OPEN ACCESS Freely available online

PLOS ONE

Involvement of Oxidative Stress in Occurrence of Relapses in Multiple Sclerosis: The Spectrum of Oxidatively Modified Serum Proteins Detected by Proteomics and Redox Proteomics Analysis

Ada Fiorini[1], Tatiana Koudriavtseva[2], Elona Bucaj[3], Raffaella Coccia[1], Cesira Foppoli[4], Alessandra Giorgi[1], M. Eugenia Schininà[1], Fabio Di Domenico[1], Federico De Marco[3]*, Marzia Perluigi[1]*

1 Department of Biochemical Sciences, Sapienza University of Rome, Rome, Italy, **2** SSD Neurology, the Regina Elena National Cancer Institute, Rome, Italy, **3** Laboratory of Virology, the Regina Elena National Cancer Institute, Rome, Italy, **4** CNR Institute of Molecular Biology and Pathology, Rome, Italy

Abstract

Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system. Several evidences suggest that MS can be considered a multi-factorial disease in which both genetics and environmental factors are involved. Among proposed candidates, growing results support the involvement of oxidative stress (OS) in MS pathology. The aim of this study was to investigate the role of OS in event of exacerbations in MS on serum of relapsing-remitting (RR-MS) patients, either in relapsing or remitting phase, with respect to serum from healthy subjects. We applied proteomics and redox proteomics approaches to identify differently expressed and oxidatively modified proteins in the low-abundant serum protein fraction. Among differently expressed proteins ceruloplasmin, antithrombin III, clusterin, apolipoprotein E, and complement C3, were up-regulated in MS patients compared with healthy controls. Further by redox proteomics, vitamin D-binding protein showed a progressive trend of oxidation from remission to relapse, respect with controls. Similarly, the increase of oxidation of apolipoprotein A-IV confirmed that levels of OS are elevated with the progression of the disease. Our findings support the involvement of OS in MS and suggest that dysfunction of target proteins occurs upon oxidative damage and correlates with the pathology.

Citation: Fiorini A, Koudriavtseva T, Bucaj E, Coccia R, Foppoli C, et al. (2013) Involvement of Oxidative Stress in Occurrence of Relapses in Multiple Sclerosis: The Spectrum of Oxidatively Modified Serum Proteins Detected by Proteomics and Redox Proteomics Analysis. PLoS ONE 8(6): e65184. doi:10.1371/journal.pone.0065184

Editor: Francisco José Esteban, University of Jaén, Spain

Received December 18, 2012; **Accepted** April 24, 2013; **Published** June 7, 2013

Funding: This work was supported by FISM- Fondazione Italiana Sclerosi Multipla grant 2010/R/29, by the Italian Ministry of Health and by the Italian Ministry of Foreign Affairs, DGSP. Dr Elona Bucaj was supported by a PhD fellowship granted by the Ministry of Foreign Affairs, DGCS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: demarco@ifo.it (FDM); marzia.perluigi@uniroma1.it (MP)

Introduction

Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system. In most MS patients, the disorder is characterized by a relapsing–remitting (RR) course [1]. During relapses new symptoms can appear and old ones resurface or worsen. The relapses are followed by periods of remission, during which the person fully or partially recovers from the deficits acquired during relapse. However, a neurodegenerative process leading to axonal loss and matrix destruction takes place over the course of years, and is implicated in sustained, irreversible neurological disability. Neuronal loss is more prominent when the disease takes a progressive course after years of RR episodes (secondary progressive, SPMS) or when clinical manifestations are progressive from onset (primary progressive, PPMS) [2]. The plurality of physio-pathological processes characterizing the disease, including inflammation, demyelination, and axonal damage among others, and the fact that they are not equally represented in MS population, determine the large heterogeneity in phenotypic expression of MS. The heterogeneity is present not only among the various MS forms (RRMS, SPMS, PPMS) but also within the same subtype, such as between the relapse and remission in RRMS. So far, what determines the exacerbation of the disease, as well as biomarkers able to give information about its progression, is yet unknown.

Since MS is considered not only an inflammatory disease but also a neurodegenerative disorder [3,4], many evidences support the crucial role of oxidative stress (OS) in the pathogenesis of MS [5,6]. Several studies highlight the presence of oxidative damage both in blood and in the nervous system of patients with MS [7,8]. Increased protein carbonyls were found in post-mortem brains of MS patients [9], in addition to elevated contents of OS markers in CSF and plasma from MS patients [6,9,10]. Further, Tasset et al. [11] showed a significant peripheral OS in RRMS patients.

The recent understanding of the patho-physiology of MS has led to the development of many drugs that counter exacerbations and the formation of new lesions in patients with relapsing remitting MS. Presently there are no therapies able to reduce neurodegenerative damages [12], therefore it is urgently needed to find new cures to contrast the progression of disability in MS.

Proteomic analysis is a powerful tool to identify putative biomarkers, to elucidate the molecular mechanisms underlying the disease, to allow monitoring of disease progression, and to find putative therapeutic targets for the treatment of the disease [13–15].

So far, most of the proteomic studies of patients affected by MS have been performed on cerebrospinal fluid (CSF) considering exclusively a single form of MS patients or a combined group of all subtypes of MS [16–18]. Recently, Stoop et al. [19] performed a proteomics study comparing CSF between RR and PP MS. Teunissen et al. [18] found novel potential biomarkers of the disease progression using proteomics technologies in CSF and serum of patients affected by all of three MS forms (RR, PP, SP). In addition, in the same study biomarkers discriminating patients with MS from others affected by different nervous disorders are shown.

In the present study, we aimed to describe the proteomic profile of the low-abundant serum protein fraction of MS patients, each one in remitting and in relapsing phase to reduce the inter-individual variability. MS patients were also compared with the sex and age-matched control group. Serum is a promising resource for biomarkers discovery, despite its complex nature and the presence of high abundant proteins masking subtle changes in the less abundant ones, can make disease biomarkers detection a very challenging task. Therefore, depletion of the most abundant proteins is an important step towards improving the analysis of serum.

In particular, in order to shed light on the role of OS in MS, we focused our attention to identify differently expressed and oxidatively modified proteins, by applying proteomics and redox proteomics approaches. Oxidative post-translational modification of proteins may produce structural changes in proteins that could be reflected on their functions, and therefore identification of these proteins could be useful in understanding which pathways are impaired in MS. The implications and the conceivable impact of the identified proteins on the molecular mechanisms and clinical outcomes of MS are discussed.

Materials and Methods

Study population was composed by patients with diagnosis of MS according to the revised McDonald criteria [20] enrolled at Multiple Sclerosis Centre, Regina Elena National Cancer Institute of Rome (Italy), and the sex- and age- matched healthy controls. The study was approved by Ethic Committee of Regina Elena National Cancer Institute and an informed written consent was obtained by all the participants.

Sample Collection

Blood samples from the same 18 RRMS patients in their relapsing (Group I) or remitting phase (Group II) and 7 healthy controls (Group III) were evaluated. A definition of MS relapse adopted was the modified Schumacher et al criteria [21]. with a minimum of 48 hours of symptom duration as well as changes in functional measures, as assessed using the Expanded Disability Status Scale (EDSS).

At the day of sampling all patients were assessed for physical and cognitive performance with followed examinations: EDSS; Multiple sclerosis severity Score (MSSS); Fatigue Severity Scale (MFIS); Beck Depression Inventory (BDI); Paced Auditory Serial Addition Test (PASAT). All patients were treated with immunomodulatory therapy in the previous 3 months. Subjects' characteristics are listed in Table 1.

Sample Preparation

Serum samples from MS patients were grouped into pools (six pools, each with three samples) to reduce the individual and biological variability that has been reported in plasma proteins [22]. Though this step involves the loss of each sample individual data, this pooling strategy has demonstrated its usefulness for biomarker discovery in shotgun proteomics approaches [23]. Serum from healthy controls were processed individually (n = 7).

In order to improve detection of low-abundant proteins, serum samples were purified with MARS-14 Column (Agilent Tech.), which removes 95 percent to 99 percent of the 14 most abundant proteins. These high-abundance proteins account for 94 percent of the total protein mass, and depletion of those facilitates the discovery and the identification of low-abundance proteins. Proteins from either the six pools, for each MS group, and the seven CTR samples were purified on a MARS-14 column and eluted according to the manufacturer's instructions. Protein determination was performed on the eluate fraction by the Bradford protein assay, using bovine serum albumin as a standard.

Measurement of Protein Carbonyls (PC)

Protein carbonyl levels were detected as adducts of 2,4-dinitrophenylhydrazine (DNPH). Five microliters of the samples (corresponding roughly to 2 μg of proteins) were treated with an equal volume of 12% SDS. Samples were then derivatized with 10 μL of 20 nM 2,4-DNPH in 2 N HCl for 20 min. After, samples were neutralized with 7.5 μL of 2 M Tris/30% glycerol buffer, pH = 8.0. Levels of protein carbonyls were measured by using the slot-blot technique with 250 ng of protein loaded per slot. The 2,4-dinitrophenylhydrazone (DNP) adducts were detected on the nitrocellulose paper using a primary DNP specific rabbit antibody (Millipore) (1:100) followed by a secondary goat alkaline phosphatase-conjugated anti-rabbit IgG (Sigma Aldrich). The reaction product was revealed by 5-Bromo-4-chloro-3-indolyl phosphate dipotassium combined with Nitrotetrazolium Blue chloride (BCIP/NBT) in ALP buffer [0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$ 6 H_2O (pH 9.5)]. After developing, blots were allowed to dry overnight, scanned on a GS800 densitometer (Biorad), and quantified by QuantityOne image software.

