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Original Contribution

Decreased expression and increased oxidation of plasma haptoglobin in Alzheimer disease: Insights from redox proteomics

A. Cocciolo [a], F. Di Domenico [a], R. Coccia [a], A. Fiorini [a], J. Cai [b], W.M. Pierce [b], P. Mecocci [c], D.A. Butterfield [d,e,f], M. Perluigi [a,*]

[a] Department of Biochemical Sciences, Sapienza University of Rome, 00185 Rome, Italy
[b] Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, Louisville, KY 40292, USA
[c] Institute of Gerontology and Geriatrics, University of Perugia, 06122 Perugia, Italy
[d] Department of Chemistry, University of Kentucky, Lexington, KY 40506-0055, USA
[e] Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055, USA
[f] Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA

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ABSTRACT

Alzheimer disease (AD) is one of the most disabling disorders of the elderly and the number of people worldwide facing dementia is expected to dramatically increase in the near future. Thus, one of the major concerns of modern society is to identify putative biomarkers that serve as a valuable early diagnostic tool to identify a subset of patients with increased risk to develop AD. An ideal biomarker should be present in blood before dementia is clinically confirmed, have high sensitivity and specificity, and be reproducible. Proteomics platforms offer a powerful strategy to reach these goals and recently have been demonstrated to be promising approaches. However, the high variability of technologies and studied populations has led to contrasting results. To increase specificity, we analyzed both protein expression profiles and oxidative modifications (carbonylation) of plasma proteins in mild cognitive impairment (MCI) and AD subjects compared with age-matched controls. Most of the proteins found to have differential levels in MCI and AD confirmed results already obtained in other cohort studies. Interestingly, we applied for the first time in MCI a redox proteomics approach to specifically identify oxidized proteins. Among them, haptoglobin, one of the most abundantly secreted glycoproteins with chaperone function, was found to be either increasingly downregulated or increasingly oxidized in AD and MCI compared with controls. We also demonstrated that in vitro oxidation of haptoglobin affects the formation of amyloid-β fibrils, thus suggesting that oxidized haptoglobin is not able to act as an extracellular chaperone to prevent or slow formation of amyloid-β aggregates. Another chaperone protein, α2-macroglobulin, was found to be selectively oxidized in AD patients compared with controls. Our findings suggest that alterations in proteins acting as extracellular chaperones may contribute to exacerbating amyloid-β toxicity in the peripheral system and may be considered a putative marker of disease progression.

Alzheimer disease (AD)[1] is a neurodegenerative disorder affecting more than 20 million people worldwide [1]. AD is characterized by progressive cognitive deficits that gradually lead to loss of memory and impaired activities in daily living. The pathological hallmarks of the disease are deposition of senile plaques resulting from the extracellular deposit of amyloid-β peptide (Aβ) and the intracellular neurofibrillary tangles caused by the aggregation of hyperphosphorylated tau protein [2,3]. Mild cognitive impairment (MCI) is considered to be an early stage of cognitive decline that precedes dementia [4]. MCI patients may progress to AD, thus suggesting MCI as a prodromal phase of the disease and a useful model to study the mechanisms of AD progression [5]. Even though the etiology of AD is still not clearly known, oxidative stress (OS) has been firmly established as one of the main pathogenic events. Numerous studies have demonstrated increased levels of OS markers including protein oxidation, lipid peroxidation, and DNA oxidation in the brain of AD subjects compared with healthy controls [6–8]. Most of these data support the view that Aβ plays a crucial role in AD pathogenesis and progression and it possibly triggers the oxidative stress-mediated damage that ultimately results in neuronal cell death. The amyloid cascade hypothesis of AD highlights that increased Aβ load is due not only to its increased release (i.e., APP mutations in familial AD) but also to the impairment of degradative systems, including

* Corresponding author.
E-mail address: Marzia.perluigi@uniroma1.it (M. Perluigi).

[1] Abbreviations used: AD, Alzheimer disease; Aβ, amyloid-β peptide; APP, amyloid precursor protein; A2M, α2-macroglobulin; 2DE, two-dimensional electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; DNP, dinitrophenylhydrazone; Hp, haptoglobin; MCI, mild cognitive impairment; UCHL1, ubiquitin C-terminal hydrolase L1.

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proteasome, autophagy, and both intracellular and extracellular chaperones.

Up to now a diagnosis of AD is mainly based on clinical features, neuropsychological tests, and family history, thus resulting in a diagnosis of "probable" AD that sometimes groups together both AD and not-AD dementias. Only postmortem brain examination allow a diagnosis of "definite" AD [9]. To improve the diagnostic tools and early diagnosis of AD, it could be of great importance to identify sensitive and reliable biomarkers that are easy to collect by noninvasive and safe procedures. Body fluids are a great source for putative biomarkers, and cerebrospinal fluid (CSF), plasma, and serum are taken into consideration to search for biological molecules that could relate to AD. Though CSF is the most studied and validated, its collection requires lumbar puncture, which is not always feasible. Thus, increasing interest is currently devoted to the analysis of plasma, which reflects the physiological and pathological status of the organs [10]. Moreover about 500 ml of CSF is absorbed every day in plasma, providing a glimpse of brain content.

One of the major limitations using plasma to find reliable markers of AD, as well as of other pathologies, is related to the extreme complexity of its composition. Indeed, albumin itself represents about 60% of the total plasma protein content. The highly abundant proteins could mask the less abundant ones, which represent the potential biomarkers of diseases. Thus, depletion of abundant plasma proteins before analysis is considered an important strategy to increase the number of detectable proteins [11].

Recent studies have proposed possible biomarkers in plasma of patients with AD [12,13]. Many studies identified proteins differentially expressed in AD by a proteomics approach [10,14]. Bennet et al. analyzed plasma samples from AD patients and age-matched controls by iTRAQ technologies coupled with LC–MS/MS [11,15].

In recent years, increasing studies have been focused on establishing a direct link between tissue-specific oxidation and systemic oxidative damage, and correlations between total levels of markers of oxidation in the brain and in the periphery have been shown [16,17].

