RESEARCH ARTICLE
Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis [version 1; referees: 1 approved, 2 approved with reservations]

Alex Lewin¹,⁵*, Shea Hamilton²*, Aviva Witkover², Paul Langford², Richard Nicholas³, Jeremy Chataway⁴, Charles R.M. Bangham ²

¹Department of Epidemiology and Biostatistics, Imperial College London, London, UK
²Division of Infectious Diseases, Department of Medicine, Imperial College London, London, UK
³Division of Brain Sciences, Department of Medicine, Imperial College London, London, UK
⁴National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Foundation Trust and Queen Square Multiple Sclerosis Centre, Department of Neuroinflammation, University College London, London, UK
⁵Present address: Department of Mathematics, Brunel University, London, UK

* Equal contributors

Abstract
Background
A major cause of disability in secondary progressive multiple sclerosis (SPMS) is progressive brain atrophy, whose pathogenesis is not fully understood. The objective of this study was to identify protein biomarkers of brain atrophy in SPMS.

Methods
We used surface-enhanced laser desorption-ionization time-of-flight mass spectrometry to carry out an unbiased search for serum proteins whose concentration correlated with the rate of brain atrophy, measured by serial MRI scans over a 2-year period in a well-characterized cohort of 140 patients with SPMS. Protein species were identified by liquid chromatography-electrospray ionization tandem mass spectrometry.

Results
There was a significant (p<0.004) correlation between the rate of brain atrophy and a rise in the concentration of proteins at 15.1 kDa and 15.9 kDa in the serum. Tandem mass spectrometry identified these proteins as alpha-haemoglobin and beta-haemoglobin, respectively. The abnormal concentration of free serum haemoglobin was confirmed by ELISA (p<0.001). The serum lactate dehydrogenase activity was also highly significantly raised (p<10⁻¹²) in patients with secondary progressive multiple sclerosis.

Conclusions
An underlying low-grade chronic intravascular haemolysis is a potential source of the iron whose deposition along blood vessels in multiple sclerosis plaques contributes to the neurodegeneration and consequent brain atrophy seen in progressive disease. Chelators of free serum iron will be ineffective in

Open Peer Review

Referee Status:  ✔  ?  ?

Invited Referees

version 1
published 15 Nov 2016

1 Hans Lassmann, Medical University of Vienna Austria, Simon Hametner, Medical University of Vienna Austria
2 George Harauz, University of Guelph Canada, Vladimir V. Bamm, University of Guelph Canada
3 Franz Fazekas, Medical University of Graz Austria, Michael Khali, Medical University of Graz Austria

Discuss this article
Comments (0)
preventing this neurodegeneration, because the iron (Fe$^{2+}$) is chelated by haemoglobin.

Corresponding authors: Alex Lewin (Alex.Lewin@brunel.ac.uk), Shea Hamilton (s.hamilton@imperial.ac.uk), Charles R.M. Bangham (c.bangham@imperial.ac.uk)

How to cite this article: Lewin A, Hamilton S, Witkover A et al. Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis [version 1; referees: 1 approved, 2 approved with reservations] Wellcome Open Research 2016, 1:10 (doi: 10.12688/wellcomeopenres.9967.1)

Copyright: © 2016 Lewin A et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Grant information: This work was supported by the Wellcome Trust [100291]; Medical Research Council [MR K019090]; National Institute of Health Research; University College London Hospitals/UCL Biomedical Research Centre. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 15 Nov 2016, 1:10 (doi: 10.12688/wellcomeopenres.9967.1)
Introduction
In multiple sclerosis (MS), progressive disease develops in over half of those who present with an initial relapsing phase – secondary progressive MS (SPMS) – but can also present as primary progressive MS (PPMS). Progressive MS, for which there is no clear disease-modifying treatment1–3, accounts for much of the disability and the cost of MS to both the person and the community4.

Unlike relapsing-remitting MS (RRMS), where an inflammatory response involving the adaptive immune system leads to episodic neurological deficits, in progressive MS neuroaxonal loss leads to an increasing neurological deficit and brain atrophy5–6. However, in all forms of the disease, both the initiating events and the mechanisms of pathogenesis remain uncertain7. Pseudoatrophy may account for some loss of brain volume8, but brain atrophy has also been associated with changes in neurofilament levels9 and sodium metabolism9.

