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The Alz-tau Biomarker for Alzheimer's Disease: Study in a Caucasian Population

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Abstract. The establishment of a molecular biomarker for early detection of Alzheimer's disease (AD) is critical for diagnosis and follow up of patients, and as a quantitative parameter in the evaluation of potential new drugs to control AD. A list of blood biomarkers has been reported but none has been validated for the Alzheimer's clinic. The changes in hyperphosphorylated tau and amyloid peptide in the cerebrospinal fluid is currently used as a tool in the clinics and for research purposes, but this method is highly invasive. Recently, we reported a non-invasive and reliable blood biomarker that correlates the increase in the ratio of heavy tau (HMWtau) and the low molecular weight tau (LMWtau) in human platelets and the decrease in the brain volume as measured by structural MRI. This molecular marker has been named Alz-tau®. Beyond the clinical trials developed with a Latin American population, the present study focuses on an evaluation of this biomarker in a Caucasian population. We examined 36 AD patients and 15 cognitively normal subjects recruited in Barcelona, Spain. Tau levels in platelets were determined by immunoreactivity and the cognitive status by using GDS and MMSE neuropsychological tests. The HMW/LMW tau ratio was statistically different between controls and AD patients. A high correlation was found between the increase in MMSE scores and HMW/LMW tau ratio. This study showed that this ratio is significantly higher in AD patients than controls. Moreover, this study on a peripheral marker of AD is valuable to understanding the AD pathogenesis.

Keywords: Alzheimer's disease, clinical study, early detection of the disease, HMWtau/ LMWtau ratio, molecular biomarkers, tau protein

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive impairment and behavioral disorders of patients, affecting around 12% of people older than 65 [1]. The World Health Organization estimated that over 48.6 million all over the world suffer from this disease. Currently, AD is the main cause of dementia in the elderly (accounts for a 60-70% of cases) [2], converting this pathology in a serious public health crisis derived from dementia at a global level.

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AD is a multifactorial disease, that not only involves the misfolded amyloid-β peptide (Aβ), but also tau oligomerization [3, 4], leading to the formation of both extracellular deposits of Aβ in plaques, and intracellular paired helical filaments (PHF) and neurofibrillary tangles (NFTs), respectively. It has been demonstrated that changes in the neuroimmunomodulation patterns [5, 6] trigger microglial and astroglial activation, neuronal dysfunction, and finally neuronal death [7–10]. Tau aggregates are the principal component of NFTs and are mainly formed as a result of its hyperphosphorylation by kinases CDK5 and GSK3β [11].

Interestingly, PHF and NFT deposition causes the loss of synaptic function and progressive neuronal

death [12]. As a result of neurodegeneration, tau aggregates are released to the extracellular domain, and evidence exists of their progressive neurotoxicity [13]. Thus, tau oligomers and PHF-tau appear to be neurotoxic agents, while the internal mechanisms associated to neurotoxicity as well as the role associated to neurodegenerative process have been part of an in-depth study [14–17]. In addition, we must consider that tau filaments and misfolded Aβ can stimulate the neuroinflammatory cascade [18, 19]. The latter can be detected by the central nervous system as a "danger signal", thus exacerbating the inflammatory response [5]. In this context, the chronic activation of the innate immune system triggers an anomalous cascade of molecular signals that eventually leads to neuronal degeneration.

