HYPERPHOSPHORYLATED TAU PROTEIN IN THE CEREBROSPINAL FLUID OF PATIENTS WITH ALZHEIMER’S DISEASE AND OTHER DEMENTIAS

Preliminary findings

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ABSTRACT - Alzheimer’s disease (AD) is pathologically characterized by the accumulation of amyloid plaques and tau-associated neurofibrillary tangles in the cerebral tissue. The search for antemortem biomarkers is intense including analysis of cerebrospinal fluid (CSF) β-amyloid and tau proteins concentrations seeking for an accurate and early diagnosis. Levels of hyperphosphorylated tau at threonine 181 were measured in the CSF of 34 patients with AD (19 with senile AD – SAD and eight with presenile AD – PSAD) and seven with other dementias (OD). The levels of CSF phosphotau were significantly higher in the AD patients compared to OD (AUC 0.812), with no association with severity of dementia, age of onset, duration of the disease or scores in the Mini-Mental State Examination. There were no differences of phosphotau levels between SAD and PSAD patients. These findings corroborate some previous studies and indicate that CSF phosphotau may help to differentiate AD from other dementias.

KEY WORDS: Alzheimer’s disease, differential diagnosis, cerebrospinal fluid, tau protein.

Alzheimer’s disease (AD) is the main cause of dementia in Western countries, being responsible for more than 50% of the cases1. The clinical diagnosis of AD is characterized by an exclusionary process. The definite diagnosis is possible only on neuropathological examination, by the observation of the senile plaques and the neurofibrillary tangles (NFTs) in the cerebral tissue2. The NFTs are intraneuronal cytoplasmic structures composed by paired helical filaments (PHFs) of hyperphosphorylated form of the microtubule-associated protein tau. In AD, these NFTs are localized preferentially in pyramidal cells of the hippocampus and entorhinal cortex, in supragranular (II and III) and infragranular layers.
nular (V and VI) layers of associative cortical areas. Subcortical areas are also affected, such as the nucleus basalis of Meynert, amygdala, locus ceruleus and dorsal raphe nuclei.

The well-established relationship between the density of NFTs and the severity of the dementia in AD has lead to many studies about protein tau levels, one of the main components of NFTs, in cerebrospinal fluid (CSF) of AD patients. Tau protein is a microtubule-associated protein (MAP) found basically in neurons and mainly localized in axons where it confers stability to microtubule components of the cytoskeleton. The protein is codified by a gene on chromosome 17 and mutations are associated to certain forms of frontotemporal dementia, especially frontotemporal dementia and parkinsonism related to chromosome 17. Microtubule stability depends on phosphorylation of tau protein. In mature CNS low phosphorylated forms of tau protein predominate, maintaining adequate neuronal homeostasis. PHF-tau concentrations has been shown to be elevated in the cortex of AD patients while normal tau concentration is decreased. Considering early and accurate diagnosis and differential diagnosis with other dementias that mimic AD symptoms, the development of a biological marker is of great value. A biological marker has a minimum of five functions: a) diagnostic confirmation; b) screening; c) predictive testing; d) monitoring disease progression and treatment; and e) analysis of the relation between brain-behavior.

In this way, an ideal biomarker for AD should be: able to detect a characteristic pathological finding of AD; validated in AD pathologically confirmed cases; precise (for differential diagnosis); trustable; not invasive; simple, reproductive; and not expensive.

Besides, it should have sensitivity and specificity above 80% and a positive predictive value above than 90%. The combination of biomarkers improves the diagnostic accuracy when compared to a single marker, thus increasing the sensitivity and specificity of the tests. Considering these factors, recent studies have been dedicated to investigate the abnormal proteins found in the CSF of AD patients. These studies have evaluated the levels of MAP-tau, primary component of the neurofibrillary tangles, and the Aβ42 form of the β-amyloid protein, the main component of the senile plaques found in the cerebral parenchyma.

