**Blood circulating microparticle species in relapsing-remitting and secondary progressive multiple sclerosis.
A case-control, cross sectional study with conventional MRI and advanced iron content imaging outcomes**

Alexander J.S.¹, Chervenak R.³, Weinstock-Guttman B.⁴, Tsunoda, I.³, Ramanathan M.⁵, Martinez, N.³, Omura, S.³, Sato, F.³, Chaitanya, G.V.¹, Minagar A.², Jennings M.H.¹, Monceaux C.¹, Becker F.¹, Cvek U.⁷, Trutschl M.⁷, Zivadinov R.^{4,6}

Key words: Multiple sclerosis, endothelial microparticles, MRI, serum, atrophy, iron deposition

Authors from:

Louisiana State University Health-Shreveport, LA ¹Departments of Molecular and Cellular Physiology, ²Neurology, ³Microbiology & Immunology, Center for Molecular and Tumor Virology, ⁴The Jacobs Neurological Institute, Department of Neurology, University of Buffalo, Buffalo, NY; ⁵Department of Pharmaceutical Sciences, State University of New York, Buffalo, NY, USA; ⁶Buffalo Neuroimaging Analysis Center, Department of Neurology, University of Buffalo, Buffalo, NY, ⁷Computer Sciences Department, Louisiana State University-Shreveport, LA

Corresponding author:

Alireza Minagar, MD
LSU Health, Shreveport
1501 Kings Highway
Shreveport, LA, 71130-3932
1-318-675-8581
Email: aminag@lsuhsc.edu

Abstract.

Background: Although multiple sclerosis (MS) is thought to represent an excessive and inappropriate immune response to several central nervous system (CNS) autoantigens, increasing evidence also suggests that MS may also be a neurovascular inflammatory disease, characterized by endothelial activation and shedding of cell membrane microdomains known as ‘microparticles’ into the circulation.

Objective: To investigate the relationships between these endothelial biomarkers and MS.

Methods: We examined the relative abundance of CD31⁺/PECAM-1, CD51+CD61+ (α V- β 3) and CD54⁺(ICAM-1) bearing microparticles in sera of healthy individuals, patients with relapsing-remitting MS, and secondary-progressive MS . We also investigated the correlation among circulating levels of different microparticle species in MS with conventional MRI (T2- and T1-lesion volumes and brain atrophy), as well as novel MR modalities [assessment of iron content on susceptibility-weighted imaging (SWI)-filtered phase].

Results: Differences in circulating microparticle levels were found among MS groups, and several microparticle species (CD31⁺/CD51⁺/CD61⁺/CD54⁺) were found to correlate with conventional MRI and SWI features of MS.

Conclusion: These results indicate that circulating microparticles’ profiles in MS may support mechanistic roles for microvascular stress and injury which is an underlying contributor to not only MS initiation and progression, but also to pro-inflammatory responses.

Introduction.

Multiple sclerosis (MS) is a presumably immune-mediated neurovascular disease whose etiology is influenced by genetic, environmental and even hemodynamic factors¹. Magnetic resonance imaging (MRI) in MS positively correlates with increased disability, (Expanded Disability Status Score, 'EDSS')² and remains the most widely applied tool for diagnosing and monitoring MS: MRI also provides important mechanistic clues to the potential bases of MS. Although MRI remains the best objective tool to calculate MS burden; it is expensive and time-consuming and alternative methods are still needed to monitor disease-activity. While lymphocytes and macrophages play important roles in MS³, additional blood biomarkers are also altered in MS which provide important clues to its pathogenesis. In particular, endothelial and platelet activation has been reported in MS⁴, suggesting that microvascular activation contributes to MS pathogenesis. In RRMS platelet activation⁵, blood-brain barrier (BBB) disruption correlate with contrast enhancing lesions (CEL) signifying diminished endothelial integrity which underlie CELs.

Microparticles' (MP) are small (<0.1µm) circulating remnants of endothelial, platelet and other immune cell membranes, which present an important new class of clinical biomarkers. The MP profile may foreshadow BBB failure^{9,6} and serve as 'surrogate' markers of vascular stress in MS. Importantly, MP carry endothelial adhesion molecules ('ECAMs'), apoptotic, and other molecular markers from their parent cells, which may link vascular activation to disease activity in MS. MP analysis, therefore, may represent a simple method to monitor MS activity and response to treatment. When correlated with specific brain MRI parameters, MPs can provide important mechanistic insights into MS initiation pathogenesis.

Several endothelial and platelet ECAMs have now been described as MP markers in MS including CD51/CD61 ($\alpha V/\beta 3$ -integrin), CD31 (platelet endothelial cell adhesion molecule-1)⁷ and CD54 (intercellular adhesion molecule-1/ICAM-1)⁷. CD31 is an immunoglobulin -superfamily ECAM constitutively expressed on apical and junctional endothelial surfaces, and on platelets, myeloid cells and lymphocytes. CD31⁺ MPs are released during MS exacerbations⁴; and appear to correlate with early changes in BBB disruption and axonal injury, often abating during remission and therapy⁷. Another MP marker, CD51 (integrin αV) forms several complexes which bind fibronectin and vitronectin and regulates vascular structure and stability⁸. Complexed with CD61, they form integrin $\alpha V/\beta 3$ which is expressed by endothelial cells, platelets but also macrophages and neutrophils. CD51⁺/61⁺ MPs increase after MS onset⁴ but does not diminish during remission or therapy⁷. MP profiling has thus suggested CD51⁺/61⁺ as a vascular marker of underlying MS disease. CD54 (ICAM-1), is

another MP marker constitutively expressed on brain endothelial cells which binds leukocyte LFA-1/Mac-1: CD54 is upregulated by T-helper (Th)-1 cytokines and may regulate BBB⁹, and vasomotion, . Consequently, the release of CD54⁺ MP has been used as a circulating biomarker of endothelial activation.