This study aimed at analyzing variations in the plasma proteome, both expression levels and posttranslational modifications, in subjects with AD and MCI compared to age-matched controls after depletion of the two most abundant proteins, albumin and IgG's. In particular, considering the role of oxidative stress in AD pathogenesis and progression, we focused our attention on oxidative modification of plasma proteins, by a redox-proteomics analysis, to obtain new insights into AD biomarker discovery.

Materials and methods

Sample collection

Blood samples were collected from 10 AD, 10 MCI, and 10 age-matched control subjects attending the Dementia Clinic, Department of Gerontology and Geriatrics, University Hospital of Perugia, Italy. In Table 1 the population demographics data are reported. Diagnosis of probable AD was accomplished according to the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's Disease (NINCDS-ADRDA) criteria [9]. MCI diagnosis was made according to the Petersen criteria [18]. Cognitively healthy subjects (CTR) were enrolled from among relatives of patients and subjects admitted to the Day Hospital of the same department for routine evaluation of the health status. All subjects underwent thorough clinical, neurological, and neuropsychological evaluation; the neuropsychological assessment included the Mini Mental State Examination (MMSE), whereas the CDR scale measured the severity of dementia. Functional status was evaluated on the basis of the activities of daily living and the instrumental activities of daily living. The presence of depressive symptoms was evaluated with the Geriatric Depression Scale, and the Hachinski Ischemic Score was used to consider ischemic risk. The investigation conformed to the principles outlined in the Declaration of Helsinki. All the patients or their relatives gave the written informed consent for blood donation. Blood was immediately centrifuged and plasma stored at $-80\ ^{\circ}C$.

Albumin and IgG plasma depletion

Plasma samples were depleted of these two most abundant proteins using a ProteoPrep Blue albumin and IgG depletion kit (Sigma–Aldrich). Briefly 50 μl of raw plasma sample was purified on a ProteoPrep column and eluted according to the manufacturer's instructions. Protein determination was performed on the eluate fraction with a Coomassie Protein Assay (Pierce).

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 bromophenol blue). Isoelectric focusing was performed with ReadyStrip IPG Strips (11 cm, pH 3–10; Bio-Rad) 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 above processes were carried out at room temperature.

After the first-dimension run the strips were equilibrated two times, first 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 and again for another 10 min in the same buffer containing 4.5% iodoacetamide in place of dithiothreitol. The second dimension was performed using 12% precast Criterion gels (Bio-Rad). The gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 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).

Table 1
General description of the population included in this study.

Patient group	*N*	Age (years)	MMSE	ADL	IADL	GDS	CDR	HIS	CHOL
CTR (female)	8	78 ± 5	27.7 ± 1.2	5.5 ± 0.5	7 ± 0.6	8 ± 1.7	0	1.5 ± 0.5	197 ± 22
CTR (male)	2	78 ± 9	28.5 ± 2.1	5 ± 0	6 ± 1.4	11.5 ± 3.5	0	1 ± 0	205 ± 5.7
MCI (female)	8	78 ± 5	23 ± 1.6	3.8 ± 0.2	4 ± 1.5	9 ± 2.7	0.5	2.2 ± 0.4	204 ± 38
MCI (male)	2	77 ± 1	22.5 ± 0.7	4 ± 1.4	6 ± 1.4	9 ± 0.7	0.5	1 ± 0	234 ± 49
AD (female)	6	83 ± 5	13.8 ± 4.9	3.8 ± 1.5	2 ± 1.4	3.8 ± 3.7	1.5 ± 0.8	1.5 ± 1.4	206 ± 31
AD (male)	4	77 ± 13	12.5 ± 2.1	2.5 ± 1.7	1 ± 2	4.5 ± 2.4	1 ± 0	1.8 ± 1.5	235 ± 26

N, number of samples for each group; age, average age ($\pm$ standard deviation) of patients; CTR, age-matched controls; AD, Alzheimer disease; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination (30 points total); ADL, activities of daily living (6 points total); IADL, instrumental activities of daily living (8 points total); GDS, Geriatric Depression Scale (15 points total); CDR, Clinical Dementia Rating; HIS, Hachinski Ischemic Score; CHOL, cholesterol.

2D gels for successive blotting were obtained by diluting 50 μg of depleted samples in a total volume of 200 μl with rehydration buffer and run as reported above except for gel fixing.

Western blotting

For 2D OxyBlot, 2D gels (50 μg of proteins) were blotted onto nitrocellulose membranes (Bio-Rad) and 2,4-dinitrophenylhydrazine (DNPH) derivatization was performed. Briefly, membranes were equilibrated in 20% methanol (5 min), then incubated in 2N 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). Finally the membranes were blocked with 3% albumin in T-TBS and incubated with the primary RabbitxDNP antibody (1:100; Millipore) and the secondary antibody alkaline phosphatase-conjugated anti-rabbit IgG (1:5000; Sigma). The colorimetric reaction was obtain using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution.

For Western blot, 30 μg of proteins from albumin- and IgG-depleted samples (CTR, AD, and MCI) were separated by 12% SDS–PAGE and blotted onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 3% bovine serum albumin in T-TBS and incubated for 1 h and 30 min at room temperature with primary anti-haptoglobin (Hp) β-chain antibody (1:100; Santa Cruz Biotechnologies) and for 1 h at room temperature with secondary antibody horseradish peroxidase-conjugated anti-mouse IgG (1:5000; Sigma–Aldrich). Membranes were developed with the Super Signal West Pico chemiluminescence substrate (Thermo Scientific).

Image analysis

2D gels 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 experimental data (CTR vs AD and CTR vs MCI). PDQuest software allows also normalization of a carbonylated spot intensity on the blot for the expression level of the same spot on the gel. One-dimensional blots were analyzed with QuantityOne software (4.6.9 version; Bio-Rad).

