Free Radical Biology and Medicine 91 (2016) 1–9

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxidative signature of cerebrospinal fluid from mild cognitive impairment and Alzheimer disease patients

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ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form
26 November 2015
Accepted 5 December 2015
Available online 8 December 2015

Keywords:
Redox proteomics
CSF
APOE
Biomarkers
Protein oxidation
Extracellular chaperones

ABSTRACT

Background: Several studies suggest that pathological changes in Alzheimer's disease (AD) brain begin around 10–20 years before the onset of cognitive impairment. Biomarkers that can support early diagnosis and predict development of dementia would, therefore, be crucial for patient care and evaluation of drug efficacy. Although cerebrospinal fluid (CSF) levels of Aβ42, tau, and p-tau are well-established diagnostic biomarkers of AD, there is an urgent need to identify additional molecular alterations of neuronal function that can be evaluated at the systemic level.
Objectives: This study was focused on the analysis of oxidative stress-related modifications of the CSF proteome, from subjects with AD and amnestic mild cognitive impairment (aMCI).
Methods: A targeted proteomics approach has been employed to discover novel CSF biomarkers that can augment the diagnostic and prognostic accuracy of current leading CSF biomarkers. CSF samples from aMCI, AD and control individuals (CTR) were collected and analyzed using a combined redox proteomics approach to identify the specific oxidatively modified proteins in AD and aMCI compared with controls.
Results: The majority of carbonylated proteins identified by redox proteomics are found early in the progression of AD, i.e., oxidatively modified CSF proteins were already present in aMCI compared with controls and remain oxidized in AD, thus suggesting that dysfunction of selected proteins initiate many years before severe dementia is diagnosed.
Conclusions: The above findings highlight the presence of early oxidative damage in aMCI before clinical dementia of AD is manifested. The identification of early markers of AD that may be detected peripherally may open new prospective for biomarker studies.

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly. The pathological hallmarks of AD are deposition of extracellular plaques formed by amyloid beta-peptide (Aβ) and neurofibrillary tangles composed of hyperphosphorylated tau (p-tau). The inaccessibility of human brain tissue for molecular studies can be partially overcome by analyzing cerebrospinal fluid (CSF), and other biofluids, for detecting molecular changes that occur in the brain but are possibly reflected outside the brain parenchyma. Within this context much efforts has been directed toward the identification of AD biomarkers in CSF. Currently, a series of CSF biomarkers, which differ between AD and control subjects reflecting multiple aspects of disease pathology, have been described [1,2]. However, a reliable method for AD diagnosis is far from being achieved.

CSF Aβ42, tau and p-tau are routinely used in AD research and drug development [3]. Reduced Aβ42 and increased tau and p-tau181 levels have been observed in CSF from AD patients in comparison with aged, cognitively-normal individuals, and these changes correlate with the pathological hallmarks of AD [4,5]. Interestingly, the change in levels of Aβ42 and tau begin 1–2 decades prior to onset of symptoms, with the change in Aβ42 preceding that of tau [6].

One important issue of studies for the identification of putative CSF biomarkers is that a specific protein isoform or peptide may be

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.12.004
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a useful marker while other isoforms or peptides from the same protein may not be indicative of disease [7]. Among candidates, apolipoprotein E (ApoE) received considerable attention in AD research. The ApoE genotype constitutes the most significant susceptibility gene for late-onset AD [8]. Two polymorphisms make up 3 different alleles—ε2, ε3, and ε4—of the ApoE gene. These polymorphisms lead to amino acid substitutions at positions 112 and 158 in the APOE protein [9]. Among the three isoforms, ApoE3 is the most common and is considered to be the wild type. ApoE2 has an R158C substitution, whereas ApoE4 has a C112R and a C158R substitution, and both are associated with different forms of hyperlipidemia [10]. One copy of the ApoE4 gene confers a three-fold increased risk of AD development, whereas two copies confer an eight-fold increased risk. One copy of the ApoE2 gene, however, reduces the risk by 60% [11]. Although the major function of ApoE in the brain is to mediate cholesterol transport through ApoE receptors, recent evidence showed that ApoE may play catalytic roles in the amyloid cascade, with binding and clearance differences between the ApoE isoforms reflecting their differing abilities to bind to Aβ and catalyze its conversion into neurotoxic macromolecular species [12].

ApoE genotype also impacts oxidative stress and oxidative stress-mediated inflammation. It has been demonstrated that plasma from ApoE4 carriers in AD was more oxidized than plasma from AD non-ApoE4 carriers [13,14]. Human ApoE allele targeted-replacement mice showed more oxidative stress in brain in ApoE4 mice than ApoE3 and ApoE2 mice [15], and Butterfield and colleagues reported that synaptic membranes from human ApoE allele targeted-replacement mice treated with human Aβ42 showed significantly greater oxidative stress in ApoE4 synaptic membranes compared to those from ApoE3 or ApoE2 mice [16].

We hypothesize a relationship between ApoE4 genotype and increased levels of oxidative stress, which affects key pathogenic mechanisms in AD brain that would be reflected by CSF biomarker changes. So far, the majority of the studies identified differential expression levels of a range of proteins in biological fluids. Indeed, variations in protein expression levels are not indicative of aberrant protein function. The experimental approaches that may provide further information on protein functionality are mostly focused on characterizing post-translational modifications (PTMs) such as oxidative modification that contribute significantly to the alteration of protein activity/ies. Several studies in the last decade have been focused on establishing a direct link between tissue-specific oxidation and systemic oxidative damage [17–19].

The current study aimed at analyzing variations in the CSF redox profile in subjects with AD and aMCI compared to age-matched control individuals. In particular, considering the role of oxidative stress in AD pathogenesis and progression, we focused our attention on oxidative modification of CSF proteins, by a redox-proteomics analysis, to obtain new insights into AD biomarker discovery that may be correlated with genetic background, such as ApoE.

2. Materials and methods

2.1. Samples

CSF samples from well-characterized healthy subjects (Control), aMCI, and probable AD patients (3 groups in total; 6 samples per group) were kindly provided by the Department of Physiology at University of Valencia. All the subjects were followed longitudinally with annual mental status testing and physical and neurological examinations. Probable AD patients met NINCDS-ADRDA criteria and demonstrated progressive intellectual decline. Subjects with aMCI met Petersen clinical criteria for diagnosis of amnestic aMCI, while Control subjects had neuropsychological test scores in the normal range and showed no evidence of memory decline. APOE genotype was also provided. Demographic data are reported in Table 1.

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

	ApoE	Date of birth	GENDER	MMSE (range)
Controls				
A	3/4	1940	M	> 30
B	3/4	1962	F	> 30
C	3/3	1946	F	> 30
D	3/3	1943	M	> 30
E	3/3	1943	F	> 30
F	3/3	1937	F	> 30
aMCI				
A	3/3	1943	F	> 20 < 30
B	4/4	1936	F	> 20 < 30
C	3/3	1927	M	> 20 < 30
D	3/4	1940	F	> 20 < 30
E	3/4	1950	F	> 20 < 30
F	4/4	1935	F	> 20 < 30
AD				
A	3/4	1952	F	< 20
B	4/4	1934	M	< 20
C	4/4	1937	M	< 20
D	3/3	1950	F	< 20
E	3/4	1948	F	< 20
F	3/3	1946	F	< 20

ApoE genotyping=presence of 3/3, 3/4 and 4/4 allele; **Age**=date of birth; **CTR**=Age-Matched Controls; **AD**=Alzheimer Desease; a**MCI**=amnestic Mild Cognitive Impairment; **Gender**=male or female.

