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# Are Plasma Levels of Vascular Adhesion Protein-1 Associated Both with Cerebral Microbleeds in Multiple Sclerosis and Intracerebral Haemorrhages in Stroke?

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Cerebral microbleeds (CMBs) are defined as small and hypointense areas which could correspond to clusters of hemosiderin-laden macrophages resulting from small self-limiting haemorrhages.[1,2] CMBs have been associated with aging, traumatic brain injury, stroke and neurodegenerative disorders, among which the cerebral amyloid angiopathy[Q4]. CMBs are potentially a radiological biomarker of the cerebral small vessel diseases prone to bleeding and developing spontaneous intracerebral haemorrhages (ICHs).[3,4] Recently, in patients with atrial fibrillation anticoagulated after ischaemic stroke or transient ischaemic attack, the presence of CMBs was independently associated with symptomatic ICH risk, and could be used to inform anticoagulation decisions.[5,6]

Failure of blood–brain barrier integrity leading to focal extravascular leakage of blood components is a decisive event in the pathogenesis of multiple sclerosis (MS), a disease characterized by multifocal demyelinated lesions within the central nervous system.[7] Extravascular leakage of blood may have the features of radiologically measurable CMBs.[8] Adhesion molecules participating in blood–brain barrier disruption and in inflammatory responses, supported by fibrinogen extravasation and promoting tissue factor expression,[9–12] in turn could be involved in the formation of CMBs.

Vascular adhesion protein-1 (VAP-1) facilitates leukocyte infiltration into inflamed tissue[13] through an enzymatic activity that mediates cell binding to the vessel wall. VAP-1 catalyzes formation of free radicals from its substrates on leukocytes, providing an inflammatory microenvironment and causing expression of additional adhesion molecules.[14] VAP-1 is both a cell surface and a circulating protein, released through cleavage mechanisms that are only partially defined.[14,15]

Higher plasma activity level of VAP-1 has been found both in consecutive patients with spontaneous ICH[16] and in patients with stroke treated with tissue plasminogen activator, who subsequently experienced ICH.[17] In animal models, VAP-1 inhibition decreased both immune cell infiltration after ICH and micro-vascular dysfunction.[18,19]

Taking advantage of MS as a disease model,[2,20,21] we aimed at extending our knowledge about the association between plasma levels of VAP-1 and occurrence of CMBs in MS patients, assessed by magnetic resonance imaging (MRI) measures in a cross-sectional study (►**Table 1** and



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**Table 1** Cerebral microbleeds in MS patients

| | All MS | RR-MS | P-MS | HI |
|---|---|---|---|---|
| Sample size, *n* | 138 | 85 | 53 | 42 |
| Female, *n* (%) | 100 (72.5) | 60 (70.6) | 40 (75.5) | 31 (73.8) |
| Age, y | 54.3 ± 10.8 | 50.1 ± 10.7 | 60.9 ± 7.2 | 51.0 ± 14.3 |
| Cerebral microbleeds (CMBs) | | | | |
| Individuals with CMB, *n* (%) | 12 (9.6) | 5 (6.3) | 7 (15.2) | 3 (7.3) |
| Number of CMBs | | | | |
| NA | 13 | 6 | 7 | 1 |
| 0 | 113 | 74 | 39 | 38 |
| 1 | 9 | 4 | 5 | 2 |
| 2 | 2 | – | 2 | – |
| ≥ 3 | 1 | 1 | – | 1 |
| CMB volume, $mm^3$ | 22.9 ± 21.04 | 33.4 ± 29 | 15.4 ± 9.8 | 14.8 ± 13.65 |
| Disease-modifying treatment (DMT) | | | | |
| No DMT | 27 (19.6) | 15 (17.6) | 12 (22.6) | – |
| Interferon-β | 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[a] | 19 (13.8) | 13 (15.3) | 6 (11.3) | |

Abbreviations: HI, healthy individuals; MS, multiple sclerosis; NA, not available; P-MS, progressive multiple sclerosis; RR-MS, relapsing remitting multiple sclerosis.
Note: 10 CMB patients were under the following DMTs: 6, glatiramer acetate; 2, interferon-β; 1, natalizumab; 1, fingolimod. Age and CMB volume are reported as mean ± standard deviation.
[a]Other DMTs included intravenous immunoglobulin, mitoxantrone and methotrexate.

