RESEARCH ARTICLE

REVISED Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis [version 2; referees: 3 approved]

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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: The results are consistent with the following hypothesis. In progressive multiple sclerosis, low-grade chronic intravascular haemolysis releases haemoglobin into the serum; the haemoglobin is subsequently translocated into the central nervous system (CNS) across the damaged blood-brain barrier. In the CNS, the haemoglobin and its breakdown products, including haem and iron, contribute to the neurodegeneration and consequent brain atrophy seen in progressive disease. We postulate that haemoglobin is a source of the iron whose deposition along blood vessels in multiple sclerosis

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plaques is associated with neurodegeneration. If so, then chelators of haemoglobin, rather than chelators of free serum iron, may be effective in preventing this neurodegeneration.

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Comments (1)

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REVISED **Amendments from Version 1**

We thank the reviewers for their comments and suggestions. In the revised version of the paper (v.2), we have taken into account the points raised by each set of reviewers. The main changes are the following:

- clarification of points of methodology (e.g. sampling; use of Top 12 protein depletion columns)

- more cautious wording on possible therapy, and to make clear the principle that we have not proved causality or claim that free serum haemoglobin is the sole correlate of brain atrophy in SPMS

- clearer and fairer representation of the results reported in previous publications

- addition of 8 papers to the bibliography, citing – as suggested - both old work (on erythrocyte fragility) and very recent work

- answering specific points concerning the normal total blood haemoglobin concentration and the kinetics of neurodegeneration

- significance values for pairwise statistical tests in Figure 3.

See referee reports

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 treatment[1–3], accounts for much of the disability and the cost of MS to both the person and the community[4].

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 atrophy[5,6]. However, in all forms of the disease, both the initiating events and the mechanisms of pathogenesis remain uncertain[5]. Pseudoatrophy may account for some loss of brain volume[7], but brain atrophy has also been associated with changes in neurofilament levels[8] and sodium metabolism[9].

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 disease[10–12]. 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, number NCT00647348, and has been described in detail elsewhere[1]. 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 volume[1]. Simvastatin treatment resulted in a highly statistically significant 43% reduction in the annualized rate of brain atrophy[1], 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

Serum samples were collected and cryopreserved from each patient at baseline, 6 months, 12 months and 24 months. Samples were not available from all patients at each time-point; a total of 475 samples were available for SELDI-TOF mass spectrometry. 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

To identify the proteins present in the 15.1 kDa and 15.9 kDa peaks, ten µL of a single patient's 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 on a 3 kDa molecular weight cut-off column (Amicon) in 25 mM Tris-HCl, pH 8.0. Twenty µL of depleted serum were separated 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[13] 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 software[14]. 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[15]. 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 from baseline in each protein peak intensity vs. treatment status, adjusting for the 5 randomization variables and MRI centre. The 3 follow-up time points (6, 12 and 24 months) were analysed separately. 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=0.001, 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[1]. 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

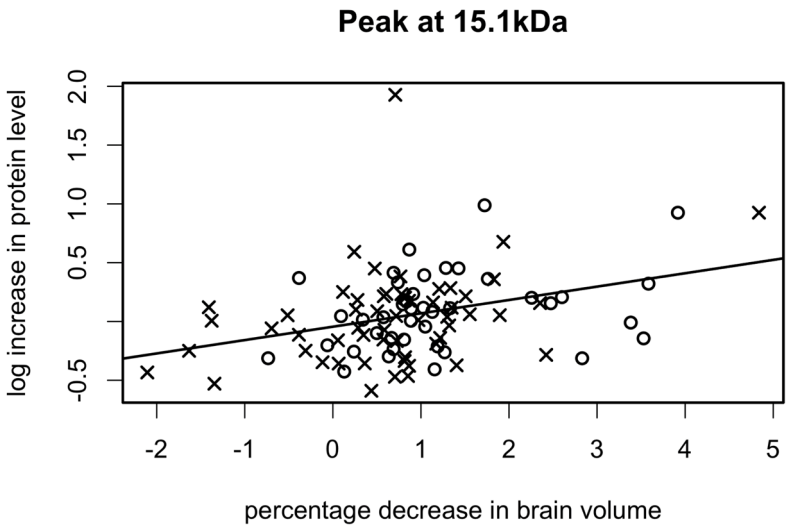

Figure 1. Change in intensity of 15.1 kDa peak correlated with rate of brain atrophy. Normalized log(change in intensity of 15.1 kDa peak between baseline and 2-year follow-up) versus percentage decrease in brain volume between baseline and 2 years. Solid line shows the best fit from the linear regression model (regression coefficient = 0.12, p=0·001). Protein ratios were normalized to all other covariates, using the linear regression model. Crosses represent treated individuals, circles represent untreated individuals. The correlation with the 15.9 kDa peak was closely similar (regression coefficient = 0.12, p=0·001).