CSF samples were taken to preserve any gradient that existed and were collected with a 25-gauge needle. All CSF samples were free of blood contamination. After collection, CSF samples were briefly centrifuged at 1000*g* to pellet any cell debris, frozen, and stored in polypropylene tubes at −80 °C in 0.5-ml aliquots until analysis. The protein content in each CSF sample was determined with the micro-BCA protein assay kit (Pierce), and it ranged from 570 to 1000 μg/ml.

2.2. ApoE genotyping

ApoE phenotype determination was performed using ApoE4/Pan-ApoE ELISA Kit (MBL International). The kit measures the amount of human ApoE4 or Pan-ApoE specifically using affinity purified polyclonal antibody against ApoE and monoclonal antibody against ApoE4. It can also measure the difference among the homozygotes (E4/E4) and the heterozygotes (E2/E4, E3/E4) of ApoE4 phenotypes, and non-ApoE4 zygotes (E2/E2, E3/E3, E2/E3) by taking a concentration ratio between ApoE4 and Pan-ApoE.

2.3. Aβ and p-Tau levels

Aβ concentration was obtained in CSF samples using the INNOTEST® β-amyloid (1–42) (Innogenetics N.V.; Fujirebio, Gent, Belgium) that is a solid-phase enzyme immunoassay in which the amyloid peptide is captured by a first monoclonal antibody, 21F12. Total tau levels were measured in CSF samples by the ELISA Human Tau (Total) kit (Life technologies, Thermo Fisher Scientific Inc.). A monoclonal antibody specific for human Tau has been coated onto the wells of the microtiter strips provided. Phosphotau was measured in CSF samples by the INNOTEST® PHOSPHOTAU (181P) (Fujirebio, Gent, Belgium) that is a solid-phase enzyme immunoassay in which the phosphorylated tau protein or

fragments are captured by a first monoclonal antibody, HT7.

2.4. 2D 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, 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, pH3–10, 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 processes above 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 after other 10 min in the same buffer containing 4.5% iodacetamide in place of dithiothreitol. Second dimension separationwas performed using 12% Precast XT-stain free criterion gels (BIO-RAD). The proteins in the gel have been visualized directly by ChemiDoc Image System, without any staining solution. The advantage of these gels is that only one gel can be used for both expression and oxidation maps.

2.5. Western blotting

For 2D oxyblot, 2D gels (after image analysis) 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 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). Finally the membranes were blocked with 3% albumin in T-TBS and incubated with the primary Rabbit × DNP antibody (1:100, Millipore) and the secondary antibody anti-Rabbit IgG alkaline phosphatase conjugated (1:5000, Sigma). The colorimetric reaction was obtained using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution.

For western blot, 30 μg of proteins from albumin and Ig-G depleted samples (CTR, AD and aMCI), were separated by 12% SDS-PAGE and blotted onto a nitrocellulose membrane (BIO-RAD). Membranes were blocked with 3% BSA in T-TBS, incubated for 1 h and 30 min at room temperature with primary anti-Haptoglobin β chain antibody (1:100, Santa Cruz Biotechnologies, Inc.) and secondary antibody anti-mouse IgG HRP conjugated, for 1 h at room temperature (1:5000, Sigma-Aldrich). Membrane were developed with the Super Signal WESTPICO Chemioluminescence Substrate (Thermo Scientific).

2.6. 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 after compared between groups using statistical analysis. Statistical significance was assessed by a two-tailed Student's *t*-test. P values ≤ 0.05 were considered significant for comparison between control and experimental data (CTR vs. AD and CTR vs. aMCI). 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).

2.7. Trypsin digestion and protein identification by mass spectrometry

Protein spots statistically different than controls were digested in-gel by trypsin. Briefly, after a progressive destaining step using 50 mM ammonium bicarbonate, 50% acetonitrile in 50 mM ammonium bicarbonate and 100% acetonitrile, about 100 ng of trypsin (Promega), solubilized in 10 μl of a 25 mM ammonium bicarbonate digestion buffer, was added to vacuum-dried gel. Digestion was performed at 37 °C overnight. 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 2014_02 (542503 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.

2.8. Immunoprecipitation

100 μg of protein extracts were dissolved in RIA buffer (10 mM Tris, pH 7.6; 140 mM NaCl; 0.5% NP40 including protease inhibitors) and then incubated with 1 μg of primary antibody (anti-gelsolin antibody), at 4 °C overnight. Immunocomplex was collected by using protein A/G beads (Santa Cruz, CA, USA) for 2 h at 4 °C. Immunoprecipitated protein was recovered by resuspending the pellets in reducing SDS buffers and subjected to electrophoresis on 12% gels followed by western blot analysis for carbonyl levels.

2.9. Statistical analysis

Statistical analyses of data obtained by PD-QUEST software were performed using Student's *t*-test. Significance was accepted if the P value < 0.05. p-Tau levels in patients, controls and between groups were compared by ANOVA; post-hoc analysis was performed using the Bonferroni adjustment, where appropriate. Differences were considered significant if $P < 0.05$.

3. Results

Subject characteristics. In total 18 CSF 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 a MMSE score indicating severe cognitive deterioration, while aMCI subjects had mild cognitive deterioration (Table 1).

3.1. ELISA assay evaluation of Aβ42 and p-tau levels in CSF samples

AD, aMCI and healthy individuals has been investigated for CSF levels of Aβ42 and p-Tau, in order to evaluate the AD-associated pathologic changes in the brain. Decreased levels of Aβ 1–42 and increased tau phosphorylated at position threonine 181 in CSF have been already established as valid biomarkers for the