Comparisons of these MP markers in different forms of MS suggest MP may provide a means of evaluating and distinguishing MS subtypes, and potentially, measuring the therapeutic effectiveness of disease modifying drugs. Lastly, annexin-V binding to MP suggests that MP can reflect early stages of apoptosis and in conjunction with endothelial MPs annexin-V⁺, reveals inflammatory injury, and in this report, were studied as markers of cell stress and activation ⁶. The present study correlated several circulating MP species in RRMS and SPMS (and healthy controls) with conventional and advanced MRI outcomes to evaluate this approach as a non-invasive metric for use by clinicians to assist in documentation of disease activity in MS.

Methods.

Subject identification and MS diagnosis. RRMS and SPMS patients and age- and sex-matched healthy controls (HC) were enrolled at the Department of Neurology, University of Buffalo, Buffalo, NY. Inclusion criteria were MS diagnosis according to McDonald criteria,¹⁰ RR or SP disease,¹¹ having an MRI exam performed ≤ 30 days of clinical examination with the standardized study protocol, age 18-80 years and EDSS = 0-8.5. Exclusion criteria included relapse/exacerbation or steroid treatment ≤ 30 days of study, pre-existing conditions associated with non-MS brain pathology or pregnancy. HC subjects were recruited from hospital personnel and respondents to local advertisements. HC underwent physical examination, and were assessed for demographic characteristics, autoimmune and other concomitant diseases, vascular and environmental risks and personal habits. Serum samples were stored -80°C . Samples of serum were analyzed at Louisiana State University Health Science Center-Shreveport (LSUHSC-S). The study was approved by Institutional Review Board at the University at Buffalo.

MP isolation, labeling, and flow cytometry analysis. Blood was drawn into serum collection tubes and sera removed and aliquots frozen at -80°C . Serum samples were first centrifuged for 10 minutes ($160\times g$) and then centrifuged for 6' minutes at $500\times g$. Cleared supernatants from these spins were then centrifuged at $14,000\times g$ for 1 h and the MP pellets resuspended in PBS. 50 μL of each sample was incubated with 4 μL of anti-CD31-PE (AbCAM), anti-CD51/61-FITC (Pharmingen), anti-CD54 PE-Cy5 (ICAM-1, BD) and anti-annexin V-APC-Cy7 for 20 minutes with orbital shaking. 1 mL of PBS was added to each sample prior to flow cytometry. MP were assayed by flow cytometry using FACSVantage SE counter (Beckman Coulter) at medium flow rate setting and 30-second stop time, with log gain on light scatter and fluorescence. Detection was set to trigger by fluorescence signal $>$ noise. Fluorescent microparticles were separated on another histogram based on size (forward light scatter). Flow cytometry analysis was performed using CellQuest for data acquisition and data analysis. Species of MP defined by flow profiling are shown in Table I.

MRI analysis.

All scans were acquired on a 3T GE Signa Excite HD 12.0 TwinSpeed 8-channel scanner (General Electric 'GE', Milwaukee, WI, USA), with a maximum slew rate of 150T/m/s and maximum gradient amplitude in each orthogonal plane of 50 mT/m (zoom mode). A multi-channel head and neck (HDNV) coil (GE) was used to acquire the following sequences: 2D multiplanar dual fast spin-echo (FSE), proton density (PD) and T2-weighted image (WI); fluid-attenuated inversion-recovery (FLAIR); 3D high resolution (HIRES) T1-WI using a fast-spoiled gradient echo (FSPGR) with magnetization-prepared inversion recovery (IR) pulse; susceptibility-weighted imaging (SWI); and SE T1-WI both with and without a single dose intravenous bolus of 0.1 mM/Kg

gadolinium (Gd)-DTPA (Gd-DTPA given only to MS subjects). All scans were prescribed in an axial-oblique orientation, parallel to the subcallosal line. One average was used for all pulse sequences.

With the exception of SWI, all sequences were acquired with a 256 x 192 matrix (freq.xphase), field-of-view (FOV) of 25.6 cm x 19.2 cm (256x256 matrix with phase FOV=0.75), for an in-plane resolution of 1x1mm. For all 2D scans (PD/T2, FLAIR and SE T1), we collected 48 slices, (3mm thickness, no gaps between slices.) For the 3D HIRES IR-FSPGR, we acquired 184 locations, (1mm thick, providing for isotropic resolution.)