Table 1. Multiple linear regression analysis of the association between change in brain volume and change in the peak intensity of the proteins whose changes were found to be significantly correlated with change in brain volume. Beta = regression coefficient. The model is adjusted for the 5 randomization variables and MRI centre.

	m/z 15885 (0 to 25 months)		m/z 15141 (0 to 25 months)	
	Beta (95% CI)	**p**	**Beta (95% CI)**	**p**
protein	0.83 (0.33, 1.33)	0.001	0.75 (0.29, 1.22)	0.002
treatment	-0·62 (-1.06, -0.19)	0.005	-0.60 (-1.03, -0.17)	0.007
	m/z 25110 (0 to 12 months)		**m/z 25402 (0 to 12 months)**	
	Beta (95% CI)	**p**	**Beta (95% CI)**	**p**
protein	-0.99 (-1.72, -0.26)	0.008	-0.73 (-1.37, -0.09)	0.025
treatment	-0.26 (-0.56, -0.04)	0.090	-0.27 (-0.58, -0.04)	0.083

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, randomly selected from the study cohort) 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).

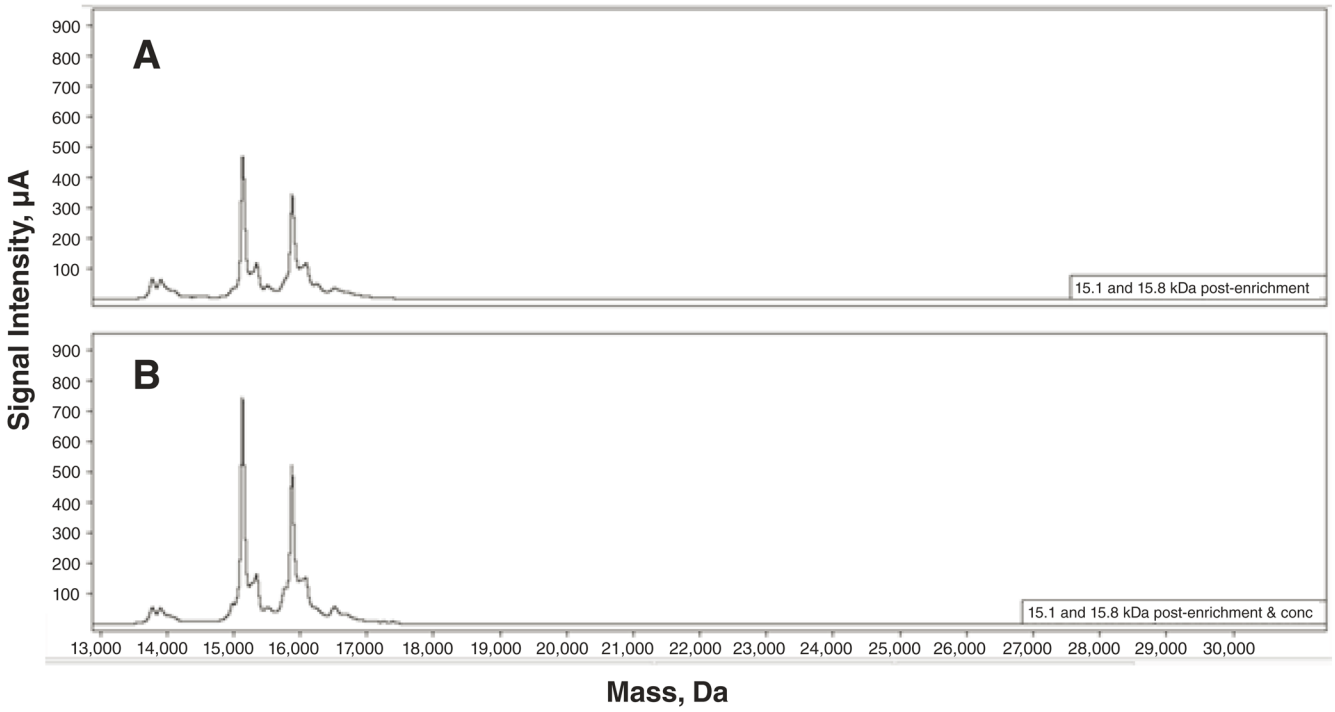

Figure 2. SELDI-TOF mass spectrometry spectra of 15.1 kDa and 15.9 kDa peaks. (**A.**) Following enrichment on Top 12 Protein Depletion column and (**B.**) Concentration of eluate on 3 kDa molecular weight cutoff column.

Table 2. Identification of peptide fragments from 15.1 kDa and 15.9 kDa protein peaks, using LC-MS/MS. *oxidation of M(1). **oxidation of M(15).

Peak	m/z	Protein name	Accession no. (UniProt)	PLGS score	Peptide matches
1	15,141	Haemoglobin alpha	P69905	2049	(R)VDPVNFK(L) (R)MFLSFPTTK(T)* (K)VGGHAAEYGAEALER(M) (R)MFLSFPTTK(T)
2	15,885	Haemoglobin beta	P68871	2579	(R)FFESFGDLSTPDAVMGNPK(V)** (R)LLVVYPWTQR(F) (K)EFTPPVQAAYQK(V) (R)FFESFGDLSTPDAVMGNPK(V) (K)LHVDPENFR(L)