Many groups have shown an increase in tau levels and a decrease in Aβ42 levels in the CSF of AD patients when compared to non-demented elderly controls. The more recent longitudinal studies use the combination of high tau and low Aβ42 to correlate their levels to the stage of the disease. Since amyloid deposition is not exclusive of AD brains, occurring in normal aging and also in other neurological diseases, additional investigation have shown a decrease in Aβ CSF levels in other conditions such as Creutzfeld-Jakob disease (CJD), some cases of frontotemporal dementia (FTD) and vascular dementia (VD).

In 1993, Vandermeer et al. developed an immunoassay able to detect tau protein in CSF and subsequent studies concluded that its levels were significantly higher in AD patients when compared to other neurological diseases and normal controls even in the early stages of the disease. As tau protein is present in blood in a very low concentration, (under the detection limit of the immunoassay), the high levels in CSF do not reflect an alteration in blood brain barrier. As elevated tau levels were also found in other neurological diseases, it was noticed that these immunoassays were measuring total tau, i.e., normal and abnormal tau protein. To solve this overlap, a group of investigators developed a method able to detect hyperphosphorylated tau (phosphotau) and obtained elevated levels when comparing AD patients to controls. Other studies reproduced these results, suggesting that phosphotau is a more specific biomarker than total tau for AD diagnosis. The abnormal phosphorylation of tau protein is an early event in AD pathophysiology and is restricted to cerebral regions affected by the disease. This hyperphosphorylation is the primary and the most critical event in PHFs and NFTs processing. More than 21 sites of abnormal tau protein phosphorylation are known. As CSF tau concentrations are low, phosphotau is only identified by highly sensitive immunoassays using phospho-specific antibodies.

Several research groups standardized synthetic phosphopeptides in order to access the proline rich region of tau protein where the phosphosites are localized. Until now, different immunoassays were developed directed to different phosphosites as serine 199, threonine 231, serine 396/404, threonine 231/serine 235 and threonine 181. Vanmechelen et al. developed an ELISA assay specific for the phosphorylation site proline-directed to Thr 181. This site has been chosen because it is a relatively isolated site in the proline rich region; 2) it is phosphorylated preferentially by kinases proline-directed; and 3) the synthetic peptide utilized for the standardization is small. The
design of this peptide was based on detailed mapping of phosphotau and its antibodies, recognizing all tau isoforms. The objectives of the present study were to compare CSF phosphotau levels between senile (SAD) and presenile (PSAD) AD groups, between AD patients and other dementias (OD), between AD patients and controls from the literature\textsuperscript{11}, and, within the AD group, to correlate phosphotau levels and severity of dementia, mini mental state examination (MMSE) scores and duration of the disease.

**METHOD**

A total number of 34 individuals were included in the study. Their main demographic and clinical characteristics are depicted in Table 1.

The subjects evaluated were consecutively selected from the population of patients followed at the Behavioral and Cognitive Neurology Unit of the Hospital das Clínicas from the University of São Paulo School of Medicine (HCFMUSP).

All patients were submitted to a diagnostic workup investigation, including clinical history, physical and neurological examination, appropriate blood tests (to exclude other causes of dementia), CT and/or brain MRI and other complementary exams if necessary.

In every case, the clinical diagnosis was made before CSF examination. No patient was on treatment with cholinesterase inhibitors or was participating in any protocol of new drugs before the lumbar puncture.

The diagnosis of probable AD was based on the NINCDS-ADRDA criteria\textsuperscript{25}. For the AD group according to DSM-III-R criteria, the severity of dementia was classified as mild, moderate or severe. The diagnosis of frontotemporal dementia (FTD) was made according to the Lund/Manchester criteria\textsuperscript{26}. Vascular dementia (VD) patients were selected according to probable VD criteria of NINDS-AIREN\textsuperscript{27} and dementia with Lewy bodies according to McKeith et al. criteria\textsuperscript{28}. Criteria used to diagnosis of primary progressive aphasia (PPA) were those defined by Mesulam\textsuperscript{29}.

