Anomalously phosphorylated tau and Aβ fragments in the CSF correlates with cognitive impairment in MCI subjects

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of extracellular amyloid deposits, consisting largely of Aβ peptide and the presence of intraneuronal aggregates of neurofibillary tangles formed by tau. Development of cerebrospinal fluid (CSF) biomarkers has become a rapidly growing research field, considering the need for diagnostic tools for AD, thus allowing therapeutic compounds to have the greatest potential for being effective. We have focused on the relationships between critical biomarkers such as tau and Aβ in the CSF and the cognitive impairment of patients, as assessed by a battery of neuropsychological tests derived from CDR and CERAD, of value in the evaluation of AD patients. As part of a longitudinal study, we analyzed by ELISA and Western blots the levels and molecular patterns of hyperphosphorylated tau in the CSF of three different groups of patients: AD patients between 69- and 73-years-old, a group characterized with mild cognitive impairment (MCI) between 65- and 70-years-old, and a non-demented neurological control group of comparable ages. The levels of AT8-reactive phosphorylated tau were significantly higher ($P < 0.05$) in AD patients (0.604 ± 0.078, $n = 23$) as compared with the control group (0.457 ± 0.086, $n = 25$). No differences between the levels of AT8-reactive tau of MCI patients (0.510 ± 0.090, $n = 45$) and controls were observed. However, when the MCI group was divided on the basis of the total box score (TBS) from CDR, those subjects with a TBS < 1.5 presented tau levels (0.456 ± 0.032, $n = 31$) similar to controls, whereas those patients with TBS ≥ 1.5 displayed tau levels (0.590 ± 0.086, $n = 14$) comparable with those of AD. Western blot analyses revealed a higher AT8 reactivity in CSF samples of AD patients as compared with MCI and control samples, indicating higher levels of AD tau phosphoepitopes in the CSF. Tau heterogeneity was observed in samples of AD and MCI with higher impairment as compared with controls. As expected from previous reports, levels of Aβ (1–42) were lower (0.052 ± 0.005) than controls (0.070 ± 0.010), whereas the levels of MCI group were 0.060 ± 0.007. The MCI group with a TBS ≥ 1.5 presented Aβ levels of 0.053 ± 0.005 similar to those of AD patients, whereas the MCI group with TBS < 1.5 exhibited Aβ levels (0.066 ± 0.007) similar to controls. Studies highlight the relationships between anomalously phosphorylated tau markers in CSF with the information from TBS analysis of the different groups of patients.

Keywords: Amyloid; Tau protein; apoE Alleles; Cerebrospinal fluid; Mild cognitive disorders; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is the commonest cause of dementia and a major public health problem that may reach epidemic proportions if no cure is found within the next decade [24]. The neuropathological features of AD are a gradual and widespread neuronal loss, the extraneuronal β-amyloid deposit formation or senile plaques (SP), alterations in cerebral

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blood vessels, and the presence of neurofibrillary tangles (NFTs). SPs are mainly made up of β-amyloid, especially the Aβ (1-42) variant. The major constituent of NFTs is the microtubule-associated protein tau, which is hyperphosphorylated in AD brains [29,30].

The diagnosis of AD is based on clinical and neuropsychological examination, identification of symptoms of AD and exclusion of other known causes of dementia [18,31,41,44], as outlined by the NINCDS-ADRDA Work Group, and the *Diagnostic and Statistical Manual* of the American Psychiatric Association. AD is characterized by a progressive decline of cognitive functions, memory, language and visuospatial orientation. Neuropsychiatric symptoms such as depression and behavioral changes are common. However, diagnosis based on these instruments is unsatisfactory, indicating the need of highly sensitive and reliable approaches, selective for AD and based on biological markers [7–9]. Ideally, such markers should reflect the pathophysiological mechanisms of AD, which according to the current hypotheses, derive from the actions of two major protein aggregates: SP and NFTs [12]. There is evidence that the CSF levels of Aβ (1–42) are significantly reduced in AD patients as compared with senile controls, while increased levels of tau have been revealed [25,32,33]. The CSF levels of these proteins reflects their metabolism in the central nervous system [14]. ELISA and immunochemical methods for quantification of these markers have been used [19,42]. A number of studies suggest that CSF markers in combination with neuroimaging and neuropsychological tools, adds to the accuracy of AD diagnosis [18]. A recent study about the correlation of CSF biomarkers and the neuropathological diagnosis, confirmed an association between elevated CSF tau levels pre-mortem and the pathological hallmarks of AD, suggesting the value of anomalously high CSF tau for AD diagnosis [12]. Besides AD [17,20,22,25,26], CSF biomarkers have also been analyzed in dementia with Lewy bodies [16] and different groups with MCI [37].