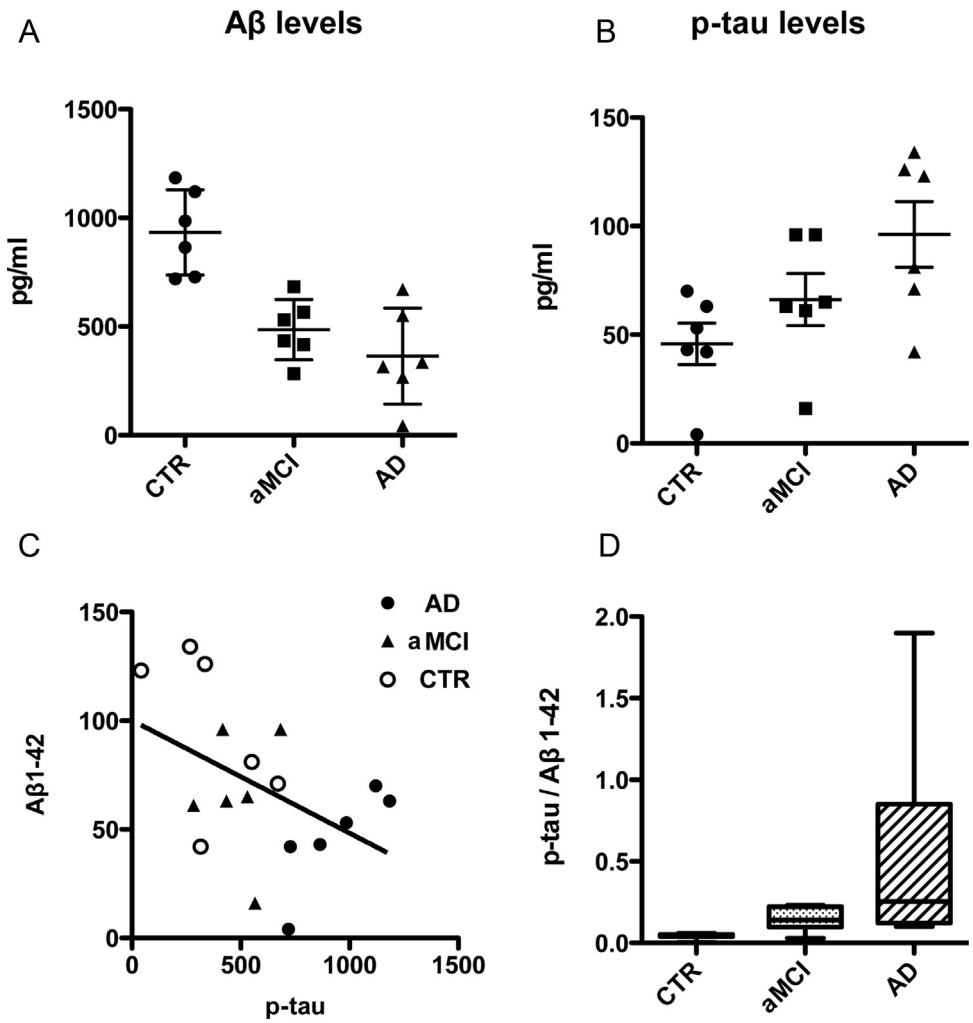

Fig. 1. Aβ (1–42) and p-Tau: total levels, ratio and correlation. Aβ (1–42) levels are reported in pg/ml (A) in CTR, aMCI and AD samples. p-Tau levels (B) are also reported in pg/ml. The correlation between Aβ (1–42) and p-Tau are showed (C).

diagnosis and prognosis of AD [20]. Our data, showed in Fig. 1 (panel A), report a clear decrease of Aβ 1–42 levels in the CSF, which inversely follows the progression of the disease (AD vs. CTR and aMCI vs. CTR). In parallel, the CSF concentrations of p-tau 181 clearly depict the increase in the concentration in both aMCI and AD vs. CTR (Panel B). A significant inverse correlation inverse correlation exists between Aβ 1–42 and p-tau values (Panel C; $R^2=0.2$ $p<0.05$). In addition, the analysis of the ratio between abnormally low CSF Aβ 1–42 levels and abnormally high CSF p-tau levels discriminate, with sufficient accuracy and specificity, among patients at different stages of AD pathology (Panel D) following their clinical evaluation, even within a small cohort.

3.2. Redox proteomics analysis of CSF samples

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 since 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 both *in vivo* and *in vitro* conditions.

In order to identify specific targets of oxidative modification occurring at the protein level, a redox proteomics approach has been used to identify carbonylated proteins in the three groups AD, aMCI and CTR. Specific carbonylated proteins in the CSF samples of patients with AD and aMCI in comparison with control group were detected immunochemically using 2D-gels and 2D-Western blots. Fig. 2 shows three representative 2D gels and the corresponding 2D blots from control, aMCI and AD respectively. Since TGX-stain free gels have been used fro all the samples, the 2D oxyblots exactly match the 2D expression profile. Relative change in carbonyl immune-reactivity, after normalization of the immunostaining intensities to the protein content, was significant for seven spots. After spot excision and in-gel trypsin digestion, proteins were identified by MS analysis.

In Table 2, the carbonylated proteins identified by MS/MS and interrogation of databases are listed. All protein identifications were consistent with comparison of proteins positions on the gel with MW and pI from databases, and are listed in Table 2 with the number of peptide sequences, the score, the coverage, MW, pI, fold-change levels, and p-value.

Interestingly, most of the identified oxidatively modified proteins were oxidized in CSF from both aMCI and AD patients compared with control patients. From the matching between **aMCI vs. CTR** the identified proteins were: α-1B-glycoprotein (3.4-fold increase), vitamin D-binding protein (DBP) (4.2-fold increase), apolipoprotein E (ApoE) (3.1-fold increase), Prostaglandin-H2 D-isomerase (PGDS) (1.9-fold increase) and alpha-1-anti-trypsin (A1AT) (2-4-fold increase).

In the analysis between **AD vs. CTR** the identified proteins were: α-1B-glycoprotein (3.2-fold increase), vitamin D-binding