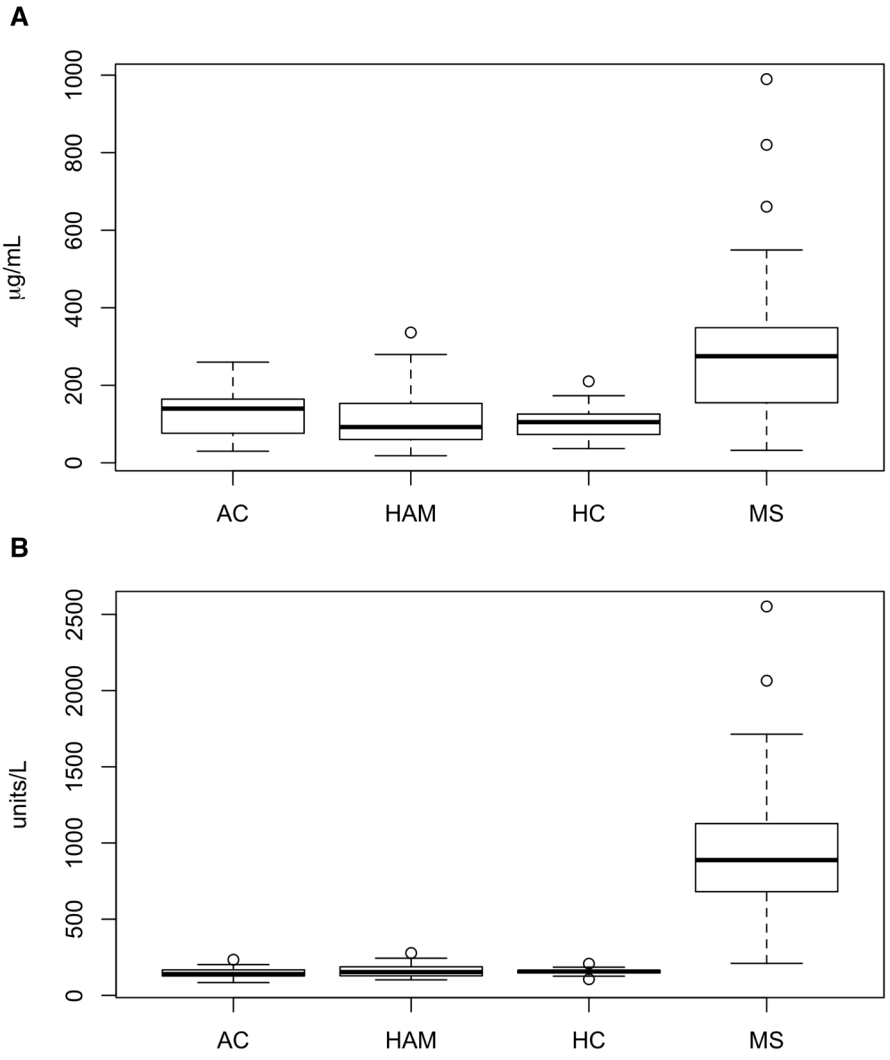

Figure 3. Free serum haemoglobin and lactate dehydrogenase are raised in secondary progressive multiple sclerosis. A. Serum haemoglobin concentration measured by ELISA in asymptomatic carriers of HTLV-1 (AC), patients with HTLV-1-associated myelopathy (HAM), uninfected, healthy controls (HC), and patients with secondary progressive MS. The concentration in the MS group was significantly greater than that in the ACs ($p = 9.9 \times 10^{-4}$), the HAM patients ($p = 1.2 \times 10^{-3}$), and the HCs ($p = 1.6 \times 10^{-4}$) (Mann-Whitney test, Bonferroni-corrected). **B.** Serum lactate dehydrogenase (LDH) activity in the same groups of subjects. The LDH activity in the MS group was significantly greater than that in the ACs ($p = 2.1 \times 10^{-12}$), the HAM patients ($p = 2.6 \times 10^{-12}$), and the HCs ($p = 2.0 \times 10^{-13}$) (Mann-Whitney test, Bonferroni-corrected).

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. Log [haemoglobin] measured by ELISA was significantly correlated with log(peak intensity)($r = 0.52$; $p = 0.02$; linear regression).

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 and haematocrit 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. The concentration of free serum haemoglobin observed here (~300 µg/mL; Figure 3A) accounted for only ~0.1% of the total blood haemoglobin, and consequently the mean total blood haemoglobin in the study cohort was also in the normal range.

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[5] include microglial activation, chronic oxidative injury, mitochondrial damage in axons, and iron accumulation. Risk factors of cardiovascular diseases are also associated with lesion burden and brain atrophy in MS[16]. A strong correlate of the neurodegeneration in MS is abnormal iron deposition in both grey and white matter, especially along veins and venules in cerebral MS plaques[17,18]. 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[19–21]. The extent of iron deposition is greater in SPMS than in relapsing-remitting disease[19]. In a longitudinal study of 144 patients of whom 62 had relapsing-remitting MS, iron deposition was most marked in early disease[22].

The source of this abnormal iron deposited in the central nervous system in MS is unknown. Iron is liberated from damaged oligodendrocytes and myelin[23] 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[24] suggested that haemoglobin might enter the parenchyma of the central nervous system as a result of either intravascular haemolysis or extravasation of red blood cells[25,26]. Both mechanisms could contribute; intravascular haemolysis is more likely to cause a detectable rise in free serum haemoglobin.