Trypsin digestion and protein identification by mass spectrometry

Protein spots statistically different from controls were digested in-gel by trypsin. Briefly, spots of interest were excised and then washed with 0.1 M ammonium bicarbonate (NH_4HCO_3) at room temperature for 15 min. Acetonitrile was added and incubated at room temperature for 15 min. This solvent mixture was then removed and gel pieces were dried. The protein spots were then incubated with 20 μl of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. The DTT solution was removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The solution was then incubated at room temperature for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH_4HCO_3 at room temperature for 15 min. Acetonitrile (200 μl) was added. After 15 min incubation, the solvent was removed, and the gel spots were dried for 30 min. The gel pieces were rehydrated with 20 ng/μl modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3 with the minimal volume necessary to cover the gel pieces. The gel pieces were incubated overnight at 37 °C in a shaking incubator. Protein spots of interest were excised and subjected to in-gel trypsin digestion, and resulting tryptic peptides were analyzed with an automated nanospray Nanomate Orbitrap XL MS/MS platform [6]. The Orbitrap MS was operated in a data-dependent mode whereby the eight most intense parent ions measured in the FT at 60,000 resolution were selected for ion trap fragmentation with the following conditions: injection time 50 ms, 35% collision energy. MS/MS spectra were measured in the FT at 7500 resolution, and dynamic exclusion was set for 120 s. Each sample was acquired for a total of 2.5 min. MS/MS spectra were searched against the IPI Worms Database using SEQUEST with the following criteria: Xcorr >1.5, 2.0, 2.5, 3.0 for $+1$, $+2$, $+3$, and $+4$ charge states, respectively, and P value (protein and peptide) <0.01. IPI accession numbers were cross-correlated with SwissProt accession numbers for final protein identification.

β-AMYLOID FIBRIL FORMATION ASsAY

$A\beta_{25-35}$ (1 mg/ml) was dissolved in 10 mM natrium hydroxide (NaOH), and stock solutions were stored at −20 °C. Thioflavin T (ThT; Sigma–Aldrich) was dissolved in ultrapure water to 1 mM for stock solutions and stored at −20 °C until use. Before each experiment an aliquot of ThT stock solution was thawed at 4 °C, shielded from light, and each stock solution was thawed only one time.

Hp β-chain lyophilized powder (Abbiotec, San Diego, CA, USA) was resuspended in 1 ml ultrapure water and its concentration was determined measuring the absorbance at 280 nm using the molar extinction coefficient of 5.1×10^4. Stock solutions of 10 μM Hp were made and stored at −20 °C. Hp was oxidized in vitro using UVB radiation. The UVB source was provided by a bank of Sankyo Denki G15T8E fluorescent tubes emitting 270–320 nm with a peak at 313 nm. The energy actually incident on the working area was measured by a UVX radiometer (UVP, Upland, CA, USA) and expressed in J/m^2. The UV dosage of 25 J/m^2 (10-s exposure time) was chosen according to previous studies we had done on cell culture.

Ten microliters of Aβ stock solution was added to 20 mM Tris–HCl, pH 7.4, alone or together with oxidized/not oxidized Hp at final concentrations of 0.5 and 1 μM (final total volume 1 ml). Ten microliters of ThT stock solution was added and the assembly of $A\beta_{25-35}$ was monitored using a ThT binding assay. ThT fluorescence intensity was monitored by fluorescence measurement using a Jasco FP-3600 fluorimeter at room temperature with constant stirring and the excitation and the emission wavelengths set at 450 and 484 nm, respectively. Changes in fluorescence were monitored every 2 min over at least 3 h.

Validation of Hp UVB oxidation in vitro

To validate the in vitro oxidation of Hp we measured the carbonyl levels both in the nonoxidized Hp solution and in the UVB-oxidized solution.

Briefly, 5 μl of each Hp solution was treated with an equal volume of 12% SDS. Samples were then derivatized with 10 μl of 20 nM DNPH for 20 min, and the derivatization was stopped by the addition of neutralizing reagent (7.5 μl of 2 M Tris/30% glycerol buffer, pH 8.0). Carbonylated Hp was measured using the slot-blot technique with 250 ng of protein loaded per slot. The 2,4-dinitrophenylhydrazone (DNP) adduct was detected on the nitrocellulose paper as reported above for 2D blot.

Statistical analysis

For comparison of values between AD and MCI 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. Significance was accepted if $P < 0.05$.

Results

Subject characteristics

In total 30 plasma samples were used for proteomics and redox proteomics analysis. The subject groups were not significantly different with respect to age, gender, or level of education. The AD group had an MMSE score indicating severe cognitive deterioration (mean 13), whereas MCI subjects had mild cognitive deterioration (mean 22; Table 1), coupled with a CDR of 1.65 and 0.5 respectively.

Identification of differentially expressed proteins in AD and in MCI

To examine the plasma proteome profile of AD, MCI, and CTR subjects, we performed a 2D-gel electrophoresis in plasma samples from each group, MCI and AD ($N=10$), comparing with age-matched controls ($N=10$), thus carrying out three different matching analyses: AD vs CTR, MCI vs CTR, and AD vs MCI. Representative 2D gels from each group are shown in Fig. 1. Statistical evaluation of relative spot densities allowed us to identify 13 proteins differentially expressed with at least 1.5-fold increase or decrease compared with respective controls. The list of all the identified proteins with the fold change and *P* values is reported in Table 2.

From the matches between AD and CTR seven proteins were found to be significantly differentially expressed. Among these, two were upregulated in AD vs CTR, α2-macroglobulin (A2M), 2.17-fold, and fibrinogen γ chain, 2.25-fold; and five were downregulated in AD, apolipoprotein 1, 1.7-fold; Hp β chain, 1.7 fold; β2-glycoprotein, 1.8-fold; fibrinogen α chain, 43-fold; and serotransferrin, 7.7-fold.

In the analysis of MCI vs CTR group the spots identified were two: inter-α trypsin inhibitor, downregulated in MCI vs CTR (2.6-fold), and fibrinogen γ chain, upregulated in MCI (2.26-fold) with respect to CTR.

