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Original Contribution

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

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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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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. Phospho-tau was measured in CSF samples by the INNOTEST® PHOSPHO-TAU (181P) (Fujirebio, Gent, Belgium) that is a solid-phase enzyme immunoassay in which the phosphorylated tau protein or

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