Two-Dimensional(2D) Gel Electrophoresis

Eluted proteins (100 μg) for each sample were diluted to a total volume of 200 μl with rehydration buffer (8 M urea, 20 mM dithiothreitol (DTT), 2.0% (w/v) Chaps, 0.2% Bio-Lyte, 2 M thiourea and bromophenolblue), and placed in agitation for 3 hours. For the first-dimension electrophoresis, 200 μL of sample solution were applied to a ReadyStrip ^{TM}IPG strip pH 4–7 (Bio-Rad). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strips were then actively rehydrated in a Protean IEF Cell Apparatus (Bio-Rad) for 16 h at 50 V. The isoelectric focusing was performed at 300 V for 2 h linearly; 500 V for 2 h linearly; 1000 V for 2 h linearly, 8000 V for 8 h linearly and 8000 V for 10 h rapidly. All the processes above were carried out at room temperature. The focused IEF strips were stored at –80°C until second dimension electrophoresis was performed.

For second dimension electrophoresis, the strips were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol (w/v), and then re-equilibrated for 15 min in the same buffer containing 4.5% iodacetamide in place of dithiothreitol. 12% Precast criterion gels (Bio-Rad) were used to perform second dimension electrophoresis. After electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10%

Table 1. Demographic and clinical characteristic of multiple sclerosis patients.

	Age	M/F	EDSS	Dis Dur	MSSS	MFIS	BDI	PASAT
Group I (Relapsing)	43.2±10	5/13	3.3±1.1	7.4±6	5.3±1.8	35.6±16.6	13.5±9.9	42.9±11.7
Group II (Remitting)	43±10.1	5/13	2.3±1.5	7.4±6.4	3.0±2.0	33.2±19.4	10.6±8.6	44.3±24.0
Group III (Control)	46.2±11.7	3/4	6.7±0.8					

EDSS = Expanded Disability Status Scale; Dis Dur = Disease duration; MSSS = Multiple sclerosis severity Score; MFIS = FatFatigue Severity Scale; BDI = Beck Depression Inventory; PASAT = Paced Auditory Serial Addition Test.
Re Reported values are ± SD.
doi:10.1371/journal.pone.0065184.t001

methanol) for 45 min, and then stained for 1 h in Bio-Safe Coomassie gel stain (Bio-Rad) and destained overnight in deionized water. The Coomassie gels were scanned using a GS 800 densitometer (Bio-Rad).

Immunochemical Detection

For immunoblotting analysis, electrophoresis was carried out in the same way as previously described, and the gels were transferred to nitrocellulose membranes. Six blots, corresponding to each single pool, for each MS group and seven blots from CTR samples were performed. To identify carbonylated proteins, blots were derivatized with 2,4-dinitrophenilhydrazine (DNPH). Briefly, membranes were equilibrated in 20% methanol (5 min), then incubated in 2 N HCl (5 min), and finally derivatized in 0.5 mM DNPH solution (5 min). After derivatization, three washes using 2 N HCl solution and five washes using methanol 50% were performed (5 min each). The membranes were then blocked with 3% albumin in T-TBS, incubated with the primary Rabbit x DNP antibody (1:100; Millipore) followed by the secondary antibody alkaline phosphatase-conjugated anti-rabbit IgG (1:5000; Sigma) and revealed by 5-Bromo-4-chloro-3-indolyl phosphate dipotassium combined with Nitrotetrazolium Blue chloride (BCIP/NBT) in ALP buffer [0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$ 6 H_2O (pH 9.5)].

Image Analysis

2D gels (a total of 12 from MS patients and 7 from CTR) and 2D blots were analyzed by PDQuest 2D Analysis (7.2.0 version; Bio-Rad). PDQuest spot-detection software allows the comparison of 2D gels as well as 2D blots from different groups. Powerful automatching algorithms quickly and accurately match gels or blots and sophisticated statistical analysis tools identify experimentally significant spots. The intensity value for each spot from an individual gel is normalized using the average mode of background subtraction. This intensity is afterward compared between groups using statistical analysis. Statistical significance was assessed using a two-tailed Student t-test. P values <0.05 were considered significant for comparison between control and MS patients data. In order to confirm data from gel comparison, each analysis was repeated three times by setting a different master gel every time according to the software instructions.

PDQuest software allows also normalization of a carbonylated spot intensity on the blot versus the expression level of the same spot on the gel. One-dimensional blots were analyzed with QuantityOne software (4.6.9version;Bio-Rad).

Trypsin Digestion and Proteins Identification by Mass Spectrometry

Selected spots were manually excised from gel and submitted to trypsin proteolysis [24]. MALDI-ToF MS analyses were performed with an AutoFlex II instrument (Bruker Daltonics, Bremen, Germany), equipped with a 337 nm nitrogen laser and operating in reflector positive mode. Two tryptic autolytic peptides were used for the internal calibration (m/z 842.5100 and 2807.3145). Data were analyzed by flex Analysis program (Bruker Daltonics, Bremen, Germany). Identification by peptide mass fingerprint (PMF), with the mono-isotopic mass list, was performed using Bio Tools program (Bruker Daltonics, Bremen, Germany), by the Mascot search engine, against human SwissProt database [(SwissProt 2012_10 (20234 sequences)]. Up to two missed cleavage, 50 ppm measurement tolerance, oxidation at methionine (variable modification) and carbamidomethylation at cysteine (fixed modification) were considered. Identifications were validated when the probability-based Mowse protein score was significant according to Mascot [25].

Statistical Analysis

For comparison of values between relapsing and remitting patients and controls, data from each group were checked for normality using the Shapiro–Wilk statistical test, and if this assumption was nonviable log transformation was applied. All statistical analyses were performed using a nonparametric one-way ANOVA with post hoc *t*-test. PDQuest software was used to select a master gel representing each group. For analysis of differentially expressed proteins among matched gels, spots whose intensities were either increased or decreased 1.5-fold or greater were marked and also confirmed by manual inspection of all relevant 2D gels to ensure consistency. Quantitative analysis was performed using the Student's *t*-test among three groups of gels. Significance was accepted if P value <0.05.

Results

Protein Carbonyl Level

Analysis of protein carbonyl levels revealed a small, but significant increase of this OS marker in serum of MS patients in relapsing phase respect to controls ($p = 0.034$). There were no significant differences in protein carbonyl levels between MS patients in remitting phase respect to MS patients in relapsing phase and respect to controls. These data are shown in Fig. 1.

The same trend was also observed by analyzing the depleted fraction (14 most abundant proteins) of serum samples (Figure S1).

Proteomics and Redox Proteomics

Proteomics analysis using 2-DE and Coomassie staining were performed in serum samples of patients with MS in relapsing (REL) or in remitting phase (REM) and in control group (CTR) to determine differently expressed proteins. In Fig. 2, 2-D gel images related to the matching between REL vs CTR, REM vs CTR, and REL vs REM are shown. Additional gels from the three groups of analysis are shown in Figure S2. Eight proteins showed expression

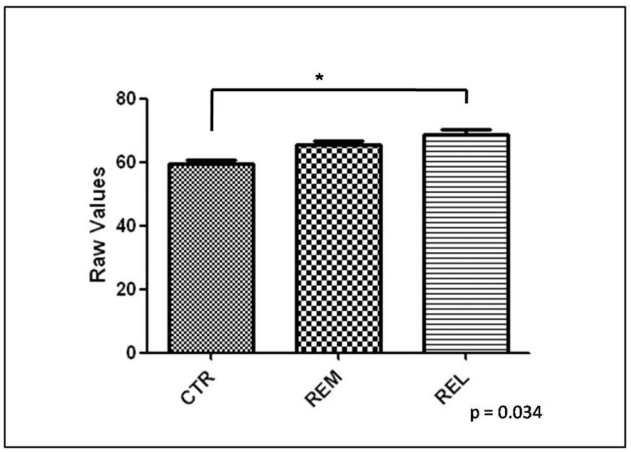

Figure 1. Levels of protein carbonyls. Protein carbonyls (PC) levels in the serum of control (CTR) and multiple sclerosis patients: remitting (REM) and relapsing (REL). Serum samples were assayed for PC by slot blot analysis. Error bars indicate SD for 6 samples per group. Densitometric values shown are given as raw values.
doi:10.1371/journal.pone.0065184.g001

differences. These protein spots were excised from the gels, and following trypsin digestion the peptides were analyzed by MS/MS. Molecular weight (MW) and isoelectric point of all identified proteins were consistent with those shown by protein positions on the gel. These are listed in Table 2 with the number of peptide sequences, the coverage, MW, pI, fold-change levels, and p-value.

From the matching between REL vs CTR, two proteins were found to be differently expressed: clusterin and apolipoprotein E, both of which showed to be over-expressed in REL compared to CTR (3.6-fold, and 1.8-fold, respectively).