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. Free haemoglobin may itself disrupt the blood-brain barrier[27]. The toxic effects of free haemoglobin[28] can be mediated by intact haemoglobin itself, by haem, or by iron, especially as Fe^{2+}[24]. 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 leukoencephalomyelitis (ADEM) or murine experimental allergic encephalomyelitis (EAE)[29]. Stahnke *et al.*[29] proposed that the role of stress-induced HO-1 is protective initially, whereas chronic upregulation might cause oligodendrocyte death. Altinoz *et al.*[30] reviewed evidence from genetic surveys and *in vitro* studies and proposed that haemoglobins could contribute to the pathogenesis of MS in both the "inside-out" and "outside-in" models[5]. Ozcan *et al.* recently reported a negative correlation between the serum level of the minor haemoglobin A2 (HbA2) and disease severity in MS[31]; HbA2 and its breakdown product hemichrome A2 bind the erythrocyte membrane with higher affinity than major haemoglobin, and may diminish erythrocyte fragility[32].

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 size[33] and fragility[34–37] of erythrocytes in the disease. Erythrocytes from patients with MS, especially those with active disease, are abnormally susceptible to lysis by both mechanical stress[36] and osmotic stress[34,35,37]. 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 conditions, 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[38]. Rithidech *et al.*[39] 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.*[40] identified an alteration in the ratio of isoforms of haptoglobin (which bind free haemoglobin) in MS. The frequency of polymorphic variants of haptoglobin varies between geographical regions, and genetic epidemiological studies are needed to test whether particular haptoglobin alleles are associated with disease severity in MS[25].

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. It is possible that other, lower-abundance markers are also correlated with brain atrophy rate. Other markers could be sought both by fractionating diluted serum and by using other surfaces in the SELDI protocol in order to select subsets of proteins with different profiles of hydrophobicity and electric charge.