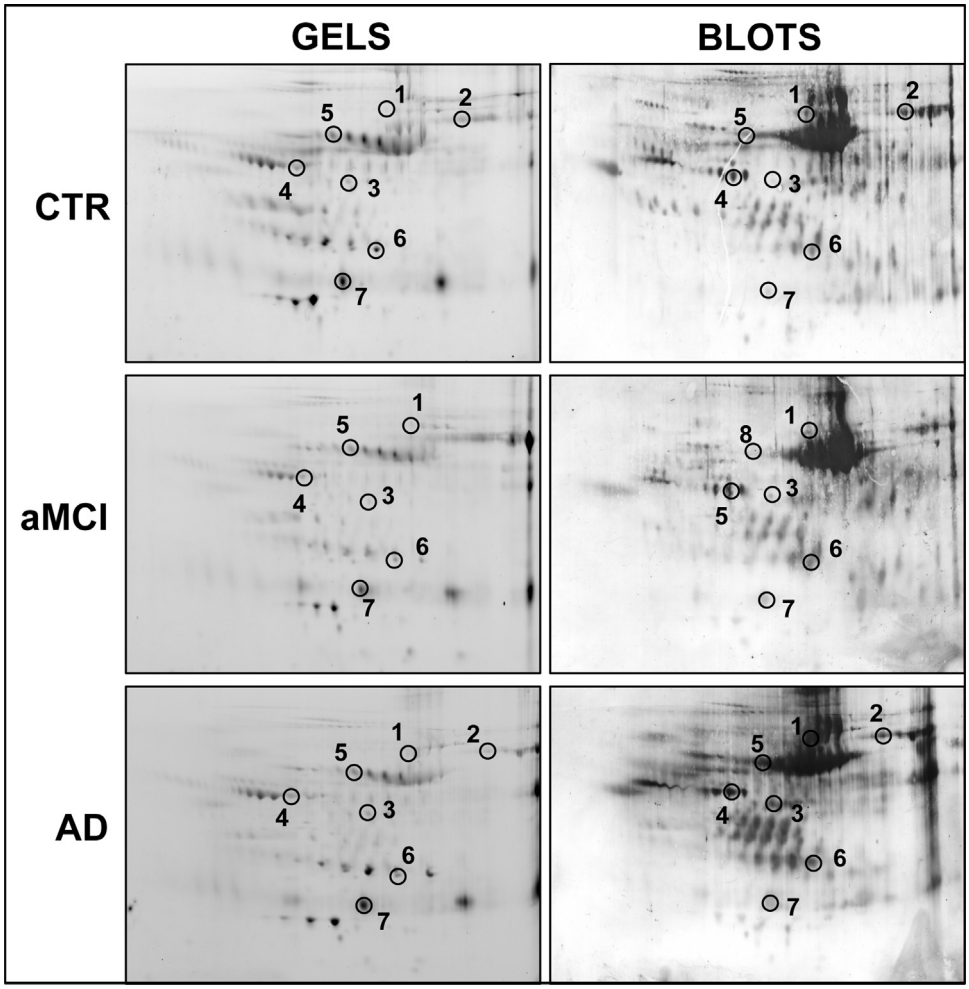

Fig. 2. 2D protein expression maps and blots. On the left: representative 2D gels for the matches AD vs. CTR, aMCI vs. CTR and AD vs. aMCI are shown. On the right: 2D carbonyl immunoblots of CTR, aMCI and AD. The spots showing significant increased carbonyl levels are labeled. The matches between AD and CTR, AD and aMCI, and aMCI and CTR, after normalization of the immunostaining intensities to the protein content, leads to seven spots significant oxidized. The spot numbers indicated in the maps are the same listed on Table 2.

protein (DBP) (6.6-fold increase), apolipoprotein E (ApoE) (3.9-fold increase), PGDS (2.8-fold increase) and A1AT (2.7-fold increase).

The match between **AD vs. aMCI** showed only one protein more oxidized: gelsolin (4.1-fold increase).

To confirm correct protein identification, immunoprecipitation followed by immunochemical detection of carbonyl levels were performed for gelsolin (Fig. 3). In addition, carbonylation of ApoE was confirmed by MS/MS as showed in Fig. 4.

4. Discussion

The identification of potential CSF biomarkers of AD is a continuous challenge and several biomarker studies are currently ongoing to evaluating specific disease risk profiles, age ranges and demographics. Three proteins that reflect the core neuropathologies are currently considered to be the most suited standards of AD CSF biomarkers [1]. Reductions in Aβ42 and increases of CSF tau and p-tau181 are observed in AD patients in comparison with aged

Table 2
List of proteins identified by redox proteomic study showing increased carbonylated levels by the matching between AD and CTR, AD and aMCI, aMCI and CTR.

Spot no.	Identified protein	SwissProt code	Sequence coverage %	Theoretical MW/pI	Score	Groups (oxidation fold)
1	**Gelsolin**	P06396	77	86043/5.9	99	AD vs. CTR (3.4) AD vs. aMCI (4.1)
2	**Serotransferrin**	P02787	25	79294/6.81	107	AD vs. CTR (3.7)
3	**Vitamin D-binding protein**	P02774	55	54526/5.40	123	AD vs. CTR (6.6) aMCI vs. CTR (4.2)
4	**Alpha-1-antitrypsin**	Q86U18	39	46878/5.37	74	AD vs. CTR (2.7) aMCI vs. CTR (2.4)
5	**Alpha-1B-glycoprotein**	P04217	63	54790/5.56	116	AD vs. CTR (3.2) aMCI vs. CTR (3.4)
6	**Apolipoprotein E**	P02649	69	36246/5.65	118	AD vs. CTR (3.9) aMCI vs. CTR (3.1)
7	**Prostaglandin-H2 D-isomerase**	P41222	51	21243/7.66	92	AD vs. CTR (2.8) aMCI vs. CTR (1.9)

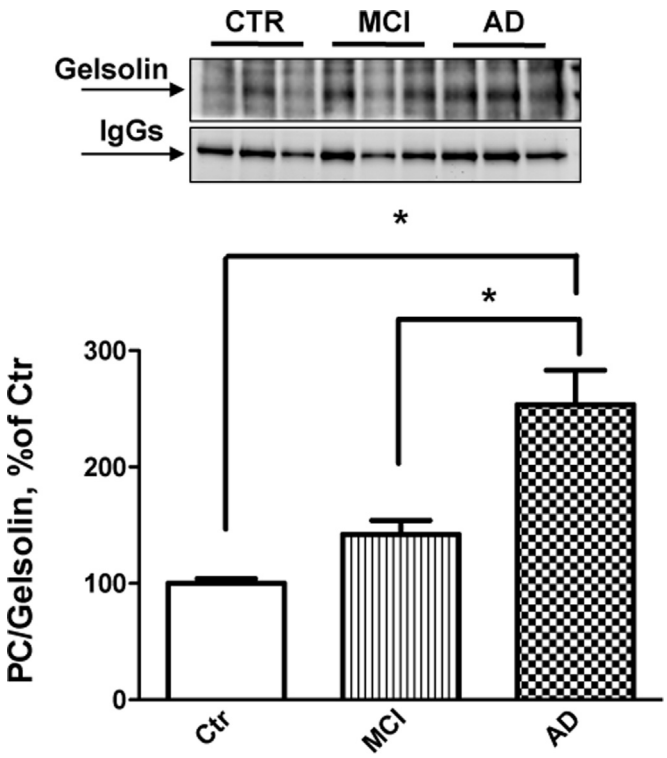

Fig. 3. Specific carbonyl levels of gelsolin. Gelsolin has been immunoprecipitated by AD, MCI and CTR samples and immunochemically stained with anti-DNP antibody. WB confirmed the increased oxidation of gelsolin in AD vs. CTR and AD vs. MCI as it was showed by redox proteomics data. The bar graph were built on all the samples (6 n per group) values normalized on CTRY that was set at 100%; Means ± SD are reported, $^{*}p < 0.05$. Three representative bands per group are showed.

cognitively normal individuals and correlate with the pathological hallmarks of AD [6]. Further, the change in levels of Aβ42 and tau begin 1–2 decades prior to onset of symptoms, with the change in Aβ42 preceding that of tau [21,22]. In parallel, proteomics approaches and targeted multi-analyte studies of CSF have been performed and lead to the identification of many proteins that are elevated or reduced in AD compared to cognitively normal controls [19,23,24]. Among these findings, identified proteins are involved in oxidative stress, synapse loss and neuroinflammation, well-recognized processes of AD neurodegeneration [25]. However, these changes may be not specific for AD.

Post-translational modifications (PTMs) have rarely been studied in CSF proteome studies. Variation of protein expression levels does not provide information on the effective activity of the protein which most likely rely on additional PTMs more than its quantity. Thus, redox proteomics allows one to evaluate specifically the oxidative modifications of proteins that are associated with oxidative-stress related pathologies [26,27]. Several studies demonstrated increased oxidative damage of proteins, in the brain form of AD and its early stages [27–29]. Based on these findings, we aimed to investigate if such damage can be reflected in CSF. Among the proteins identified to be increasingly oxidized in AD and aMCI vs. CTR, we identified: A1AT, 1 α-1B-glycoprotein, serotransferrin, APOE, gelsolin, PGDS and DBP.