The search of methods for AD detection in the pre-clinical phase could provide a hope for its prevention and treatment [11]. Early diagnosis would allow treatment with agents that delay cognitive deterioration in the initial phase of the disease, thus slowing its progression. The use of biomarkers in the pre-clinical phase operate as instruments of early diagnosis and differentiate AD from other similar pathologies [8,10]. This is applicable to patients with MCI without a differential diagnosis. In recent years a variety of syndromes have been proposed to characterize subjects with cognitive decline without dementia. Among them MCI has gained increasing attention [36,37,43], and refers to a transitional state between the cognition of normal aging and mild dementia, characterized by memory impairment, or a mild decline in some abilities [13]. The concept of MCI is one of the clinical entities proposed to characterize a heterogeneous group of individuals cognitively impaired but not demented. The causes of MCI are not yet clearly understood. No genetic link has yet been found for MCI, although that like AD, a genetic component in addition to sporadic components, might be a risk factor for people with MCI to develop AD [37,38,43]. Thus, the study of the potential markers in the cerebrospinal fluid (CSF) constitutes an important source of information in different neurological disorders. In these processes, brain pathological and structurally altered proteins among other normal components are released from the central nervous system toward the CSF, an appropriate target for the analysis of markers in AD [3,4].

We focused on the relationships between CSF biomarkers and the levels of cognitive impairment of three human subpopulations, by evaluating the relationships between the levels of hyperphosphorylated tau and the Aβ (1–42) peptide, and data from neuropsychological tools derived from CDR and CERAD, of proven diagnostic efficiency for AD. This research is part of a longitudinal study derived from the screening of over 600 patients, and in which a group of 93 patients was evaluated with the entire battery of neuropsychological tests and biological assays.

2. Methods and subjects for the study

2.1. Sampling

The subjects in the study consisted of 93 elderly patients, evaluated in the *Hospital El Salvador*, from the general population resident in the eastern metropolitan area of Santiago, Chile, who fulfilled the inclusion criteria of the study. Subjects were divided into three different groups: 23 patients with probable AD, 45 subjects with mild cognitive impairment (MCI) as defined by Petersen et al. [36,37], and 25 non-demented neurological controls. Participants were recruited through the printed media and underwent a multistage screening procedure. To be included in the study, participants needed to be more than 60 years old, to be free of significant medical illness, and to be willing to participate in the study. The study and the experimental protocols were approved by the Committee on Ethical Issues of the Faculty of Medicine, University of Chile, and all subjects provided informed consent prior to the initiation of the study. In the cases of demented subjects, in agreement with the guidelines of this Committee, the informed consent for participation in the study was obtained from their caregivers. The clinical diagnosis of AD was made according with the criteria for AD as outlined by the National Institute of Neurologic, Communicative Disorders and Stroke, AD and Related Disorders Association (NINCDS-ADRDA) Work Group [31].

