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# Multiplexed Proteomic Analysis of Oxidation and Concentrations of Cerebrospinal Fluid Proteins in Alzheimer Disease

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***Background:*** **Carbonylation is an irreversible oxidative modification of proteins that has been linked to various conditions of oxidative stress, aging, physiological disorders, and disease. Increased oxidative stress is thus also considered to play a role in the pathogenesis of age-related neurodegenerative disorders such as Alzheimer disease (AD). In addition, it has recently become evident that the response mechanisms to increased oxidative stress may depend on sex. Several oxidized carbonylated proteins have been identified in plasma and brain of AD patients by use of 2-dimensional oxyblotting.**

***Methods:*** **In this pilot study, we estimated the concentrations and carbonylation of the most abundant cerebrospinal fluid proteins in aging women and men, both AD patients suffering from mild dementia and individuals exhibiting no cognitive decline. Oxidized carbonylated proteins were analyzed with 2-dimensional multiplexed oxyblotting, mass spectrometry, and database searches.**

***Results:*** **Signals for β-trace, λ chain, and transthyretins were decreased in probable AD patients compared with controls. The only identified protein exhibiting an increased degree of carbonylation in AD patients was λ chain. The concentrations of proteins did not generally differ between men and women; however, vitamin D–binding protein, apolipoprotein A-I, and α-1-antitrypsin exhibited higher extents of carbonylation in men.**

***Conclusions:*** **None of the brain-specific proteins exhibited carbonylation changes in probable AD patients compared with age-matched neurological controls showing no cognitive decline. The carbonylation status of proteins differed between women and men. Two-dimensional multiplexed oxyblotting is applicable to study both the concentrations and carbonylation of cerebrospinal fluid proteins.**

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Oxidative stress is an integral part of physiological functions in all organisms living in an aerobic environment. An increase in oxidative stress, especially in the brain, is a part of normal aging and is related directly to decreased neurological activities and inversely to lifespan *(1, 2)*. In addition, oxidative stress may be an essential feature in a variety of neurodegenerative diseases, including Alzheimer disease (AD)[5] *(3)*. In AD, increased oxidative stress has been associated with aggregation of proteins, calcium dysregulation, mitochondrial malfunction, chronic inflammation, altered antioxidant function, and accumulation of redox-active metals *(3–5)*. Accumulating evidence also points to significant sex differences with respect to oxidative stress and antioxidant defense systems, including the response to antioxidant treatment *(6–8)*. Taken together, the factors that are known to be related to an increase in oxidative stress, such as age, and differences in antioxidant response mechanisms, such as sex, may also

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[5] Nonstandard abbreviations: AD, Alzheimer disease; ROS, reactive oxygen species; 2-D, 2-dimensional; 2-DE, 2-D gel electrophoresis; CSF, cerebrospinal fluid; MMSE, Mini-Mental State Examination; IPG, immobilized pH gradient; DBP, vitamin D–binding protein; α-1-AT, α-1-antitrypsin; TTR, transthyretin.

influence the risk for an individual to develop AD *(3, 9–11)*.

Oxidative stress occurs as a result of an imbalance between the generation and scavenging of reactive oxygen species (ROS) *(12)*. This increase in the amount of ROS may be oxidatively damaging to a variety of macromolecules, leading to fatal cellular effects. Proteins are considered to be the major targets of oxidative stress. In general, the complexity of oxidative modifications of proteins is still not well understood *(12)*. Some modifications may be considered reversible and beneficial against attacks caused by ROS; however, some modifications, such as carbonylation, are irreversible and may thus be destructive, resulting in alterations in the structure and function of proteins. Carbonyl groups are introduced to proteins by oxidizing amino acid residue side-chain hydroxyls into ketone or aldehyde derivatives *(13)*. A variety of oxidative pathways lead to carbonylation of proteins *(12)*. Carbonyl groups can be introduced to proteins by direct oxidation of lysine, arginine, proline, and threonine residues, or from the cleavage of peptide bonds by α-amidation pathway or by oxidation of glutamyl residues. ROS can also react with other molecules, such as sugars (glycation/ glycoxidation) and lipids (lipid peroxidation), resulting in generation of reactive carbonyl derivatives and aldehydes, which may then react with proteins, followed by formation of protein-bound carbonyls. Measurement of protein carbonylation is thought to be a good estimation for the extent of oxidative damage of proteins associated with various conditions of oxidative stress, aging, physiological disorders, and disease *(14, 15)*.

The traditional approach in proteomics, 2-dimensional (2-D) gel electrophoresis (2-DE) in combination with mass spectrometry, enables simultaneous identification and quantification of multiple protein isoforms *(16)*. A number of studies have reported differences in the cerebrospinal fluid (CSF) proteome of AD patients compared with controls *(17–20)*. Two-dimensional oxyblotting is a method that permits examination of both the concentrations and carbonylation of multiple proteins by slightly modifying 2-DE and combining it with immunoblotting. This method can be used to assess the concentrations of oxidative stress via the increase in the formation of protein-bound carbonyls, which are measured based on the covalent reaction of the carbonylated protein side chains with 2,4-dinitrophenylhydrazine followed by detection with a specific antidinitrophenyl antibody *(21)*. The amount of protein-bound carbonyls is increased in AD brains *(14)*, and several oxidized carbonylated proteins have recently been characterized by 2-D oxyblotting in AD plasma and brain *(5, 22–25)*.