A1AT, a serine protease inhibitor, is the only inflammatory response protein identified to be more oxidized in both AD and aMCI vs. CTR [30]. It was previously found to be increased in AD plasma and serum [31] compared to controls and oxidized in its precursor form in AD [32]. In addition, A1AT is localized in both senile plaques and neurofibrillary tangles (NFT) [33]. Considering that the physiological role of A1AT is to break down overexpressed proteases during inflammatory conditions, oxidation of this protein can impair its activity, thus contributing to exacerbation of inflammatory processes. We found that A1AT is more oxidized both in aMCI vs. CTR, AD vs. CTR and AD vs. MCI.

The function of α-1β-glycoprotein is unknown; however, this protein has sequence similarities with immunoglobulins. Previous studies demonstrated reduced level of this protein in AD vs. CTR, thus suggesting that alteration of both levels and PTMs of this protein may be indicative of AD [34]. Serotransferrin, an iron binding transport protein that can bind two Fe^{3+} ions in association with the binding of an anion is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization. The carbonylation of transferrin has also been already found in patients with AD [32].

Among the results, a particularly intriguing finding was DBP. DBP was found to be more oxidized in both aMCI vs. CTR and AD vs. CTR thus showing a full concordance with the course of the

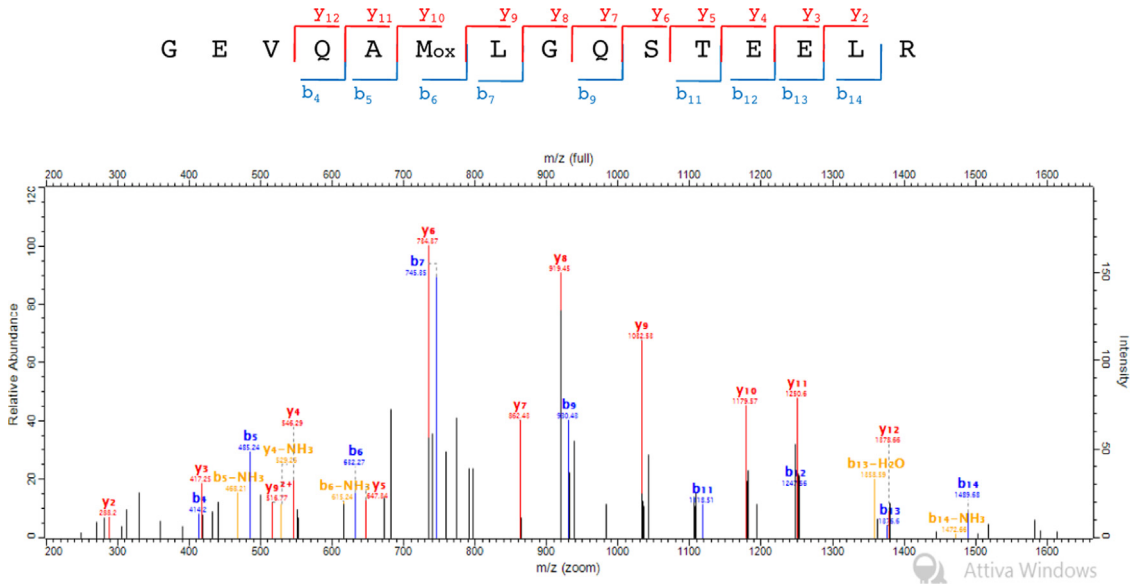

Fig. 4. MS/MS spectrum of tryptic GEVQAMoxLGQSTEELR peptide (2+—charged precursor 832.401 m/z) from ApoE. Red lines represent y-type ions, blue lines represent b-type ions. MS/MS experiments were carried out by an LTQ-Orbitrap Discovery (Thermo Fisher Scientific GmbH, Bremen, Germany) mass spectrometry platform. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

disease. DBP is known to have a variety of physiological functions, including the transport of vitamin D, fatty acid transport, immune modulation among others [35,36]. Recently, Gressner et al. [37] reported that intrathecal synthesis of DBP is increased in patients with severe neurodegeneration, including AD, and suggested that up-regulated DBP may act as an actin scavenger. Other studies also have shown that the level of DBP in CSF is increased in AD patients [38], however the functions of extracellular DBP in AD have not yet been elucidated. The emerging idea is that DBP either from human plasma and CSF interacts with Aβ, possibly binding to and sequestering Aβ to form DBP–Aβ complexes under physiological conditions [39]. Further, DBP altered function would affect the CNS level of VitD, whose deficiency may accelerate cognitive decline in aging [40]. Indeed, local conversion of VitD to the active form in the brain may be a direct neuroprotective response to CNS inflammation followed by inhibition of NF-κB related iNOS induction, and conversely, low vitamin D leads to cognitive decline associated with elevated brain protein nitration [41].

Particularly relevant results of this study highlight the disturbance of members of "anti-aggregating" protein family including gelsolin, ApoE and PGDS. CSF and plasma contain several circulating Aβ-binding proteins (transthyretin, ApoE, cystatin C, and albumin), which have been demonstrated to influence Aβ-mediated pathology. Indeed, the formation of Aβ fibrils is a well-recognized pathological event in AD that leads to neuronal cell death. Several plasma and CSF constituents have been shown to bind Aβ and to serve as potential anti-aggregating proteins [42–44]. However, when Aβ overload overcomes the endogenous defense system, formation of fibrils occurs and accumulation of toxic aggregates become a continuous threat for the organism. One of the members of peripheral anti-aggregating protein family is gelsolin [45]. Gelsolin is present as both intracellular and secreted protein in the plasma and CSF [46]. Gelsolin acts as a major actin-binding protein in the cytoplasm, where it regulates the assembly of actin filaments [47]. Besides its function in actin polymerization, recent studies demonstrated the role of gelsolin in several other activities, including modulation of calcium channel and NMDA receptor, apoptosis, and in tumor suppression [48]. However, the function of gelsolin in the plasma and CSF is not yet elucidated. Gelsolin, like other Aβ binding proteins, is also present in the plasma and CSF, but was not detected in the amyloid plaques of AD. We found that gelsolin is more oxidized in AD vs. aMCI and AD vs. CTR, thus indicating that its structural modification and therefore alteration of its activity is an AD-specific changes, that is not detected in aMCI patients. Considering that gelsolin is a major Aβ-sequestering protein in the plasma and CSF where it prevents Aβ from fibrillization and maintains it in the soluble form, an imbalance of this highly complex regulatory pathway may result in increased Aβ fibrillization in AD [45].