2.2. Application of the semi-structured interview

The CDR ratings were obtained using a semi-structured interview specially adapted by Daly et al. [13], from the validated original version of Hughes et al. [21]. This interview was specially adapted to be used with a population with very mild impairments. It includes a standardized medical,

neurologic, and psychiatric examination. A Spanish adaptation of the English version of the semi-structured interview was used in a pilot study. Subjects distributed across a range of cognitive function from no-impairment to mild impairment, and their collaterals were evaluated by three independent interviewers. The questions that the three interviewers considered were not well understood by the subjects or collaterals, were modified according to the language skills of our population. The rating of the overall CDR score and the ratings of the each six CDR domains were analyzed with a kappa index. A very high concordance was obtained. For the overall CDR the κ was 0.957, and for Memory, Orientation, Judgment, Problem Solving, Community Affairs, Home and Hobbies, and Personal Care this index were 0.924, 0.964, 0.966, 0.929, 0.926 and 1.00, respectively.

The subjects of this study underwent a comprehensive medical and neurologic examination to ascertain that they were free of any significant medical condition. The subjects could be using psychoactive medications, and disabilities and co-morbid illnesses could be present, but the neurologists did not judge that these factors were causing clinically significant cognitive impairments. Once the interview was completed and rated, the subjects in the study were administered a neuropsychological battery. The CDR ratings [6] were completed with the interviewers blinded to the results of the neuropsychological test. The written interviews were scored by a reviewer who made his own rating judgment, blinded to the interviewer. A weekly consensus conference was carried out among the interviewers in order to reach an agreement regarding the rating of each of the CDR subcategories. In this discussion an explicit reference was made to CDR ratings coded by Daly et al. [13], to be sure that the final rating of each of the CDR subcategories adhered as closely as possible to the CDR expanded criteria.

The subjects were categorized into the following groups: (i) non-demented neurological controls with normal cognition and CDR rating 0.0 ($n = 25$); (ii) mild cognitive impairment (MCI) group that met the Petersen's criteria of questionable dementia with CDR rating 0.5 ($n = 45$), and AD patients ($n = 23$) with CDR rating 1.0 or higher, and met the NINCDS/ADRDA criteria for probable AD. According with the total box score (TBS) of CDR, the MCI subjects were divided into two subgroups [13]: those subjects with TBS ≥ 1.5 ($n = 14$) exhibiting greater cognitive impairment, and those with TBS < 1.5 ($n = 31$) with a lesser impairment.

2.3. Neuropsychological battery of tests

The neuropsychological evaluation consisted in a neuropsychological battery of CERAD [6] that include: Folstein's MiniMental Test, Verbal Fluency, Boston Nomination Test (15 items), Learning Word List (10 items), and Praxis.

2.4. Statistical analysis

One-way ANOVA was used to test differences in mean values, and Dunn's post-hoc test was used for comparisons (In Stat program from GraphPad). Differences were considered significant if $P < 0.05$ as statistical inference criteria. ANCOVA was used to analyze the covariance of age and education in the variables under study.

2.5. CSF samples

Lumbar CSF samples were obtained using a standardized protocol. The lumbar punctures were performed early in the morning, the subjects were kept at bed rest and had nothing by mouth until the procedure was completed. While the patient was in the lateral decubitus or sitting position, lumbar punctures were performed with a 20 or 22 gauge needle after application of local anaesthesia with 1% lidocaine. Headache rates following lumbar punctures were less than 5% (ranging from mild discomfort to severe headache). Approximately, 5.0 mL of CSF was withdrawn during lumbar puncture; the CSF was separated in aliquots into individual polypropylene tubes without preservative and frozen at the bedside on dry ice within minutes of withdrawal. Samples were then transferred to -70° freezers.

2.6. Determinations of total and hyperphosphorylated tau pools in the CSF

Quantitative ELISA assays with the monoclonal Tau-5 antibody for total tau determination, and with the monoclonal AT8 antibody for measurements of the hyperphosphorylated forms of tau protein, were used in this study [2]. Tau-5 tag the different tau variants, independently of their level of posttranslational modifications, while AT-8 recognizes the AD epitope on tau containing Ser202 and Thr205, when they are anomalously hyperphosphorylated. In order to simplify, these will be referred to as AD epitopes. Tau-1 antibody was used to assess tau species un-phosphorylated at the AD epitopes. Both Tau-5 and Tau-1 antibodies were generously donated by Dr. Lester Binder, while AT8 is a commercial antibody from Innogenetics, Belgium. The Aβ (1–42) peptide (sequence NH2-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIACOOH) and Aβ (1–40) amyloid peptide in the CSF fluid were evaluated by ELISA using different commercial specific monoclonal antibodies (Lab Chemicon) against these two peptides. All ELISA data are expressed as absorbance units at 492 nm based on ELISA reaction.