►**Supplementary Material**, available in the online version). In turn, this could underline potential biological relations between CMBs and ICH.

Concentration of soluble VAP-1 molecule (►**Fig. 1**) was measured in ethylenediaminetetraacetic acid plasma samples using a multiplex assay (see ►**Supplementary Material**, available in the online version), which permitted the parallel investigation of several adhesion molecules.

Analysis of VAP-1 concentration by non-parametric Mann–Whitney U test revealed a trend for higher VAP-1 in MS(+)CMB versus MS(−)CMB (median = 300.9, interquartile range [IQR] =192.2–401.5 ng/mL vs. median = 237.2, IQR = 195.8–276.1 ng/mL, $p = 0.076$, ►**Fig. 1A**). VAP-1 levels were not associated with CMB volume ($p = 0.86$, linear regression). Interestingly, mean levels of this soluble adhesion molecule were numerically 32% higher in MS patients with CMBs than in healthy individuals (HIs) (300 ± 105 ng/mL vs. 227.3 ± 50 ng/mL), and 35% higher in ICH patients than in controls,[16] a very similar proportion.

None of the other MRI measures (T2-LV, T1-LV, normalized brain, normalized cortical, lateral ventricular, deep grey matter and thalamus volumes) in MS patients were associated with VAP-1 concentration by regression analyses (data not shown) adjusting for age, sex and type of disease-modifying treatment (DMT).

Taking into account that DMTs could potentially influence VAP-1 levels, the proportional distribution of DMT's use between MS(+)CMB versus MS(−)CMB was also assessed ($p = 0.761$, chi-square). Analysis among MS patients on DMTs, patients without treatment and HI groups provided a significant variation in VAP-1 levels ($p = 0.001$, Kruskal–Wallis test, ►**Fig. 1B**). In particular, MS patients on DMTs had lower VAP-1 levels than those without treatment (median = 231.1, IQR = 190.3–276.3 ng/mL vs. median = 272.7, IQR = 251.6–324 ng/mL; $p = 0.002$, Dunn's multiple comparison test). Further, VAP-1 levels in MS patients without treatment were higher than in HI (median = 272.7, IQR = 251.6–324 ng/mL vs. median = 233.2, IQR = 181.1–263.5 ng/mL; $p = 0.002$, Dunn's multiple comparison test, ►**Fig. 1B**).

Modulation of VAP-1 levels by these drugs (►**Table 1**) was further investigated in MS patients ($p = 0.002$, Kruskal–Wallis test, ►**Fig. 1C**). This comparison showed that the decrease in VAP-1 levels was mostly influenced by glatiramer acetate treatment compared with MS without treatment (median = 225.3, IQR = 181.9–253.3 ng/mL vs. median = 272.7, IQR = 251.6–324 ng/mL; $p = 0.002$, Dunn's multiple comparison test, ►**Fig. 1C**).

Finding higher VAP-1 concentration in plasma of MS patients without any DMT and lower in those on DMTs, clearly indicated that DMTs were not responsible for the higher VAP-1 levels in patients with CMBs. Instead, DMTs could mask even higher VAP-1 levels in patients with CMBs as indicated by comparison of patients not on DMTs (MS(+)CMB no DMT, median = 400.8, IQR = 333.2–468.4 ng/mL vs. MS(−)CMB no