The match between AD and MCI showed four proteins differentially expressed, among which three were downregulated in AD vs MCI, Hp β, 1.9-fold decrease; fibrinogen α chain (2.5-fold); and complement factor B (2.5-fold); and one was upregulated in AD vs MCI, retinol-binding protein 4, 1.9-fold increase.

Validation of proteomics result for Hp

To verify the alterations in Hp β-chain levels obtained by spot-density comparison on 2D gels by PDQuest software (Fig. 2A), we also analyzed by Western blotting the expression level of Hp in AD, MCI, and CTR plasma samples (Fig. 2B). Western blot confirmed the results obtained by 2DE and Hp was found to be downregulated in AD with respect to CTR. Similarly, by comparing AD vs MCI Hp was found to be downregulated in AD.

Identification of carbonylated proteins in AD and MCI

It has been firmly established that AD is caused by a combination of risk factors, among which oxidative stress plays an important role. Protein carbonyls are a common marker of protein oxidation and because they are chemically stable compared with other by-products of oxidative stress, they are generally used as markers to determine the extent of oxidative modification under both in vivo and in vitro conditions.

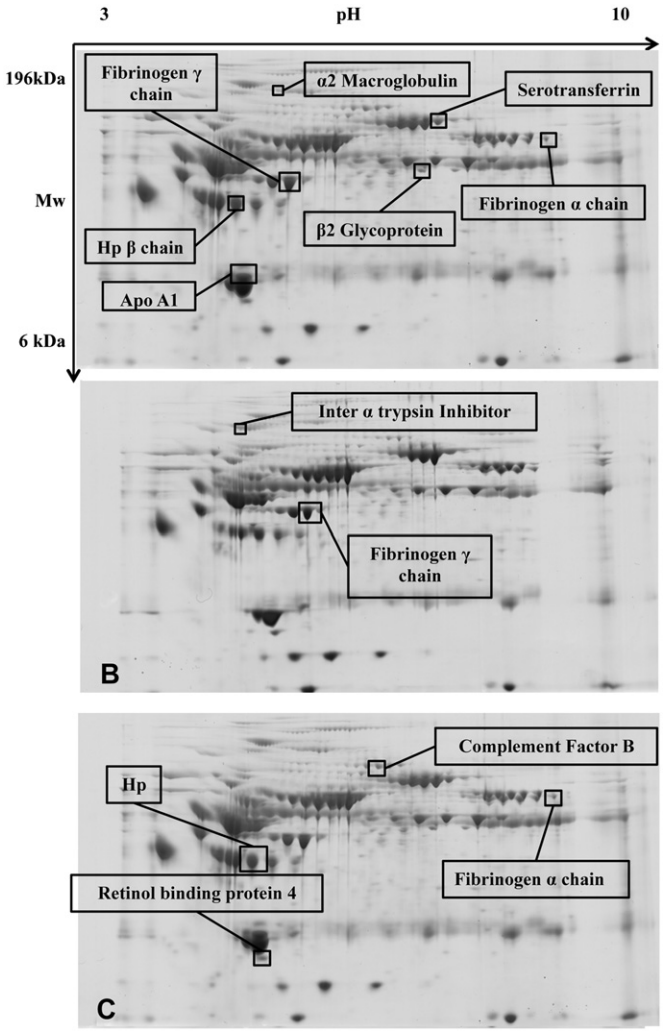

Fig. 1. 2D protein expression maps. Representative 2D gels for the matches AD vs CTR, MCI vs CTR, and AD vs MCI are shown, together with molecular weight and pH. Gels were stained using Coomassie dye. The proteomic analysis allowed us to find 13 spots corresponding to differentially expressed proteins in the three matches. Proteins identified by mass spectrometry are reported.

To identify specific targets of oxidative modification occurring at the protein level, a redox proteomics approach was used to identify carbonylated proteins in the three groups AD, MCI, and CTR. Representative 2D blots from CTR, MCI, and AD samples are shown in Fig. 3. We identified four proteins specifically carbonylated in AD and MCI compared with age-matched controls (Table 3). Three proteins were more oxidized in AD with respect to CTR: Hp β chain, 4.8-fold; serotransferrin, 3.4-fold; and A2M, 2.5-fold.

Interestingly, among the identified proteins Hp β chain was found to be more oxidized both in MCI vs CTR (3.34-fold) and in AD vs MCI with 2.67-fold oxidation. Hp β-chain oxidation gradually increased from MCI to AD, as shown in Fig. 4, and its expression was downregulated with the same pattern, thus allowing us to hypothesize a progressive impairment of its function during disease progression. Complement factor B was 1.9-fold increased in oxidation when AD blots were compared to MCI blots.

Table 2
Summary of the proteins identified as differentially expressed using the proteomic approach.

Protein	SwissProt Accession No.	AD vs CTR (fold change)	MCI vs CTR (fold change)	AD vs MCI (fold change)	Theoretical MW (kDa)/p*I*	Peptide (hits)[a]	*P* value[b]
Apolipoprotein A1	P02647	0.6	–	–	30.76/5.46	3 (4)	0.03
Serotransferrin	P02787	0.13	–	–	77.1/6.81	54 (101)	0.05
α2-Macroglobulin	P01023	2.17		–	163.29/6.03	4 (29)	0.02
β2-Glycoprotein	P02749	0.55			38.298/8.34	8 (15)	0.006
Fibrinogen α chain	P02671	0.02[c]	–	0.4[d]	69.76/8.23	32 (73)[c] 20 (43)[d]	0.03[c] 0.02[d]
Haptoglobin β chain	P00738	0.6[c]		0.5[d]	45.177/6.13	8 (38)[c] 3 (15)[d]	0.05[c] 0.02[d]
Inter-α trypsin inhibitor	Q14624	–	0.38	–	103.2/6.53	3 (5)	0.02
Fibrinogen γ chain	P02679	2.25	2.26	–	49.49/5.70	23 (56)	0.04[c] 0.004[d]
Retinol-binding protein 4	P02753	0.76	0.41	0.5	23.01/5.76	3 (3)	0.01
Complement factor B	P00751	0.6	–	0.4	85.48/6.66	3 (6)	0.01

[a] The number of peptide sequences identified by ESI–MS/MS. The total number of peptide hits is indicated in parentheses.
[b] The *P* value associated with the fold change using a Student *t* test.
[c] Match between AD and CTR.
[d] Match between AD and MCI.