One of the major drawbacks regarding AD, is that currently there are no effective biomarkers that can aid to diagnose preclinical AD or even at the clinical phase. Potential biomarkers for AD have been reported, but these candidates are expensive and/or highly invasive [20]. The latter includes putative disease markers obtained by magnetic resonance imaging (MRI) [21], positron emission tomography (PET) [22–24], and those that requires the collection of cerebrospinal fluid (CSF) [21, 25, 26] via lumbar puncture. While all the latter made a considerable progress demonstrating how these biomarkers relate to the pathophysiology of AD, their invasiveness prevent the routine use in the follow up of patients and also for the search of new drugs. In this context, a growing set of potential blood-based biomarkers have been reported, and there is a hope that those can support the preclinical diagnosis of AD, as well as to predict disease progression. Aβ peptide is a widely studied plasma biomarker for AD, although the extent to which blood (plasma, serum, cell-bound, or free) Aβ levels accurately reflect the presence or state of AD is not clear. A meta-analysis by Koyama et al. [27] of thirteen studies and 10,303 subjects examining plasma $A\beta_{1\text{-}42}$ and the ratio of $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ as predictors of dementia and AD, has concluded that a decrease in the plasma $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$ ratio is a statistically significant and clinically meaningful predictor of subsequent cognitive decline. However, significant heterogeneity underlines the need for further investigation of plasma Aβ levels as a preclinical biomarker.

Tau protein has become a target in order to obtain a successful novel biomarker for AD [28]. Indeed, CSF tau markers were successfully correlated with the hippocampal volume on AD [29]. Nonetheless, in an effort to obtain a non-invasive biomarker, tau protein was detected on patients' platelets by immuno-western blot [30] and showed different patterns on AD patients compared to controls. The reason for using platelets stems from the fact that these cells provide well-defined protein patterns with the anti-tau antibodies, with low levels of contamination. The use of serum material, however, presents the problem of the need to eliminate the high content of serum albumin and other proteins from the samples. We have information from the studies of Padovani et al. [31,32] on the presence of amyloid. Thus, considering that several brain proteins are expressed in platelets, this appear to be an appropriate system for these studies. Moreover, tau markers correlated better with the clinical observations in patients with AD. Thus, platelets are a major peripheral reservoir of the amyloid-β protein precursor, so they have been considered as a potential biological marker of AD, and tau is also present in them. Also, platelet tau correlated with brain atrophy in AD patients [31]. The difference in the detection of high-molecular weight (HMW) and low-molecular weight (LMW) isoforms could provide an accurate plasma biomarker for preclinical AD [32], as well as a disease progression predictor tool. It has been demonstrated its accuracy, as it correlates with the Clinical Dementia Rating (CDR) value, in a regional study performed with a Chilean population in Santiago [31]. This biomarker showed a sensitivity of a 75.7% and a specificity of a 73.7% [33], which is quite acceptable for a novel technique. No correlation was found with age, educational level, and other demographic parameters [31].

In this work, we present novel evidence based on a Caucasian population, consistent with the previously reported data obtained in a Latin American cohort of patients, to support Alz-tau® as a reliable biomarker and an early predictor for AD.

METHODS

Subjects

The subjects for the study were recruited on the basis of the purchase of medical services from Ace Foundation, Barcelona, Spain. For the purpose of this study, we examined 36 probable AD subjects (Mini-Mental Status Exam (MMSE) score < 28 and Global Deterioration Scale (GDS) >2) and 15 cognitive healthy subjects (MMSE score > 28 and GDS < 2). The range of patients age in both groups of

Table 1
Demographic and clinical features of the groups of Alzheimer's disease and healthy subjects.

	Alzheimer's disease patients	Healthy subjects
Total subjects	36	15
Men	7	6
Women	29	9
GDS	3.97 ± 0.77	1.53 ± 0.52
CDR	1.11 ± 0.57	0.07 ± 0.18
MMSE	21.69 ± 3.90	29.47 ± 0.74
Age (mean)	74.92 ± 10.89 (52-91)	65.87 ± 6.63 (55-78)
Ratio HMW/LMWtau	1.445 ± 0.439	1.098 ± 0.349

the study was between 52 to 91 years old (for more details see Table 1). It should be noted that for the present study, the presence of familial AD was ruled out, since the group of patients incorporated corresponded to the group of sporadic AD patients followed during years even before we initiated the study. In this context, no familial AD patients were followed in that way. The procedures were approved by the Committee on Ethical Issues of the Hospital Salvador (Santiago, Chile), the established Medical Ethics Committee of the International Center for Biomedicine and the Medical Ethics Committee of the Ace Foundation, and all of the participants and/or their legal guardian signed informed consents.