Other relevant parameters were as follows: for dual FSE PD/T2, echo and repetition times (TE and TR) TE1/TE2/TR=9/98/5300ms, flip angle (FA)=90°, echo train length ETL=14; for FLAIR, TE/TI/TR=120/2100/8500 ms (inversion time, IT), FA=90°, ETL=24; for SE T1-WI, TE/TR=16/600 ms, FA=90°; for 3D HIRES T1-WI, TE/TI/TR=2.8/900/5.9 ms, FA=10°.

All analyses were performed by operators blinded to participants' disease status. SWI was acquired using a 3D flow-compensated gradient echo (GRE) sequence with 64 partitions, 2mm thickness, a 512x192 matrix, FOV= 25.6cm x 19.2cm (512x256 matrix with Phase FOV=0.75), for an in-plane resolution of 0.5x1 mm, flip angle FA=12°, TE/TR=22/40 ms.

Global atrophy and lesion analyses. The SIENAX cross-sectional software tool (version 2.6) was used, with correction for T1-hypointensity misclassification, for brain extraction and tissue segmentation on 3D-T1-WI¹². We acquired normalized measures of whole brain volumes (NBV), gray matter volumes (NGMV) and white matter volumes (NWMV). T2- and T1- lesion volumes (LV) were measured on FLAIR and SE T1-WI, respectively, using a semi-automated edge detection contouring/thresholding technique, (previously described¹³). Normalized volumes were obtained for all subcortical deep gray matter (SDGM) structures with FMRIB's integrated registration and segmentation tool (FIRST) on 3D-T1-WI¹⁴.

Abnormal phase identification. SDGM structures were segmented using a combination of semi-automated edge-contouring and FIRST on 3D T1-WI¹⁵. The thalamus, caudate, putamen, globus pallidus, hippocampus, amygdala, and nucleus accumbens were identified using this approach.¹⁴ Structures not identifiable by FIRST, (red nucleus, pulvinar nucleus of the thalamus, substantia nigra, were identified semi-automatically using JIM5 (Xinapse Systems, Northamptonshire, UK) on the most representative slice for each subject¹⁴ Using these methods, it has been determined that segmentation of separate SDGM regions can be reliably reproduced¹².

A detailed overview of SWI processing, reproducibility and analysis methods has been previously described¹². Briefly, using SWI-filtered phase images, processing was performed to identify voxels likely to contain iron, based on their mean phase values. Images were thresholded to retain only voxels with phase values ≤ 2 SDs below the reference mean-values of each structure. As a measure of the degree of phase decrease, mean values of sub-threshold voxels of the mean phase of low phase voxels (MP-LPV) were calculated. More negative (decreased) MP-LPV values suggest increased regional iron content. Mean values are presented in radians.

Statistical analysis. We analyzed correlations between MRI and MPs using the SPSS 11.5 statistical package (SPSS Inc. Chicago, IL). Quantitative data are presented as mean $\pm$ SD or median. Data were compared by 2-tailed t- test and non-parametric Mann–Whitney *U*-testing between groups. Data are expressed as rate and compared by χ^2 test. Pearson or Spearman-correlation analyses were conducted for variables with trends towards correlation. Multivariate-linear regressions were adopted for multiple factor analysis. A $p < 0.05$ was considered statistically significant.

Results.

Demographic, clinical and MRI characteristics of participants.

44 patients with RRMS, 16 with SPMS, and 36 healthy controls (HC) participated in the study. Study subjects with MS had a mean age of 48.1 years ($\pm$ SD=10.6, median 50), at study entry, with mean disease duration of 139.2 months (RRMS) and 277.2 months (SPMS). The mean age of HC subjects was 42.8 $\pm$ 12.7 (SD) years (median=47). 45 MS patients were female and 15 were male. Demographic features are presented in [Table 2](#).

Different MP types identified. Several different MP types were identified in this study based on their surface expression of CD31 (PECAM-1), CD51/61 (integrin α V/ β 3) and CD54 (ICAM-1) (shown in Table 1). The different combination of these biomarkers expressed on these microparticles indicates different cellular (platelet, endothelial or immune cells) and spatial origins (apical, lateral or basolateral surfaces) of the MPs based on the combinations of markers identified on the MP surface by FACS analysis. For example, type 1 MPs were CD31⁺/CD51⁻/CD61⁻/CD54⁻ consistent with these MPs originating from either platelets or from endothelial apical and lateral surfaces. Type 2 MPs were CD31⁺/CD51⁺/CD61⁺/CD54⁻ which could be derived from apical, lateral and basolateral surfaces of non-activated endothelial cells. Conversely, type 3 MPs were CD31⁺/CD51⁺/CD61⁺/CD54⁺ and would be expected to be derived from apical, lateral and basolateral surfaces of

activated endothelial cells. Type 4 MPs were $CD31^+/CD51^-/CD61^-/CD54^+$ which should derive from endothelial basolateral surfaces. Type 5 MPs were $CD31^-/CD51^+/CD61^+/CD54^-$ and could be derived from either activated endothelial apical surfaces or from the surface of immune cells. Type 6 MPs were $CD31^-/CD51^+CD61^+/CD54^+$ indicating their potential origin from the basolateral surfaces of activated endothelial cells. Lastly, type 7 MPs were $CD31^-/CD51^-/CD61^-/CD54^+$ which should be derived from either the apical surfaces of activated endothelial cells or immune cells.