2.7. Western blot assays

The antigenic behavior of CSF tau protein variants was further analyzed by *Western blot* assays in 5 different CSF samples from each of the three groups of patients, and the blot patterns compared with highly purified tau from

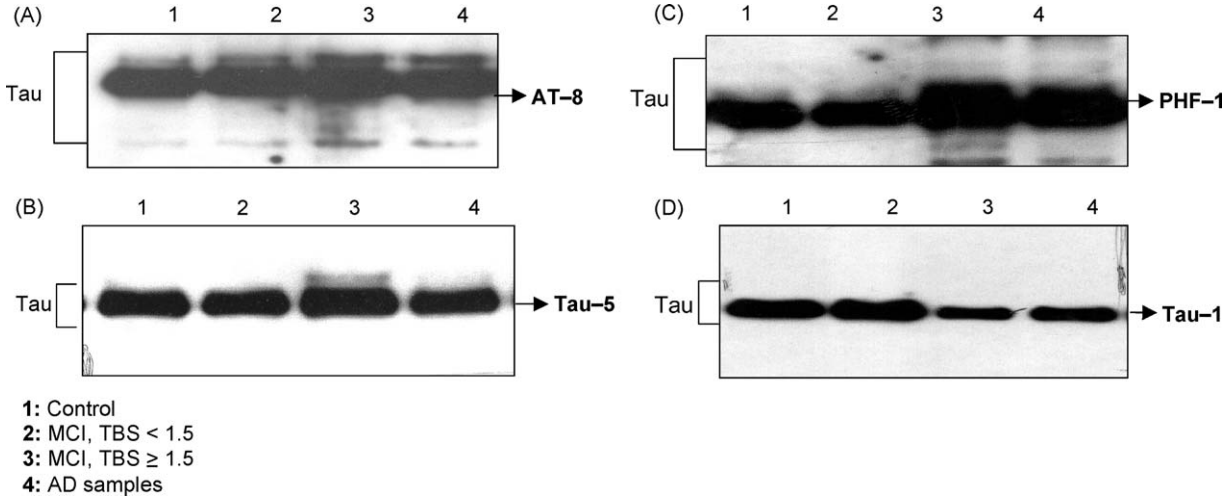

Fig. 1. Western blots from the CSF samples. Blots were developed by using antibodies that recognize hyperphosphorylated AD epitopes on tau isoforms (AT8 and PHF1), total tau (Tau-5) and Tau-1 that recognizes the de-phosphorylated tau. AT8 tag Ser202 and Thr205 epitopes on tau, PHF1 antibody tag Ser396 and Ser404 epitopes, while Tau-1 recognizes Ser202 and Thr205 when they are de-phosphorylated. A: AT8 antibody. B: PHF-1. C: Tau-5 antibody, and D: Tau-1 antibody. The proteins migrating between Mw. 61.5 and 67.0 kDa stained with the respective anti-tau antibodies are denoted by the vertical lines at the left of each panel.