In the analysis of REM vs CTR we identified three proteins: clusterin and complement C3 were up-regulated in REM compared to CTR (4.0-fold, and 1.5-fold, respectively), while antithrombin III was down-regulated in REM with respect to CTR (3.1-fold).

The match between REL vs REM showed three differently expressed proteins: the inter-α-trypsin inhibitor heavy chain H4 and the zinc-α-2-glycoprotein were, up-regulated (3.9-fold, and 2.8-fold, respectively), and ceruloplasmin was down-regulated (6.0-fold).

Specific carbonylated proteins were detected immunochemically using 2D-gels and 2D-Western blots. 2D-gels and 2D-blots probed with DNP- antibody were matched using the PD-Quest software and the specific protein carbonyl levels were obtained by dividing the protein carbonyl level of a single spot on the blot by the protein level of its corresponding protein spot on the gel. Fig. 3 shows three representative 2D gels and the corresponding 2D blots of REL vs CTR, REM vs CTR, and REL vs REM.

Relative change in carbonyl immune-reactivity, after normalization of the immunostaining intensities to the protein content, was significant for eleven spots. After spot excision and in-gel trypsin digestion, proteins were identified by MS/MS analysis.

In Table 3, the carbonylated proteins identified by MS/MS and interrogation of databases are listed. Interestingly, most of these proteins were more oxidized in relapsing patients compared with control patients. This result confirms our data about the measure of total levels of carbonylated proteins.

From the matching between REL vs CTR the identified proteins were: nebulin-related-anchoring protein (115-fold increase), inter-α-trypsin inhibitor heavy chain H4 (103-fold increase), α-1B-glycoprotein (326-fold increase), hemopexin (118-fold increase and 307-fold increase), antithrombin-III (305-fold increase), gelsolin (215-fold increase), vitamin D-binding protein (DBP) (213-fold increase), serum amyloid P-component (841-fold increase), and apolipoprotein A-IV (346-fold increase).

In the analysis between REM vs CTR one protein was found more oxidized in REM compared to CTR (65.2-fold): DBP.

The match between REL vs REM showed only one protein more oxidized in REL with respect to REM: apolipoprotein A-IV (23-fold).

As shown in Fig. 3, two spots with different MW and pI, were identified as DBP. A rational explanation to our findings is that in relapse phase the higher OS levels result in increased oxidation of the protein which also causes its subsequent fragmentation [26]. Indeed, in the matching between REL vs CTR solely C-terminal portion of DBP was revealed by mass spectrometry. To corroborate our notion, spectra obtained by MALDI-ToF MS analysis related to spots of DBP are shown in Fig. 4. This result indicates that the two spots correspond to the same protein, DPB, which in the case of REL group was identified as a fragment, most likely due to its oxidative modification. The same consideration can be also applied to hemopexin which has been identified by two different spots as well.

DBP seems to be the only protein differently carbonylated in both relapsing patients compared to controls ($p = 0.028$), and in remitting patients compared to controls ($p = 0.025$). In either matching, DBP is more oxidized in pathological samples, as shown in Fig. 5 and in Fig. 6.

Another protein, namely apolipoprotein A-IV, was found more oxidized in relapsing group compared to both control and remitting group (Fig. 7).

The proteins identified as differently expressed or oxidized are listed in Table 4 correlated with their functions.

Discussion

The etiology of MS is not yet completely known. Growing results support a pivotal role of OS in MS pathogenesis and progression [5,27]. Increased levels of OS markers and decreased levels of antioxidant molecules have been described in patients with MS [6]. This imbalance has been implicated in demyelination and axonal damage and a positive correlation between OS markers and expanded disability status scale was recently observed [28]. Pathological and clinical data collected in the past decade reveale that MS pathological mechanisms may vary according to the stage of the disease [29]. It is likely that there is on-going OS during different phases of MS. Recently the concept of MS as a biphasic disease has been divided into an inflammatory RR phase and a degenerative SP phase [30]. However, it is not clear how OS contributes to the occurrence of relapse in RRMS patients or eventually to further degeneration in SP. By evaluating total protein carbonyl levels in serum, we confirmed that oxidative damage is increased in relapse phase of MS patients. Indeed, we found higher protein carbonyl levels in relapsing phase compared with both remitting and healthy groups. In order to better understand this trend, we performed both proteomics and redox proteomics approaches to identify changes in serum proteome profiles in subjects of these three groups. Serum is a rich source of disease related proteins. Detecting them is extremely challenging due to the body fluid's characteristics. One particular feature is the risk that abundant proteins may interfere with identification and quantification of the less abundant ones, which are usually recognized as putative disease biomarkers. In order to improve

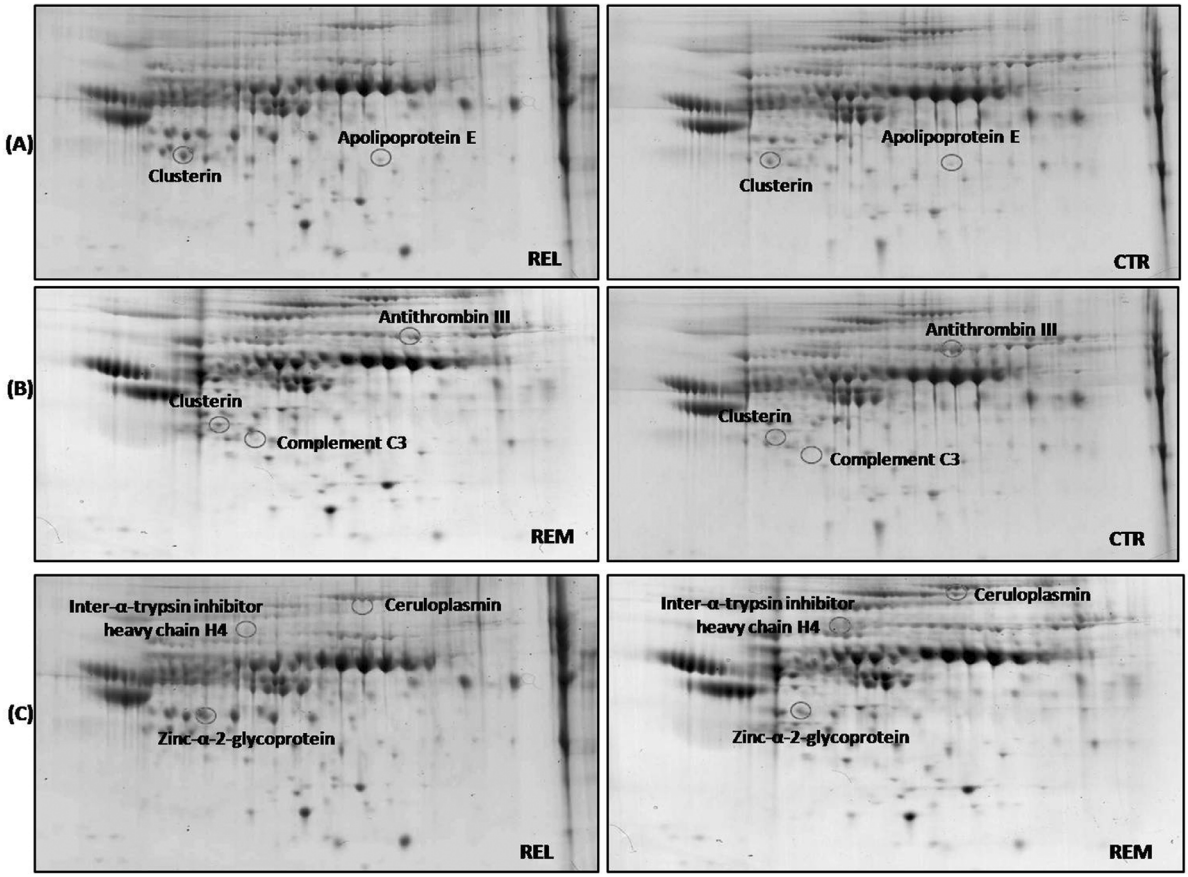

Figure 2. 2D protein expression maps. Proteomic profile of representative 2D-gels with proteins differently expressed in the three groups of matching: REL vs CTR (A), REM vs CTR (B) and REL vs REM (C). The identified proteins by mass spectrometry are listed in Table 2.
doi:10.1371/journal.pone.0065184.g002

the detection of low abundant proteins, we depleted the most-abundant protein fraction in our serum samples.

By comparing serum proteome profile of three groups of analysis, CTR, REM and REL, we identified differently expressed proteins, obtaining results in part consistent with data already showed in literature [31], thus validating our experimental approach. Interestingly, many of these proteins were up-regulated in MS patients compared to healthy controls, and in particular clusterin showed an intriguing trend, since its expression was increased already in remitting phase and kept up-regulated in relapsing phase. Clusterin is demonstrated to be implicated in several disorders characterized by increased OS and high cell death [32]. One of the most investigate areas among its functions, is chaperone activity, thanks to which clusterin binds to partly

Table 2. Summary of the proteins identified as differently expressed using the proteomics approach.