Platelet tau analyses

6 mL of venous blood samples were obtained and subjected to differentiated centrifugation to separate the platelets. First, the blood samples were centrifuged at 250 g for 10 min at room temperature (RT), to obtain a platelet-rich plasma. Next, the plasma was centrifuged at 1,750 g for 10 min at RT in order to obtain the isolated platelets. Then, the platelets were carefully resuspended in 200 μL of NH_4Cl 0.83% and incubated for 5 min at RT, in order to eliminate the red cells that are still in suspension. It was then centrifuged at 1,750 g for 10 min at 4°C. The supernatant is discarded, and the precipitated platelets washed with 200 μL of 100 mM EGTA. In the last step, the platelet proteins were extracted by the addition of 85 μL lysis buffer, supplemented with 15 μL of a cocktail of protease inhibitors (Roche). Finally, the proteins were stored at $^{-}$80°C until further study of the tau variants carried out in our laboratory in Santiago, Chile. Before continuing with the experimental procedure, protein concentration was determined through the microBradford method.

Second stage (SDS-PAGE and western blot)

Once the total protein concentration measurements are made, 50 μg of total protein per sample is loaded in 10% polyacrylamide minigels. Once the electrophoresis is finished, the proteins are transferred to a nitrocellulose membrane at 330 mA for 90 min in an appropriate transfer system (BioRad). The membranes were blocked with 5% BSA in 1X TBS for 1 h at RT. As specific primary antibodies, we used Tau 5 (1 : 1000) (Calbiochem), PHF-1 1 : 100 (generous gift from Dr. Peter Davies, Albert Einstein College of Medicine, NY, USA), allowing to incubate in constant agitation overnight at 4°C. The membranes were incubated with the conjugated secondary antibody HRP 1 : 10000 (Pierce) for 1 h at 25°C. Immunoreactive bands were detected using the appropriate the chemiluminescent reagent, on X-ray plates. The films were scanned, and the intensities of the bands quantified using the ImageJ 1.50i software (National Institutes of Health, USA). For the determination of the ratio of HMW and LMW molecular species of platelet tau, the relative intensities of the tau bands were quantified and a ratio of tau of high molecular weight (HMW-tau> 80 kDa) and of low molecular weight (LMW-tau, <80 kDa) was calculated for each sample, both control subjects and AD patients.

Statistical analysis

The levels of the biomarker, age, and cognitive measurements between the different groups were compared using the Student's t-statistic for unrelated samples or by one-way analysis of variance (ANOVA) to compare more than two averages, considering each measurement as the independent variable comparing the groups. The Bonferroni post hoc test was used to evaluate significant differences between averages. The Graph Pad Prism program, version 6.01 for Windows was used for all the analyzes. The level of significance was set at 0.05 to consider the significant differences in the statistical analyzes.

RESULTS

The main demographic and clinical characteristics of the cohort of patients with AD and healthy subjects are presented in Table 1. The typical western blot pattern of both AD and control subject, after stained with tau5 antibody in show in Fig. 1. There were significant statistical differences between

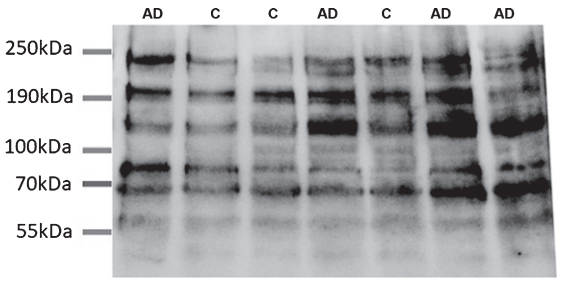

Fig. 1. Representative western blot of platelet tau with tau5 monoclonal antibody. AD patients showed higher immunoreactivity in high molecular weight fragment of tau than healthy patients (C). On the other hand, low molecular weight tau bands have similar immunoreactivity between both study groups (AD and C).