Microparticle characteristics of study participants. We found that $CD31^+/CD51^+/CD61^+/CD54^+$ MPs (type 3) increased in RRMS ($p<0.05$, figure 1a) but not SPMS. Conversely, $CD31^+/CD51^-/CD61^-/CD54^-$ MPs (type 1) decreased in SPMS ($p<0.05$, figure 1b) but not RRMS. **Group 3** ($CD31^+/CD51^+/CD61^+/CD54^+$) MP. Group 3 MPs also positively correlated with group 7 ($C = 0.563$, $p=0.001$) and group 2 MP ($C = 0.353$, $p<0.05$). Group 3 MPs may have predictive value in evaluating RRMS.

Relationship between microparticle characteristics and conventional MRI and advanced iron content imaging outcomes. Of the 7 types of Annexin-V⁺ MPs identified by flow cytometry analysis, and described in **Table 1**, groups 5 ($CD31^-/CD51^+/CD61^+/CD54^-$) and 7 ($CD31^-/CD51^-/CD61^-/CD54^+$) were correlated with several MRI parameters in MS including T1-LV, NBV, NGMV and MP-LPV (iron deposition) in the caudate nucleus.

T1-LV. Table 4 shows that while T1-LV was positively correlated with group 5 MPs in RRMS (Corr.= 0.358, $p=0.035$). No T1-LV correlation was observed in HC. T1-LV was not correlated with group 5 (or other MP species) in MS. This suggests that the group 5 MP - T1-LV association seen in RRMS may differ from MS in general or SPMS. Several clinical phenomena could cause T1-hypointensity in RRMS including elevated tissue methemoglobin, lipid and protein levels and calcium and iron deposition¹⁶. The relationship between T1-LV and group 5 MP may signify one or more of these MRI imaging features in RRMS.

NBV. Similarly, group 5 MPs were also significantly (positively) correlated with normalized brain volume (NBV) in MS ($C=0.32$, $p<0.05$, Spearman's rho); there was no significant correlation in HC. Similarly, NBV and group 5 MPs were also significantly (positively) correlated in RRMS ($C=0.37$, $p<0.05$). This finding was unexpected and may indicate that this MP specie may reflect brain edema often a characteristic of MS¹⁷, also seen in experimental MS¹⁸. Such MP changes could precede BBB disturbances caused by aquaporin-4 or VEGF-A activation in neuromyelitis optica and MS, respectively.

MP-LPV. A striking finding in comparisons between MP species and MS/RRMS was that group 7 MPs were significantly and negatively correlated with MP-LPV, indicating iron deposition in the caudate of patients with MS ($C = -0.304$, $p < 0.05$, Spearman's ρ) and in RRMS ($C = -0.35$, $p < 0.05$); (no correlation in HC) (**Table-5**). Elevated iron in the caudate and globus pallidus has been previously reported in Alzheimer's disease¹⁹, and in the caudate (and putamen) in cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)²⁰.

In healthy controls, some significant negative associations between tissue iron loading and microparticle species were also observed. While not certain, these data suggest that these MP may be associated with iron transport, or potentially reduce overall iron burden in MS. Group 4 ($CD31^+/CD51^-/CD61^-/CD54^+$) MP were negatively correlated with globus iron deposition ($C = -0.53$, $p = 0.002$) in healthy controls (**Table-5**). It is intriguing to speculate that if MPs can carry iron away from the CNS, elevated microparticles may represent an early adaptive response to oxidative stress. Therefore, both elevations and reduction in particular MP species could prove useful as indicators of MS disease activity or response to therapy.

Discussion.

Currently, MRI of the neuroaxis is the only accepted objective tool for measuring disease activity in MS. However, MRI remains a complex and time-consuming clinical test. On the other hand, measurement of circulating MP in MS patients could provide an additional simple and accessible instrument to supplement MRI of the brain and spinal cord. While MRI remains a powerful imaging tool, MP analysis may help to refine interpretation for functional and biochemical information in MS. Perivascular deposits of iron in MS suggest MS pathophysiology involves forms of vascular stress that include iron deposition. Iron deposition signatures in MS, combined with evidence of endothelial stress could help to mechanistically link these phenomena. MP profiling with MR iron imaging may assist neurologists and clinicians in making decisions about: 1) which groups of patients might respond to or fail specific therapies. Here we have seen evidence of several forms of endothelial stress in MS association with several conventional and novel MR imaging findings.

RRMS. In RRMS, several interesting relationships were observed between circulating microparticles and iron content in several brain regions. Table 5 shows that Group 2 ($CD31^+/CD51^+/CD61^+/CD54^-$) MP were significantly correlated with deep GM MP-LPV (Corr. 0.344, $p = 0.035$), there was not a significant correlation in healthy controls. Group 2 MP were also correlated with thalamus MP-LPV (Corr. coeff. 0.413, $p = 0.01$) in RRMS, also

indicating a significant elevation of iron content in these regions in RRMS. Again group 2 MP were not correlated with thalamus MP-LPV in healthy controls.