CSF is a secreted product of choroid plexus and a highly specific repository of cellular byproducts, metabolites, neurotransmitters, and proteolytic fragments *(26)*. CSF is also connected with the extracellular space in brain, and is protected from the peripheral circulation thanks to the highly regulated blood–CSF barrier. Therefore, the composition of CSF partially reflects cerebral metabolic changes and enables screening of ongoing pathophysiological processes in brain. Approximately 20% of the proteins in CSF are considered to be brain derived, although they may not be brain specific *(27)*. Physiologically, proteins that are small and/or highly lipophilic can be passively transported across the blood–CSF barrier from the peripheral circulation. Alternatively, proteins can be actively transported across the blood–CSF barrier via pinocytosis. Because CNS is normally protected from the peripheral circulation, the presence of some blood-derived macromolecules in CSF may indicate a disruption of blood–CSF barrier that is thought to occur during aging as well as in some neurodegenerative disorders *(17, 28)*.

Based on the current knowledge of the CSF proteome and oxidation of proteins in AD, the aim of this pilot study was to find out whether alterations in carbonylation status of proteins may also be detected in CSF. By use of 2-D multiplexed oxyblotting, we measured the concentrations and carbonylation of major CSF proteins in aging women and men, both neurological controls with no cognitive decline and AD patients *(29)*. These proteins were further identified by in-gel digestion, mass spectrometry, and database searches.

## Materials and Methods

### STUDY POPULATION AND SAMPLES

We obtained lumbar CSF samples, collected as a part of the ongoing biomarker project, from 11 probable AD patients and 8 neurological control patients showing no cognitive decline (Table 1). All participants and caregivers of dementia patients gave informed consent for participation in the study. The study was approved by the local ethics committees of University of Kuopio and Kuopio University Hospital. The diagnosis of probable AD was established according to National Institute of Neurological and Communicative Disorders and Stroke Alzheimer's Disease and Related Disorders Association criteria *(30)*. We used the Mini-Mental State Examination (MMSE) to assess the severity of AD *(31)*.

We performed lumbar puncture according to a standardized protocol. Samples that contained visible blood were excluded. CSF samples were immediately centrifuged at 4000*g* for 10 min. Supernatants were removed, divided into aliquots, and immediately frozen and stored at −70 °C until use. In addition, we routinely measured protein content in the supernatant after lumbar puncture for all samples. Two patients (Table 1, nos. 4 and 12) had abnormal (>600 mg/L) protein concentrations in CSF, indicating a possible disruption of the blood–CSF barrier.

### 2-D OXYBLOTTING

We measured total protein content by use of a $D_C$ protein assay reagent set according to the manufacturer's instructions (Bio-Rad). Individual CSF samples containing 1 mg protein were precipitated with ice-cold acetone (1:2, vol/vol), incubated overnight at −20 °C, and centrifuged

**Table 1. Patient demographics.**[a]

| Patient no. | Diagnosis | Sex | ApoE ϵ4 allele | Age, years | Duration of cognitive impairment, years | MMSE score | Clinical history |
|---|---|---|---|---|---|---|---|
| 1 | Control | M | − | 61 | | 29 | Depression, medication for hypertension |
| 2 | Control | M | + | 52 | | ND | Alcoholism and diabetes, medication for bipolar disorder |
| 3 | Control | M | − | 65 | | 22 | Essential tremor, medication for depression |
| 4 | Control | M | + | 69 | | 25 | Multisystem atrophy, medication for diabetes and atherosclerosis |
| 5 | Control | M | − | 57 | | ND | Schizophrenia with medication |
| 6 | Control | M | + | 55 | | 29 | No medication, nonsmoker, nondrinker |
| 7 | Control/MCI | F | − | 79 | | 21 | Stable MCI, medication for chronic epilepsy and hypertension |
| 8 | Control | F | − | 79 | | 24 | Medication for hypertension, atherosclerosis, and essential tremor |
| 9 | MCI/AD | F | − | 66 | 0 | 26 | Transient ischemic attack, mild depression, medication for hypertension, conversion to AD 4 years after lumbar puncture |
| 10 | AD | M | + | 77 | 5 | 14 | Despite evidence of dementia, healthy without any medication |
| 11 | AD | M | + | 90 | 1 | 18 | Mild hypothyroidism, chronic myeloid leukemia |
| 12 | AD | M | + | 71 | 4 | 23 | Dementia-related epilepsy treated with sodium-valproate, asthma, hypertension, |
| 13 | AD | F | − | 79 | 2 | 20 | Medication for hypertension and atherosclerosis, type 2 diabetes treated with diet |
| 14 | MCI/AD | F | + | 68 | 2 | 21 | Despite evidence of mild dementia, healthy without any medication, conversion to AD 3 years after lumbar puncture |
| 15 | AD | F | − | 54 | 1 | 22 | Smoker, estrogen treatment |
| 16 | AD | F | + | 74 | 1 | 16 | Despite evidence of mild dementia, healthy without any medication |
| 17 | MCI/AD | F | + | 78 | 3 | 24 | Mild medication for depression, conversion to AD 1 year after lumbar puncture |
| 18 | AD | F | + | 78 | 2 | 22 | Medication for type 2 diabetes and atherosclerosis |
| 19 | AD | F | + | 74 | 3 | 23 | Medication for atherosclerosis and hypercholesterolemia |

[a] AD, probable Alzheimer disease; ApoE ϵ 4 allele, 1 or 2 copies of apolipoprotein E ϵ 4 allele; MCI, mild cognitive impairment; ND, not defined. Score range, 0–30.