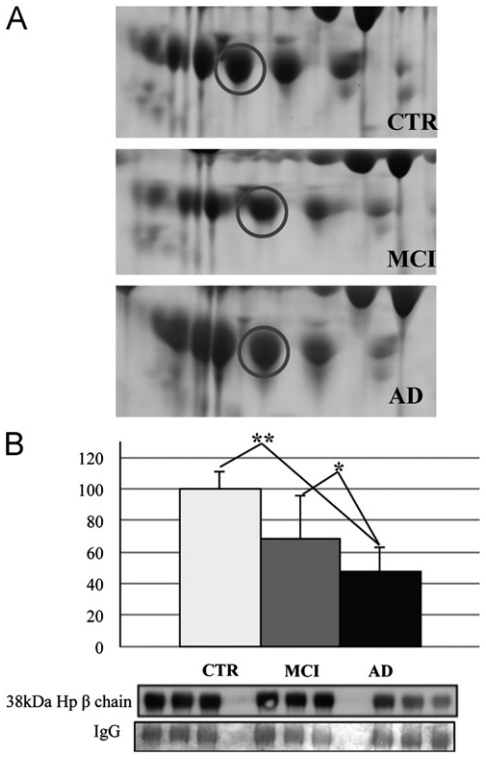

Fig. 2. Hp β-chain expression by proteomic approach and its validation by Western blot analysis. (A) The expanded view of the "area of magnification" corresponding to the Hp train of spots in 2D gels from CTR, MCI, and AD is shown. The spot identified as Hp *β* chain is labeled. (B) Immunoblot of Hp *β* chain to validate data from 2D gels on Hp expression. Both Hp β-chain and IgG bands are shown. $^{**}P=0.009$; $^{*}P=0.02$.

Aβ fibril formation assay, influence of Hp β chain

Hp has been recently identified as a member of a small family of extracellular mammalian chaperones present in human plasma at levels of about 100 μg/ml [19]. This protein seems to play a critical role in defense mechanisms against the clinically dangerous consequences of inappropriate extracellular protein aggregation. In addition Hp is found associated with Aβ deposits in vivo [20]. We investigated Hp's role in Aβ fibril formation with ThT assay and how Hp β-chain oxidation can affect the assay. As shown in Fig. 5A the ThT fluorescence at 484 nm increased as a function of time in the presence of $A\beta_{25-35}$ 10 μM. $A\beta_{25-35}$ was chosen as a model of fibrillogenesis because it has already been demonstrated that it contains a highly hydrophobic region, thus forming β-sheet aggregates [21,22].

The curve is characterized by a lag phase followed by a rapid growth phase that finally leads to a plateau. When $A\beta_{25-35}$ is added together with Hp *β* chain (1 μM) a decreased fluorescence intensity is observed and the curve loses its sigmoid trend according to what was already shown by Yerbury et al. [23].

The same figure also shows the effect of Hp β-chain oxidation on Aβ fibrillation. Once the Hp *β* chain is oxidized it is likely that it loses its chaperone activity as indicated by the recovery of ThT fluorescence at 484 nm to the same trend observed in the absence of Hp.

Further, to confirm Hp β-chain in vitro oxidation with UVB we performed a slot-blot analysis to measure carbonyl levels both in Hp β-chain standard solution and in UV-treated Hp β-chain solution. As shown in Fig. 5B, carbonyl levels of UV-treated Hp were 1.64 times higher than those of untreated Hp β chain.

Discussion

AD is a progressive dementing disorder, which is sometimes preceded by a prodromal phase, amnestic MCI. However, not all MCI patients necessarily develop AD [24,25]. Recently, proteomics strategies have been increasingly used to search for unique biochemical changes in peripheral tissue that may represent putative biomarkers and allow early diagnosis and prognosis. Although some of the reported results for AD are controversial, most of them support the presence of peripheral oxidative damage and of a characteristic panel of systemic oxidation that correlates with the occurrence of the disease [11]. One of the major challenges of research in this field is to identify specific alterations at the systemic level that may reflect brain damage. We recently demonstrated that impairment of heme degradation pathways, due to oxidative modifications of the main components, occurs in the postmortem brain from MCI and AD patients [26,27] as well as in plasma samples [28].

Here, we applied both proteomics and redox proteomics approaches to analyze modifications of the plasma proteome profiles from AD, MCI, and CTR subjects. The analysis of plasma