Protein Identified	SwissProt Code	% sequence coverage	Theoretical mw/pI (kDa)	Groups	P value[a]	Fold[b]
Ceruloplasmin	P00450	10	122.98/5.44	Rel-Rem	0.025	6.0 ↑ Rem
Inter-α-trypsin inhibitor heavy chain H4	Q14624	15	103.521/6.51	Rel-Rem	0.037	3.9 ↑ Rel
Zinc-α-2-glycoprotein	P25311	32	34.465/5.71	Rel-Rem	0.049	2.8 ↑ Rel
Antithrombin III	Q9UC78	40	53.025/6.32	Rem-Ctr	0.044	3.1 ↑ Ctr
Clusterin	P10909	24	53.031/5.89	Rem-Ctr	0.034	4.0 ↑ Rem
Clusterin	P10909	14	53.031/5.89	Rel-Ctr	0.038	3.6 ↑ Rel
Apolipoprotein E	P02649	45	36.246/5.65	Rel-Ctr	0.017	1.8 ↑ Rel
Complement C3	P01024	7	188.569/6.02	Rem-Ctr	0.034	1.5 ↑ Rem

[a]The p-value associated with fold-change calculated using a Student's t-test.
[b]The fold-change in spot density from three groups of matching: Rel vs Ctr; Rem vs Ctr; Rel vs Rem. The arrow indicates the direction of change.
doi:10.1371/journal.pone.0065184.t002

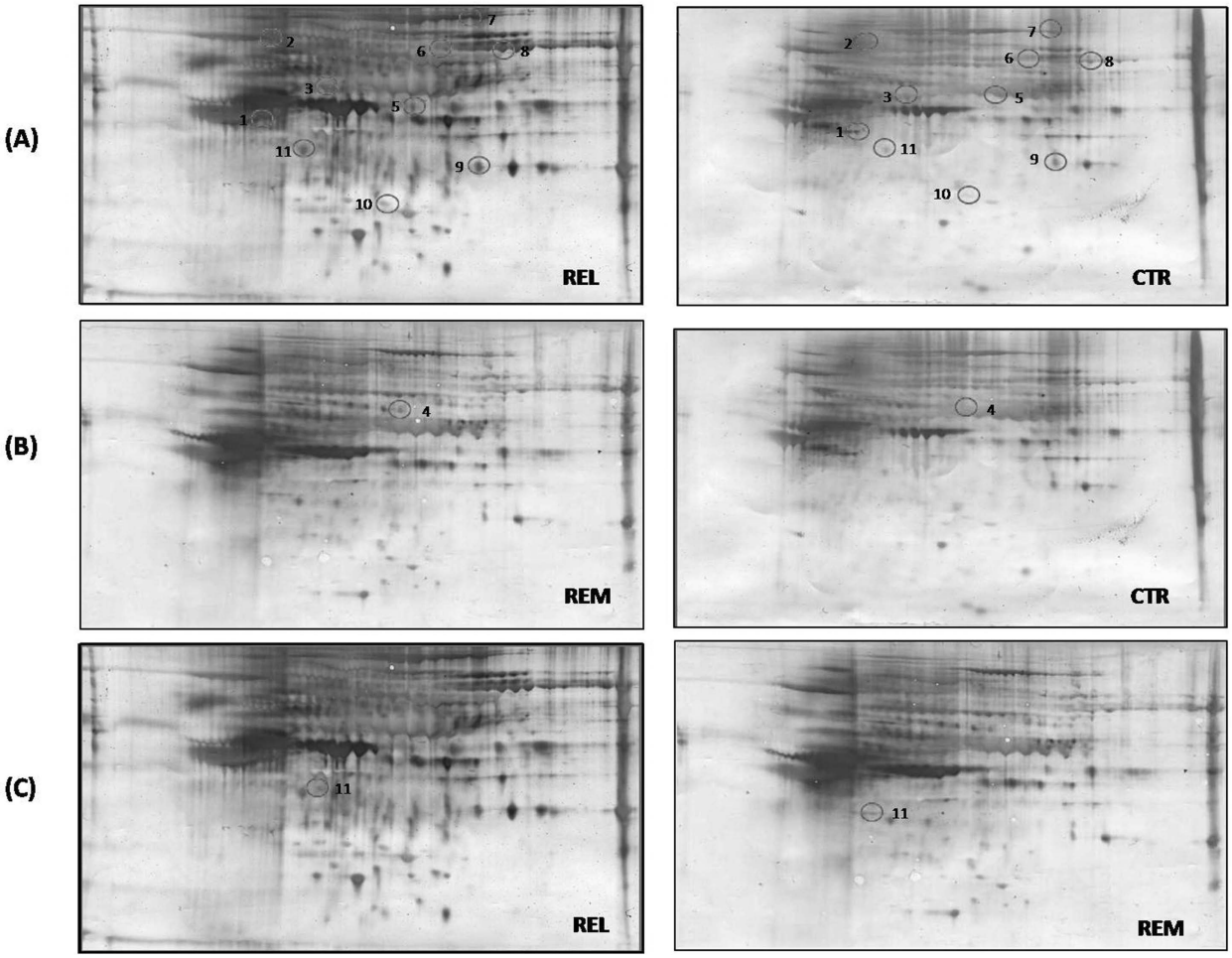

Figure 3. Oxidized protein detection by redox proteomics. Proteomic profile of representative 2D-blots with proteins differently oxidized in the three groups of matching: REL vs CTR (A), REM vs CTR (B) and REL vs REM (C).The identified proteins are listed in Table 3.
doi:10.1371/journal.pone.0065184.g003

Table 3. Summary of the proteins identified as differently oxidized using the redox proteomics approach.

Protein Identified	Spot	SwissProt code	% sequence coverage	Theoretical mw/pI (kDa)	Groups	P value[a]	Fold[b]
Nebulin-related-anchoring protein	1	Q80XB4	22	194.0/9.4	Rel-Ctr	0.003	115 ↑ Rel
Inter-α-trypsin inhibitor heavy chain H4	2	Q14624	29	103.52/6.51	Rel-Ctr	0.002	103 ↑ Rel
α-1B-glycoprotein	3	P04217	29	54.79/5.56	Rel-Ctr	0.0004	326 ↑ Rel
Vitamin D-binding protein	4	P02774	33	54.52/5.4	Rem-Ctr	0.025	65.2 ↑ Rem
Vitamin D-binding protein	9	P02774	9	54.52/5.4	Rel-Ctr	0.028	213 ↑ Rel
Hemopexin	5	P02790	23	52.38/6.55	Rel-Ctr	0.049	118 ↑ Rel
Hemopexin	7	P02790	25	52.38/6.55	Rel-Ctr	0.0004	307 ↑ Rel
Antithrombin-III	6	Q9UC78	22	53.025/6.32	Rel-Ctr	0.019	305 ↑ Rel
Gelsolin	8	P06396	21	86.04/5.9	Rel-Ctr	0.023	215 ↑ Rel
Serum amyloid P-component	10	P02743	27	25.48/6.1	Rel-Ctr	0.012	841 ↑ Rel
Apolipoprotein A-IV	11	P06727	60	45.37/5.28	Rel-Rem Rel-Ctr	0.012 0.012	23 ↑ Rel 346 ↑ Rel

[a]The p-value associated with fold-change calculated using a Student's t-test.
[b]The fold-change in spot density from three groups of matching: Rel vs Ctr; Rem vs Ctr; Rel vs Rem. The arrow indicates the direction of change.
doi:10.1371/journal.pone.0065184.t003