bovine brain or tau present in the paired helical filaments extracted from postmortem human brains. Data on Fig. 1 represents one of these blots, with samples obtained from every group of subjects. Protein in all samples was quantified by the Bradford protein assay, using bovine serum albumin as standard (Bio-Rad), then dissolved in Laemmli SDS-sample buffer in the presence of protease inhibitors: leupeptin (2 μg/mL), pepstatin (2 μg/mL), aprotinin (1 μg/mL), and PMSF (50 μg/mL), heat denatured, reduced by addition of 5% β-mercaptoethanol, and electrophoresed on 10% SDS-PAGE minigels. The electrophoresis-separated proteins were then transferred by electroblotting onto nitrocellulose filters for 1 h at 100 V. After blocking all specific sites on the membrane by incubation with 5% low fat milk, the presence of hyperphosphorylated tau was analyzed by Western blots by current procedures in our laboratory [2], and using the fully characterized Tau-5, Tau-1 AT8 and PHF1 (donated by Dr. P. Davis) as primary antibodies. PHF1 antibody tag epitope around Ser396 and Ser404 in the C-terminal tau domain, and is a marker for AD type phosphorylation. The ELC system (Amersham) was used to detect the proteins in the Western blots nitrocellulose strips. Quantification of blots was carried out by scanning the photographic films of nitrocellulose membranes by using a Kodak digital Science densitometry program. Statistical analysis was performed by the Sigma plots software.

3. Results

By using quantitative ELISA and Western blots we analyzed the molecular patterns of hyperphosphorylated tau and the levels of Aβ (1–42) in the CSF samples of the three groups of subjects. In a first approach, the levels of total tau tagged by the monoclonal antibody Tau-5 indicated no differences between the three groups analyzed. However, the assays with the AT8 antibody that recognizes the AD epitope containing Ser202 and Thr205, when they are anomalously hyperphosphorylated on tau, showed significant differences between the AD group (0.604 ± 0.078) as compared with the control group (0.457 ± 0.086) ($P < 0.05$). According with the absorbance units of ELISA, AT8-reactive tau was 0.12 ng/mL for AD subjects, and 0.09 ng/mL for controls. When the levels of hyperphosphorylated tau of the MCI group (0.510 ± 0.090) were compared with the control group, no significant differences were evidenced (Table 1). The different demographic variables and data on neuropsychological evaluations of the three groups of subjects are described in Table 1. In regard with MMSE examination, significant differences were found between the AD (16 ± 5.72) with controls (29.11 ± 0.83), as well as when AD and MCI were compared ($P < 0.05$)

It was important to evaluate the two subsets of individuals, those with TBS ≥ 1.5 that exhibit greater cognitive impairment, and those with TBS < 1.5 with lesser impairment. In this context, the MCI group with TBS ≥ 1.5 showed hyperphosphorylated tau levels (0.59 ± 0.086, $n = 14$) which were similar to those of AD patients, and significantly higher that the cognitively normal controls, while the MCI group with TBS < 1.5 presented tau levels (0.456 ± 0.032, $n = 31$) similar to controls but significantly lower than those of AD patients (Table 2). In this context, the covariance of age and education were analyzed on the different group. Data indicated that none of these co-variables affected the relationships previously established, and therefore the differences observed for MCI with respect to AD and controls are not due age or education.

Table 1
Demographic and neuropsychological variables, and biological markers in CSF of the groups of patients: AD, MCI and Controls[a]

Determinations	Controls CDR: 0.0	MCI CDR: 0.5	Alzheimer CDR ≥ 1.0
No of subjects (*n*)	25	45	23
Age (years)	67.94 (6.94)	69.24 (5.78)	71.41 (6.68)
Education (years)	12.11 (3.23)	13.32 (3.63)	9.76 (3.29)
MMSE	29.11 (0.83)[b]	27.97 (1.95)[d]	16.06 (5.72)[c,d]
Delayed Word Recall	7.44 (1.34)[b,c]	5.30 (2.05)[c,d]	0.69 (1.32)[c,d]
Boston Naming	14.40 (0.80)	13.50 (1.4)	9.40 (2.80)[c,d]
Verbal Fluency	14.90 (3.6)	12.30 (4.5)	6.73 (4.60)[c,d]
Constructional Praxis	9.33 (1.64)[b]	8.78 (1.60)[d]	5.24 (2.25)[c,d]
AT-8	0.457 (0.086)[b]	0.510 (0.090)[d]	0.604 (0.078)[c,d]
Aβ(1-42)	0.070 (0.010)[b,c]	0.060 (0.007)[c]	0.052 (0.005)[b]
Tau-5	0.404 (0.040)	0.390 (0.065)	0.390 (0.070)
Aβ(1-40)	0.060 (0.010)	0.068 (0.010)	0.076 (0.032)

Data are expressed as means ± S.D. (in parentheses). *Abbreviations:* MCI: mild cognitive impairment; MMSE: Mini-Mental State Examinations; CDR Clinical Dementia Rating.