The objective of the present study was to use an unbiased, high-throughput technique to identify protein biomarkers of brain atrophy in a longitudinal cohort of patients with SPMS. We used surface-enhanced laser desorption-ionization time-of-flight (SELDI-TOF) mass spectrometry to analyse serial serum samples from the population that participated in the MS-STAT study (described below)1, to identify proteins whose abundance was associated with MRI-measured brain atrophy rate. Serum proteomics in MS has previously been investigated in small cross-sectional studies to compare relapsing MS and progressive disease10–12. However, these previous studies were neither designed nor powered to identify correlates of neurodegeneration in SPMS.

We found that the rate of brain atrophy in this cohort was associated with an increase in the concentration of free haemoglobin in the serum. This association was independent of the beneficial effect of simvastatin treatment, which remained significant in the present analysis. An ELISA assay confirmed the presence of abnormal concentrations of free haemoglobin in the serum of patients with SPMS. In addition, the serum lactate dehydrogenase (LDH) activity was significantly greater in patients with SPMS than in three different groups of control subjects. These results suggest that chronic intravascular haemolysis releases haemoglobin into the serum in SPMS; we postulate that this haemoglobin is a source of the abnormal iron deposition along blood vessels in the central nervous system that is associated with neurodegeneration in progressive MS.

Methods
Ethical approval
The study was done in accordance with Good Clinical Practice and the Declaration of Helsinki. The protocol was approved by the UK National Research Ethics Service (Berkshire Research Ethics Committee; reference 07/Q1602/73), and every patient gave written informed consent before entering the study.

Subjects
The MS-STAT clinical trial was registered with ClinicalTrials.gov (NCT00647348) and has been described in detail elsewhere1. In this phase 2 placebo-controlled double-blind trial, 140 patients with SPMS were randomized 1:1 to simvastatin 80 mg/day (40 mg for the first month) or matched placebo. The patients were in trial for 2 years. The primary outcome was change in whole brain volume as measured by the Brain Boundary Shift Integral (BBSI), with MRI data acquired at baseline, 12 months and 25 months; the last MRI scan (25 months) was carried out 1 month after last medication to minimize any potential artefactual changes in volume1. Simvastatin treatment resulted in a highly statistically significant 43% reduction in the annualized rate of brain atrophy1, and significant changes were also seen in certain clinician- and patient-reported outcome measures. As control groups in the haemoglobin assays, we studied healthy adult volunteers (n=20); patients with human T-lymphotropic virus (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP), which closely resembles chronic spinal forms of multiple sclerosis (n=20); and asymptomatic HTLV-1 carriers (n=20).

Protein profiling of serum by SELDI-TOF mass spectrometry
SELDI-TOF mass spectrometry was performed on 475 serum samples collected at baseline, 6 month, 12 months and 24 months. Samples were randomized, and staff were blinded to the treatment arms. CM10 ProteinChip arrays (Bio-Rad Laboratories) were primed with binding buffer (50 mM ammonium acetate, 0.01% Triton X-100, pH 4.0) and incubated at room temperature (RT) for 5 min. A 1:10 dilution of serum in binding buffer was then applied to the array and incubated at RT for 1 hr. The arrays were washed twice with binding buffer and deionized water. Saturated sinapinic acid (0.7 µL) was applied twice to each spot on the arrays. Time-of-flight spectra were generated using a PCS–4000 mass spectrometer (Bio-Rad). Low-range spectra (mass/charge (m/z) ratio 0 – 20,000) were obtained at a laser energy of 3000 nJ, with a focus mass of 6000 and the matrix attenuated to 1000. High-range spectra (m/z 10,000 – 75,000) were obtained at a laser energy of 3900 nJ, with a focus mass of 30,000 and the matrix attenuated to 10,000. Mass accuracy was calibrated externally using All-in-One Peptide or Protein molecular mass standards (Bio-Rad).

Proteomics data processing
Spectra were analysed using ProteinChip Data Manager (Bio-Rad version 4.1.0) and normalized using total ion current. Peaks were auto-detected using a peak threshold of 12.5% and a mass window of 0.3%, and the resulting data were converted for subsequent analysis using R software. The abundance (intensity) of a given protein peak was quantified as the area under the peak; peak intensities were log-transformed before analysis. After exclusion of one contaminated sample and 4 technical failures, the proteomics data consisted of 470 spectra from 138 patients.