Interestingly, in RRMS, $CD31^{-}/CD51^{-}/CD61^{-}/CD54^{+}$ MP were negatively correlated with caudate nucleus MP-LPV ($C=-0.354$, $p<0.05$) with no correlation in healthy controls. **(Table-5)**. $CD54^{+}$ MP (without $CD31/CD51/CD61$) in RRMS could indicate endothelial or immune cell fragmentation. Correlation of these particles with caudate nucleus iron could thus reflect MS-associated loss of barrier function allowing erythrocyte extravasation or other forms of iron accumulation (microglia, macrophages) in the brain. RRMS showed a correlation between group 1 ($CD31^{+}/CD51^{-}/CD61^{-}/CD54^{-}$) MP and red nucleus MP-LPV ($C =0.354$, $p<0.05$, no correlation in HC.)

SPMS. In SPMS, group 7 MP ($CD31^{-}/CD51^{-}/CD61^{-}/CD54^{+}$) were positively correlated with globus pallidus MP-LPV ($C= 0.556$, $p<0.05$), consistent with elevated iron in this region in RRMS and SPMS **(Table-5)**. Interestingly, in SPMS, $CD31^{-}/CD51^{+}/CD61^{+}/CD54^{-}$ MP were correlated with putamen MP-LPV ($C=0.697$, $p<0.05$,) **(Table-5)**. These findings were also consistent with elevated putamen iron content. Because $CD31^{-}/CD51^{+}/CD61^{+}/CD54^{-}$ MP may reflect endothelial denudation, correlation of this species with iron deposition could reflect endothelial barrier failure that permits leakage of iron-bearing cells and molecules into the parenchyma to intensify neurovascular injury. Correlation of specific circulating MP may therefore be useful indicators of MS activity relevant to particular lesions and iron deposition signatures in particular regions of the brain.

In MS, $CD51^{+}/CD61^{+}$ MPs were associated with CNS iron signatures consistent with vascular injury reducing the barrier against iron-bearing blood cells and components. Inappropriate iron uptake into the brain parenchyma generates oxidants species that perturb barrier function and exacerbate immune-cell extravasation in MS. The differing patterns of circulating endothelial MPs in RRMS and SPMS suggest independent vascular etiologies for these conditions. While perivascular iron deposits are linked with MS lesions, it is still not entirely clear whether iron deposits initiate injury or are markers of later barrier instability.²¹

Iron accumulation in the CNS in MS has been described since the 1980s,^{22–24}; but its significance in MS pathogenesis remains unclear. It is still controversial whether iron deposits are an epiphenomenon (following demyelination, neuronal/axonal loss or venous injury) or an initiating/exacerbating factor underlying inflammatory cascades in MS. The most important issues regarding iron in MS are the source(s) and scheduling of its deposition. Does iron trigger and modify the course of MS neuroinflammation? Iron is essential to many CNS processes including neurotransmitter synthesis and myelination²⁵, yet several studies correlate iron deposits with

neurodegenerative diseases, including MS^{23,25,26}. In MS, free-iron derived from myelin/oligodendrocyte debris, as a byproduct of micro-hemorrhages, or released during macrophage decomposition²⁵ generates oxidants, activates macrophage and T-cell recruitment to maintain MS inflammation. Both macrophages and T-cells are linked with iron overload in MS²⁷. Vascular congestion and diminished blood flow in MS also increase the burden of inflammatory cells, fibrin deposits and other factors which retain iron^{24,28}. MR imaging profiles are effective in determining how iron deposits fit into this model: as a cause or consequence of MS inflammatory reactions.

Deep GM structures are affected by iron accumulation, which triggers unremitting inflammatory responses and damage in MS²⁹. We previously correlated endothelial MP levels in RRMS with contrast-enhancing lesions⁴ and later demonstrated that MP binding to monocytes facilitates trans-endothelial extravasation. A concept emerging from these studies is that in MS, circulating MP and possible MP of endothelial origin may identify and exacerbate CNS inflammatory responses. Our findings now correlate several circulating MP groups (some of possible vascular origin) with iron deposits in subcortical deep GM structures. Elevated circulating MP and iron deposition in the MS-inflamed CNS may cooperatively initiate, and sustain immune cascades via inflammatory cell (macrophage/microglia) derived-oxidants, release of MMPs and inflammatory cytokines³⁰. Although CNS iron accumulation increases susceptibility to neurovascular injury proportionate with age, the point at which brain iron-content crosses an irreversible threshold, triggering persistent neurovascular inflammation is unclear. Larger prospective, longitudinal studies correlating iron-imaging with more focused MP analysis to pinpoint the parent cells of the circulating MP, are required to more accurately validate these findings and establish a timeline for these events in MS pathophysiology.

Literature Cited

1. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest*. 2012;122(4):1180-1188. doi:10.1172/JCI58649.
2. Prosperini L, Gianni C, Barletta V, et al. Predictors of freedom from disease activity in natalizumab treated-patients with multiple sclerosis. *J Neurol Sci*. 2012;323(1-2):104-112. doi:10.1016/j.jns.2012.08.027.
3. Wee Yong V. Inflammation in neurological disorders: a help or a hindrance? *Neuroscientist*. 2010;16(4):408-420. doi:10.1177/1073858410371379.
4. Minagar A, Jy W, Jimenez JJ, et al. Elevated plasma endothelial microparticles in multiple sclerosis. *Neurology*. 2001;56(10):1319-1324.
5. Steinman L. Platelets provide a bounty of potential targets for therapy in multiple sclerosis. *Circ Res*. 2012;110(9):1157-1158. doi:10.1161/CIRCRESAHA.112.269050.