[a] A population of 93 patients (25 control, 45 MCI and 23 AD) were included in the present study. Information on age of subjects, their educational level, performance in the interview and neuropsychological examinations is provided. CSF samples were obtained as indicated in Section 2 and analyzed by ELISA for tau protein reactive with AT8 and Tau-5, and Aβ (1–42) and Aβ (1–40) reactive with the respective antibodies. ELISA data are expressed as a fraction of one absorbance unit. As reference, each absorbance unit corresponds statistically to a concentration around 0.2 ng/mL for AT8-reactive tau and 5 ng/mL for Aβ (1–42).

[b] Significant differences between AD and Control subjects ($P < 0.05$).

[c] Significant differences between MCI and non-demented neurological controls ($P < 0.05$).

[d] Significant differences between MCI and AD subjects ($P < 0.05$).

Therefore, it was important to analyze the tau molecular species detected in the CSF, and for this purpose the immunoreactivity of CSF tau was assessed by Western blots. The hyperphosphorylated variants of tau were significantly increased in CSF samples of the AD group as compared with those of cognitively relatively normal controls, when stained with the AT8 or PHF1 antibodies that recognize anomalously hyperphosphorylated epitopes on tau (Fig. 1). In addition, a higher level of molecular heterogeneity was detected in the CSF samples of AD subjects as compared with controls.

Table 2
Demographic and neuropsychological variables, and biological markers in CSF of the MCI subgroups of patients with TBS < 1.5 and TBS ≥ 1.5[a]

Determinations	TBS < 1.5	MCI TBS ≥ 1.5
Numbers of subjects (*n*)	31	14
Age (years)	66.56 (4.37)[b]	72.58 (5.94)[c]
Education (years)	13.56 (4.08)[b]	12.89 (3.25)[c]
MMSE	28.56 (1.72)[b]	26.89 (2.56)[d,e]
Delayed Word Recall	5.94 (1.66)[b,c]	4.21 (2.23)[c,d,e]
Boston Naming	13.50 (1.4)[b]	13.50 (1.40)[e]
Verbal Fluency	12.30 (4.5)[b,c]	11.40 (4.70)[c,d,e]
Constructional Praxis	9.11 (1.45)[c]	8.37 (1.67)[e]
AT-8	0.456 (0.032)[b]	0.590 (0.086)[d]
Aβ (1–42)	0.066 (0.007)[b]	0.053 (0.005)[d]
Tau-5	0.401 (0.040)	0.390 (0.060)
Aβ (1–40)	0.062 (0.010)	0.060 (0.020)

Data are expressed as means ± S.D. (in parentheses). *Abbreviations:* MCI: mild cognitive impairment; MMSE: Mini-Mental State Examinations; CDR Clinical Dementia Rating; TBS: Total box score. Evaluation criteria: TBS < 1.5: relative lower cognitive impairment. TBS ≥ 1.5: higher cognitive impairment.

[a] The data on the population of 45 MCI patients included in the present study was divided into two subgroups (31 with TBS < 1.5 and 14 with TBS ≥ 1.5). Information on the age of subjects, their educational level, performance in the interview and neuropsychological examinations is provided. CSF samples were obtained and analyzed by ELISA for tau protein reactive with AT8 or Tau-5, and Aβ fragments with their respective antibodies.

[b] Significant differences between MCI with TBS < 1.5 and AD ($P < 0.05$).

[c] Significant differences between MCI with TBS < 1.5 and MCI TBS ≥ 1.5 ($P < 0.05$).