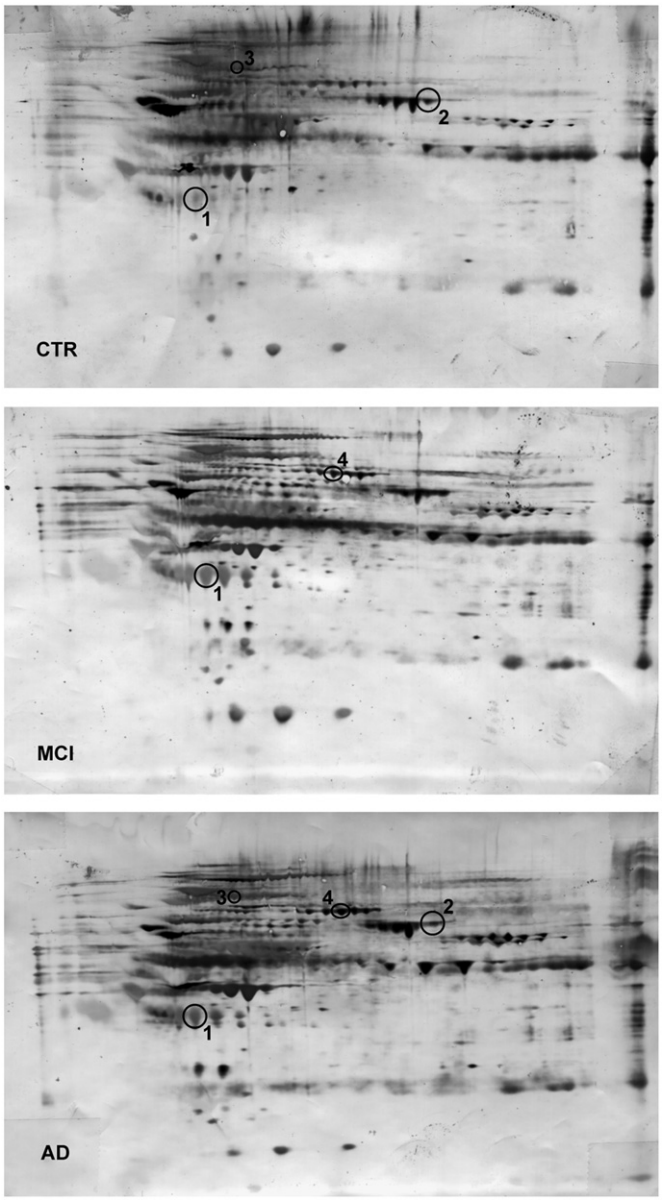

Fig. 3. Oxidized protein detection by redox proteomics. 2D carbonyl immunoblots of CTR, MCI, and AD. The spots showing significantly increased carbonyl levels are labeled. The matches between AD and CTR, AD and MCI, and MCI and CTR, after normalization of the immunostaining intensities to the protein content, lead to four spots significantly oxidized. The spot numbers indicated on the maps are the same as those listed in Table 3.

Table 3
List of proteins identified by redox proteomic study showing altered carbonylated levels by the matching between AD and CTR, AD and MCI, MCI and CTR.

Protein (spot number)	AD vs CTR (oxidation fold)	MCI vs CTR (oxidation fold)	AD vs MCI (oxidation fold)	*P* value[a]
Hp β chain (1)	4.8	3.34	2.67	<0.05
Serotransferrin (2)	3.4	–	–	<0.05
α2-Macroglobulin (3)	2.5	–	–	<0.05
Complement factor B (4)	–	–	1.9	<0.05

Spot number (1 to 4) is shown on the 2D blot image.

[a] The *P* value associated with the carbonylation fold using a Student *t* test.

composition is complicated because of the large dynamic range of protein concentration. This range, indeed, may spread over more than 10 orders of magnitude, and disease-specific biomarkers are mostly found within the less abundant proteins that represent around 1% of proteins in whole blood [29,30]. By taking advantage of plasma purification strategies, we performed depletion of two major abundant proteins, IgG and albumin, which together constitute about 80–85% of the plasma proteome. This step allowed us to improve detection of less abundant proteins and also to improve gel separation. Our proteomics results allowed us to identify specific proteins that are differentially expressed among the three groups of comparison. Consistent with the literature, our findings confirm most of the data already published by other groups (see for review [13]), thus demonstrating the soundness of our experimental approach. We found that a trend toward decreased expression was evident for apolipoprotein A1, serotransferrin, β2-glycoprotein, Hp β chain, and fibrinogen α chain, whereas A2M was increased in AD vs CTR. These proteins seem to be significantly differentially expressed only in AD patients compared with CTR, whereas no significant variations were evidenced in MCI. It is reasonable to speculate that these results may be due to low sensitivity of the experimental approach or may simply indicate that changes in the proteome profile are not statistically relevant in the transition of adults to MCI.

Two proteins, however, fibrinogen α chain and Hp β chain, showed a decreased expression already in MCI compared with controls. Both Hp β chain and fibrinogen α chain expression levels were further reduced in AD vs MCI, suggesting that their progressive decrease could be related to the progression of the disease. In the case of Hp β chain, we suggest that its decrease is disease specific and may represent a discerning factor between AD and other inflammatory diseases. Indeed, we found that Hp levels are increased in abdominal aortic aneurysm patients as a marker of increased inflammatory state in these patients [31]. Because Hp is an acute-phase protein, it is usually found to be induced and released in the periphery as a marker of inflammation [32,33].

A step forward for our research was to apply redox proteomics to identify oxidatively modified proteins in plasma that can discriminate MCI and AD subjects and to compare them with CTR. The use of a redox proteomics approach may represent an advanced tool in the search for changes in the blood proteome. In fact, whereas expression data are usually difficult to decipher, the data obtained by the study of oxidative postsynthetic modifications allow the identification of target proteins that are dysfunctional and may correlate with the pathology. Studies from Butterfield's group demonstrated that many brain proteins in MCI and AD subjects are oxidatively modified compared with controls and that this irreversible, posttranslational modification leads to their dysfunction [8,34]. Indeed, alterations in specific pathways, including energy metabolism, axonal integrity, chaperone machinery, antioxidant systems, etc., as a consequence of oxidative damage of proteins involved in the above-reported pathways correlate with the pathology of MCI and AD.

There are a few clinical 2DE-based studies focusing on post-translational modifications in AD blood. Only one group reported increased carbonylation of α1-antitrypsin and fibrinogen γ-chain precursor in AD compared with controls [35]. The lack of data is probably due to the difficulty of analyzing complex samples such as biological fluids with a wide variability among patients. This variability becomes even more critical mostly in the case of MCI subjects, which, although diagnosed, present with a diversity of symptoms and with diverse severity.