(5000*g* at 4 °C) for 15 min. The precipitates were dissolved in rehydration buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 20 mmol/L dithiothreitol, and 1% 3–10 nonlinear immobilized pH gradient (IPG) buffer (Amersham Biosciences). We performed isoelectric focusing 18-cm 3–10 nonlinear IPG gels (Amersham Biosciences) with an IPGphor apparatus (Amersham Biosciences). The samples were focused until 32 500 volt-hours.

To detect carbonylated proteins, IPG gels were treated with 2,4-dinitrophenyl hydrazine (Sigma) and equilibrated according to the in-strip derivatization method described by Conrad et al. *(32)*. In the 2nd dimension, proteins were separated on 12.5% sodium dodecyl sulfate polyacrylamide gel with Protean II (Bio-Rad) followed by an electrotransfer to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences) in Towbin transfer buffer by use of a Protean II Transfer apparatus (Bio-Rad). We detected proteins as described *(29)*. Briefly, we stained proteins with Sypro Ruby membrane stain according to the manufacturer's instructions (Bio-Rad) followed by the detection of hydrazone groups with an antidinitrophenyl antibody (Dako, V0401). We detected fluorescence signals with fluoroimager Storm 860 (Amersham Biosciences).

### Image analysis

We analyzed light intensities of detected proteins on membranes by use of ImageMaster 2D Elite, version 4.1 (Amersham Biosciences). Two-dimensional images of relative concentrations and carbonylation of proteins were

analyzed separately. The relative concentrations of proteins are given as normalized values. To compare the concentrations of proteins, the normalized value was justified since the total amount of protein loaded to each gel was the same. The extents of protein-bound carbonyls, however, are given as light intensity values, since 2-D oxyblots could not be normalized according to the total CSF carbonylation. In other words, the equal amount of total protein was not expected to exhibit the same degree of carbonylation in individuals. We transferred values of light intensities to SPSS software to calculate the degree of carbonylation.

#### protein identification

We performed 2-DE as described above, except that IPG gels were not treated with 2,4-dinitrophenyl hydrazine. We manually cut out protein spots from the Sypro Ruby–stained 2-D gels on a ultraviolet light table. Proteins were reduced and alkylated before in-gel digestion with trypsin (Sequencing Grade Modified trypsin; Promega), and we analyzed the resulting peptides with automated nano–light chromatography/tandem mass spectrometry essentially as described *(25)*. The nano–light chromatography/tandem mass spectrometry runs were performed using either Ultimate™ capillary LC system, Famos autosampler, and Switchos II (Dionex/LC Packings) coupled to QStarPulsar (ABI/MDS-SCIEX) or CapLC coupled to Q-TOF Global (Waters/Micromass). Database searches were performed using the publicly available Mascot search engine (http://www.matrixscience.com) against the NCBInr database.

#### statistical analysis

We used the Mann–Whitney *U*-test to compare normalized volumes of light intensities of the amount of protein-bound carbonyls and the amount of each protein. The degree of carbonylation was calculated as the ratio of protein-bound carbonyls to the amount of protein. The sensitivity for the detection of protein spots is limited when using image analysis software. For a few spots, zero values were obtained for light intensities although these proteins were visible by eye. Because we could not obtain background values by image analysis, we replaced zero values with an empirical minimum value for the calculation of the degree of carbonylation. The empirical minimum was the lowest light intensity value of 0.01 detected by image analysis.

All protein identification scores obtained using Mascot search engine were significant ($P < 0.05$).

## Results

In this pilot study, we found 30 oxidized carbonylated protein spots in the CSF of probable AD patients and controls and identified 27 of them. In 3 spots, the protein amount was too low for identification, even when spots were pooled from several 2-DE gels before in-gel digestion (Fig. 1 and Table 2, proteins 16, 17, and 18). In addition, the largest spot in Fig. 1 represents human serum albumin, which was heavily oxidized. Human serum albumin was not identified by mass spectrometry in this study, nor did we measure its carbonylation or concentrations because of its high abundance and low resolution. The most abundant identified carbonylated proteins in the CSF were vitamin D–binding protein (DBP), $\alpha$-1-antitrypsin ($\alpha$-1-AT), transthyretin (TTR), $\beta$-trace, apolipoprotein A-I, and apolipoproteins E and J (Fig. 1 and Table 2). Although some of the proteins, including apolipoproteins E and J, were identified as oxidized proteins, they were not reliably matched during image analysis owing to individual variation and insufficient resolution in 2-D images and therefore not further quantified. These proteins are indicated as "not quantified" in Table 2. We matched and quantified carbonylation status and concentrations of a total of 22 protein isoforms in probable AD patients vs neurological controls exhibiting no cognitive decline, and in women vs men (Fig. 1 and Table 2).

In patients with probable AD, there was a significant decrease in the protein concentrations of $\beta$-trace, TTR isoforms, $\lambda$ chain, and 1 unidentified protein compared with controls (Table 2). Generally, the degrees of carbonylation were similar in probable AD and control samples. $\lambda$ Chain and 1 unidentified protein exhibited increased carbonylation in probable AD (Fig. 1 and Table 2, proteins 17 and 18).