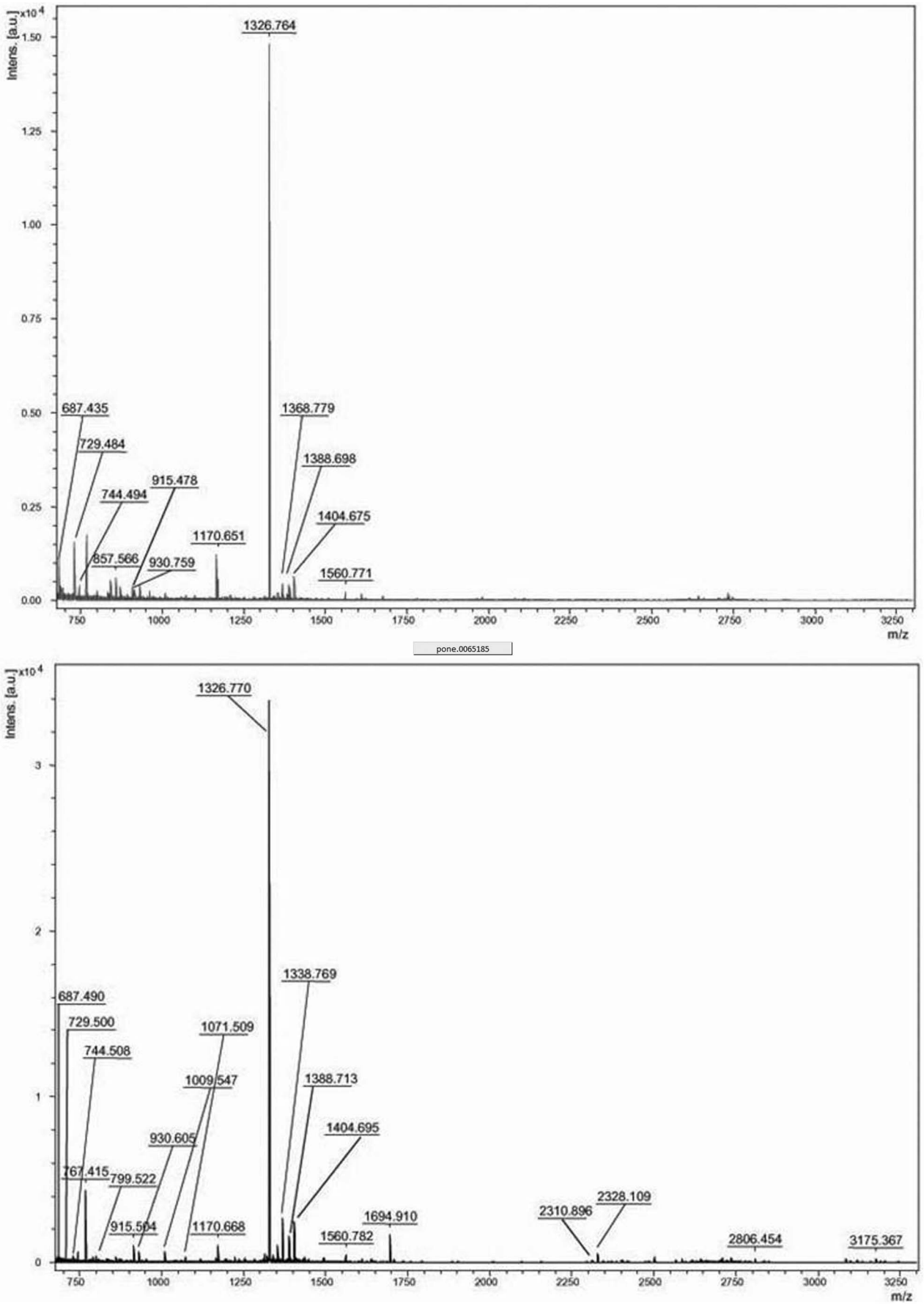
Intens. [a.u.]
x10^4
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1326.764
687.435
729.484
744.494
915.478
857.566
930.759
1170.651
1368.779
1388.698
1404.675
1560.771
750
1000
1250
1500
1750
2000
2250
2500
2750
3000
3250
m/z
pone.0065185
Intens. [a.u.]
x10^4
3
2
1
0
1326.770
687.490
729.500
744.508
767.415
799.522
915.504
930.605
1009.547
1071.509
1170.668
1338.769
1388.713
1404.695
1560.782
1694.910
2310.896
2328.109
2806.454
3175.367
750
1000
1250
1500
1750
2000
2250
2500
2750
3000
3250
m/z

Figure 4. Spectra obtained by MALDI-ToF MS analysis related to spots of vitamin D-binding protein (DBP). DBP C-terminal region spectrum (upper panel) and major coverage DBP spectrum (lower panel).
doi:10.1371/journal.pone.0065184.g004

unfolded proteins preventing their precipitation and protecting cells from the cytotoxic consequences [33]. For its protective capacity, clusterin expression has been shown to be up-regulated in cells under OS conditions [32]. Therefore, the increased expression levels of clusterin in REM and REL compared to CTR indicate that its up-regulation is an indirect "measure" of OS, which is found to occur already in remission and further increase in relapse. Thus, it could be used to assess the levels of OS in different phases of MS.

To more thoroughly investigate changes in the serum proteome, we also performed a redox proteomics analysis to reveal OS specific proteins potentially dysfunctional and linked to the pathological course of the disease.

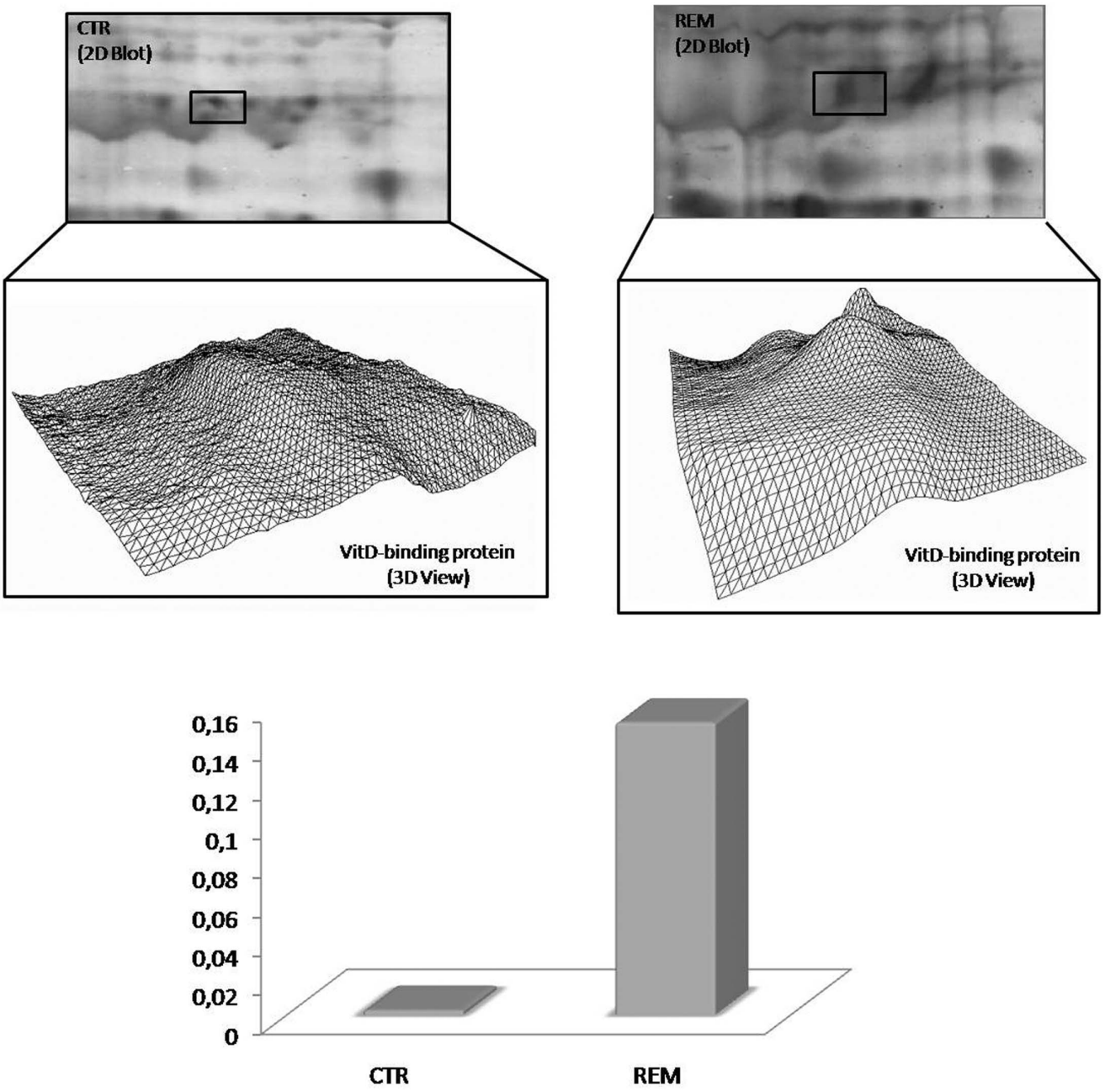

Figure 5. Vitamin D-binding protein oxidation fold in REM vs CTR matching. In the upper part of the figure, an enlarged area of 2D blots from CTR and REM groups is shown corresponding to the vitamin D-binding protein (DBP). DBP identified as differently oxidized in the matching between REM vs CTR is labeled. 3D density graphs are elaborated by PD-Quest from DBP spot on 2D blot. In the lower part of the figure the histogram reports oxidation fold for DBP.
doi:10.1371/journal.pone.0065184.g005