This is the first report applying redox proteomics to plasma from two different stages of the disease, AD and MCI, compared to healthy subjects. Interestingly, our redox proteomics analysis led to the identification of a few distinct oxidized proteins among AD, MCI, and CTR, with only one protein showing a significant and

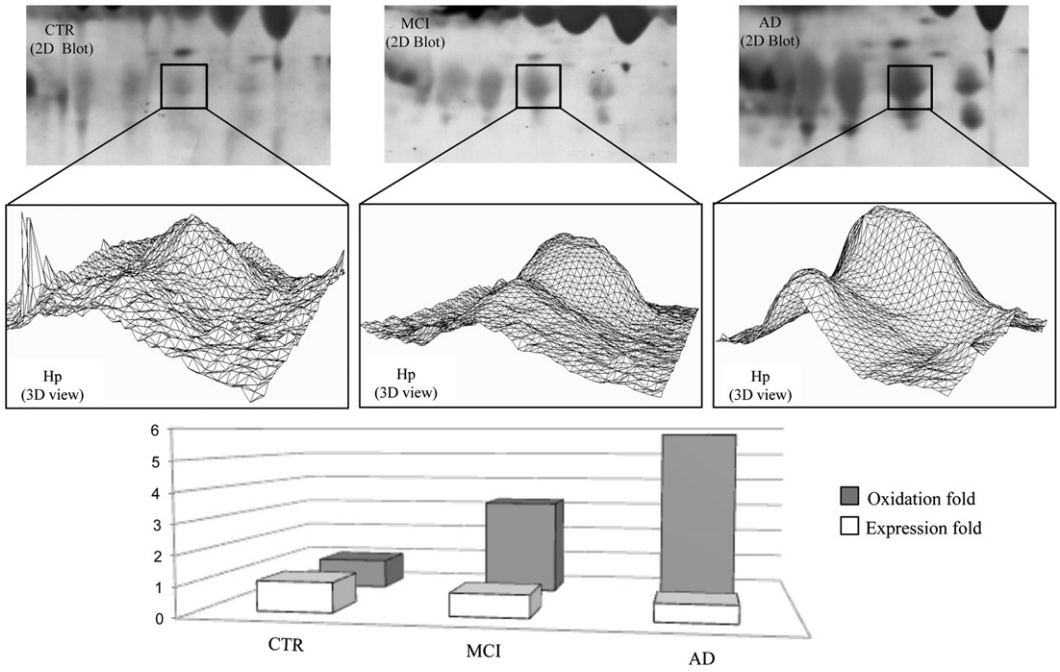

Fig. 4. Hp β-chain oxidation fold. At the top an enlarged area of 2D blot from each group, CTR, MCI, and AD, is shown corresponding to the Hp train of spots. The Hp β-chain spot identified as differentially oxidized in the three groups is labeled. 3D density graphs were elaborated by PDQuest from the Hp spot on the 2D blot. At the bottom the histogram reports both expression levels and oxidation fold for Hp β chain (data obtained from proteomics and redox proteomics approaches).

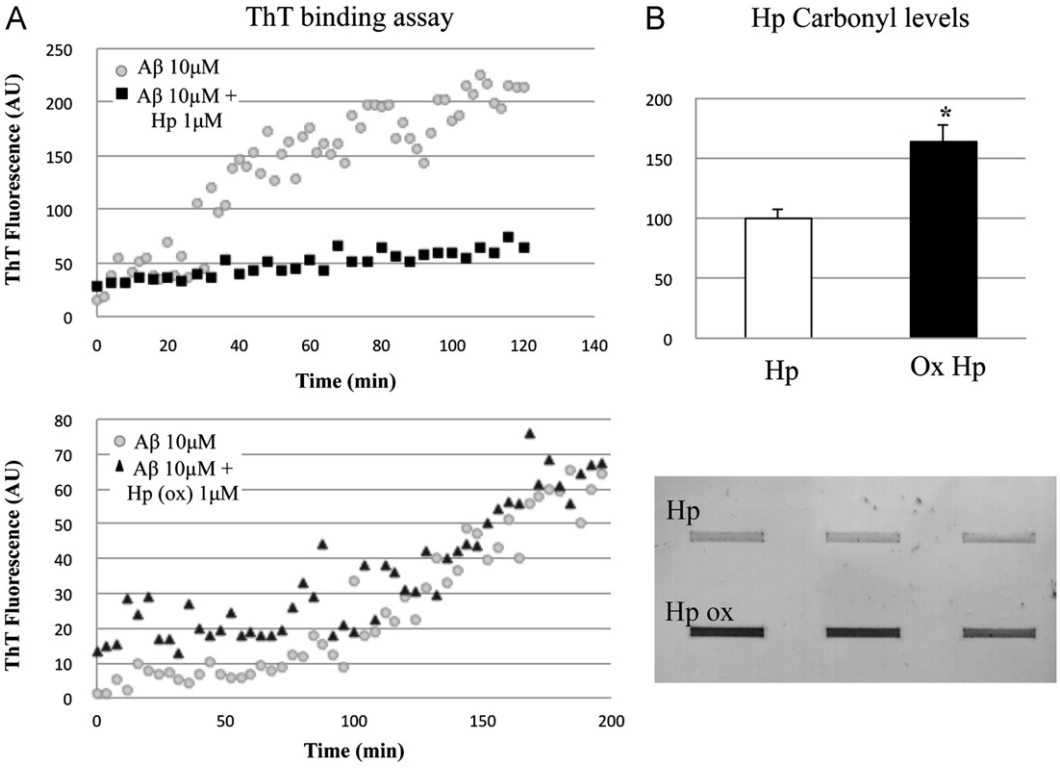

Fig. 5. Oxidation of Hp affects its the ability to suppresses the aggregation of $A\beta_{25-35}$. Samples of $A\beta_{25-35}$ were incubated as described under Materials and methods. Amyloid aggregation was measured by in situ measurements of ThT fluorescence. (A) Gray circles, data for amyloid aggregating in the absence of Hp; black squares, Aβ aggregation inhibited by Hp; black triangles, restoration of Aβ aggregation when Hp is damaged by oxidation. (B) Hp was oxidized by UV exposure (UV dosage of 25 J/m^2); after UV treatment Hp carbonylation was verified by slot-blot technique.

consistent trend of altered expression and oxidative modification, Hp β chain. Among identified proteins, serotransferrin and A2M were increasingly oxidized only in AD vs CTR. It is worth noting that complement factor B is specifically more oxidized only in AD vs MCI. In addition, this protein was also downregulated in AD vs MCI.