In addition to gelsolin, ApoE has been extensively studied for its role in Aβ fibrillogenesis. Indeed, the ApoE4 allele is associated with increased Aβ deposition in the brain, and a distinct neuropathological phenotype, whereas a lack of ApoE reduces Aβ plaque formation in mice [9]. Further, amyloid plaques bind anti-ApoE antibodies, and also ApoE co-purifies with Aβ from amyloid plaques and may exist as a bound complex with Aβ proteins [49]. Interestingly, others studies have suggested that risk of AD may be associated with APOE isoforms, based on difference of isoform-specific antioxidant activities. ApoE4 has no Cys residues and, hence, no free thiol groups; ApoE3 has one Cys, whereas ApoE2 has two. Free thiol groups are members of the "total antioxidant pool" but through mechanisms different from those of glutathione. It has been demonstrated that in the case of ApoA-I, variants with a free Cys residue side chain have greater antioxidant activity than variants without a free Cys residue [9]. Therefore, it is intriguing to speculate that ApoE4 confers increased risk of AD, whereas ApoE2 confers decreased risk, because Cys residue side chains protect lipids in lipoprotein E particles against oxidative stress.

In light of these observations, we might propose that increased oxidation of ApoE, observed in this study, affects thiol-mediated antioxidant activity; in turn this deficiency would allow excess oxidative damage to the lipids in lipoprotein particles that likewise promote Aβ protein aggregation. It is also important to underlie that ApoE accelerates neuronal Aβ uptake, lysosomal trafficking and degradation in an isoform-dependent manner with ApoE3 more efficiently facilitates Aβ trafficking and degradation than ApoE4, a risk factor for AD. These considerations become more relevant in light of the fact some of the subjects enrolled in this study carry the ApoE ε4 variants. It is likely that ApoE plays a catalytic role in the AD amyloid cascade and consequent cognitive decline, with differences between the APOE isoforms in binding and clearance of Aβ that ultimately promote formation of neurotoxic species [49]. Although no significant correlation between Abeta and ApoE ε4 as well as Tau and ApoE ε4 was found among groups, the increased oxidation of ApoE already in aMCI patients (which show similarities of ApoE4 genotype with AD) suggest that defective ApoE favors Aβ oligomerization and the subsequent increase of oxidative stress at CNS and periphery.

Within this context, it worth's to be mentioned also the finding of increased carbonylation of PGDS. PGDS catalyzes the isomerization of prostaglandin H2 (PGH2) to produce prostaglandin D2 (PGD2), which is involved in sleep regulation, platelet aggregation, allergy, inflammatory response, and chemoattractant activity [50,51]. PGDS, the first member of the important lipocalin family to be recognized as an enzyme, is also able to bind and transport small hydrophobic molecules and was formerly known as β-trace protein, the second most abundant protein in human CSF. Intriguingly, recent evidence suggests that PGDS acts as a β-amyloid chaperone and may play a role in the deposition of Aβ plaques in Alzheimer's disease [52]. Based on our result, the incidence of Aβ aggregation would be expected to be further accelerated when PGDS is impaired. It is reasonable, therefore, to posit that functional disturbances of PGDS/beta-trace may contribute to AD progression.

These findings suggest that extracellular chaperones, in addition to the set of intracellular quality control system, are important elements against Aβ toxicity and accumulation. We speculate that the imbalance in the expression level or failure as a result of increased oxidation of A*β*-sequestering proteins, including most of the proteins identified by this study, may significantly push the development of late-onset AD. Taken together these findings point out the importance of redox proteomics to identify specific protein biomarkers and potential molecular mechanisms that contribute to pathological changes of AD.

Acknowledgments

All authors state that they have no conflicts of interest. This work was partially supported by fondi di Ateneo Sapienza to Dr. Perluigi and Dr. Di Domenico. This work was supported by grants SAF2010-19498 and SAF2013-44663-R, ISCIII2012-RED-43-029 from (RETICEF); RS2012-609 INCLIVA Intramural Grant and EU Funded CM1001 and FRAILOMIC-HEALTH.2012.2.1.1-2. The study has been co-financed by FEDER funds from the European Union. Dr. Giraldo holds a Sara Borrell grant from ISCIII.

References

[1] A.M. Fagan, C. Xiong, M.S. Jasielec, R.J. Bateman, A.M. Goate, T.L. Benzinger, B. Ghetti, R.N. Martins, C.L. Masters, R. Mayeux, J.M. Ringman, M.N. Rossor,