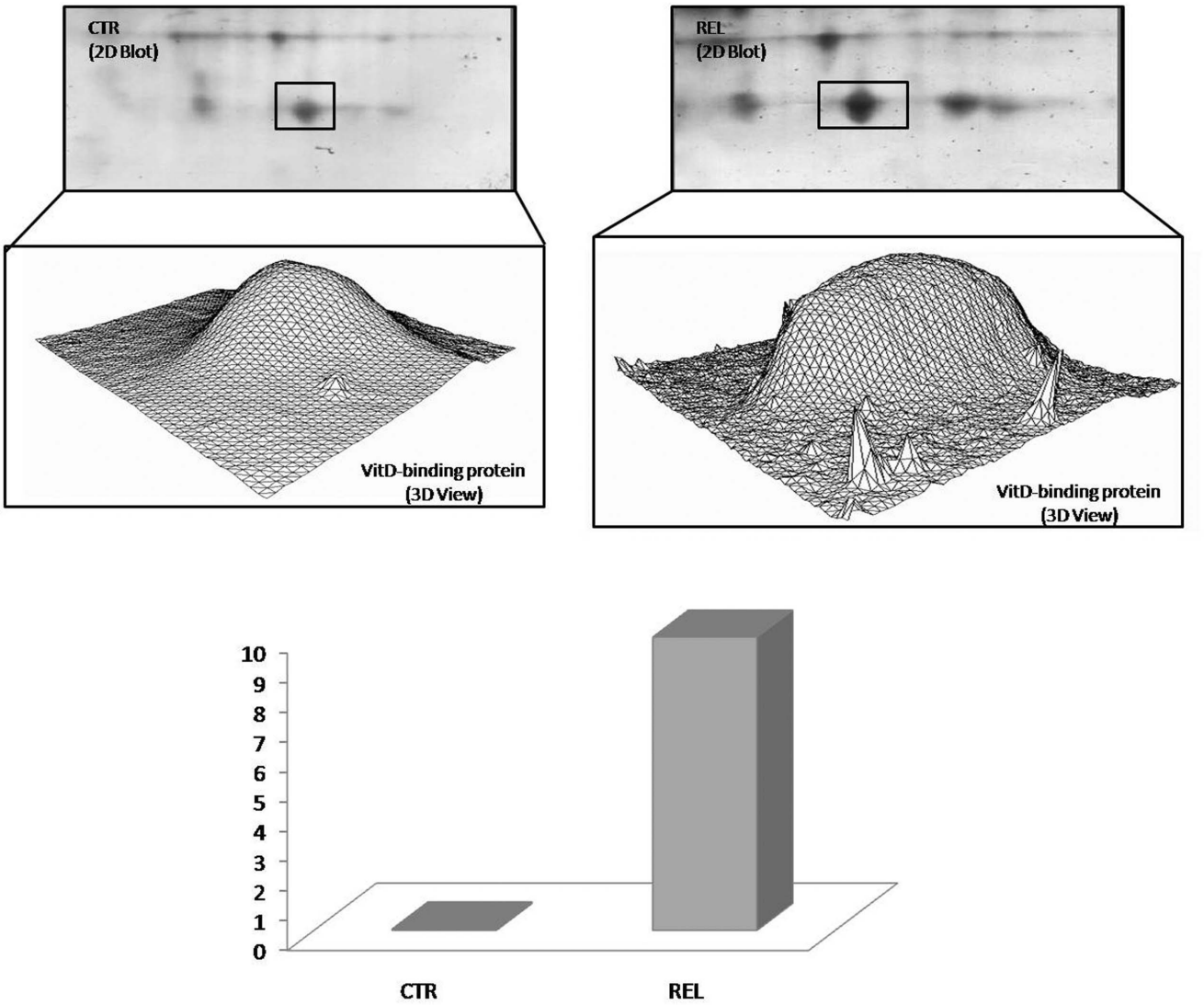

Figure 6. Vitamin D-binding protein oxidation fold in REL vs CTR matching. In the upper part of the figure, an enlarged area of 2D blots from CTR and REL groups is shown corresponding to the DBP. DBP identified as differently oxidized in the matching between REL vs CTR is labeled. 3D density graphs are elaborated by PD-Quest from DBP spot on 2D blot. In the lower part of the figure the histogram reports oxidation fold for DBP. (Data obtained from Proteomics and redox proteomics approach).
doi:10.1371/journal.pone.0065184.g006

Among our results, particularly intriguing appear the modifications shown by vitamin D-binding protein (DBP). DBP was found to be more oxidized in both the remitting and relapsing phases compared to controls, further showing a progressive rate of oxidation from control, to remission and to relapse., thus showing a full concordance with the course of the disease. DBP is the major plasma carrier protein of vitamin D metabolites [30]. It is synthesized by the liver in an estrogen dependent manner and is removed by several tissues such as kidney, skeletal muscle, intestine, bone, lung and the liver itself. The balance between production and clearance constantly maintains DBP plasmatic levels in molar excess respect to its major ligand, the 25-OH-vitamin D. A close connection between DBP level and the clinical course of MS has been recently suggested by a number of proteomic studies [29]. In their complex these studies indicate reduced DBP levels in CSF of patients with clinically isolated syndrome and RRMS relapses, while increased levels are found in patients with the progressive form [34]. The reported evidence of sharply relevant changes in DBP oxidation occurring during relapses introduce a further level of complexity in DBP activity during MS and consequently in the modulation of the whole vitamin D axis. It is to be stressed however that it is still unknown whether the role of DBP is dependent on its vitamin D binding activity, s on the other hand, it is well-established that DBP has a distinct immune-modulatory effect independent by vitamin D binding [35].

In light of the importance of immune response on the development of MS, oxidation of DBP seems to highlight the dysfunction of this protein as a selective alteration of this pathology. Moreover, the increased oxidation rate of DBP already in the remitting phase, supports the notion that some molecular pathways are not completely suppressed during remission. They may represent potential targets to reduce the severity of relapse and slowdown reoccurrence.

MS is mainly considered an inflammatory disease of the central nervous system, but several evidence further support an involve-

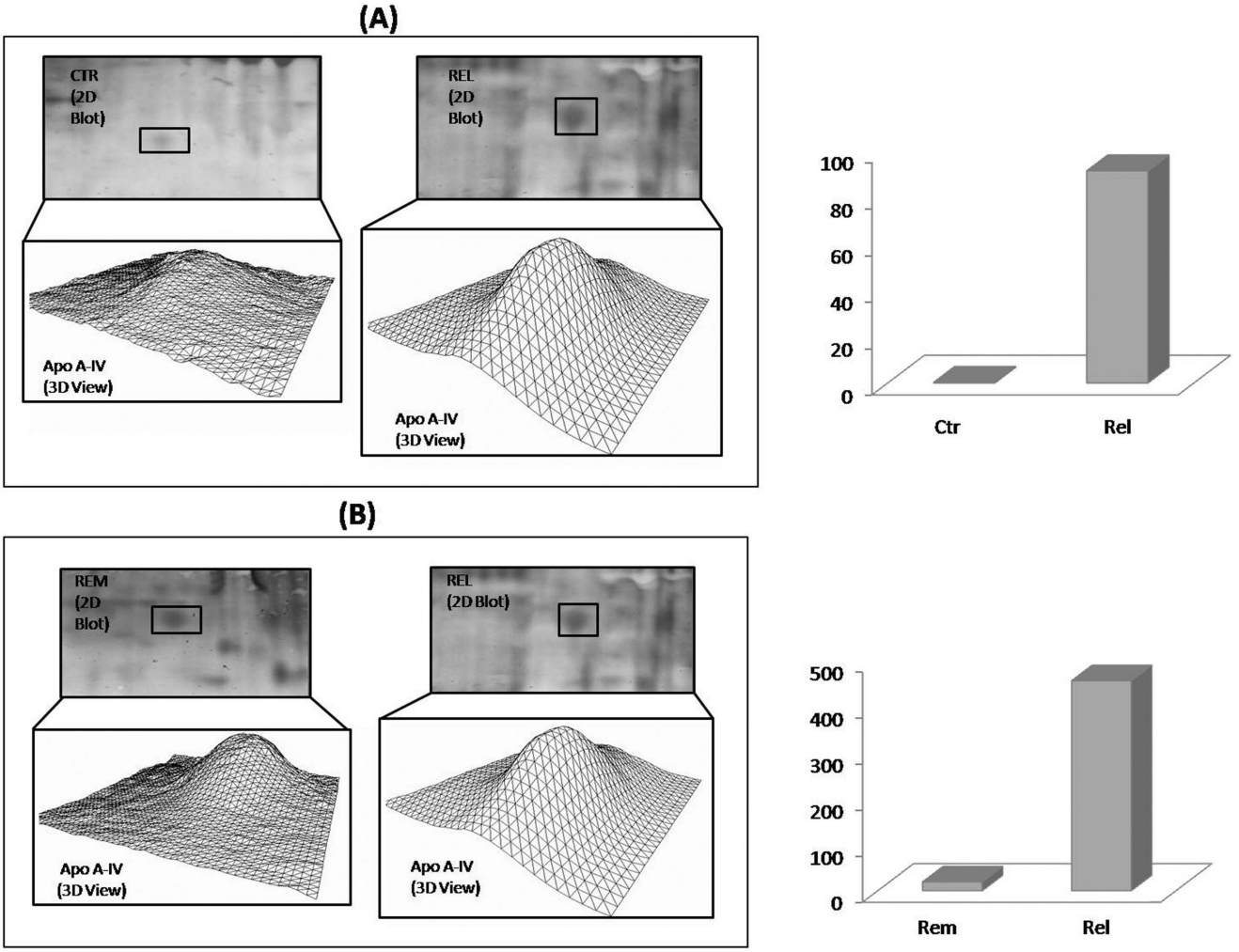

Figure 7. Apolipoprotein A-IV oxidation fold. On the left side of the figure, an enlarged area of 2D blots from CTR and REL groups (A), and from REM and REL (B),are shown corresponding to the Apolipoprotein A-IV (Apo A-IV) spot. Apo A-IV spot identified as differently oxidized in the matching between REL vs CTR, and REL vs REM is labeled. 3D density graphs are elaborated by PD-Quest from Apo A-IV spot on 2D blot. On the right side of the figure the histogram reports oxidation fold for Apo A-IV.
doi:10.1371/journal.pone.0065184.g007

ment of the coagulation system in its pathogenesis [36]. A link between the inflammation and coagulation system is well established [37,38]. High levels of thrombin and its plasma inhibitor, AT-III [39], were found in experimental autoimmune encephalomyelitis (EAE) brains during the peak of the disease. This is due to an increase of blood-brain barrier (BBB)

Table 4. Functions of Identified Proteins Differently Expressed and Oxidized.