Previous studies of serum iron have shown normal concentrations in the serum in patients with MS[41]; 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 may be ineffective, again because the iron is sequestered in haemoglobin[42]. Scavengers of haemoglobin and haemin[43] might be more effective.

Data availability

Links to each respective dataset are given below. The data on SELDI-TOF peaks are derived from the BioRad software package ProteinChip Data Manager.

Zenodo: Raw data for SELDI-TOF low range from article: Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis, http://doi.org/10.5281/zenodo.160737[44]

Zenodo: Raw data for SELDI-TOF high range from article: Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis, http://doi.org/10.5281/zenodo.160743[45]

Zenodo: Nano LC MS-MS peptide matches from article: Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis, http://doi.org/10.5281/zenodo.160744[46]

Zenodo: Raw data for RBC, Hb and haematocrit from article: Free serum haemoglobin is associated with brain atrophy in secondary progressive multiple sclerosis, http://doi.org/10.5281/zenodo.161532[47]

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

The authors declare that they have no competing interests.

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

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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.

Click here to access the data

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Data Source

Open Peer Review

Current Referee Status: ✔ ✔ ✔

Version 2

Referee Report 30 December 2016

doi:10.21956/wellcomeopenres.11315.r18853

✔ **George Harauz**, **Vladimir V. Bamm**
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We are happy to approve this article in in its revised form. The authors responded to all points of concern and their contribution is an important one.

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.

Referee Report 28 December 2016

doi:10.21956/wellcomeopenres.11315.r18826

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The authors have adequately addressed all our comments, questions and suggestions.

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.

Version 1

Referee Report 12 December 2016

doi:10.21956/wellcomeopenres.10742.r17822

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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.

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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.

Referee Report 21 November 2016

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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 6month 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 lowgrade 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 6month 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 SELDITOF 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 lowerabundance 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 haptoglobinbound 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 [(CostaMallen *et al.* 2015; Delanghe *et al.* 2016; CostaMallen *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.

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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.

Referee Report 18 November 2016

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Center for Brain Research, Medical University of Vienna, Vienna, Austria

This is a very interesting study providing convincing evidence for an association between the serum level increases of free haemoglobin with the extent of brain atrophy progression, determined by MRI, in secondary progressive multiple sclerosis. This was originally observed by the authors using an unbiased proteomics approach, aimed at determining potential serum biomarkers for disease progression. The patient collective derives from a well-controlled clinical trial investigating the effect of simvastatin on the rate of brain atrophy progression in secondary progressive MS. Having found this association, the authors then confirmed free hemoglobin increase in SPMS patients by an independent approach using ELISA.

The study is very well performed, based on a sound and innovative technology and the results have major implications for the understanding of the neurodegenerative process in MS. Interestingly, the association between haemoglobin serum level increase and atrophy rate occurred independently from the effect of simvastatin treatment. Thus increased haemoglobin in the serum may contribute to the neurodegenerative process, but there are other mechanisms additionally involved. By showing also increased levels of serum LDH the authors further support the concept that there is a low degree of hemolysis in the peripheral blood of MS patients, which however is not reflected in reduction of erythrocyte counts, haematocrit or total blood haemoglobin. However, it would also be interesting to address whether there is an association between actual free hemoglobin levels at any of the single time points (not their change over time), as determined by mass spectrometry, and the observed brain atrophy rates. Authors only show a positive association of free hemoglobin increase with brain atrophy levels, which might have a different interpretation than what the authors conclude. Furthermore, the quantitative ELISA data are not linked with MRI brain atrophy rates. Were the 20 MS patients included in the ELISA quantification taken from the big cohort of the proteomics part of the study? Then MRI data would be available, and the analysis could be easily performed.