S. Salloway, P.R. Schofield, R.A. Sperling, D. Marcus, N.J. Cairns, V.D. Buckles, J. H. Ladenson, J.C. Morris, D.M. Holtzman, Dominantly Inherited Alzheimer Network, Longitudinal change in CSF biomarkers in autosomal-dominant Alzheimer's disease, Sci. Transl. Med. 6 (2014) 226ra230.
[2] K. Blennow, B. Dubois, A.M. Fagan, P. Lewczuk, M.J. de Leon, H. Hampel, Clinical utility of cerebrospinal fluid biomarkers in the diagnosis of early Alzheimer's disease, Alzheimer's Dement.: J. Alzheimer's Assoc. 11 (2015) 58–69.
[3] O. Harari, C. Cruchaga, J.S. Kauwe, B.J. Ainscough, K. Bales, E.H. Pickering, S. Bertelsen, A.M. Fagan, D.M. Holtzman, J.C. Morris, A.M. Goate, Alzheimer's Disease Neuroimaging, I. Phosphorylated tau-Abeta42 ratio as a continuous trait for biomarker discovery for early-stage Alzheimer's disease in multiplex immunoassay panels of cerebrospinal fluid, Biol. Psychiatry 75 (2014) 723–731.
[4] T. Sunderland, G. Linker, N. Mirza, K.T. Putnam, D.L. Friedman, L.H. Kimmel, J. Bergeson, G.J. Manetti, M. Zimmermann, B. Tang, J.J. Bartko, R.M. Cohen, Decreased beta-amyloid1-42 and increased tau levels in cerebrospinal fluid of patients with Alzheimer disease, J. Am. Med. Assoc. 289 (2003) 2094–2103.
[5] C.M. Clark, S. Xie, J. Chittams, D. Ewbank, E. Peskind, D. Galasko, J.C. Morris, D. W. McKeel Jr, M. Farlow, S.L. Weitlauf, J. Quinn, J. Kaye, D. Knopman, H. Arai, R. S. Doody, C. DeCarli, S. Leight, V.M. Lee, J.Q. Trojanowski, Cerebrospinal fluid tau and beta-amyloid: how well do these biomarkers reflect autopsy-confirmed dementia diagnoses? Arch. Neurol. 60 (2003) 1696–1702.
[6] K.R. Wildsmith, S.P. Schauer, A.M. Smith, D. Arnott, Y. Zhu, J. Haznedar, S. Kaur, W.R. Mathews, L.A. Honigberg, Identification of longitudinally dynamic biomarkers in Alzheimer's disease cerebrospinal fluid by targeted proteomics, Mol. Neurodegener. 9 (2014) 22.
[7] Y.S. Choi, S. Hou, L.H. Choe, K.H. Lee, Targeted human cerebrospinal fluid proteomics for the validation of multiple Alzheimer's disease biomarker candidates, J. Chromatogr. B, Anal. Technol. Biomed. Life Sci. 930 (2013) 129–135.
[8] K.D. Coon, A.J. Myers, D.W. Craig, J.A. Webster, J.V. Pearson, D.H. Lince, V. L. Zismann, T.G. Beach, D. Leung, L. Bryden, R.F. Halperin, L. Marlowe, M. Kaleem, D.G. Walker, R. Ravid, C.B. Heward, J. Rogers, A. Papassotiropoulos, E.M. Reiman, J. Hardy, D.A. Stephan, A high-density whole-genome association study reveals that APOE is the major susceptibility gene for sporadic late-onset Alzheimer's disease, J. Clin. Psychiatry 68 (2007) 613–618.
[9] P.H. Axelsen, H. Komatsu, I.V. Murray, Oxidative stress and cell membranes in the pathogenesis of Alzheimer's disease, Physiology 26 (2011) 54–69.
[10] R.W. Mahley, S.C. Rall Jr., Apolipoprotein E: far more than a lipid transport protein, Annu. Rev. Genom. Hum. Genet. 1 (2000) 507–537.
[11] E.H. Corder, A.M. Saunders, W.J. Strittmatter, D.E. Schmechel, P.C. Gaskell, G. W. Small, A.D. Roses, J.L. Haines, M.A. Pericak-Vance, Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families, Science 261 (1993) 921–923.
[12] L.M. Tai, S. Mehra, V. Shete, S. Estus, G.W. Rebeck, G. Bu, M.J. LaDu, Soluble apoE/Abeta complex: mechanism and therapeutic target for APOE4-induced AD risk, Mol. Neurodegener. 9 (2014) 2.
[13] L. Chico, C. Simoncini, A. Lo Gerfo, A. Rocchi, L. Petrozzi, C. Carlesi, L. Volpi, G. Tognoni, G. Siciliano, U. Bonuccelli, Oxidative stress and APO E polymorphisms in Alzheimer's disease and in mild cognitive impairment, Free Radic. Res. 47 (2013) 569–576.
[14] M.A. Fernandes, M.T. Proenca, A.J. Nogueira, M.M. Grazina, L.M. Oliveira, A. I. Fernandes, B. Santiago, I. Santana, C.R. Oliveira, Influence of apolipoprotein E genotype on blood redox status of Alzheimer's disease patients, Int. J. Mol. Med. 4 (1999) 179–186.
[15] C. Ramassamy, D. Averill, U. Beffert, S. Bastianetto, L. Theroux, S. Lussier-Cacan, J.S. Cohn, Y. Christen, J. Davignon, R. Quirion, J. Poirier, Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype, Free Radic. Biol. Med. 27 (1999) 544–553.
[16] C.M. Lauderback, J. Kanski, J.M. Hackett, N. Maeda, M.S. Kindy, D.A. Butterfield, Apolipoprotein E modulates Alzheimer's Abeta(1–42)-induced oxidative damage to synaptosomes in an allele-specific manner, Brain Res. 924 (2002) 90–97.
[17] E. Straface, P. Matarrese, L. Gambardella, R. Vona, A. Sgadari, M.C. Silveri, W. Malorni, Oxidative imbalance and cathepsin D changes as peripheral blood biomarkers of Alzheimer disease: a pilot study, FEBS Lett. 579 (2005) 2759–2766.
[18] F. Di Domenico, R. Coccia, D.A. Butterfield, M. Perluigi, Circulating biomarkers of protein oxidation for Alzheimer disease: expectations within limits, Biochim. Biophys. Acta 1814 (2011) 1785–1795.
[19] D. Galasko, T.J. Montine, Biomarkers of oxidative damage and inflammation in Alzheimer's disease, Biomarkers Med. 4 (2010) 27–36.
[20] N. Mattsson, H. Zetterberg, O. Hansson, N. Andreasen, L. Parnetti, M. Jonsson, S.K. Herukka, W.M. van der Flier, M.A. Blankenstein, M. Ewers, K. Rich, E. Kaiser, M. Verbeek, M. Tsolaki, E. Mulugeta, E. Rosen, D. Aarsland, P.J. Visser, J. Schroder, J. Marcusson, M. de Leon, H. Hampel, P. Scheltens, T. Pirttila, A. Wallin, M.E. Jonhagen, L. Minthon, B. Winblad, K. Blennow, CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment, J. Am. Med. Assoc. 302 (2009) 385–393.
[21] R.J. Bateman, C. Xiong, T.L. Benzinger, A.M. Fagan, A. Goate, N.C. Fox, D. S. Marcus, N.J. Cairns, X. Xie, T.M. Blazey, D.M. Holtzman, A. Santacruz, V. Buckles, A. Oliver, K. Moulder, P.S. Aisen, B. Ghetti, W.E. Klunk, E. McDade, R. N. Martins, C.L. Masters, R. Mayeux, J.M. Ringman, M.N. Rossor, P.R. Schofield, R.A. Sperling, S. Salloway, J.C. Morris, Dominantly Inherited Alzheimer Network, Clinical and biomarker changes in dominantly inherited Alzheimer's disease, New. Engl. J. Med. 367 (2012) 795–804.
[22] P. Buchhave, L. Minthon, H. Zetterberg, A.K. Wallin, K. Blennow, O. Hansson, Cerebrospinal fluid levels of beta-amyloid 1–42, but not of tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia, Arch. Gen. Psychiatry 69 (2012) 98–106.
[23] R. Ghidoni, L. Benussi, A. Paterlini, V. Albertini, G. Binetti, E. Emanuele, Cerebrospinal fluid biomarkers for Alzheimer's disease: the present and the future, Neuro-Degener. Dis. 8 (2011) 413–420.
[24] K. Blennow, H. Hampel, M. Weiner, H. Zetterberg, Cerebrospinal fluid and plasma biomarkers in Alzheimer disease, Nat. Rev. Neurol. 6 (2010) 131–144.
[25] A.M. Fagan, R.J. Perrin, Upcoming candidate cerebrospinal fluid biomarkers of Alzheimer's disease, Biomarkers Med. 6 (2012) 455–476.
[26] D.A. Butterfield, M. Perluigi, R. Sultana, Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics, Eur. J. Pharmacol. 545 (2006) 39–50.
[27] D.A. Butterfield, F. Di Domenico, A.M. Swomley, E. Head, M. Perluigi, Redox proteomics analysis to decipher the neurobiology of Alzheimer-like neurodegeneration: overlaps in Down's syndrome and Alzheimer's disease brain, Biochem. J. 463 (2014) 177–189.
[28] D.A. Butterfield, T. Reed, S.F. Newman, R. Sultana, Roles of amyloid beta-peptide-associated oxidative stress and brain protein modifications in the pathogenesis of Alzheimer's disease and mild cognitive impairment, Free Radic. Biol. Med. 43 (2007) 658–677.
[29] D.A. Butterfield, M. Perluigi, T. Reed, T. Muharib, C.P. Hughes, R.A. Robinson, Sultana, R. Redox proteomics in selected neurodegenerative disorders: from its infancy to future applications, Antioxidants Redox Signal. 17 (2012) 1610–1655.
[30] C.D. Aluise, R.A. Sowell, D.A. Butterfield, Peptides and proteins in plasma and cerebrospinal fluid as biomarkers for the prediction, diagnosis, and monitoring of therapeutic efficacy of Alzheimer's disease, Biochim. Biophys. Acta 1782 (2008) 549–558.
[31] O.C. Maes, S. Kravitz, Y. Mawal, H. Su, A. Liberman, K. Mehindate, D. Berlin, D. J. Sahlas, H.M. Chertkow, H. Bergman, C. Melmed, H.M. Schipper, Characterization of alpha1-antitrypsin as a heme oxygenase-1 suppressor in Alzheimer plasma, Neurobiol. Dis. 24 (2006) 89–100.
[32] H.L. Yu, H.M. Chertkow, H. Bergman, H.M. Schipper, Aberrant profiles of native and oxidized glycoproteins in Alzheimer plasma, Proteomics 3 (2003) 2240–2248.
[33] P.A. Gollin, R.N. Kalaria, P. Eikelenboom, A. Rozemuller, G. Perry, Alpha 1-antitrypsin and alpha 1-antichymotrypsin are in the lesions of Alzheimer's disease, Neuroreport 3 (1992) 201–203.
[34] M. Puchades, S.F. Hansson, C.L. Nilsson, N. Andreasen, K. Blennow, P. Davidsson, Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease, Brain Res. Mol. brain Res. 118 (2003) 140–146.
[35] P.T. Gomme, J. Bertolini, Therapeutic potential of vitamin D-binding protein, Trends Biotechnol. 22 (2004) 340–345.
[36] R.F. Chun, New perspectives on the vitamin D binding protein, Cell. Biochem. Funct. 30 (2012) 445–456.
[37] O.A. Gressner, M.C. Schifflers, P. Kim, L. Heuts, B. Lahme, A.M. Gressner, Questioning the role of actinfree Gc-Globulin as actin scavenger in neurodegenerative central nervous system disease: relationship to S-100B levels and blood-brain barrier function, Clin. Chim. Acta; Int. J. Clin. Chem. 400 (2009) 86–90.
[38] J. Zhang, I. Sokal, E.R. Peskind, J.F. Quinn, J. Jankovic, C. Kenney, K.A. Chung, S. P. Millard, J.G. Nutt, T.J. Montine, CSF multianalyte profile distinguishes Alzheimer and Parkinson diseases, Am. J. Clin. Pathol. 129 (2008) 526–529.
[39] M. Moon, H. Song, H.J. Hong, D.W. Nam, M.Y. Cha, M.S. Oh, J. Yu, H. Ryu, I. Mook-Jung, Vitamin D-binding protein interacts with Abeta and suppresses Abeta-mediated pathology, Cell Death Differ. 20 (2013) 630–638.
[40] C.S. Latimer, L.D. Brewer, J.L. Searcy, K.C. Chen, J. Popovic, S.D. Kraner, O. Thibault, E.M. Blalock, P.W. Landfield, N.M. Porter, Vitamin D prevents cognitive decline and enhances hippocampal synaptic function in aging rats, Proc. Natl. Acad. Sci. USA 111 (2014) E4359–E4366.
[41] J.T. Keeney, S. Forster, R. Sultana, L.D. Brewer, C.S. Latimer, J. Cai, J.B. Klein, N. M. Porter, D.A. Butterfield, Dietary vitamin D deficiency in rats from middle to old age leads to elevated tyrosine nitration and proteomics changes in levels of key proteins in brain: implications for low vitamin d-dependent age-related cognitive decline, Free Radic. Biol. Med. 65 (2013) 324–334.
[42] R.B. DeMattos, J.R. Cirrito, M. Parsadanian, P.C. May, M.A. O'Dell, J.W. Taylor, J. A. Harmony, B.J. Aronow, K.R. Bales, S.M. Paul, D.M. Holtzman, ApoE and clusterin cooperatively suppress Abeta levels and deposition: evidence that ApoE regulates extracellular Abeta metabolism in vivo, Neuron 41 (2004) 193–202.
[43] W. Mi, M. Pawlik, M. Sastre, S.S. Jung, D.S. Radvinsky, A.M. Klein, J. Sommer, S. D. Schmidt, R.A. Nixon, P.M. Mathews, E. Levy, Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models, Nat. Genet. 39 (2007) 1440–1442.
[44] B. Bohrmann, L. Tjernberg, P. Kuner, S. Poli, B. Levet-Trafit, J. Naslund, G. Richards, W. Huber, H. Dobeli, C. Nordstedt, Endogenous proteins controlling amyloid beta-peptide polymerization. Possible implications for beta-amyloid formation in the central nervous system and in peripheral tissues, J. Biol. Chem. 274 (1999) 15990–15995.
[45] I. Ray, A. Chauhan, J. Wegiel, V.P. Chauhan, Gelsolin inhibits the fibrillization of amyloid beta-protein, and also defibrillizes its preformed fibrils, Brain Res. 853 (2000) 344–351.
[46] D. Wen, K. Corina, E.P. Chow, S. Miller, P.A. Janmey, R.B. Pepinsky, The plasma