Because our study participants were not sex-matched, we also wanted to look for possible differences in the carbonylation and protein concentrations between men and women. Amounts of proteins did not generally differ between men and women, apart from 1 isoform of $\beta$-trace [mean (SE) 390 (130) in all women vs 843 (204) in all men; $P = 0.04$; Fig. 1, protein 25]. However, there were differences in the degrees of carbonylation between women and men. The degrees of carbonylation were significantly increased in all men compared with all women for DBP, apolipoprotein A-I, and $\alpha$-1-AT (Fig. 1 and Table 3, proteins 4, 8, 9, 10, and 22). In probable AD men compared with probable AD women, DBP exhibited a higher degree of carbonylation (Fig. 1 and Table 3, protein 4). The degrees of carbonylation did not vary significantly between control women and men.

## Discussion

Carbonylation of several abundant proteins has been characterized in plasma and brain of AD patients in different stages compared with defined controls *(5, 22–25)*. Therefore, the major aim of our study was to determine whether carbonylation changes in abundant proteins can also be detected in CSF. Altogether, we were able to assess the carbonylation and concentrations of a total of 22 abundant CSF proteins in probable AD patients and neurological control patients exhibiting no cognitive decline. In general, the extent of carbonylation did not vary between probable AD patients and controls; however, 2 proteins did exhibit a higher extent of carbonyla-

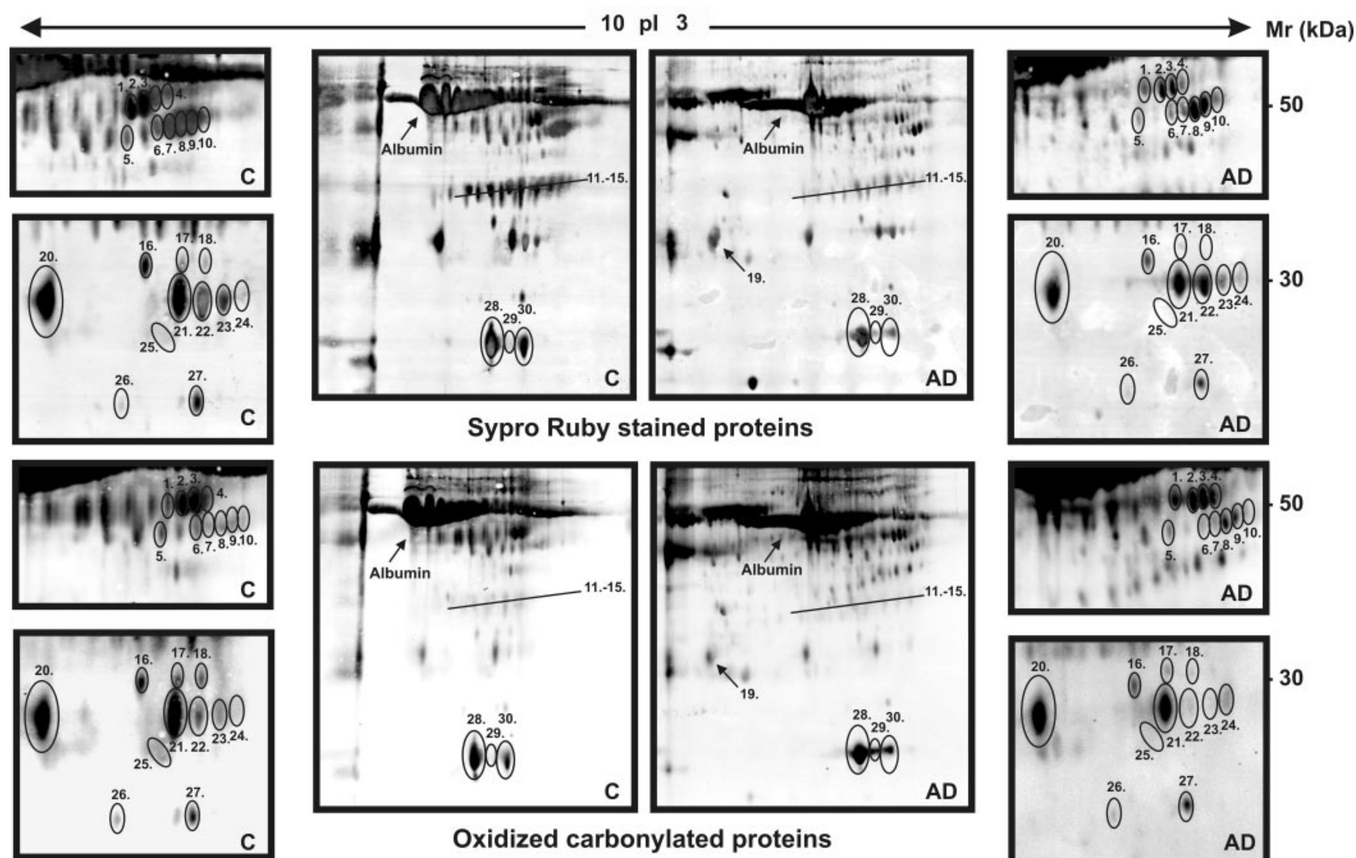


Fig. 1. Two-dimensional images of the most abundant proteins stained with Sypro Ruby and major carbonylated proteins detected by immunoblotting in the CSF of a female AD patient (Table 1, no. 15) and a male control patient (Table 1, no. 5).

*Numbers* of spots correspond to proteins listed in Tables 2 and 3. *AD*, probable AD; *C*, control.

tion in probable AD patients, showing that differences in carbonylation status of proteins are detectable in CSF. It must be noted that in this study, we selected probable AD patients suffering from mild dementia with relatively short durations of clinical impairment. Alterations in CSF protein oxidation status may vary according to disease stage and be more pronounced in patients suffering from more severe dementia.