6. Jung K-H, Chu K, Lee S-T, et al. Circulating endothelial microparticles as a marker of cerebrovascular disease. *Ann Neurol*. 2009;66(2):191-199. doi:10.1002/ana.21681.
7. Lowery-Nordberg M, Eaton E, Gonzalez-Toledo E, et al. The effects of high dose interferon- β 1a on plasma microparticles: correlation with MRI parameters. *J Neuroinflammation*. 2011;8:43. doi:10.1186/1742-2094-8-43.
8. Bader BL, Rayburn H, Crowley D, Hynes RO. Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all α v integrins. *Cell*. 1998;95(4):507-519.
9. Achen MG, Clauss M, Schnürch H, Risau W. The non-receptor tyrosine kinase Lyn is localised in the developing murine blood-brain barrier. *Differentiation*. 1995;59(1):15-24. doi:10.1046/j.1432-0436.1995.5910015.x.
10. Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol*. 2011;69(2):292-302. doi:10.1002/ana.22366.
11. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology*. 1996;46(4):907-911.
12. Zivadinov R, Heinen-Brown M, Schirda CV, et al. Abnormal subcortical deep-gray matter susceptibility-weighted imaging filtered phase measurements in patients with multiple sclerosis: a case-control study. *Neuroimage*. 2012;59(1):331-339. doi:10.1016/j.neuroimage.2011.07.045.
13. Zivadinov R, Rudick RA, De Masi R, et al. Effects of IV methylprednisolone on brain atrophy in relapsing-remitting MS. *Neurology*. 2001;57(7):1239-1247.
14. Batista S, Zivadinov R, Hoogs M, et al. Basal ganglia, thalamus and neocortical atrophy predicting slowed cognitive processing in multiple sclerosis. *J Neurol*. 2012;259(1):139-146. doi:10.1007/s00415-011-6147-1.
15. Patenaude B, Smith SM, Kennedy DN, Jenkinson M. A Bayesian model of shape and appearance for subcortical brain segmentation. *Neuroimage*. 2011;56(3):907-922. doi:10.1016/j.neuroimage.2011.02.046.
16. Ginat DT, Meyers SP. Intracranial lesions with high signal intensity on T1-weighted MR images: differential diagnosis. *Radiographics*. 2012;32(2):499-516. doi:10.1148/rg.322105761.
17. Balashov KE, Aung LL, Dhib-Jalbut S, Keller IA. Acute multiple sclerosis lesion: conversion of restricted diffusion due to vasogenic edema. *J Neuroimaging*. 2011;21(2):202-204. doi:10.1111/j.1552-6569.2009.00443.x.
18. Lepore S, Waiczies H, Hentschel J, et al. Enlargement of cerebral ventricles as an early indicator of encephalomyelitis. *PLoS ONE*. 2013;8(8):e72841. doi:10.1371/journal.pone.0072841.
19. Bartzokis G, Sultzer D, Mintz J, et al. In vivo evaluation of brain iron in Alzheimer's disease and normal subjects using MRI. *Biol Psychiatry*. 1994;35(7):480-487.

20. Liem MK, Lesnik Oberstein SAJ, Versluis MJ, et al. 7 T MRI reveals diffuse iron deposition in putamen and caudate nucleus in CADASIL. *J Neurol Neurosurg Psychiatr.* 2012;83(12):1180-1185. doi:10.1136/jnnp-2012-302545.
21. Zivadinov R, Weinstock-Guttman B, Pirko I. Iron deposition and inflammation in multiple sclerosis. Which one comes first? *BMC Neurosci.* 2011;12:60. doi:10.1186/1471-2202-12-60.
22. Drayer B, Burger P, Hurwitz B, Dawson D, Cain J. Reduced signal intensity on MR images of thalamus and putamen in multiple sclerosis: increased iron content? *AJR Am J Roentgenol.* 1987;149(2):357-363. doi:10.2214/ajr.149.2.357.
23. Craelius W, Migdal MW, Luessenhop CP, Sugar A, Mihalakis I. Iron deposits surrounding multiple sclerosis plaques. *Arch Pathol Lab Med.* 1982;106(8):397-399.
24. Adams CW. Perivascular iron deposition and other vascular damage in multiple sclerosis. *J Neurol Neurosurg Psychiatr.* 1988;51(2):260-265.
25. Stankiewicz J, Panter SS, Neema M, Arora A, Batt CE, Bakshi R. Iron in chronic brain disorders: imaging and neurotherapeutic implications. *Neurotherapeutics.* 2007;4(3):371-386. doi:10.1016/j.nurt.2007.05.006.
26. LeVine SM. Iron deposits in multiple sclerosis and Alzheimer's disease brains. *Brain Res.* 1997;760(1-2):298-303.
27. Petry KG, Boiziau C, Dousset V, Brochet B. Magnetic resonance imaging of human brain macrophage infiltration. *Neurotherapeutics.* 2007;4(3):434-442. doi:10.1016/j.nurt.2007.05.005.
28. Wakefield AJ, More LJ, Difford J, McLaughlin JE. Immunohistochemical study of vascular injury in acute multiple sclerosis. *J Clin Pathol.* 1994;47(2):129-133.
29. Al-Radaideh AM, Wharton SJ, Lim S-Y, et al. Increased iron accumulation occurs in the earliest stages of demyelinating disease: an ultra-high field susceptibility mapping study in Clinically Isolated Syndrome. *Mult Scler.* 2013;19(7):896-903. doi:10.1177/1352458512465135.
30. Williams R, Rohr AM, Wang W-T, et al. Iron deposition is independent of cellular inflammation in a cerebral model of multiple sclerosis. *BMC Neurosci.* 2011;12:59. doi:10.1186/1471-2202-12-59.