Protein enrichment and identification
Ten µL serum were applied to Top 12 Abundant Protein Depletion Spin Columns (Thermo Scientific Pierce) according to the manufacturer’s protocol. Five hundred µL of the eluate were concentrated by 1D SDS-PAGE on an 18% Tris-glycine denaturing gel (TGX, Bio-Rad) at 150 V for 70 min and compared against SeeBlue Plus 2 pre-stained protein standard (Life Technologies). The gel was rinsed 5 times with deionized water and stained overnight in See Band staining solution (Gene Bio-Application Ltd.) A band...
corresponding to 15 to 16 kDa was excised and an in-gel trypsin digest was carried out.

Samples were analysed by nanoscale liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS), using a nanoAcquity UPLC system (Waters MS Technologies, Manchester, UK). Peptide identification was performed using ProteinLynx Global SERVER v3.1 (Waters).

Serum haemoglobin concentration
Free haemoglobin levels were assayed by ELISA (Abcam ab157707) according to the manufacturer’s protocol. Samples were analysed in random order, and staff were blinded to the treatment arms. Absorbance was measured at 450 nm on a SpectraMax microplate reader (Molecular Devices).

Serum lactate dehydrogenase (LDH) activity
Serum LDH activity was assayed by the conversion of lactate to pyruvate, using the absorption of light at 340 nm by the reaction product NADH (Abbott Laboratories, ref. 7D69).

Statistics
All statistical models were carried out using R 3.2.1 software. To test for associations between SELDI-TOF mass spectrometry peak intensity changes and treatment group, linear regression models were fitted separately for each spectral peak at each follow-up time (6, 12 and 24 months), modelling log(peak intensity change from baseline) as a function of baseline log(peak intensity), treatment group, and the five randomization variables (age, gender, EDSS [Expanded Disability Severity Scale], neuroscience centre, and assessing physician).

To test for associations between peak intensity changes and brain volume changes, for each pair of time points (0–12 months, 0–25 months and 12–25 months) the BBSI was compared with the change in each peak intensity. Linear regression was used to model the log(change in peak intensity) as a function of BBSI (expressed as a percentage of baseline whole-brain volume), adjusted for baseline log(peak intensity), MRI centre, and the five randomization variables.

For both treatment and brain volume analyses, sensitivity analysis was carried out using repeated-measures models including all four time points. Protein peaks whose regression coefficients differed significantly from zero (Wald test) were selected for further analysis. To take into account multiple testing, the p-value for each peak was converted into the False Discovery Rate (FDR: expected proportion of false positives) for that p-value threshold, using the R package fdrtool. Peaks at FDR ≤ 0.2 were retained for further analysis.

The results of the haemoglobin ELISA and the serum LDH assay were analysed using two-tailed Mann-Whitney tests to test for pairwise differences between the subject groups.

Results
Intensity of specific protein peaks was associated with change in brain volume
Expression-difference mapping of all longitudinal serum samples (peak threshold of 12.5%; mass window of 0.3% minimum) resulted in detection of 145 peaks that were differentially expressed within individual subjects over time.

To determine whether changes in protein levels (SELDI-TOF peak intensity) were associated with simvastatin treatment, we ran regression models for the change in each protein peak intensity vs. treatment status, adjusting for the 5 randomization variables and MRI centre. No association remained significant after adjusting for multiple comparisons (the lowest level at which the FDR could be controlled was 0.3).

We next ran regression models for change in protein intensity v. brain volume loss, for each interval in which the BBSI was measured (0 to 12 months; 12 to 25 months) and over the whole trial period (0 to 25 months). The changes in intensity of peaks at m/z = 25,110 and 25,402 were significantly associated with the change in brain volume between baseline and 12 months (p=0.003 and 0.001 respectively, corresponding to FDR = 0.08). The regression coefficients for the association were negative, i.e. an increase in these protein intensities was associated with a smaller decrease in brain volume. The change in intensity of the peaks at m/z = 15,141 and 15,885 between baseline and 25 months was significant in each case (p=0.003 and 0.001 respectively), corresponding to a FDR of 0.2. For these peaks the regression coefficients were positive, i.e. an increase in these peaks was associated with a larger decrease in brain volume (Figure 1). There were no significant regression coefficients for the 12 to 25 month time period. Repeated-measures models for the whole time period of the trial also identified the peaks at m/z = 15,141 and 15,885 as significant at an FDR of 0.2.