Differences in protein oxidation may provide a diagnostic tool in neurodegenerative diseases. A recent study showed that a peptide whose mass corresponded to oxidized β-amyloid 1–40 could be used to differentiate patients with Lewy body dementia from patients with Parkinson disease dementia *(33)*. In our 2-D study, the only identified protein exhibiting increased carbonylation in probable AD was blood-derived λ chain. In addition, protein concentrations of λ chain were significantly decreased in probable AD patients compared with controls. λ Chains, also known as immunoglobulin λ light chains, are involved in systemic and localized primary immunoglobulin-related (amyloid light chain or AL) amyloidosis *(34, 35)*. Interestingly, immunohistopathological studies have indicated that λ chains may be associated with amyloid deposits *(36, 37)*, whereas IgG and IgM have been shown to deposit in epithelial basement membrane of choroid plexus in AD brain *(38)*. It is possible that the increased carbonylation and the decreased amount of λ chain in CSF may reflect the oxidative stress present in probable AD. However, the origin of λ chain in CSF is difficult to ascertain, and it may infiltrate to CSF directly from blood.

The prevalent trend we found of the decrease in the protein concentrations of β-trace and TTR is consistent with previous proteomic studies, although we were not able to confirm the prominent decreases in the concentrations of apolipoprotein A-I *(19, 20)*. The similar findings increase the credibility of the results in the different studies. Proteomic studies are difficult to compare with each other because of insufficient characterization of the study material, the small number of patients involved in studies, and variations in experimental designs. The selection of patients and experimental design differed between our studies as well *(19, 20)*. For example, Puchades et al. *(20)* measured the concentrations of CSF proteins in non–age-matched elderly people with MMSE scores of 29 to 30 and older probable AD patients, in contrast to our age-matched neurological controls showing no cognitive decline (with MMSE scores between 21 and 29) and

**Table 2. List of the most abundant oxidized CSF proteins including concentrations and carbonylation of proteins that were reliably quantified and matched with each other.[a]**

| | | | Amount of protein, light intensity | | | | Degree of carbonylation | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| No. | Carbonylated proteins | Acc. no. | Control, mean (SE) | AD, mean (SE) | *P* | AD vs controls[b] | Control, mean (SE) | AD, mean (SE) | *P* | AD vs controls[b] |
| **I. Significant changes** | | | | | | | | | | |
| 17 | λ Chain precursor | 33395 | 250 (78) | 103 (57) | 0.02 | ↓ | 0.59 (0.14) | 1199.15 (668.73) | 0.03 | ↑ |
| 18 | Unidentified | | 44 (17) | 2 (2) | 0.01 | ↓ ↓ | 1309.80 (930.85) | 2646.28 (1328.936) | 0.04 | ↓ |
| 25 | β-Trace | 18028972 | 934 (223) | 366 (105) | 0.03 | ↓ | 0.48 (0.16) | 790.62 (602.40) | 0.10 | – |
| 29 | TTR | 14719497 | 848 (218) | 243 (139) | 0.02 | ↓ | 8519.97 (8517.62) | 17 102.46 (7314.15) | 0.16 | – |
| 30 | TTR | 999653 | 3591 (718) | 1522 (492) | 0.03 | ↓ | 1.46 (0.45) | 2.46 (1.01) | 0.56 | – |
| **II. Nonsignificant changes** | | | | | | | | | | |
| 1 | DBP | 21730549 | 3232 (424) | 2859 (332) | 0.41 | – | 1.25 (0.29) | 1.28 (0.26) | 0.87 | – |
| 2 | DBP | 22219267 | 4872 (953) | 3676 (459) | 0.30 | – | 1.36 (0.49) | 1.25 (0.42) | 0.93 | – |
| 3 | DBP | 22219267 | 3596 (665) | 3815 (557) | 0.79 | – | 1.23 (0.21) | 0.89 (0.11) | 0.16 | – |
| 4 | DBP | 72105 | 2193 (428) | 2306 (316) | 0.82 | – | 1.34 (0.29) | 0.82 (0.14) | 0.12 | – |
| 5 | α-1-AT | 1942629 | 977 (193) | 695 (130) | 0.16 | – | 1.06 (0.17) | 1.10 (0.28) | 0.56 | – |
| 7 | α-1-AT | 7245932 | 2322 (271) | 2704 (318) | 0.51 | – | 0.19 (0.03) | 0.15 (0.05) | 0.19 | – |
| 8 | α-1-AT | P01009 | 2777 (521) | 2856 (252) | 0.80 | – | 0.40 (0.18) | 0.25 (0.07) | 0.41 | – |
| 9 | α-1-AT | P01009 | 5918 (554) | 3638 (360) | 0.16 | – | 0.20 (0.05) | 0.26 (0.11) | 0.56 | – |
| 10 | α-1-AT | P01009 | 4389 (613) | 4770 (701) | 0.32 | – | 0.08 (0.02) | 0.10 (0.05) | 0.93 | – |
| 16 | Unidentified | | 1211 (274) | 604 (234) | 0.10 | – | 0.47 (0.0.17) | 0.43 (0.09) | 0.68 | – |
| 20 | β-Trace | 18028972 | 6662 (687) | 4946 (461) | 0.07 | – | 0.30 (0.06) | 0.30 (0.06) | 0.93 | – |
| 21 | β-Trace | 18028972 | 4410 (472) | 3439 (508) | 0.32 | – | 0.28 (0.05) | 1.29 (1.02) | 0.93 | – |
| 22 | Proapolipoprotein A-I | 178775 | 2350 (425) | 1740 (265) | 0.19 | – | 0.06 (0.02) | 0.05 (0.02) | 0.41 | – |
| 23 | Proapolipoprotein A-I | 178775 | 1368 (207) | 898 (194) | 0.19 | – | 0.02 (0.01) | 0.04 (0.02) | 0.46 | – |
| 26 | Cu/Zn-superoxide dismutase | 1237406 | 102 (35) | 72 (44) | 0.20 | – | 534.88 (366.20) | 2281.25 (1040.58) | 0.12 | – |
| 27 | Unidentified | | 762.84 (220) | 442 (179) | 0.19 | – | 0.34 (0.11) | 673.04 (459.12) | 0.48 | – |
| 28 | TTR | 339685 | 13 271 (1684) | 10 811 (1001) | 0.25 | – | 1.50 (0.32) | 1.34 (0.14) | 0.93 | – |
| **III. Proteins that were not quantified** | | | | | | | | | | |
| 6 | α 1-AT precursor | 7245932 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 11 | Apolipoprotein E3 | 178853 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 12 | Apolipoprotein E3 | 1942471 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 13 | Apolipoprotein E | 338305 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 14 | Apolipoprotein E | 178853 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 15 | Apolipoprotein J | 338305 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 19 | β-Trace | 410564 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |
| 24 | Proapolipoprotein A-I | 178775 | NQ | NQ | NQ | – | NQ | NQ | NQ | – |