There is good indirect evidence that iron loading of the brain may be involved in neurodegeneration in MS through the amplification of oxidative injury. So far, the mechanisms how the human brain is loaded with iron during aging and in the disease process of MS is not fully understood. This study indicates that heme iron, derived from free circulating haemoglobin, may be of major importance in brain iron accumulation in the course of multiple sclerosis. Free hemoglobin, presumably bound to the haemoglobin scavenger haptoglobin, might diffuse into the brain via a partially compromised blood-brain barrier and might lead to iron accumulation within MS basal ganglia and microglia at edges of chronic lesions. The key question, 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
1. Altinoz MA, Ozcan EM, Ince B, Guloksuz S: Hemoglobins as new players in multiple sclerosis: metabolic and immune aspects.*Metab Brain Dis*. 2016; **31** (5): 983-92 PubMed Abstract | Publisher Full Text
2. Caspary EA, Sewell F, Field EJ: Red blood cell fragility in multiple sclerosis.*Br Med J*. 1967; **2** (5552): 610-1 PubMed Abstract
3. Prineas J: Red blood cell size in multiple sclerosis.*Acta Neurol Scand*. 1968; **44** (1): 81-90 PubMed Abstract

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.

Discuss this Article

Version 1

Author Response 15 Dec 2016

Charles Bangham, Department of Immunology, Imperial College London, UK

Lewin *et al.* – response to reviewers
We thank the three pairs of reviewers of our article, each of whom made helpful suggestions and raised salient points for clarification or further discussion. We have revised the article in the light of these comments, and cite further relevant literature (8 references have been added). The response to individual points is given below.

Review 1
Referee Report 18 Nov 2016
Hans Lassmann, Center for Brain Research, Medical University of Vienna, Vienna, Austria
Simon Hametner, Center for Brain Research, Medical University of Vienna, Vienna, Austria

Approved

DOI: 10.21956/wellcomeopenres.10742.r17609

We thank the reviewers for their comments. We have included the three papers suggested in the revised version of the paper.

Review 2
Approved with Reservations
DOI: 10.21956/wellcomeopenres.10742.r17825
READ ALL

Comments on title and abstract –

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

In standard usage, "associated" has a broader, less specific meaning than "correlation", and it does not imply causation. We would like to keep the term.

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

We like the phrasing used by the reviewers at the end of their summary, and we have adapted it in the Conclusions section of the revised version of the abstract, to clarify the hypothesis and to avoid speculation.

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.

The proteomic screen was carried out on the 140 SPMS patients in the MS-STAT trial, which did not include subjects without MS. There were 4 time-points, giving a potential total of 560 serum samples; however, samples were not available from all patients at each time-point. We have clarified these points in the revised text.
It will indeed be interesting to study the serum proteome in other neurodegenerative diseases, as the reviewers indicate.

2. The 6month time point was not mentioned in the paragraph describing the study design, and there were no results reported for it.

Thank you for pointing this out. We have amended the relevant section - paragraph 2 in the Results – to explain that data from each of the three follow-up visits (including the 6-month time point) were compared against the baseline.

3. For protein profiling by SELDITOF 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 lowerabundance markers, such as hemopexin, as we indicate next.

The reviewers are of course correct that we cannot exclude the possibility that other, lower-abundance markers are also correlated with brain atrophy rate, in addition to the correlation with free serum haemoglobin reported in the present study. Other markers could be sought both by fractionating diluted serum and by using other surfaces in the SELDI protocol, in order to select subsets of proteins with different profiles of hydrophobicity, charge etc. We have added a sentence to the text (Discussion section) to point out these considerations.

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.

We apologize for not making this clear. The SELDI procedure, which led to the detection of the two correlated peaks, was carried out on whole sera, not after depletion of the abundant serum proteins; the Top 12 Protein Depletion Spin Columns were used subsequently, to enrich the peaks for identification by LC-MS/MS. We have added a sentence to the beginning of the relevant paragraph in the Materials and Methods section to clarify this.

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

The reviewers are correct that the anti-human haemoglobin polyclonal antibody in the ELISA kit should detect both free and haptoglobin-bound Hb, although the manufacturers have not tested this. In order to detect only free Hb, Top 12 depletion columns could be used to remove haptoglobin-bound Hb. However, in our results the Top 12 depletion columns increased the relative abundance of the two candidate peaks (15.1 kDa and 15.9 kDa), rather than depleting them, suggesting that the bulk of the Hb was not bound to haptoglobin.

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

The ELISA was carried out for two purposes. First, to test whether the concentration of free serum haemoglobin in these samples was in the range measurable by a standard commercial assay, and was not only detectable by mass spectrometry. Second, to test whether the ELISA measurements correlated with the intensity of the protein peaks in the SELDI data. For these purposes we assayed a randomly-chosen subset of 20 sera; the results established both of the points we set out to test.

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.

As noted above, we use the term "associated" in its standard, broad sense; the term does not imply that this association proves a causal connection, although we postulate this later.