Multiple regression analysis, modelling brain volume change as a function of protein peak intensity, treatment status, and the five randomization variables and MRI centre as covariates confirmed (Table 1) that simvastatin treatment and the protein peak intensity were independently associated with the rate of brain atrophy.

The multiple regression model (Table 1) explained 25% of the observed variation in the rate of brain atrophy over the two-year observation period; the protein peak at 15.1 kDa alone explained 10% of this variation. The regression coefficient of -0.6 for simvastatin treatment indicates a mean difference in brain atrophy rate between treatment groups of -0.6% over the 2-year trial period; this estimate (-0.3%/year) is consistent with the rate of -0.25%/year previously reported in the MS-STAT trial. The regression coefficient of 0.75 for BBSI v. protein change means that two patients whose protein increases differ by 30% have an expected difference in brain atrophy rate of 0.1% over two years (the patient with higher increase in protein 15.1kDa expects a greater decrease in brain volume).
Identification of proteins associated with brain atrophy

After enrichment, the intensity of the protein peaks at 25.1 kDa and 25.4 kDa remained insufficient to allow their isolation and identification. However, the peaks at 15.1 kDa and 15.9 kDa remained at high intensities and distinct from nearby peaks (Figure 2). LC-MS/MS identified twenty-six peptide fragments matching human proteins: 15 fragments corresponded to human α-haemoglobin and the remaining 11 fragments corresponded to β-haemoglobin (Table 2; Supplementary Table 1). Of the remaining 364 sequence matches (after exclusion of bacterial sequences and the common contaminant keratin), the top 360 were partial matches to haemoglobin subunits of other mammalian species.

ELISA confirms the presence of free serum haemoglobin in MS patients

We assayed free haemoglobin by ELISA in MS patients (n=20) and in three control groups (n=20 in each group; Materials and Methods). The results (Figure 3A) showed significantly higher...
concentrations of free haemoglobin in the serum from MS patients, when compared to each control group (p<0.001 in each comparison; Mann-Whitney). Of the 20 MS patients assayed, 17 had a serum haemoglobin concentration greater than the mean + 2 standard errors of the healthy adult controls. No significant difference was observed between the three control groups.

Abnormally high serum LDH activity in MS patients
The presence of free haemoglobin in the serum in MS patients suggested a degree of intravascular haemolysis in these individuals. To seek corroborative evidence of haemolysis, we assayed the serum LDH activity. The median LDH activity in the patients with MS was significantly greater than that in each of the three control groups (Figure 3B; p<10^-12 in each case; Mann-Whitney); no significant difference was found between the three control groups, in each of which the LDH was within the normal range.

The mean erythrocyte count, haematocrit and total blood haemoglobin in the cohort were within the normal range (see Data availability), and there was no association between these parameters and the rate of brain atrophy.

![Figure 2. SELDI-TOF mass spectrometry spectra of 15.1 kDa and 15.9 kDa peaks. (A.) Following enrichment on Top 12 Protein Depletion column and (B.) Concentration of eluate on 3 kDa molecular weight cutoff column.](image)

<table>
<thead>
<tr>
<th>Peak</th>
<th>m/z</th>
<th>Protein name</th>
<th>Accession no. (UniProt)</th>
<th>PLGS score</th>
<th>Peptide matches</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.141</td>
<td>Haemoglobin alpha</td>
<td>P69905</td>
<td>2049</td>
<td>(R)VDPVNFK(L) (R)MFLSFPTTK(T) KVVGHAAEYGAEALER(M) (R)MFLSFPTTK(T)</td>
</tr>
<tr>
<td>2</td>
<td>15.885</td>
<td>Haemoglobin beta</td>
<td>P68871</td>
<td>2579</td>
<td>(R)FFESFGDLSTPDAVMGPNK(V) (R)ILVYVPTQR(F) KIEFTPPQAAYQK(V) (R)FFESFGDLSTPDAVMGPNK(V) KLVDPENFR(L)</td>
</tr>
</tbody>
</table>
Figure 3. Free serum haemoglobin and lactate dehydrogenase are raised in secondary progressive multiple sclerosis. A. Serum haemoglobin concentration measured by ELISA in asymptomatic carriers of HTLV-1 (AC), patients with HTLV-1-associated myelopathy (HAM), uninfected, healthy controls (HC), and patients with secondary progressive MS. Log [haemoglobin] by ELISA was significantly correlated with log(peak intensity): r = 0.52; p = 0.02; linear regression. B. Serum lactate dehydrogenase (LDH) activity in the same groups of subjects.