and cytoplasmic forms of human gelsolin differ in disulfide structure, Biochemistry 35 (1996) 9700–9709.
[47] J.H. Hartwig, D. Brown, D.A. Ausiello, T.P. Stossel, L. Orci, Polarization of gelsolin and actin binding protein in kidney epithelial cells, J. Histochem. Cytochem.: Off. J. Histochem. Soc. 38 (1990) 1145–1153.
[48] V. Chauhan, L. Ji, A. Chauhan, Anti-amyloidogenic, anti-oxidant and anti-apoptotic role of gelsolin in Alzheimer's disease, Biogerontology 9 (2008) 381–389.
[49] H. Potter, T. Wisniewski, Apolipoprotein e: essential catalyst of the Alzheimer amyloid cascade, Int. J. Alzheimer's Dis. 2012 (2012) 489428.
[50] C.R. Beasley, C. Robinson, R.L. Featherstone, J.G. Varley, C.C. Hardy, M. K. Church, S.T. Holgate, 9 alpha,11 beta-prostaglandin F2, a novel metabolite of prostaglandin D2 is a potent contractile agonist of human and guinea pig airways, J. Clin. Investig. 79 (1987) 978–983.
[51] B.J. Whittle, S. Moncada, J.R. Vane, Comparison of the effects of prostacyclin (PGI2), prostaglandin E1 and D2 on platelet aggregation in different species, Prostaglandins 16 (1978) 373–388.
[52] T. Kanekiyo, T. Ban, K. Aritake, Z.L. Huang, W.M. Qu, I. Okazaki, I. Mohri, S. Murayama, K. Ozono, M. Taniike, Y. Goto, Y. Urade, Lipocalin-type prostaglandin D synthase/beta-trace is a major amyloid beta-chaperone in human cerebrospinal fluid, Proc. Natl. Acad. Sci. USA 104 (2007) 6412–6417.