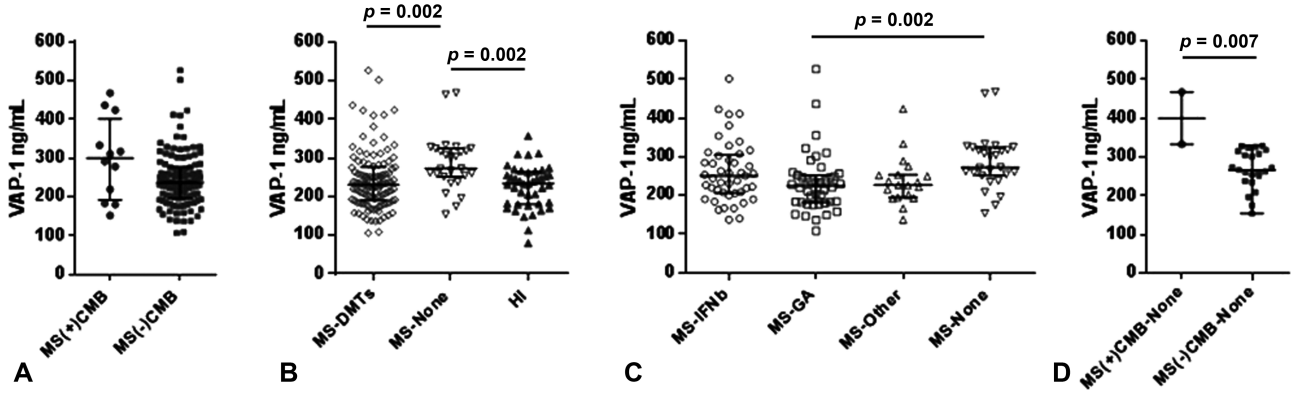


**Fig. 1** (**A**) Soluble vascular adhesion protein 1 (VAP-1) concentration in multiple sclerosis (MS) patients with cerebral microbleeds (CMBs) (MS (+)CMB, $n = 12$), MS without CMB (MS(−)CMB, $n = 113$). Mann–Whitney U test showed a trend of $p = 0.076$. (**B**) VAP-1 levels among patients on disease-modifying treatments (MS-DMTs, $n = 111$), patients without treatment (MS-none, $n = 27$) and healthy individuals (HIs, $n = 42$) groups. The adjusted *p*-values from Dunn's multiple comparison test are provided for comparisons between groups where Kruskal–Wallis test was significant. (**C**) VAP-1 levels in MS cohort according to the DMT (IFNb: Interferon-β,, $n = 45$; GA: glatiramer acetate, $n = 42$; Other: other DMTs, $n = 19$; None: no DMT, $n = 27$). Since very few patients (►**Table 1**) had been treated with natalizumab, we did not include them in this panel of analysis. The adjusted *p*-values from Dunn's multiple comparison test are provided for comparisons between groups where Kruskal–Wallis test resulted significant. (**D**) VAP-1 concentration in MS patients without treatment (none DMTs, $n = 25$) grouped for presence of CMBs (MS(+)CMB-none, $n = 2$ and MS(−)CMB-none, $n = 23$). The significant *p*-value from Mann–Whitney U test is shown. In all panels, median values and interquartile ranges are shown.

DMT, median = 266.5, IQR = 238–315, $p = 0.007$, Mann–Whitney U test, ►**Fig. 1D**).

Of note, in patients with CMBs, plasma concentration differences were observed for VAP-1 and not for other soluble adhesion molecules (neural cell adhesion molecule, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), investigated in multiplex assays (unpublished data).

Our observations have some limitations: (1) CMBs are heterogeneous as suggested by their radiographic appearance,[22] and in MS they are likely smaller in size as compared with CMBs investigated in ICH[6]; (2) we have evaluated in peripheral blood the soluble VAP-1, the level and role of which in brain vessel endothelium are only inferred; (3) the VAP-1 activity values have the potential to reveal the presence of a functional enzyme in plasma,[16,17] whereas we measured the VAP-1 protein concentration, which however corresponds well to the level of enzymatic activity found in serum or plasma[14]; (4) the number of patients with CMBs in our study was low; and (5) we are unaware of the effects and/or the biological implications of VAP-1 levels on CMBs over time. To confirm and detail this association, further investigation in prospective studies of VAP-1 levels in larger cohorts of patients with CMBs is needed, including cerebral amyloid angiopathy, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy and small vessel disease patients.