Figure 1) A. CD31⁺/CD51⁺/CD61⁺/CD54⁺ microparticles were elevated in RRMS, but not in SPMS. B. CD31⁺/CD51⁻/CD61⁻/CD54⁻ microparticles were decreased in SPMS, but not RRMS. (Mann-Whitney U-test, 2-tailed.)

figure 1

Table 1. Possible origins of MPs observed in flow cytometry

Species	Microparticle type	PECAM/ CD31 ⁺	aV/b3 CD51+CD61 ⁺	ICAM-1 CD54 ⁺	Possible MP Origin
1	CD31 ⁺ /CD51 ⁻ /CD61 ⁻ /CD54 ⁻	+	-	-	Platelet or endothelial apical/lateral surfaces
2	CD31 ⁺ /CD51 ⁺ /CD61 ⁺ /CD54 ⁻	+	+	-	Non-activated endothelial apical / lateral/basolateral surfaces
3	CD31 ⁺ /CD51 ⁺ /CD61 ⁺ /CD54 ⁺	+	+	+	Activated endothelial apical / lateral / basolateral surfaces
4	CD31 ⁺ /CD51 ⁻ /CD61 ⁻ /CD54 ⁺	+	-	+	Endothelial basolateral surface
5	CD31 ⁻ /CD51 ⁺ /CD61 ⁺ /CD54 ⁻	-	+	-	Activated endothelial apical surface / immune cell surface
6	CD31 ⁻ /CD51 ⁺ /CD61 ⁺ /CD54 ⁺	-	+	+	Activated endothelial basolateral surfaces
7	CD31 ⁻ /CD51 ⁻ /CD61 ⁻ /CD54 ⁺	-	-	+	Activated endothelial apical surface / immune cells

Table 2. Demographic, clinical and conventional MRI characteristics of healthy controls and multiple sclerosis patients.

	HC (n = 36)	MS (n = 60)	p value	RRMS (n = 44)	SPMS (n = 16)	p value
Sex, female, n (%)	18 (50)	45 (75)	0.028	32 (72.7)	13 (81.2)	0.378
Age, yrs, mean (SD) median	42.8 (12.7) 47	48.1 (10.6) 50	0.03	45 (10.2) 46.5	56.6 (6.1) 58	<0.001
Age at onset, yrs, mean (SD) median	NA	33.4 (9.8) 33	NA	33.5 (8.8) 33	33.5 (12.6) 34	0.988
Disease duration, yrs, mean (SD) median	NA	14.6 (10.9) 11	NA	11.6 (8.3) 9	23.1 (12.7) 21	<0.001
EDSS, mean (SD) median	NA	3.1 (2.2) 2.5	NA	2.0 (1.2) 2.0	5.8 (1.5) 6.0	<0.001
Duration of DMT in months, mean (SD) median	NA	47.6 (47.4) 29	NA	42.8 (47.2) 25.5	62.4 (46.5) 52	0.163
T2-LV, mean (SD) median	1.2 (1.6) 0.8	15.1 (16.2) 10.6	<0.001	13.9 (14.4) 10.2	18.6 (20.4) 14.1	0.318
T1-LV, mean (SD) median	NA	2 (5.6) 0.5		1.2 (2.1) 0.3	4.3 (9.9) 1.5	0.05
NBV, mean (SD) median	1602.4 (87.4) 1605	1527.6 (89.6) 1533	<0.001	1546.4 (72.4) 1544	1473.6 (111.2) 1504	0.004
NGMV, mean (SD) median	847.9 (69) 824.9	769.7 (63.5) 792.9	0.01	806.4 (56.3) 799	773.7 (77) 776.1	0.07
NWMV, mean (SD) median	754.5 (65.8) 756	729.3 (60.8) 728.5	0.06	740 (61.7) 730	700 (61.6) 716	0.023

Legend= HC: healthy controls; MS: multiple sclerosis; RRMS: relapsing-remitting; SPMS: secondary-progressive MS; EDSS: Expanded Disability Status Scale; NA: not available; DMT: disease-modifying treatment; LV: lesion volume; NBV: normalized brain volume; NGMV: normalized gray matter volume; NWMV: normalized white matter volume; SD: standard deviation. The volumes are expressed in milliliters. Statistical analysis between MRI variables of MS patients and HC groups was performed using analysis of co-variance adjusted for age and gender, while the analysis between RRMS and SPMS patients was performed using Student's *t*-test, test and Mann-Whitney *U* test, as appropriate. Categorical differences were tested using chi-square.