Discussion
The characteristic pathological feature of early, active multiple sclerosis lesions is primary demyelination, with partial preservation of axons. But the dominant feature in progressive disease is neurodegeneration, which results in brain atrophy. Factors associated with this neurodegeneration include microglial activation, chronic oxidative injury, mitochondrial damage in axons, and iron accumulation. A strong correlate of neurodegeneration in MS is abnormal iron deposition in both grey and white matter in MS, especially along veins and venules in cerebral MS plaques\textsuperscript{16,17}. Iron can potentiate oxidative damage by generating hydroxyl radicals by the Fenton reaction. The extent of iron accumulation, as indicated by T2 signal hypointensity on MRI, is correlated with disease progression, lesion accumulation and cell death of oligodendrocytes\textsuperscript{18-20}. The extent of iron deposition is greater in SPMS than in relapsing-remitting disease\textsuperscript{18}.

The source of this abnormal iron deposited in the central nervous system in MS is unknown. Iron is liberated from damaged oligodendrocytes and myelin\textsuperscript{21} and accumulates in macrophages and microglia at the margin of active lesions, but it remains unclear whether this is the principal source of the iron that accumulates in the vessel walls and perivascular space. Bamm and Harauz\textsuperscript{22} proposed that chronic extravasation of red blood cells is a source
of the abnormal iron deposits; however, neuropathological evidence does not show frequent or widespread extravascular erythrocytes in the MS brain.

The results presented here show that a rise in the concentration of free haemoglobin in the serum was associated with the rate of brain atrophy in this cohort of patients with SPMS. This effect was independent of the beneficial treatment effect of simvastatin, because there was no association between free haemoglobin concentration and simvastatin treatment. Since a successful response to simvastatin treatment was not associated with the free serum haemoglobin concentration, we infer that the change in free serum haemoglobin was not a consequence of brain atrophy but preceded brain atrophy in the causal pathway.

These results suggest the hypothesis that chronic, low-grade intravascular haemolysis releases haemoglobin into the serum, which is then translocated into the CNS parenchyma across the impaired blood-brain barrier and potentiates oxidative damage to oligodendrocytes. Cytotoxicity by free haemoglobin can be mediated by intact haemoglobin itself, by haem, or by iron, especially as Fe^{2+}. Free haemoglobin is degraded by haem oxygenase-1 (HO-1), producing biliverdin and Fe^{2+} ions. HO-1 is upregulated in glia by oxidative stress, and HO-1 is expressed in oligodendrocytes in actively demyelinating areas in MS, but not in two other CNS diseases, human acute disseminated leukencephalomyelitis (ADEM) or murine experimental allergic encephalomyelitis (EAE). Stahnke et al. proposed that the role of stress-induced HO-1 is protective initially, whereas chronic upregulation might cause oligodendrocyte death.

The observation (Figure 3B) of abnormally high serum LDH activity is consistent with the presence of haemolysis in these patients. LDH is present in all cell types, and serum LDH is raised in many inflammatory conditions; however, erythrocytes are particularly rich in LDH, and serum LDH is a sensitive marker of haemolysis. The notion that chronic intravascular haemolysis might serve as a source of the iron deposited in MS is also consistent with earlier reports of abnormal fragility of erythrocytes. Erythrocytes from patients with MS, especially those with active disease, are abnormally susceptible to lysis by both mechanical stress and osmotic stress. The causes of this erythrocyte fragility remain to be identified. Possible artefactual causes of haemolysis, such as venepuncture, cannot explain the significant association observed here between brain atrophy and free serum haemoglobin.

If intravascular haemolysis indeed occurs in SPMS, the rate of red cell destruction is insufficient to reduce the total blood haemoglobin, which remained within normal limits in this cohort. Neurodegeneration is not a feature of other chronic haemolytic anemias, such as spherocytosis or elliptocytosis; however, in these conditions the blood-brain barrier is intact, and most erythrocyte destruction occurs in the spleen, where efficient phagocytosis may prevent the release of the toxic breakdown products into the circulation.

Polymorphisms in genes encoding iron-binding and iron-transporting proteins are associated with disability, disease severity and early progression in MS. Rithidech et al. used 2D electrophoresis to identify plasma biomarkers in paediatric MS: the haem-binding protein haemopexin was 1 of 12 proteins found to be upregulated in 9 MS patients. Robotti et al. identified an alteration in the ratio of isoforms of haptoglobin (which bind free haemoglobin) in MS.