Functions	Proteins involved
Inflammatory response	Hemopexin; Alpha-1-B glycoprotein; Inter-α-trypsin inhibitor heavy chain H4;Complement C3
Thrombin inhibition	Antithrombin III
Innate response	Serum amyloid P-component
Actin-binding	Nebulin-related anchoring protein; Gelsolin
Lipid metabolism	Apoliprotein A-IV; Apolipoprotein E; Zinc-α-2-glycoprotein
Vitamin D carrier; Actin binding; Immune response	Vitamin D-binding protein
Chaperone-activity	Clusterin
Fe homeostasis	Ceruloplasmin; Hemopexin

doi:10.1371/journal.pone.0065184.t004

permeability occurring in the acute phase of EAE allowing coagulation factors and their inhibitors to enter in the CNS [40]. Moreover, it has been shown that thrombin exacerbates EAE, thus inhibiting thrombin may have potential benefit for MS treatment [41]. Based on these notions, thrombin is considered a new therapeutic target for the cure of MS, and thrombin inhibitors, such as AT-III, a possible candidate drug [41]. In our study, AT-III has been found either down-regulated in the remitting phase compared to controls or oxidatively modified in the relapse phase compared to healthy patients, reinforcing the importance of the coagulation system in MS course according to current literature. Our results suggest that the balance restoration of the coagulation system may reduce the inflammatory damage typical of MS, and also may prevent the progression of the disease.

In addition, a number of acute phase proteins including: hemopexin, α-1-B-glycoprotein, inter-α-trypsin inhibitor heavy chain H4, and complement C3 have been found to be differently expressed and more oxidized in pathological versus controls samples. These same proteins were already detected differently expressed in inflammatory disease such as mastitis [42] thus further underscoring the link between MS pathogenesis and the disruption of the inflammatory response regulation.

Recently, a new aspect of MS is emerging which supports the involvement of altered phospholipid metabolism in the etiology of the disease [43]. Detection of biomarkers related to the deregulation of lipid metabolism may offer advances in diagnosis of the disease. In our study three modified proteins: apolipoprotein E (Apo E), zinc-α-2-glycoprotein, and apolipoprotein A-IV, implicated in lipid homeostasis, were found. apolipoprotein A-IV (Apo A-IV), a glycoprotein formed in intestine and then released in plasma, showed an increased oxidation in patients in relapsing phase compared with both remitting phase and controls. ApoA-IV is associated with triglyceride-rich lipoproteins and high cholesterol lipoprotein (HDL), and is also found as a lipoprotein-free form [44]. It has been demonstrated that Apo A-IV has an important role in lipid metabolism, and a defensive function against atherosclerosis acting as endogenous inhibitor of lipid oxidation [45]. Moreover, considering its role in the myelination [44,46], the oxidative damage of Apo A-IV could cause myelin dysfunction, thus contributing to demyelination process characteristic of MS. Interestingly, since Apo A-IV was found to be more oxidized in relapse compared to healthy patients, and to patients in remitting phase, it underlies that relapsing phase is characterized by high OS levels, in contrast to remitting phase that seems to be indistinguishable from controls. This result is in line with the above reported results on total protein carbonyl levels, and led us to hypothesize that Apo A-IV may be considered a putative biomarker to predict the occurrence of relapses.

By the comparison of serum proteome profile of MS patients and healthy controls, cytoskeletal proteins, such as nebulin-related anchoring protein and gelsolin, were found increasingly oxidized in relapse compared to controls. Gelsolin is a cytoplasmic actin-binding protein involved in actin assembly and filament remodeling [47]. It is also known to have an extracellular isoform called plasma gelsolin [48]. The main investigated role of gelsolin is its extracellular actin scavenging activity, resulting in actin removal from plasma with consequent consumption of gelsolin [49]. During MS, axonal degeneration leads to a release of actin within the CNS, which may mobilize gelsolin from plasma to CNS [50], as demonstrated by studies showing low levels of gelsolin in blood and in CSF of MS patients [50,51]. In contrast, in a recent study gelsolin was found over-expressed in CSF of MS patients [31], thus suggesting that during the development of the disease there may be a change in the concentration of some proteins [50]. Furthermore, gelsolin inhibits apoptosis [52], and, during physiological conditions, binds amyloid-β reducing its amount and preventing its fibrillation [53]. In light of these considerations, we propose that oxidized gelsolin may have a patho-physiological relevance in MS, including axonal degeneration and alteration of amyloid-β metabolism.

In autoimmune diseases, such as MS, there is a substantial increment of apoptotic cells [54], and an impairment of their clearance. In our study, serum amyloid P component (SAP) was increasingly oxidized during relapsing phase. Considering the SAP role in binding apoptotic cell and favoring their elimination [55], it is reasonable to propose that alteration of this protein is a specific trait of MS.

In conclusion, our findings support the hypothesis that OS plays a central role in MS. Indeed, the oxidized proteins found in MS correlate with some aspects of the clinical course of the pathology, and may be considered specific blood-based diagnostic and prognostic markers. Interestingly remission seems to be a "healthy-like" condition where apparently almost no significant accumulation of oxidative damage occurs. Accordingly, the clinical evaluation often fails to diagnose MS when patients are in the remission phase. It is reasonable to hypothesize that OS occurs at a level insufficient for immediate toxic effect. However it is still able to initiate a cascade of reactions affecting the function of several proteins that progressively become impaired and eventually culminate in relapse outburst. At this level, severe disability and inflammatory events occur and oxidative damage seems to affect multiple pathways. Further, large studies are needed to confirm our results and to support the above proposed hypotheses.

Supporting Information

Figure S1 Levels of protein carbonyls of high-abundant proteins. High-abundant proteins were removed from serum of control (CTR) and remitting (REM) and relapsing (REL) multiple sclerosis patients, and assayed for protein carbonyls by western blot analysis using a pool for each group of samples as described in Material and Methods and running them in triplicate. Representative immunoblot is shown in the upper part of the figure. The bar graph shows the densitometric evaluation and values are expressed as mean ± SD (*p = 0,02 REL vs CTR).
(TIF)

Figure S2 2D-gels. Three different 2D-gels for every group of analysis are shown (control, CTR; remitting, REM; and relapsing REL). Differentially expressed proteins are circled and labeled with their names.
(TIF)

Acknowledgments

We wish to thank Ms Tania Merlino for editing the English of this manuscript.

Author Contributions

Conceived and designed the experiments: MP FDM TK. Performed the experiments: AF EB AG. Analyzed the data: CF RC MES. Contributed reagents/materials/analysis tools: FDD. Wrote the paper: AF MP.