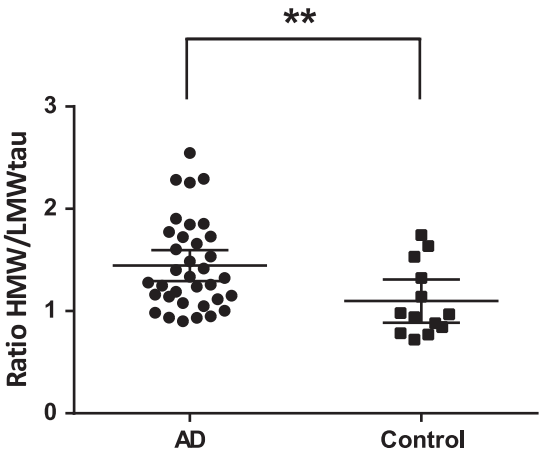

Fig. 2. Significant difference between healthy subjects versus AD patients, with respect to the tau ratio of HMW/LMWtau. The ratio corresponds to arbitrary units of densitometric quantification of tau species, both monomeric and oligomeric, using Tau5 as a primary antibody. Bars: mean with a 95% CI (p = 0.0059).

both study groups, the AD versus healthy controls, with respect to the severity of dementia, measured through CDR (p <0.0001) and also by other tools of general cognitive functions such as MMSE and GDS (both with a p <0.0001) (Fig. 2). As observed in previous studies [33, 34], immunoblots presented an electrophoretic pattern immunoreactive to tau5, with molecular weights ranging from approximately 60 kDa (the expected molecular weight for monomeric tau) to sizes as large as 240 kDa (approximately), the latter presenting a greater reactivity in comparison with cognitively healthy subjects. The ratio of platelet tau was as much for healthy subjects as for AD, according to those described by Neumann et al. [34], with some modifications. When probed with PHF-1 antibodies, no significant differences were found in the HMWtau/LMWtau ratio, when AD samples and controls were compared (not shown). This suggest that tau5 is the appropriated antibody to be used in the biomarker's reagents. In the present study, according to the western blots densitometric analyzes of each subject under study, it was observed that the ratio HMW/LMWtau is significantly higher in the AD group in relation to the healthy subjects (p = 0.0059) (Fig. 2). It should be noted that no significant correlation was found between the age of the subjects included in the study (AD and control subjects) versus the neuropsychological batteries (MMSE, CDR, and GDS). In turn, also no correlation between age and ratio HMW/LMWtau, which is supported by previously obtained data in our laboratory [33].

According to the correlation between the biomarker and the clinical study, the relationship of HMW/LMWtau was correlated with the cognitive deterioration of the subjects under study, through the evaluation of MMSE (r = -0.3454 and p = 0.0162)

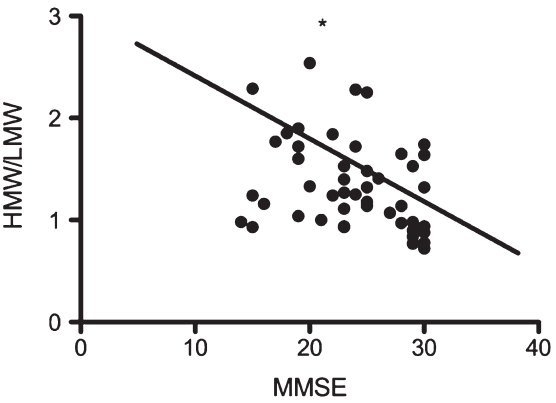

Fig. 3. Variation of the ratio HMW/LMW tau of platelet tau in relation to the cognitive performance of the AD cohort of patients in the study. The cognitive parameters were determined by the Mini-Mental State Examination (MMSE) applied to every patient in the study.