Among our findings, the trend of modification we observed for Hp β chain is perhaps most interesting. Indeed, Hp β chain was both downregulated and increasingly oxidized in a disease-dependent way. Hp is a secreted acidic glycoprotein produced mainly in the liver and found in most body fluids. As previously stated, the levels of Hp are usually upregulated in response to

both endogenous and exogenous stress, mostly inflammation, and it is commonly know as an "acute-phase protein" [32]. The most important physiological role of Hp is its binding activity to hemoglobin, leading to the formation of an Hp–Hb complex, which functions as a way to prevent Hb-mediated production of reactive oxygen species, as it occurs during inflammatory events [23,36]. Interestingly, in addition to its well-established inflammatory role, Hp has a specific capacity to inhibit aggregation/precipitation of a wide variety of proteins induced by various stress conditions. Hp forms stable and soluble high-molecular-weight complexes with misfolded proteins but has no independent ability to refold misfolded proteins [23]. However, although Hp has been found associated with Aβ plaques in AD patients [20], few studies are available on Hp effects on amyloid formation and its role in AD pathogenesis. The finding of increased oxidation of Hp in both MCI and AD patients compared with controls led us to hypothesize that dysfunction of Hp in human plasma may affect the rate of extracellular Aβ fibril formation. To confirm our hypothesis, we have oxidized in vitro Hp β chain, as demonstrated by increased level of carbonylation, and tested if this chemical modification might affect amyloid fibril formation. Consistent with this notion, we found that in vitro oxidation of Hp β chain suppresses its ability to inhibit the in vitro formation of amyloid fibrils. From our results, it is reasonable to describe a condition under which lower levels of a more oxidized/dysfunctional protein are not efficient in preventing or delaying Aβ aggregation.

The same considerations can also be applied to another chaperone, A2M. However, we found that expression levels of A2M were increased in AD vs CTR, coupled with increased levels of carbonylation. Studies from Hye et al. [10] demonstrated increased expression of A2M in AD vs CTR plasma samples. In addition, a study from the same group showed a significant association between plasma A2M concentration and hippocampal metabolite abnormalities quantified by ^{1}H magnetic resonance spectroscopy in patients with AD [14]. Interestingly, Akuffo and colleagues reported that the plasma concentration of A2M was associated with changes in ADAS-Cog scores in AD patients treated with rosiglitazone [14,37] We found that in contrast to the increased expression, A2M is more oxidized in AD vs CTR patients. Specific oxidation of both Hp β chain and A2M points to the fact that alterations in the extracellular chaperone machinery occur in AD patients and that this dysfunction is already present in MCI patients (exclusively Hp).

Our results are consistent with the Aβ hypothesis and further highlight the central role of Aβ toxicity in the pathogenesis and progression of AD. In fact, it is thought that an increase in Aβ production and/or a reduced rate of clearance from extracellular central nervous system fluids may be contributing factors to the development of AD pathology [38,39]. One or more types of soluble Aβ species are likely to be responsible for the neurotoxicity associated with AD, as the pathology is correlated with elevated levels of Aβ in the brain [40] but does not correlate well with the location of insoluble Aβ plaques [41]. Although total plasma levels of Aβ are thought to decrease as AD progresses [42], our findings are consistent with the idea that the plasma of AD patients is less efficient at sequestering the remaining Aβ than healthy individuals because of impaired functionality of extracellular chaperones. This is exactly what occurs in the brain, where altered activity of intracellular chaperones such as heat shock proteins (Hsp's) has been demonstrated [43,44]. Butterfield and co-workers showed for the first time that HSC71 is oxidatively modified in AD brain compared with age-matched controls [45]. HSC-71, the constitutive form of Hsp70, is involved in the degradation of proteins with abnormal conformation and might be involved in the structural maintenance of the proteasome and conformational recognition of misfolded proteins by proteases. In addition to altered function of chaperones, selective oxidative modification of the proteasome component UCHL1 has also been demonstrated in AD brain by redox proteomics studies [46]. Taken together, these data indicate that failure to eliminate misfolded proteins can lead to the formation of potentially toxic aggregates, inactivation of functional proteins, and ultimately cell death. The number of disease states linked to aberrant protein conformations highlights the importance of effective quality control for cell survival. The correct balance between folding and degradation of misfolded proteins is critical for cell viability [47]. Indeed, AD is currently considered a "protein deposition disorder," in which inappropriate protein deposits arise when the normally efficient protein folding quality control system is overwhelmed [48–50]. However, an analogous system exists also in the extracellular space that recognizes nonnative proteins and promotes their removal from the extracellular fluid via receptor-mediated endocytosis [19,49,51,52]. Members of this extracellular quality control system are Hp, A2M, serum amyloid P, and also clusterin. Thambisetty et al. discovered that the plasma concentration of clusterin is associated with disease severity, pathology, and clinical progression in AD, as well as with brain fibrillar Aβ burden in nondemented older individuals [14].

Within this frame, altered function of extracellular chaperone might play a crucial role in AD pathogenesis and progression. Our results suggest that increasing the concentration of extracellular chaperones in Alzheimer brain fluid may alleviate Aβ toxicity by promoting its removal from the brain and may represent a potential therapeutic strategy for the treatment of AD. Hp β-chain expression and oxidative modification levels may possibly be considered putative markers of the disease, because they can be detected at the early stage and persist during the whole progression of the pathology, being recognizable as a major signature of AD in plasma.

In conclusion, our findings support the hypothesis that peripheral oxidative damage may be considered a specific and sensitive marker for current diagnostic criteria for AD and MCI. One of the major limits of clinical routine analysis is the lack of prognostic markers, which may discriminate between progressive and stable MCI. Our laboratory is giving continuous effort to the search for reliable systemic markers that may support current diagnostic criteria. Further studies are needed to confirm our results on a large population and toward a better understanding of the molecular mechanisms exerted by extracellular chaperones in AD pathology.

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