Table 3. Mean phase of low phase voxels (MP-LPV) in multiple sclerosis patients and in healthy controls.

	HC (n = 36)	MS (n = 60)	p value	RRMS (n = 44)	SPMS (n = 16)	p value
Total DGM	-0.148 (0.02) - 0.147	-0.159 (0.02) - 0.155	0.025	-0.154 (0.02) - 0.153	-0.172 (0.03) - 0.172	0.011
Caudate	-0.169 (0.01) - 0.169	-0.182 (0.02) - 0.180	0.001	-0.179 (0.02) - 0.176	-0.190 (0.01) - 0.190	0.05
Putamen	-0.178 (0.03) - 0.169	-0.197 (0.04) - 0.193	0.02	-0.192 (0.04) - 0.186	-0.213 (0.04) - 0.218	0.085
Globus Pallidus	-0.183 (0.02) - 0.178	-0.187 (0.03) - 0.181	0.425	-0.182 (0.02) - 0.180	-0.202 (0.04) - 0.199	0.019
Thalamus	-0.094 (0.01) - 0.095	-0.099 (0.02) - 0.098	0.123	-0.097 (0.01) - 0.097	-0.103 (0.02) - 0.102	0.236
Hippocampus	-0.171 (0.05) - 0.149	-0.169 (0.05) - 0.159	0.846	-0.163 (0.04) - 0.151	-0.186 (0.05) - 0.160	0.102

Legend= HC: healthy controls; MS: multiple sclerosis; RRMS: relapsing-remitting; SPMS: secondary-progressive MS; SDGM: subcortical deep gray matter; SD: standard deviation. The data are presented as mean (SD) median. The volumes are expressed in radians. Statistical analysis between MP-LPV variables of MS patients and HC groups was performed using analysis of co-variance adjusted for age and gender, while the analysis between RRMS and SPMS patients was performed using Student's t-test.

Table 4. Correlation of CD31⁺/CD51⁺/CD61⁺/CD54⁻ and CD31⁻/CD51⁻/CD61⁻/CD54⁺ MP groups with conventional MRI measures and MP-LPV (iron deposition) in MS and RRMS.

	T1-LV CD31- /CD51+/CD61+/CD54-	NBV CD31- /CD51+/CD61+/CD54-	NGMV CD31- /CD51+/CD61+/CD54-	Caudate MP-LPV CD31 ⁻ /CD51 ⁻ /CD61 ⁻ /CD54 ⁺
MS	(No correlation)	HC C=-.083, P=0.64 N=33	HC C= -.081, P=0.65 N=33	HC C=0.118, P=0.51 N=33
		MS C=0.32, P=0.02 N=52	MS C=0.31, P=0.026 N=52	MS C=-0.304, p=0.028 N=52
RRMS	HC (No correlation)	HC C= -.083, P=0.64 N=33	(No correlation)	HC C= -0.011 p=0.951 N=33
	RRMS C=0.358, P=0.035 N=35	RRMS C=0.37, P=0.02 N=38		RRMS C= -.35, p=0.029 N=38

Legend= HC: healthy controls; MS: multiple sclerosis; RRMS: relapsing-remitting; N- number; LV-lesion volume; NBV-normalized brain volume; NGMV-normalized gray matter volume; MP-LPV-mean phase of low phase voxels. Comparison of T1-LV, and normalized brain volume (NBV), normalized gray matter volume (NGMV) and caudate iron content with different microparticle species in multiple sclerosis (MS) and relapsing-remitting multiple sclerosis (RRMS). CD31⁺/CD51⁺/CD61⁺/CD54⁻ microparticles and CD31⁻/CD51⁻/CD61⁻/CD54⁺ was performed using *Spearman's rho test*.

MP Profile	Particle type	Brain Region	Correlation	P-value	Condition
CD31+/CD51+/CD61+/CD54+	3	White Matter	0.276	0.011	All
CD31+/CD51+/CD61+/CD54+	3	Thalamus	0.348	0.047	HC
CD31+/CD51-/CD61-/CD54+	4	Globus	-0.53	0.002	HC
CD31-/CD51-/CD61-/CD54+	7	Caudate	-0.304	0.028	MS
CD31+/CD51+/CD61+/CD54+	3	White Matter	0.292	0.036	MS
CD31+/CD51+/CD61+CD54-	2	Deep Gray Matter	0.374	0.035	RRMS
CD31+/CD51+/CD61+/CD54-	2	Thalamus	0.413	0.01	RRMS
CD31-/CD51-/CD61-/CD54+	7	Caudate	-0.354	0.029	RRMS
CD31-/CD51-/CD61-/CD54+	7	Globus	0.556	0.039	SPMS
CD31-/CD51+/CD61+/CD54-	5	Putamen	0.697	0.006	SPMS

Table 5. MP-LPV correlations with microparticle profile, brain region, correlation, p-value and clinical condition.