Patients were divided in three groups: SAD (senile AD), PSAD (presenile AD) and OD (other dementias), with the latter including one patient with the diagnosis of PPA, two cases of FTD, one with VD, one with corticobasal degeneration (CBD) and two with dementia with Lewy bodies (DLB).

CSF phosphotau analysis was performed using the kit INNOTEST\textsuperscript{TM} PHOSPHO-TAU (181P) (Innogenetics, Ghent, Belgium). The phosphotau levels from each of the patients’ groups were compared to a subset of controls (composed of 32 individuals) extracted from a previously published study.\textsuperscript{11} These individuals were aged 63 ± 9 years and had no history, symptoms or signs of psychiatric or neurological disease, malignant or systemic disorders. The mean MMSE score in this group was 28.3 ± 2.7.

**CSF analysis** - All CSF samples were obtained by lumbar puncture. An approximate volume of 12 ml was collected in polypropylene tubes and submitted to routine analysis (cytology, biochemistry and protein electrophoresis, as well as immunology for syphilis, cysticercosis and ADA measurement), always within a six-hour interval after the collection procedure. Samples with more than 500 erythrocytes/mm\textsuperscript{3} were not included. After routine analysis and centrifugation of the material at 1500 rpm for 10 minutes, the samples destined to phosphotau measurement were identified and stored at - 70°C freezer in the CSF laboratory.

An enzymatic immunoassay in solid phase for the quantitative determination of phosphotau in human CSF standardized in the kit INNOTEST\textsuperscript{TM} PHOSPHO-TAU (181P) (Innogenetics, Ghent, Belgium) was the method used for this evaluation.

The study was approved by the Ethics Committee of the HCFMUSP and all participants signed a written informed consent.

**Statistical analysis** - The comparison between phosphotau levels in AD groups and controls from literature was made by the Student’s t test. The comparison between AD and OD groups was made by the Mann-Whitney test. In the AD group, the correlation between phosphotau levels and severity of dementia, MMSE scores and duration of the disease was made by the Spearman’s rank correlation test.

**RESULTS**

Due to difficulties in obtaining CSF samples from normal controls, we have been unable to

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>Gender (M: F)</th>
<th>Age (years) mean</th>
<th>Schooling (years) mean</th>
<th>Duration disease (years) mean</th>
<th>MMSE scores mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>S AD</td>
<td>19</td>
<td>08: 11</td>
<td>75.5</td>
<td>5.7</td>
<td>5.0</td>
<td>16.5</td>
</tr>
<tr>
<td>PS AD</td>
<td>08</td>
<td>1: 7</td>
<td>62.5</td>
<td>7.5</td>
<td>4.0</td>
<td>16.5</td>
</tr>
<tr>
<td>OD</td>
<td>07</td>
<td>6: 1</td>
<td>67</td>
<td>8.9</td>
<td>2.1</td>
<td>12.5</td>
</tr>
</tbody>
</table>

N, number of patients; M, male; F, female; MMSE, Mini-Mental Status Examination; S AD, senile Alzheimer’s disease; PS AD, presenile Alzheimer’s disease; OD, other dementias.
establish sensitivity and specificity of the method
as we could not define cut-off values for the analysis
of the results obtained in this study. We tried
to overcome this limitation by comparing our re-
sults of phosphotau levels with controls described
in the literature\textsuperscript{11}.

The statistical analysis showed no significant dif-
ference between SAD, PSAD and OD groups accord-
ing to duration of the disease (p=0.644), education
(p=0.830), severity of dementia (p=0.481) and
MMSE scores (p=0.651). As expected, there was a
significant age difference between SAD and PSAD
groups (p=0.000). Significant differences were also
observed between the AD and OD groups for age
(p=0.013), which was lower in the latter, and gen-
der (p=0.035) with women predominating in the
AD group and men in OD.

Using the mean of phosphotau levels of con-
trol individuals obtained from a previous study
(32.8 pg/ml)\textsuperscript{11}, we obtained a significant differ-
ce (p=0.013) between AD patients and controls.
The mean phosphotau concentration in the AD
group was 50.4 pg/ml. No differences were obser-
vied between phosphotau levels of SAD and PSAD
groups (p=0.549) as it is depicted in Table 2.

