells, add further interest to this observation. This relation, that we report here for the first time, could reflect a coordinated regulation of NCAM and VCAM-1 expression and/or shedding, present in healthy and disease conditions. The correlation coefficient, lower in MS patients than in HI, suggests that the biological pathways linking these adhesion molecules are altered in MS by still undefined molecular components, which deserves further investigation. Differently, the correlation between sICAM-1 and sVCAM-1 was observed only in MS patients, which might be explained by the role of their membrane forms, involved in the coordinated multistep leukocytes adhesion process in disease [8].

We detected higher plasma levels of sNCAM in P-MS patients compared to HI, a novel observation in the MS literature, in which data for the soluble form of NCAM in plasma are scanty. Interestingly, increased levels of sNCAM were recently detected in sera of patients with various types of peripheral neuropathies [33] and peripheral neuropathy has been reported in MS without being associated to EDSS [34–36]. The hypothesis that increased levels of sNCAM in progressive MS could be associated to the presence of peripheral neuropathy deserves further investigation. On the other hand in CSF, sNCAM levels were found either reduced in MS [37] as well as in SPMS as compared to RRMS [38], or increased in MS patients in the acute phase of the disease and undergoing steroid treatment [16]. However, one of the limitations of our study is that we did not investigate sNCAM levels in CSF, which makes it difficult to speculate further.

Although sICAM-1 and sVCAM-1 have been extensively investigated in plasma [7,10,11,39,40], comparison with the previous and often conflicting observations is difficult because of different recruitment criteria of patients, and differently grouped clinical MS phenotypes. Our cross-sectional study did not include patients with signs of relapse provided by Gd-contrast, and thus the relapse-associated disease activity was not explored, a potential limitation of the present investigation.

In this study we explored brain atrophy, which is emerging as a meaningful indicator of neurodegeneration and clinical disease progression in MS patients and is at least partially independent of the effects of conventional MRI lesions [23,41]. Through extensive analysis of lesion volumes as MRI indicators of brain inflammation and with global and regional atrophy as neurodegenerative mark, we did not find any correlation with adhesion molecules plasma levels. In addition, association of levels with disability and disease duration did not emerge. As a matter of fact, a longitudinal study failed to find a correlation between mean levels of sICAM-1 and sVCAM-1 with MRI data of disease progression [21]. On the

other hand, patients with diagnosis of progressive MS in the present study presented higher levels of sVCAM and sNCAM and significant differences in MRI measures of disease severity as compared to HI.

We provide the first evaluation for sNCAM in relation to several DMTs, which did not appear to influence plasma levels of this adhesion molecule. This would support relation of the higher sNCAM concentration with MS progressive phenotype.

Although we found a noticeable correlation between sNCAM and sVCAM-1 plasma levels, only for sVCAM-1 we observed level differences in patients grouped by DMTs and higher levels in patients without treatment than in HI. Moreover, patients treated by drugs other than IFN-b and GA displayed the lowest levels. The study design and the heterogeneity of DMTs in this group do not enable to relate this observation to a specific treatment, which requires further investigation.

5. Conclusions

In a large cohort of patients characterized for multiple MRI measures of disease severity, sNCAM levels in plasma were evaluated for the first time, and compared with those of sICAM-1 and sVCAM-1. Plasma levels of sNCAM, sICAM-1 and sVCAM-1 did not correlate with clinical and MRI measures of disease severity.

Whereas correlation between plasma levels of sNCAM and sVCAM-1 were detectable both in patients and HI, that between sICAM-1 and sVCAM-1 was observed only in MS patients. In progressive MS, as compared with HI, increased levels of sNCAM and sVCAM-1 were detected. This association was confirmed even after evaluation of adhesion molecules levels in patients grouped by DMTs, which appear to modulate only sVCAM-1 plasma levels.

Conflicting interests

Nicole Ziliotto, Dejan Jakimovski, Marcello Baroni, Veronica Tisato, Paola Secchiero, Niels Bergsland, Deepa P. Ramasamy, Francesco Bernardi and Giovanna Marchetti have nothing to disclose.

Dr. Robert Zivadinov has received speaker honoraria and consultant fees from Genzyme-Sanofi, Novartis, Claret Medical, Celgene and EMD Serono. He has received research support from EMD Serono, Genzyme-Sanofi, Claret Medical, Protembis, QuintilesIMS and Novartis.

Dr. Bianca Weinstock-Guttman received honoraria for serving in advisory boards and educational programs from Teva Pharmaceuticals, Biogen Idec, Novartis, Accorda EMD Serono, Pfizer, Novartis, Genzyme and Sanofi. She also received support for research activities from the National Institutes of Health, National Multiple Sclerosis Society, National Science Foundation, Department of Defense, EMD Serono, Biogen Idec, Teva Neuroscience, Cyberonics, Novartis, Acorda and the Jog for the Jake Foundation.

Dr. Murali Ramanathan received research funding the National Multiple Sclerosis Society, the National Institutes of Health and Otsuka Pharmaceutical and Development. These are unrelated to the research presented in this report.

Ethics approval and consent to participate

The study protocol was approved by the local Institutional Review Boards of University of Buffalo, USA (CEG-MS study; IRB ID: MODCR00000352) and of University/Hospital of Ferrara, Italy (IRB ID: 170585). All participants gave their written informed consent.

The adjusted *p*-values from Dunn's multiple comparison test are provided. The error bars indicate median and the interquartile range.

HI: healthy individuals; RR-MS: relapsing-remitting multiple sclerosis; P-MS: progressive multiple sclerosis; sNCAM: soluble neural cell adhesion molecule; sICAM-1: soluble intercellular adhesion molecule 1; sVCAM-1: soluble vascular cell adhesion molecule 1; GA: Glatiramer acetate; IFN-b: interferon-beta; None: no disease-modifying therapy; Other: other disease-modifying therapy.

Acknowledgements

This study was funded in part by the Annette Funicello Research Fund for Neurological Diseases and internal resources of the Buffalo Neuroimaging Analysis Center. In addition, we received support from the Jacquemin Family Foundation.

Research reported in this publication was also funded in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under award Number UL1TR001412. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

This study was partially supported by the grant 1786/2012 from the strategic 2010–2012 Research Program of Emilia Romagna Region, Italy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2018.10.023.

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