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.

We accept that current evidence does not exclude extravasation of erythrocytes as a possible cause of haemoglobin in the CNS parenchyma. We have amended the text of the Discussion to reflect more accurately the balanced views expressed in the Bamm and Harauz 2014 paper, and to include citations of the recent papers noted above. In the following paragraph, we have also added a citation of the useful experimental study of haemoglobin toxicity (Bamm et al 2015).

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)].

Thank you for these suggestions. Indeed we did cite Caspary *et al.* (1967) in an earlier version of the manuscript. We have now re-inserted this citation, and the first report of which we are aware of erythrocyte fragility in MS (Laszlo, 1964); and we have also added references to the very interesting papers recently published by Altinoz *et al.* and Ozcan *et al.*

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 + cell free 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.

Thank you for raising this point, which is important to clarify. As shown in Figure 3A, the mean concentration of free Hb in the serum of the 20 patients assayed was ~300 micrograms/mL, i.e. 30 mg/dL. Since the haematocrit is ~50%, this concentration is equivalent to ~15 mg/dL of whole blood. This compares with the total [Hb] in whole blood, which is of the order of 15g/dL. Thus, even the abnormally high [Hb] accounts for only 0.1% of the total blood haemoglobin, and consequently does not significantly

alter the mean [Hb]. We have added a sentence to the Discussion to note this.

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 [(CostaMallen et al. 2015; Delanghe et al. 2016; CostaMallen 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.

We have added a sentence to the paragraph in the Discussion on genetic polymorphisms to discuss the possible role of haptoglobin variants, citing Bamm *et al.* 2016. We have also added a sentence to the first paragraph of the Discussion to cite the evidence of Kappus *et al.* (2016) that risk factors of cardiovascular diseases are also associated with lesion burden and brain atrophy in MS.

Review 3
Referee Report 12 Dec 2016
Franz Fazekas, Department of Neurology, Medical University of Graz, Graz, Austria
Khalil Michael, Department of Neurology, Medical University of Graz, Graz, Austria

Approved with Reservations

Thank you for the thoughtful comments. The point about the rate at which iron deposition might cause neurodegeneration is well taken, and in the revised version of the paper we cite the very interesting longitudinal study of brain iron levels (Khalil *et al.* 2015: Neurology 84, 2396). However, in our study we were not able to test hypotheses on the kinetics or the mechanisms of neurodegeneration, which have been intensively studied by others, including the reviewers. Our results do not imply that the free haemoglobin detected during the course of the two-year trial caused neural damage during the same period: it is perhaps more likely that both iron deposition and neurodegeneration were occurring continously in these patients.

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.***

As noted above, we use the term "associated" in its standard, broad sense; the term does not imply that this association proves a causal connection: although we postulate the direction of causation later, it is certainly not proved by our present results.

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

We have added a conditional clause to the Abstract (last sentence) to make it clear that chelators of haemoglobin may be useful therapeutically if indeed free haemoglobin contributes to the pathogenesis of the neurodegeneration. Similarly, in the final paragraph of the Discussion, we state "However, if an

important source of iron is free serum haemoglobin, standard iron-chelating agents such as desferrioxamine may be ineffective..." We think it is important to discuss the possible therapeutic implications, with due caution, for two reasons: first, because iron chelators are currently being tested therapeutically in MS, and second, because new therapeutic options are needed.

- ***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.***

Thank you for pointing this out, which was also noted by reviewer 1. Please see response to Reviewer 1, point 2 above.

- ***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.***

Please see response to Reviewer 1, point 6 above.

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

We have added further text to make it clear we do not postulate (still less prove) that free haemoglobin is the sole cause of neurodegeneration in SPMS. We write in the Discussion “These results suggest the hypothesis...”; “It is possible that other, lower-abundance markers are also correlated with brain atrophy rate”; “If intravascular haemolysis indeed occurs in SPMS...”; [these results] identify a potential contributor to the pathogenesis...”; “...if an important source of iron is free serum haemoglobin, standard iron-chelating agents such as desferrioxamine may be ineffective...”.

Minor comments

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

We have now included the Bonferroni-corrected p values in the figure legend.

- ***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.***

For clearer presentation, we have expanded the sentence reporting this correlation and moved it to the text in the Results section.

Competing Interests: No competing interests were disclosed.