[d] Significant differences between MCI with TBS ≥ 1.5 and Controls ($P < 0.05$).

[e] Significant differences between MCI with TBS ≥ 1.5 and AD ($P < 0.05$).

As a reference, and based on the evidence that AD subjects display significantly lower levels of Aβ (1–42) peptide variant of the amyloid, we analyzed the levels of the soluble peptide in the CSF of the three groups of patients. The levels of Aβ (1–42) showed differences between the control group as compared with AD and MCI. As a matter of fact, the levels of Aβ (1–42) were significantly lower in the subjects from the AD group (0.052 ± 0.005) than controls (0.070 ± 0.010). For the statistical analysis, the significance level in accordance with the null hypothesis was $P \leq 0.05$. The comparison of the MCI group (0.060 ± 0.007) with the control group did not show statistically significant differences (see Table 1). When the MCI group of patients, of greater interest in this study, was divided into two subpopulations according to TBS criteria, data showed that the MCI group with TBS ≥ 1.5 exhibited Aβ (1–42) levels (0.053 ± 0.005) almost identical to those of the AD patients, whereas the MCI group with TBS < 1.5 exhibited Aβ (1–42) levels (0.066 ± 0.007) similar to those of controls (Table 2). Studies to quantify the levels of Aβ (1–40) did not show any differences among the three different groups of patients indicating that this peptide cannot be considered a candidate for a selective biomarker.

The battery of antibodies against hyperphosphorylated forms of tau, and particularly the analysis of the subsets of an important population of MCI patients provide interesting new information toward an evaluation of tau patterns as potential biomarkers for the degree of cognitive impairment.

On the other hand, data on the amyloid confirm previous findings based on studies carried out with different amyloid fragments.

4. Discussion

These studies complementing the molecular and neuropsychological analysis open an avenue for research on potential biomarkers, especially with the purpose of optimizing early diagnostic tools for AD, and the groups at risk of a neurodegenerative disease [5,7,10,12,16,17,20,32,39]. Therefore, research on biological markers in general is of interest for therapeutic approaches, but also for the design of new drugs for AD. Previous studies have used ELISA for tau determinations in normal and AD samples of CSF [22,23,39,42], and found correlation with neuropathological observations [15,40]. In this context, the present analysis on the changes in anomalously phosphorylated tau provides information on the nature of post-translational modifications of tau species [28] released to the CSF, as well as on the subtle alterations on AD tau epitopes in the three groups of patients, namely those with AD, MCI and the normal controls.

The MCI population includes a fraction of subjects that can progress into AD, while others exhibit TBS values from CDR lower than 1.5, that imply that a minor cognitive impairment [36] may have statistically occurred. In agreement with clinical evaluations, significantly higher levels of hyperphosphorylated tau in CSF of the MCI patients with a TBS > 1.5 as compared with normal controls was found. However, tau levels were similar to controls in the case of the MCI subset with TBS < 1.5. It is worth mentioning on the possible limitation of the sample size in the MCI subgroups with higher and lower impairment. On the other hand, consistent with these data, the levels of anomalously phosphorylated tau were significantly higher in the CSF of AD patients as compared with controls (Table 1). CSF levels of tau and Aβ have been studied in the pre-clinical phase. At autopsy, subjects with MCI show a broad spectrum of morphological brain changes including typical AD features [37]. Thus, MCI probably may represent a pre-dementia stage of AD. Moreover, high CSF phosphorylated tau and low Aβ (1–42) was found of value for MCI subjects that later progressed to AD [4,5].