[a] Degree of carbonylation, light intensities of protein-bound carbonyls divided by light intensities of the amount of corresponding protein spots. *P* value obtained by Mann–Whitney *U*-test. No., number of protein spots; Acc. no, identification of proteins indicated as NCBI or SWISS-PROT accession numbers; AD, probable AD; NQ, not quantified owing to individual variation, unreliable matching, or insufficient resolution.

[b] Arrows indicate the concentrations in probable AD compared with controls: –, no difference; ↓ or ↑, $P \leq 0.05$; ↓ ↓, $P \leq 0.01$.

probable AD patients suffering from mild dementia. In addition, the concentrations of proteins were measured on a Sypro Ruby–stained 2-D gel *(20)* in contrast to our Sypro Ruby–stained 2-D membranes. The congruencies in the results obtained in our studies support the usefulness of 2-DE when studying CSF proteome profiles in AD patients.

CSF is composed mainly of soluble proteins. Therefore, the decrease in the amounts of several brain proteins in CSF may be due to decreased production and/or increased aggregation in brain that may occur during the early stages of AD. Notably, decreased concentrations of proteins in CSF may also be a secondary event to increased oxidative stress, since excessive carbonylation is generally thought to lead to enhanced aggregation of proteins.

Previous studies have revealed sex-specific differences related to oxidative stress and antioxidant defenses *(6–8)*. We also found significant differences in carbonylation of proteins between men and women regardless of diagnosis. Men had increased carbonylation of an isoform of DBP, an isoform of apolipoprotein A-I, and α-1-AT. Increased carbonylation of several isoforms of α-1-AT in plasma from AD patients compared with healthy nondemented controls has been reported *(22)*. However, we did not find any difference in the carbonylation of α-1-AT in CSF between probable AD patients and neurological controls. Several possible reasons may explain differences between our studies, such as the selection of study participants, e.g., numbers of women and men. For example, our study participants were not sex-matched; based on our findings on sex differences in the carbonylation of proteins, this may have influenced our results when comparing protein carbonylation between AD patients and controls. Moreover, variations in experimental design may have contributed to the differences in the results. For

**Table 3. Differences in the degrees of carbonylation between men and women.[a]**

| No. | Carbonylated proteins | Degree of carbonylation: Women, n = 10, mean (SE) | Degree of carbonylation: Men, n = 9, mean (SE) | *P* | Men vs women[b] |
|---|---|---|---|---|---|
| 4 | DBP | 0.67 (0.09) | 1.45 (0.25) | 0.01 | ↑↑ |
| 7 | α-1-AT precursor | 0.14 (0.05) | 0.21 (0.03) | 0.09 | – |
| 8 | α-1-AT precursor | 0.16 (0.04) | 0.49 (0.15) | 0.01 | ↑↑ |
| 9 | α-1-AT precursor | 0.04 (0.01) | 0.15 (0.06) | 0.02 | ↑ |
| 10 | α-1-AT precursor | 0.14 (0.04) | 0.34 (0.12) | 0.05 | ↑ |
| 22 | Proapolipoprotein A-I | 0.03 (0.02) | 0.08 (0.02) | 0.04 | ↑ |
| | | **Control women, n = 2, mean (SEM)** | **Control men, n = 6, mean (SEM)** | ***p*** | **Men vs Women** |
| 4 | DBP | 1.00 (0.09) | 1.45 (0.38) | 0.18 | – |
| 7 | α-1-AT precursor | 0.19 (0.07) | 0.20 (0.04) | 1.00 | – |
| 8 | α-1-AT precursor | 0.12 (0.04) | 0.50 (0.23) | 0.10 | – |
| 9 | α-1-AT precursor | 0.03 (0.003) | 0.10 (0.03) | 0.10 | – |
| 10 | α-1-AT precursor | 0.09 (0.05) | 0.24 (0.07) | 0.10 | – |
| 22 | Proapolipoprotein A-I | 0.02 (0.02) | 0.07 (0.02) | 0.31 | – |
| | | **AD women, n = 8, mean (SEM)** | **AD men, n = 3, mean (SEM)** | ***p*** | **Men vs Women** |
| 4 | DBP | 0.59 (0.09) | 1.44 (0.06) | 0.01 | ↑↑ |
| 7 | α-1-AT precursor | 0.12 (0.06) | 0.22 (0.07) | 0.15 | – |
| 8 | α-1-AT precursor | 0.17 (0.05) | 0.47 (0.15) | 0.07 | – |
| 9 | α-1-AT precursor | 0.05 (0.01) | 0.25 (0.19) | 0.10 | – |
| 10 | α-1-AT precursor | 0.15 (0.04) | 0.55 (0.37) | 0.22 | – |
| 22 | Proapolipoprotein A-I | 0.03 (0.02) | 0.10 (0.04) | 0.10 | – |