These results do not suggest that free serum haemoglobin concentration is useful in the differential diagnosis of neurological disease; rather, they identify a potential contributor to the pathogenesis of neurodegeneration in progressive multiple sclerosis.

Previous studies of serum iron have shown normal concentrations in the serum in patients with MS; however, standard assays of free serum iron do not detect iron that is sequestered in haemoglobin. Iron chelation has been proposed as a therapy to approach to reduce neurodegeneration in MS. However, if an important source of iron is free serum haemoglobin, standard iron-chelating agents such as desferrioxamine will be ineffective, again because the iron is sequestered in haemoglobin. Scavengers of haemoglobin and haemin might be more effective.

Data availability

Author contributions
AL analysed data, wrote the manuscript; SH designed experiments, conducted experiments, analysed data, wrote the manuscript; AW designed experiments, conducted experiments, analysed data; PL designed experiments, wrote the manuscript; RN designed experiments, wrote the manuscript; JC designed MS-STAT trial, designed experiments, wrote the manuscript; CB conceived the project, designed experiments, analysed data, wrote the manuscript.

Competing interests
No competing interests were disclosed.

Grant information
This work was supported by the Wellcome Trust [100291]; Medical Research Council [MR K019090]; National Institute of Health Research; University College London Hospitals/UCL Biomedical Research Centre.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Supplementary material

Supplementary Table 1. Protein peaks associated with brain atrophy: identification by liquid chromatography-electrospray ionization tandem mass spectrometry.

Relative molecular mass, protein score and identity of the genes with sequence matches to peptide fragments from the protein peaks that were significantly correlated with the rate of brain atrophy. The common contaminant keratin and partial matches to contaminating bacterial sequences were excluded.

References


This is an interesting study linking an increase of serum hemoglobin to brain atrophy in patients with secondary progressive multiple sclerosis. In this study 140 patients have been included, who were initially recruited for the phase 2, placebo-controlled MS-STAT trial, investigating the effect of simvastatin on brain volume loss after 2 years of treatment. A total of 475 serum samples collected at baseline, 6 months, 12 and 24 months have been analyzed using an unbiased search for protein profiles by SELDI-TOF mass spectrometry. Brain volume changes were measured by MRI Brain Boundary Shift Integral (BBSI) at baseline, 12 months and 25 months.

An increased abundance of serum proteins at 15.1 kDa and 15.9 kDa were significantly correlated to the rate of brain atrophy after 2 years. These proteins have then been identified as alpha-hemoglobin and beta hemoglobin using tandem mass spectrometry. In addition, higher serum hemoglobin concentration in SPMS patients compared to controls has been confirmed using ELISA. The authors further studied serum lactate dehydrogenase activity, which has also been shown to be significantly increased in SPMS compared to controls.

It was concluded that a low-grade chronic intravascular hemolysis, which is a potential source of iron whose deposition along blood vessels in multiple sclerosis plaques contributes to neurodegeneration and brain atrophy in progressive MS.

The results of this study are intriguing and underline other reports on the possible implication of hemoglobin in the pathogenesis of multiple sclerosis, reviewed in Altinoz et al. (2016). Lewin et al. hypothesize that increased serum levels of free hemoglobin may trigger iron deposition along blood vessels in MS, which could then propagate neurodegeneration and brain atrophy. While this speculation brings in a new idea, it appears biologically difficult how these processes (e.g. iron deposition along blood vessels in MS lesions and cortical atrophy) should be so tightly linked in time if at all. Otherwise increased iron deposition has been shown to occur in MS using several MRI techniques although due to technical reasons robust quantification of brain iron deposition is currently limited to deep gray matter areas (Ropele et al. 2011). Longitudinal studies are still scarce, but a recent report indicates that increased brain iron deposition is more pronounced in early phases of the disease (Khalil et al. 2015). Less information exists on progressive forms of MS but one would speculate that the damaging effects from iron deposition are a long-standing rather than an immediate effect as suggested by Lewin et al.. Observed correlation thus are likely not able to clarify if and to which extent increased brain iron deposition really amplifies neurodegeneration in MS or merely reflects an epiphenomenon of the disease. There is also some evidence for alterations of iron related proteins in CSF and serum of MS patients, including transferrin.
(Khalil et al. 2014) and lipocalin 2 (Khalil et al. 2016), and which may contribute to iron accumulation.