In light of the previously detected association between CMBs and ICH occurrence,[6] finding similarly increased plasma levels of VAP-1 both in patients with ICH[16] and, at least as a trend, in MS with CMBs, is hypothesis generating. VAP-1, present in endothelium and smooth muscle cells of the brain vessel,[23] promotes inflammatory cell recruitment,[24] which might be associated with the conveyance of pro-coagulant mediators to the sites of vascular injury.[25] It is tempting to speculate that VAP-1 contributes both to cerebral microvascular endothelial cells dysfunction and to small and self-limiting haemorrhages, revealed by MRI. Our data foster the investigation in prospective studies of VAP-1 and other molecules potentially bridging ICH and CMBs.

### Ethical Approval

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.

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### Conflict of Interest

Nicole Ziliotto, Dejan Jakimovski, Niels Bergsland, Deepa P. Ramasamy, Giovanna Marchetti and Francesco Bernardi 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

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## Supplementary Materials and Methods

The study population comprised of subjects recruited in a case–control study of cardiovascular, environmental and genetic risk factors for disease progression in patients with multiple sclerosis (MS) (CEG-MS study; IRB ID: MODCR00000352).[1]

Subjects with the following characteristics were included: having MS according to the revised McDonald criteria[2] or being a healthy individual (HI), having a magnetic resonance imaging (MRI) scan at the 3T 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 study protocol was approved by the local Institutional Review Board and all participants gave their written informed consent. MRI acquisition and image analysis have been previously described in detail.[1]

The cerebral microbleeds (CMBs) analysis was performed on susceptibility weighted imaging (SWI) minimum intensity projection images and susceptibility maps by two experienced neuroimagers who were also blinded to MR images were obtained with other sequences. CMBs were classified as focal, small, round to ovoid punctuate areas of signal hypo-intensity on SWI minimum intensity projection images, as previously reported.[1] Signal voids caused by sulcal vessels, calcifications and signal averaging from bone were considered mimics of microbleeds. The presence and number of definite CMBs were determined on SWI minimum intensity projection images by using the Microbleed Anatomic Rating Scale.[3] The CMB volume was calculated on susceptibility maps by using a semi-automated edge detection contouring and thresholding technique.[4]

Soluble vascular adhesion protein-1 (sVAP-1) levels were measured in ethylenediaminetetraacetic acid plasma samples obtained at the follow-up visit, using Luminex Screening Assays magnetic bead kits (R&D Systems Inc., Minneapolis, Minnesota, United States). Samples were processed following the manufacturer's recommended protocols and read on a MAGPIX instrument equipped with the MILLIPLEX-Analyst Software 5.1 (Merk Millipore) using a five-parameter non-linear regression formula to compute sample concentrations from the standard curves.[5] Concentrations were expressed as ng/mL. The calculated inter-assay coefficient of variations for sVAP-1 was 3.7%.

SPSS (IBM Corp., Armonk, New York, United States, version 24.0) statistical software was used for all statistical analyses and GraphPad (GraphPad Software, Inc., La Jolla, California, United States, prism version 6.01) for the figures. Fisher's exact test was used to compare differences in categorical variables, Student's *t*-test was used to compare age and analysis of covariance, with age and gender as covariates, was used to compare brain volume measurements between the total MS and HI groups. Spearman's rank correlation was used to assess associations of sVAP-1 levels with demographic characteristics, Expanded Disability Status Scale and disease duration. The associations of presence/absence of CMBs with VAP-1 levels were performed using logistic regression analysis. The associations of MRI measures with sVAP-1 were assessed using linear regression analysis. All regression analyses included the MRI measure of interest as the dependent variable; the predictor variables were age, gender, drug treatment and sVAP-1. Differences in sVAP-1 levels between MS clinical sub-groups and HI were determined by the Kruskal–Wallis test, followed by Dunn's multiple comparison test. The same statistical tests were used to analyse variations of sVAP-1 levels according to the disease-modifying treatments. *p*-Values of $\leq 0.05$ were considered as statistically significant.

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