References

1. Polman CH, Reingold SC, Edan G, Filippi M, Hartung H-P, et al. (2005) Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". Annals of Neurology 58: 840–846.
2. Compston A, Coles A (2008) Multiple sclerosis. The Lancet 372: 1502–1517.
3. Silber E, Sharief MK (1999) Axonal degeneration in the pathogenesis of multiple sclerosis. Journal of the Neurological Sciences 170: 11–18.
4. Trapp BD, Ransohoff R, Rudick R (1999) Axonal pathology in multiple sclerosis: relationship to neurologic disability. Curr Opin Neurol 12: 295–302.
5. Gonsette RE (2008) Neurodegeneration in multiple sclerosis: The role of oxidative stress and excitotoxicity. Journal of the Neurological Sciences 274: 48–53.
6. Miller E, Walczak A, Saluk J, Ponczek MB, Majsterek I (2012) Oxidative modification of patient's plasma proteins and its role in pathogenesis of multiple sclerosis. Clinical Biochemistry 45: 26–30.
7. Ortiz GG, Macías-Islas MÁ, Pacheco-Moisés FP, Cruz-Ramos JA, Sustersik S, et al. (2009) Oxidative stress is increased in serum from Mexican patients with relapsing-remitting multiple sclerosis. Disease Markers 26: 35–39.
8. Stavropoulou C, Zachaki S, Alexoudi A, Chatzi I, Georgakakos VN, et al. (2011) The C609T inborn polymorphism in NAD(P)H:quinone oxidoreductase 1 is associated with susceptibility to multiple sclerosis and affects the risk of development of the primary progressive form of the disease. Free Radical Biology and Medicine 51: 713–718.
9. Bizzozero OA, DeJesus G, Callahan K, Pastuszyn A (2005) Elevated protein carbonylation in the brain white matter and gray matter of patients with multiple sclerosis. Journal of Neuroscience Research 81: 687–695.
10. Greco A, Minghetti L, Sette G, Fieschi C, Levi G (1999) Cerebrospinal fluid isoprostane shows oxidative stress in patients with multiple sclerosis. Neurology 53: 1876.
11. Tasset I, Agüera E, Sánchez-López F, Feijóo M, Giraldo AI, et al. (2012) Peripheral oxidative stress in relapsing–remitting multiple sclerosis. Clinical Biochemistry 45: 440–444.
12. Luessi F, Siffrin V, Zipp F (2012) Neurodegeneration in multiple sclerosis: novel treatment strategies. Expert Review of Neurotherapeutics 12: 1061–1077.
13. Butterfield DA, Perluigi M, Sultana R (2006) Oxidative stress in Alzheimer's disease brain: New insights from redox proteomics. European Journal of Pharmacology 545: 39–50.
14. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, et al. (2005) Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. Mass Spectrometry Reviews 24: 55–99.
15. Singh V, Hintzen RQ, Luider TM, Stoop MP (2012) Proteomics technologies for biomarker discovery in multiple sclerosis. Journal of Neuroimmunology 248: 40–47.
16. Chiasserini D, Di Filippo M, Candeliere A, Susta F, Orvietani PL, et al. (2008) CSF proteome analysis in multiple sclerosis patients by two-dimensional electrophoresis. European Journal of Neurology 15: 998–1001.
17. Liu S, Bai S, Qin Z, Yang Y, Cui Y, et al. (2009) Quantitative proteomic analysis of the cerebrospinal fluid of patients with multiple sclerosis. Journal of Cellular and Molecular Medicine 13: 1586–1603.
18. Teunissen C, Koel-Simmelink M, Pham T, Knol J, Khalil M, et al. (2011) Identification of biomarkers for diagnosis and progression of MS by MALDI-TOF mass spectrometry. Multiple Sclerosis Journal 17: 838–850.
19. Stoop MP, Singh V, Dekker LJ, Titulaer MK, Stingl C, et al. (2010) Proteomics Comparison of Cerebrospinal Fluid of Relapsing Remitting and Primary Progressive Multiple Sclerosis. PLoS ONE 5: e12442.
20. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, et al. (2011) Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Annals of Neurology 69: 292–302.
21. Schumacher GA, Beebe G, Kibler RF, Kurland LT, Kurtzke JF, et al. (1965) Problems Of Experimental Trials Of Therapy In Multiple Sclerosis: Report By The Panel On The Evaluation Of Experimental Trials Of Therapy In Multiple Sclerosis. Annals of the New York Academy of Sciences 122: 552–568.
22. Nedelkov D, Kiernan UA, Niederkofler EE, Tubbs KA, Nelson RW (2005) Investigating diversity in human plasma proteins. Proceedings of the National Academy of Sciences of the United States of America 102: 10852–10857.
23. Alsaif M, Guest PC, Schwarz E, Reif A, Kittel-Schneider S, et al. (2012) Analysis of serum and plasma identifies differences in molecular coverage, measurement variability, and candidate biomarker selection. Proteomics Clin Appl 6: 297–303.
24. Perluigi M, Giorgi A, Blarzino C, De Marco F, Foppoli C, et al. (2009) Proteomics analysis of protein expression and specific protein oxidation in human papillomavirus transformed keratinocytes upon UVB irradiation. Journal of Cellular and Molecular Medicine 13: 1809–1822.
25. Pappin DJ (2003) Peptide mass fingerprinting using MALDI-TOF mass spectrometry. Methods Mol Biol 211: 211–219.
26. Berlett BS, Stadtman ER (1997) Protein Oxidation in Aging, Disease, and Oxidative Stress. Journal of Biological Chemistry 272: 20313–20316.
27. Gilgun-Sherki Y, Melamed E, Offen D (2004) The role of oxidative stress in the pathogenesis of multiple sclerosis: The need for effective antioxidant therapy. Journal of Neurology 251: 261–268.
28. Oliveira SR, Kallaur AP, Simão ANC, Morimoto HK, Lopes J, et al. (2012) Oxidative stress in multiple sclerosis patients in clinical remission: Association with the expanded disability status scale. Journal of the Neurological Sciences 321: 49–53.
29. Bradl M, Lassmann H (2009) Progressive multiple sclerosis. Semin Immunopathol 31: 455–465.
30. Trapp BD, Nave KA (2008) Multiple sclerosis: an immune or neurodegenerative disorder? Annu Rev Neurosci 31: 247–269.
31. Rithidech K, Honikel L, Milazzo M, Madigan D, Troxell R, et al. (2009) Protein expression profiles in pediatric multiple sclerosis: potential biomarkers. Multiple Sclerosis 15: 455–464.
32. Trougakos IP, Gonos ES (2006) Regulation of clusterin/apolipoprotein J, a functional homologue to the small heat shock proteins, by oxidative stress in ageing and age-related diseases. Free Radical Research 40: 1324–1334.
33. Humphreys DT, Carver JA, Easterbrook-Smith SB, Wilson MR (1999) Clusterin Has Chaperone-like Activity Similar to That of Small Heat Shock Proteins. Journal of Biological Chemistry 274: 6875–6881.
34. Disanto G, Ramagopalan S, Para A, Handunnetthi L (2011) The emerging role of vitamin D binding protein in multiple sclerosis. Journal of Neurology 258: 353–358.
35. Roussel BD, Macrez R, Jullienne A, Agin V, Maubert E, et al. (2009) Age and albumin D site-binding protein control tissue plasminogen activator levels: neurotoxic impact. Brain 132: 2219–2230.
36. Chapman J (2006) Thrombin in inflammatory brain diseases. Autoimmunity Reviews 5: 528–531.
37. Cicala C, Cirino G (1998) Linkage between inflammation and coagulation: An update on the molecular basis of the crosstalk. Life Sciences 62: 1817–1824.
38. Esmon CT (2001) Role of coagulation inhibitors in inflammation. Thromb Haemost 86: 51–56.
39. Beresford CH, Owen MC (1990) Antithrombin III. Int J Biochem 22: 121–128.
40. Beilin O, Karussis DM, Korczyn AD, Gurwitz D, Aronovich R, et al. (2005) Increased thrombin inhibition in experimental autoimmune encephalomyelitis. Journal of Neuroscience Research 79: 351–359.
41. Inaba Y, Ichikawa M, Koh C-S, Inoue A, Itoh M, et al. (1999) Suppression of Experimental Autoimmune Encephalomyelitis by Dermatan Sulfate. Cellular Immunology 198: 96–102.
42. Turk R, Piras C, Kovačić M, Samardžija M, Ahmed H, et al. (2012) Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. Journal of Proteomics 75: 4412–4428.
43. Del Boccio P, Pieragostino D, Di Ioia M, Petrucci F, Lugaresi A, et al. (2011) Lipidomic investigations for the characterization of circulating serum lipids in multiple sclerosis. Journal of Proteomics 74: 2826–2836.
44. D'Aguanno S, Barassi A, Lupisella S, d'eril GM, Del Boccio P, et al. (2008) Differential cerebro spinal fluid proteome investigation of Leber hereditary optic neuropathy (LHON) and multiple sclerosis. Journal of Neuroimmunology 193: 156–160.
45. Qin X, Swertfeger DK, Zheng S, Hui DY, Tso P (1998) Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. American Journal of Physiology - Heart and Circulatory Physiology 274: H1836–H1840.
46. Olmarker K, Nordborg C, Larsson K, Rydevik B (1996) Ultrastructural changes in spinal nerve roots induced by autologous nucleus pulposus. Spine (Phila Pa 1976) 21: 411–414.
47. Sun HQ, Yamamoto M, Mejillano M, Yin HL (1999) Gelsolin, a Multifunctional Actin Regulatory Protein. Journal of Biological Chemistry 274: 33179–33182.
48. Kwiatkowski DJ, Stossel TP, Orkin SH, Colten HR, Yin HL (1986) Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. Nature 323: 455–458.
49. Lind SE, Smith dB, Janmey PA, Stossel TP (1986) Role of plasma gelsolin and the vitamin D-binding protein in clearing actin from the circulation. J Clin Invest 78: 736–742.
50. Kulakowska A, Ciccarelli NJ, Wen Q, Mroczko B, Drozdowski W, et al. (2010) Hypogelsolinemia, a disorder of the extracellular actin scavenger system, in patients with multiple sclerosis. BMC Neurol.
51. Kulakowska A, Drozdowski W, Sadzynski A, Bucki R, Janmey PA (2008) Gelsolin concentration in cerebrospinal fluid from patients with multiple sclerosis and other neurological disorders. European Journal of Neurology 15: 584–588.
52. Koya RC, Fujita H, Shimizu S, Ohtsu M, Takimoto M, et al. (2000) Gelsolin Inhibits Apoptosis by Blocking Mitochondrial Membrane Potential Loss and Cytochrome c Release. Journal of Biological Chemistry 275: 15343–15349.
53. Antequera D, Vargas T, Ugalde C, Spuch C, Molina JA, et al. (2009) Cytoplasmic gelsolin increases mitochondrial activity and reduces Aβ burden in a mouse model of Alzheimer's disease. Neurobiology of Disease 36: 42–50.
54. Zipp F (2000) Apoptosis in multiple sclerosis. Cell Tissue Res 301: 163–171.
55. Bijl M, Horst G, Bijzet J, Bootsma H, Limburg PC, et al. (2003) Serum amyloid P component binds to late apoptotic cells and mediates their uptake by monocyte-derived macrophages. Arthritis & Rheumatism 48: 248–254.