Reiterating some of the concerns and comments of the other reviewers we thus see the need to put reported findings in a more cautious context. Also some methodological issues deserve clarification.

**Major comments**

- The authors should rather use the term “correlated” instead of “associated” and discuss that presented observations do not prove causality as outlined above.

- For the same reasons the authors should also avoid to embark on therapeutic speculations.

- The authors state that they analyzed 475 serum samples at different time points (0, 6, 12 and 24 months), however they do not indicate later on how they used the time point a 6 months. Please clarify.

- It is not clear why the confirmative analysis of free serum hemoglobin using ELISA has only been performed in 20 SPMS patients. It would have been advantageous and the authors a strongly encouraged to determine free serum hemoglobin in all 475 serum to see if increased free serum hemoglobin as determined by ELISA was also correlated with increased brain atrophy.

- The Discussion should also reflect still existing uncertainties in a more comprehensive manner.

**Minor comments**

- In Figure 3, p-values (corrected for multiple comparisons) of group differences should be included.

- In Figure legend 3 it is mentioned that “Log [haemoglobin] by ELISA was significantly correlated with log(peak intensity): r = 0.52; p = 0.02; linear regression...”. For better understanding and reading this information should be presented by a separate scatter plot.

**References**


We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.

---

**Summary** – This article describes a proteomics analysis of serum proteins derived from 140 patients with secondary progressive multiple sclerosis (MS), 20 healthy adult volunteers, 20 patients with human Tlymphotropic virus (HTLV1) causing symptoms resembling spinal MS, and 20 asymptomatic HTLV1 carriers. Half of the MS patients were undergoing treatment with simvastatin, a drug used to lower blood cholesterol and shown to have immunomodulatory and anti-inflammatory properties. Protein profiling of sera was achieved by mass spectrometry of 475 serum samples collected at 0, 12, and 24 months. (A 6 month time point is mentioned in some places and is queried below). The MS patients had concurrent MRI scans at 0, 12, and 25 months to measure whole brain volume (BBSI – brain boundary shift integral), presumably amongst other measures. Serum samples were “enriched” and analysed by 1D SDSPAGE followed by LCMS/MS of in gel digested protein. Free haemoglobin (Hb) levels were assessed by ELISA, and activity of lactate dehydrogenase (LDH), an indicator of general tissue damage, and particularly of haemolysis was measured. The proteomics analysis suggested that a 15.1kDa protein peak correlated with the rate of brain atrophy in seemingly all MS patients, regardless of treatment regime. Following protein enrichment, 15.1kDa and 15.9kDa peaks were observed and confirmed to represent the α and β-chains of haemoglobin, respectively. In all MS patients, levels of both free Hb chains and of LDH activity were elevated compared to all controls. The results are consistent with the idea that Hb is released into serum by chronic and low-grade intravascular haemolysis, with subsequent translocation into the CNS where it has great potential to cause oxidative damage.

**Comments on title and abstract** –

1. We suggest that the word “associated” needs to be substituted by “correlated”

2. Conclusions in the abstract must be linked to the objectives of the study rather than be a speculative claim.

**Comments on study design and data interpretation** – Several points require clarification, in our view.

1. There were 140 patents, and 60 controls (3 groups of 20). So the total number is supposed to be 200 serum samples per time point. What are the other 275 samples? The question of sample numbers, both of patients and controls, arises again later when 138 patients are mentioned. Additionally, a valuable control could be a group of patients with another neurodegenerative disease characterised by brain atrophy.

2. The 6 month time point was not mentioned in the paragraph describing the study design, and there were no results reported for it.
3. For protein profiling by SELDI-TOF mass spectrometry, after the 1:10 serum dilution, one would expect signal suppression effect on the lower abundance proteins. To remove this effect it would have been advised to fractionate the serum first, and then use protein chip arrays. Since the authors did not fractionate the sera, it is not possible to rule out other lower abundance markers, such as hemopexin, as we indicate next.

4. The Top 12 Protein Depletion Spin Columns are a good way to partially fractionate the serum or to enrich the protein of interest. However, several very important proteins (haptoglobin, transferrin, and Apo AI) related to iron homeostasis will be removed by this procedure. In the context of this study, it is important to see the specific expression patterns of haptoglobin, hemopexin, and HO1 since they represent different levels of defence mechanisms against extracellular Hb. Also, it could be beneficial to try and correlate different haptoglobin phenotypes with BBSI.