[a] Degree of carbonylation, light intensities of protein-bound carbonyls divided by light intensities of the amount of corresponding protein spot. *P* value obtained by Mann–Whitney *U*-test. No., number of protein spots.

[b] Arrows indicate a comparison of the degree of oxidation between men and women: –, no difference, ↑, $P \leq 0.05$, ↑↑, $P \leq 0.01$.

example, Choi et al. *(22)* detected the concentrations of plasma proteins by staining one 2-DE gel and electrotransferred plasma proteins from another 2-D gel to a membrane for oxyblotting. In contrast, we applied a multiplexed oxyblotting approach that enables the detection of both the concentrations and carbonylation of proteins using a single membrane *(29)*. Carbonylation changes in CSF proteome may not be analogous to those in plasma even if matching the selection criteria of study participants and experimental designs. Finally, we found that DBP was more carbonylated in probable AD men compared with probable AD women. Taken together, our results indicate that the extent of protein carbonylation may vary between men and women, and that the impact of sex on protein oxidation needs to be further evaluated. In addition, our results emphasize the importance of having sex-matched patients when studying protein carbonylation in disease states.

Recent studies suggest that oxidized proteins may offer a diagnostic tool for differentiation of neurodegenerative diseases *(33)*. This is the first proteomic study on carbonylation of CSF proteins using 2-D multiplexed oxyblotting. Only 2 proteins, λ chain and 1 unidentified protein, exhibited a higher extent of carbonylation in probable AD patients compared with controls. Hereafter, it will be important to go deeper into the proteome and to study carbonylation of less abundant CSF proteins. Yet the exploration of the deep CSF proteome presents a number of challenges. For example, the protein content in blood is much higher than in CSF, and thus even a minor contamination during collection may influence the results *(39)*. Therefore, in CSF proteomic studies as well as in our study, the possibility of blood contamination is common and difficult to control. Although we excluded samples that contained visible blood and centrifuged the samples, and all patients except 2 had CSF protein concentrations in the normal interval, suggesting that they did not have any major damage to the blood–brain barrier, minor blood contaminations cannot be excluded. Moreover, examination of the brain-derived deep CSF proteome would require either the removal of the most abundant proteins (albumin and immunoglobulins) that account for ~80% of the total protein content in CSF or prefractionation of the sample *(40)*. One problem with the removal of abundant CSF proteins is the concomitant omission of some additional less abundant proteins. A further limitation is that the volume of CSF needed for the studies of less abundant proteins using 2-DE is high. Nevertheless, our results indicate that there are differences in carbonylation statuses of CSF proteins and further studies are warranted. It is likely that additional changes in CSF protein carbonylation will be revealed by methodologically modifying 2-D oxyblotting or by using quantitatively more sensitive methods.