(Fig. 3). However, there is no correlation between HMW/LMWtau with CDR (r = 0.05254, with p > 0.05).

Fig. 4 shows the ROC curve data curve for platelet tau ratio as a biomarker for AD. In this analysis, a cut-off point of 1.146 for HMW-tau/L-tau ratio displayed a sensitivity of 71.43% and a specificity of 69.23% to discriminate between AD and control subjects.

DISCUSSION

We have described an increase in the human platelets HMW tau variant and a concomitant

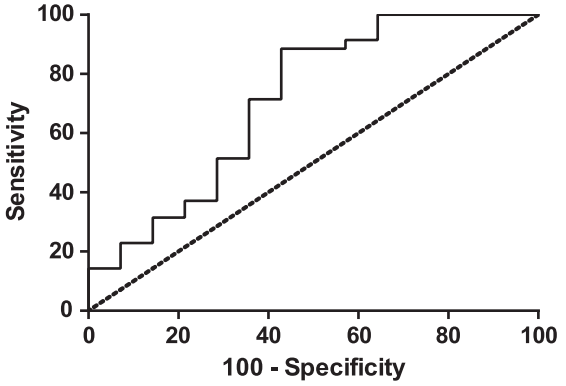

Fig. 4. ROC curve data showing sensitivity and specificity of platelets tau ratio as a potential biomarker for detecting AD. The area under the curve corresponds to 0.7560 with a cut-off point of 1.146; a sensitivity of 71.43% and a specificity of 69.23% (IC 95 : 0.5886 to 0.9235 - $p = 0.0067$).

decrease in the normal LMW tau species in the blood samples of AD patients [33]. The HMW species appear to correspond to oligomeric forms of tau and not to the expression of a different gene as described for "heavy tau" of 130 kDa [34]. According to our methodological approach to this biomarker, tau oligomers can be visualized in Western blots under both native and denaturing conditions, as described for patients with frontotemporal dementia [35]. In turn, the increase in tau oligomers seems to be correlated with the pathophysiology of AD that occurs in the brain, and is also evidenced in peripheral blood cells [26]. As in the previous studies, the HMW/LMWtau relationship in the Caucasian population does not depend on age [33]. Interestingly, previous studies have shown a correlation between the HMW/LMWtau ratio and the brain volume in the mesial temporal lobe region, the cingulate cortex, the pulvinar nucleus, the frontal cortex, and the cerebellum [31].

This study on the tau blood biomarker showed that the HMW/LMW tau ratio was significantly higher in AD subjects as compared to those with subjects with normal cognition as a control. Clearly, the tau ratio in isolated platelets correlated with global measures of cognitive and functional performance in all subjects. These data are in agreement with those obtained in precedent clinical trials for this marker [31, 32, 34]. This is highly relevant considering that all previous studies on Alz-tau® biomarker were carried out in a Chilean population (major Amerindian and minority Caucasian population), and the present findings correspond to a clinical study in a Caucasian cohort of patients and also normal counterparts developed in Barcelona, Spain. Thus, validation of this biomarker technology is extended to a broader population of AD patients, independently of ethnic and sex factors. It is worth pointing out that previous reports on the genetic features of the Chileans population used in the CSF biomarker showed unique characteristics in the distribution of ApoE alleles [36] which are different than the Caucasian population. This was no evidenced in the present study on a blood biomarker. In this context, the Alz-tau® marker appears to be a rather universal marker that can be applied without restrictions to a broad population of patients throughout the world. Moreover, this marker has the advantage that it can be obtained from a simple venous blood sample, and the cost will be significantly lower than that for a PET scan for example. The latter is important as it can give access to a window of patients that did not have access to the diagnostic tools available due to their invasiveness and costs. The combination of high sensitivity, efficiency and replicability of the tau platelet biomarker turn this in a reliable and accurate AD detection tool to add to the different approaches for a diagnosis of this disease.

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Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/18-0637r1).

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