Studies on the usefulness of tau as a potential marker requires integrated cell biological, molecular and genetic studies on tau alterations and their relationships with the clinical features of the different groups of subjects [5,10]. It is noteworthy that phosphorylated tau is not significantly different in the MCI group with TBS < 1.5 respect to controls, which may be a consequence that at early stages of neurodegeneration, the levels of hyperphosphorylated tau varies slowly due to the possible counteracting effects of protein phosphatases [46]. The observation that the sub-group of MCI with less cognitive impairment (TBS < 1.5) exhibits tau patterns similar to those of normal controls, find additional support from studies on hippocampal cells in primary cultures that clearly distinguish early and late phases in the sequence of events during neurodegeneration. In early stages of the neurodegenerative process, a dephosphorylation of tau on the typical AD epitopes has been found after exposing cells to oxidative agents, due to changes in the ratios of enzymatic activities of protein phosphatases and protein kinases involved in tau phosphorylations in AD [46], demonstrating that both the Aβ fragments and oxidative stress operate through an activation of the cdk5/p35 signaling pathway [2,45,46]. At early stages of the process, an activation of PP1 phosphatase occurs as a consequence of the blockage of inhibitor-2 by its phosphorylation by cdk5 [46]. Therefore, hyperphosphorylations may occur at a more advanced stage, which could be the case of MCI with TBS ≥ 1.5. The increased AT8 staining of tau species in AD and MCI group of higher cognitive impairment was corroborated with PHF1 antibody that tag C-terminal epitopes on tau, different than those of AT8. The higher heterogeneity in the AD and MCI samples as compared with controls, may be a result of tau truncation by proteases such as caspase-3 found in AD brains [1].

Studies on tau modifications in the CSF were complemented with the analysis of changes in Aβ (1–42) in the CSF of the three groups of patients, while Aβ (1–40) remained stable. Actually, our data corroborated a series of studies [4,12,15,25] that indicate a decrease in the CSF of the Aβ fragments with a higher self-aggregation capacity such as Aβ (1–42) in AD subjects. This can be explained considering that an increase in these two fragments results in a significantly higher capacity to generate senile plaques, and therefore most of Aβ (1–42) pools are utilized in SP thus decreasing its content in the CSF. The studies with Aβ (1–40) antibody did not show significant differences between AD, MCI and controls, observation that can be explained considering the lower capacity of this fragment to generate SP. However, these observations should be evaluated with caution considering observations reported in other studies [25]. Interestingly, when the MCI subgroup was divided into two subsets defined by TBS lower or higher than 1.5, the levels of Aβ (1–42) in the CSF of subjects with TBS ≥ 1.5 were similar to those of AD patients, and therefore significantly lower than controls. However, when the subset of MCI with TBS < 1.5 was analyzed, the data showed Aβ (1–42) levels similar to normal controls. In sum, all the different measurements in the CSF of the three groups of patients has led to findings that contribute to clarify the involvement of tau and Aβ peptides in the etiopathogenesis of AD. Most interestingly, these studies provide important clues on the correlation between the levels of hyperphosphorylated tau and Aβ (1–42) in the CSF with the degree of cognitive impairment of different groups of patients. Observations that anomalously hyperphosphorylated tau levels of MCI with TBS < 1.5 are similar to controls, and those cases of MCI with TBS > 1.5 are similar to the AD population, are consistent with the idea that a gradual cognitive impairment correlates with an increase in the hyperphoshorylated tau at advanced stages of the neurodegenerative process. In addition, levels of tau in

the CSF correlates with neurofibrillary tangles in AD [40]. These studies find support in our previous investigations using hippocampal cells in primary culture as a cell model and with the transgenic animal model with the human Swedish mutation Tg2576, that favor the hypothesis on tau pathology in AD [34,35], and that early and late events in the neurodegenerative process are indicated by differential changes in tau protein post-translational modification patterns.

Considering that this report is part of a longitudinal study that requires a follow-up of the selected groups of patients, the continuation of this research and later evaluation of behavioral and neuropsychological and clinical features will be of importance for this research. Since neuropathology is the final confirmation of AD and the nature of a dementia, it is expected that post-mortem neuropathological studies [15] on the brains of subjects in the study will supply further evidence for this analysis. Studies could also provide clues toward therapeutic approaches based on the idea that preventing protein aggregates could help in controlling neurodegenrative disorders such as AD [27].

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