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## References

1. Navarro A, Sanchez Del Pino MJ, Gomez C, Peralta JL, Boveris A. Behavioral dysfunction, brain oxidative stress, and impaired mitochondrial electron transfer in aging mice. Am J Physiol Regul Integr Comp Physiol 2002;282:R985–92.
2. Navarro A, Emerit J, Edeas M, Bricaire F. Mitochondrial enzyme activities as biochemical markers of aging, neurodegenerative diseases and oxidative stress. Mol Aspects Med 2004;25:37–48.
3. Mariani E, Polidori MC, Cherubini A, Mecocci P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B Analyt Technol Biomed Life Sci 2005;827:65–75.
4. Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR. Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. Curr Drug Targets CNS Neurol Disord 2005;4:383–403.
5. Butterfield DA. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. Brain Res 2004;1000:1–7.
6. Mendoza-Núñez VM, Sánchez-Rodriguez MA, Retana-Ugalde R, Vargas-Guadarrama LA, Altamirano-Lozano MA. Total antioxidant levels, gender, and age as risk factors for DNA damage in lymphocytes of the elderly. Mech Ageing Dev 2001;122:835–47.
7. Schuessel K, Leutner S, Cairns NJ, Muller WE, Eckert A. Impact of gender on upregulation of antioxidant defence mechanisms in Alzheimer's disease brain. J Neural Transm 2004;111:1167–82.
8. Liu H, Harrell LE, Shenvi S, Hagen T, Liu RM. Gender differences in glutathione metabolism in Alzheimer's disease. J Neurosci Res 2005;79:861–7.
9. Jorm AF, Korten AE, Henderson AS. The prevalence of dementia: a quantitative integration of the literature. Acta Psychiatr Scand 1987;76:465–79.
10. Turner RS. Alzheimer's disease in man and transgenic mice: females at higher risk. Am J Pathol 2001;158:797–801.
11. Rocca WA, Hofman A, Brayne C, Breteler MM, Clarke M, Copeland JR, et al. Frequency and distribution of Alzheimer's disease in Europe: a collaborative study of 1980–1990 prevalence findings: the EURODEM-Prevalence Research Group. Ann Neurol 1991;30:381–90.
12. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 2006;10:389–406.
13. Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997;272:20313–6.
14. Smith MA, Sayre LM, Anderson VE, Harris PL, Beal MF, Kowall N, et al. Cytochemical demonstration of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine. J Histochem Cytochem 1998;46:731–5.
15. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346–57.
16. Görg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004;4:3665–85.
17. Mattila KM, Pirttilä T, Blennow K, Wallin A, Viitanen M, Frey H. Altered blood-brain-barrier function in Alzheimer's disease? Acta Neurol Scand 1994;89:192–8.
18. Choe LH, Dutt MJ, Relkin N, Lee KH. Studies of potential cerebrospinal fluid molecular markers for Alzheimer's disease. Electrophoresis 2002;23:2247–51.
19. Davidsson P, Westman-Brinkmalm A, Nilsson CL, Lindbjer M, Paulson L, Andreasen N, et al. Proteome analysis of cerebrospinal fluid proteins in Alzheimer patients. Neuroreport 2002;13:611–5.
20. Puchades M, Hansson SF, Nilsson CL, Andreasen N, Blennow K, Davidsson P, et al. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease. Brain Res Mol Brain Res 2003;118:140–6.
21. Nakamura A, Goto S. Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. J Biochem (Tokyo) 1996;119:768–74.
22. Choi J, Malakowsky CA, Talent JM, Conrad CC, Gracy RW. Identification of oxidized plasma proteins in Alzheimer's disease. Biochem Biophys Res Commun 2002;293:1566–70.
23. Yu HL, Chertkow HM, Bergman H, Schipper HM. Aberrant profiles of native and oxidized glycoproteins in Alzheimer plasma. Proteomics 2003;3:2240–8.
24. Choi J, Rees HD, Weintraub ST, Levey AI, Chin LS, Li L. Oxidative modifications and aggregation of Cu/Zn superoxide dismutase associated with Alzheimer's and Parkinson's diseases. J Biol Chem 2005;280:11648–55.
25. Korolainen MA, Goldsteins G, Nyman TA, Alafuzoff I, Koistinaho J, Pirttilä T. Oxidative modification of proteins in the frontal cortex of Alzheimer's disease brain. Neurobiol Aging 2006;27:42–53.
26. Romeo MJ, Espina V, Lowenthal M, Espina BH, Petricoin EF 3rd, Liotta LA. CSF proteome: a protein repository for potential biomarker identification. Expert Rev Proteomics 2005;2:57–70.
27. Reiber H. Dynamics of brain-derived proteins in cerebrospinal fluid. Clin Chim Acta 2001;310:173–86.
28. Skoog I, Wallin A, Fredman P, Hesse C, Aevarsson O, Karlsson I, et al. A population study on blood-brain barrier function in 85-year-olds: relation to Alzheimer's disease and vascular dementia. Neurology 1998;50:966–71.
29. Korolainen MA, Goldsteins G, Alafuzoff I, Koistinaho J, Pirttilä T. Proteomic analysis of protein oxidation in Alzheimer's disease brain. Electrophoresis 2002;23:3428–33.
30. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 1984;34:939–44.
31. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state": a practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res 1975;12:189–98.
32. Conrad CC, Choi J, Malakowsky CA, Talent JM, Dai R, Marshall P, et al. Identification of protein carbonyls after two-dimensional electrophoresis. Proteomics 2001;1:829–34.
33. Bibl M, Mollenhauer B, Esselmann H, Lewczuk P, Klafki HW, Sparbier K, et al. CSF amyloid-beta-peptides in Alzheimer's disease, dementia with Lewy bodies and Parkinson's disease dementia. Brain 2006;129:1177–87.
34. Hamidi Asl K, Liepnieks JJ, Nakamura M, Benson MD. Organ-specific (localized) synthesis of Ig light chain amyloid. J Immunol 1999;162:5556–60.

**35.** Schröder R, Linke RP. Cerebrovascular involvement in systemic AA and AL amyloidosis: a clear haematogenic pattern. Virchows Arch 1999;434:551–60.

**36.** Eikelenboom P, Stam FC. Immunoglobulins and complement factors in senile plaques: an immunoperoxidase study. Acta Neuropathol (Berl) 1982;57:239–42.

**37.** Kametani F, Tanaka K, Sato M, Haga S, Saitoh T, Shinoda T, et al. A monoclonal antibody Hy20–54-16–3L to lambda (λ) light chain of human immunoglobulin reacts with amyloid in Alzheimer's disease brain. Neurosci Lett 1990;117:62–7.

**38.** Serot JM, Bene MC, Faure GC. Comparative immunohistochemical characteristics of human choroid plexus in vascular and Alzheimer's dementia. Hum Pathol 1994;25:1185–90.

**39.** You JS, Gelfanova V, Knierman MD, Witzmann FA, Wang M, Hale JE. The impact of blood contamination on the proteome of cerebrospinal fluid. Proteomics 2005;5:290–6.

**40.** Righetti PG, Castagna A, Antonucci F, Piubelli C, Cecconi D, Campostrini N, et al. Proteome analysis in the clinical chemistry laboratory: myth or reality? Clin Chim Acta 2005;357:123–39.