There was no significant correlation, in the AD
group, between levels of phosphotau and severi-
ty of dementia (r=−0.082), duration of disease
(r=0.015) and MMSE scores (r=−0.020).

A significant difference was found between
phosphotau levels of AD and OD groups (p= 0.023).
Plotting the values of phosphotau levels from the
AD and OD groups in a Receiving Operator
Characteristics (ROC) curve we found that phosho-
tau levels differentiated AD from OD patients,
what was confirmed with an area under the curve
(AUC) of 0.812 (Fig 1).

DISCUSSION

In the present study CSF levels of phosphotau
were increased in AD patients when compared to
normal controls from the literature and to patients
with OD, a finding that is similar to previous re-
ports\textsuperscript{11,20,21,23,24}. However, It is important to reinfor-
ce that phosphotau levels in a normal range do not
exclude AD. The absence of a correlation between
phosphotau levels and severity of dementia sug-
gests that this elevation is an early event in AD pa-
thogenesis. Indeed, some studies demonstrated
the clinical value of this biomarker in the early
stages of AD\textsuperscript{11,30}.

Two patients with other dementias in the pres-
ent study presented high levels of phosphotau
(one case with FTD and one case with DLB).
Although it is not possible to exclude the hypoth-
esis of diagnostic errors, the long follow-up of all
these patients, before and after the CSF analysis,
is a feature that certainly increases the diagnostic
confidence within this sample.

The utilization of additional biomarkers, such
as the combination A\textsubscript{β}1-42 protein with phospho-
tau, and its correlation with the clinical presenta-
tion, certainly would have given a higher diagno-
tic sensitivity and specificity. This fact was recent-
ly confirmed in a meta-analysis that included all
the studies that evaluated the combination of bio-
markers in an expressive number of patients and
using an adequate diagnostic method of the cause
of dementia\textsuperscript{14}. Unfortunately, it has not been pos-
sible to verify the diagnostic values of this combina-
tion in the present study.

<table>
<thead>
<tr>
<th>Table 2. Mean (± SD) levels of phosphotau.</th>
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<tr>
<td>Levels of CSF phosphotau (pg/ml)</td>
</tr>
<tr>
<td>------------------------------------------</td>
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<tr>
<td>S AD 49.78 (± 36.09)</td>
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<tr>
<td>PS AD 44.06 (± 33.29)</td>
</tr>
<tr>
<td>OD 15.01 (± 27.05)</td>
</tr>
</tbody>
</table>

S AD, senile Alzheimer’s disease; PS AD, presenile Alzheimer’s disease; OD, other dementias.

Fig 1. Values of phosphotau levels from AD and OD groups.
Additional studies are necessary to establish a methodological standardization of CSF immunoassays between the research centers and to observe if they represent a very high specificity for AD diagnosis, mainly in relation to forms of dementia that present an important overlap with AD either on clinical and on pathological examination (such as DLB and VD). Moreover, the conflicting findings from the literature, correlating CSF biomarkers with clinical severity measures of dementia, suggest the need of larger samples to establish a confident statistical significance. The reason for this variability is probably due to the fact that a single marker is not able to reflect precisely the central pathological process of each stage of the disease.

Maybe a potential utility of such biomarkers is in the follow-up of individuals at risk of developing AD in prospective studies. However, much work is still necessary to standardize assay methods before the determination of the prognostic value attributable to these biomarkers. With the definition of well standardized values, in large populations, it is possible that gradual alterations in these levels can be interpreted as suggestive evidences of incipient AD. To test this hypothesis, longitudinal studies including large samples of elderly individuals are necessary.

In conclusion, the present study found that CSF levels of phosphotau analysis is a good biomarker for AD and is able to help in differentiating AD from other dementias, independently of the age of onset, severity of dementia or MMSE scores.

REFERENCES