5. In the same vein, the ELISA kit will detect extracellular Hb from two sources: free Hb and haptoglobin-bound Hb. The latter form could have been removed by the spin column that was used for protein enrichment.

6. Why and how were only 20 patients selected for ELISA?

Comments on discussion – We believe that the Discussion can be augmented to give a broader picture as follows.

1. Association versus correlation of brain volume and Hb levels. In our opinion the results show correlation between brain volume and free Hb levels and are insufficient to claim that these two factors are “associated” per se.

2. In reference to [(Bamm and Harauz 2014)], the authors state that “neuropathological evidence does not show frequent or widespread extravascular erythrocytes in the MS brain”. This statement is not strictly correct. Firstly, in [(Bamm and Harauz 2014)] we did not suggest that chronic extravasation of red blood cells is an exclusive source of the abnormal iron deposits. In fact, we said: “Any type of seemingly minor yet chronic cerebrovascular abnormality and/or damage to the blood–brain barrier, … , can potentially lead to intravascular hemolysis, or to extravasation of erythrocytes and extravascular hemolysis”. Secondly, as we reviewed recently [(Bamm et al. 2016)], extracellular Hb could arise from blood extravasation due to capillary and venous microhemorrhages, which ARE being documented in MS lesions, as possibly are cerebral microbleeds (CBMs) [(Zivadinov et al. 2016)]. Such events can be difficult to detect at the histological level, especially in the early stages of lesion formation that can arise from molecular dysfunction rather than gross structural damage. The potential molecular mechanisms of Hb toxicity in myelin are described in a subsequent experimental paper [(Bamm et al. 2015)] and do not rely on largescale iron deposition. The damage inflicted by free Hb can arise at many different levels and per se will not necessarily be associated with the number of extravasated erythrocytes in MS tissue.

3. In reference to erythrocyte fragility in MS, we suggest also citing one of the earlier papers to indicate that this is an old idea that has been insufficiently explored [(Caspary et al. 1967)], and two newer papers that describe the association of haemoglobin variants with MS severity [(Altinoz et al. 2016; Ozcan et al. 2016)].

4. It is not clear how the finding of higher free Hb in the sera of MS patients can be explained. The authors reported that the mean erythrocytes count and total blood Hb did not differ between MS
patients and control groups. However, the MS patients were characterized by the increased presence of free Hb. If a simple formula for total Hb is: Hb within the erythrocytes + cellfree Hb, and the erythrocyte count was similar, then the amounts of cellfree Hb should be similar. Is the morphology of erythrocytes from MS patients and controls different? Perhaps the erythrocytes from MS patients were hypochromic (less intracellular Hb)? We believe that the authors should clarify this point.

5. The first line of defense against extracellular haemoglobin is haptoglobin (Hpt). We have determined recently that the frequency of the hpt1 allele is lower in Australia, and in European and North American countries with a high reported prevalence of MS [(Bamm et al. 2016)]. We also recommended an epidemiological study to evaluate the potential association of Hpt phenotype with disease severity and/or comorbidity with cardiovascular disorders, as is being done for Parkinsonism [(Costa-Mallen et al. 2015; Delanghe et al. 2016; Costa-Mallen et al. 2016)]. It should also be pointed out that cardiovascular disorders have been found to be associated with disease severity and brain atrophy in MS [(Kappus et al. 2016)]. This latter reference is certainly relevant to the Discussion of this current study.

References
We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.
which however remains unresolved here, concerns the cause of increased free haemoglobin in the blood of MS patients. Interestingly, it has already been shown in the late 1960ths that erythrocyte diameters are larger in MS patients (J. Prineas, 1968) and that there is a higher fragility of erythrocytes in comparison to controls, which may result in liberation of haemoglobin (E.A. Caspary et al., 1967). These observations together with more recent findings, which relate them to other disturbances of iron homeostasis in MS, have been recently discussed in a comprehensive review article (Altinoz et al., 2016). Whether the increased erythrocyte fragility is a genuine metabolic problem or a consequence of a general systemic chronic pro-inflammatory environment in the MS patients is currently unresolved.

References

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

*Competing Interests:* No competing interests were disclosed.