

Presence of Phosphorylated Tau Protein in the Skin of Alzheimer's Disease Patients

Rodríguez-Leyva Ildelfonso^{1,5}, Chi-Ahumada Erika², Calderón-Garcidueñas Ana Laura³, Medina-Mier Verónica¹, Santoyo Martha E², Martel-Gallegos Guadalupe⁴, Zarazúa Sergio⁴, Carrizales Juan² and Jiménez-Capdeville María E^{2*}

¹Department of Neurology, Central Hospital "Ignacio Morones Prieto", San Luis Potosí, Mexico

²Department of Biochemistry, Faculty of Medicine, Universidad Autónoma de San Luis Potosí, Mexico

³Institute of Forensic Medicine, Universidad Veracruzana, Boca del Río, Mexico

⁴Laboratory of Neurotoxicology, Faculty of Chemistry, Universidad Autónoma de San Luis Potosí, Mexico

⁵Graduate program in Applied Sciences, Universidad Autónoma de San Luis Potosí, Mexico

*Corresponding author: Jiménez-Capdeville, ME Faculty of Medicine, University of San Luis Potosí. Av. V. Carranza 2405 Col. Los Filtrós, 78210 San Luis Potosí, SLP, Mexico, Tel: +52 (444) 826 23 00, Ext: 6630; E-mail: mejimenez@uaslp.mx

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Abstract

Background: The presence of misfolded proteins in the brain is the hallmark of neurodegenerative diseases. Protein aggregates could have systemic expression and might be found in several tissues including the skin.

Objective: To demonstrate the presence of phosphorylated Tau (p-Tau) in the skin cells of patients with Alzheimer's Disease (AD).

Material and methods: Antibodies against p-Tau (PHF, phosphorylated at S296 and AT8, phosphorylated at S202) were assayed in biopsied tissue from the retro-auricular area in 49 subjects: 20 with AD, 12 with non-degenerative dementia and 17 age-matched controls. Light and confocal microscopies were employed to localize Tau protein by immunohistochemistry and their presence in the skin was confirmed through Western blots.

Results: The skin biopsy taken from AD patients presented significantly higher levels of p-Tau (AT8: hyperphosphorylated at Ser 202) when compared both to control subjects and patients with non-degenerative dementia ($p < 0.001$).

Conclusion: This study demonstrates the presence of p-Tau in skin biopsies by immunoreactivity. This procedure could be used to support the clinical diagnosis of AD in living patients.

Keywords: Alzheimer's disease; Tau protein; Tau phosphorylation; Alzheimer's disease diagnosis; Neurodegenerative diseases

Introduction

The increase in life expectancy of the population is associated with a higher incidence of neurodegenerative diseases. Their main feature is the expression of misfolded proteins [1-3]. The clinical differentiation among them is not always easy, since different proteinopathies can be expressed simultaneously. They are accompanied clinically by dementia, parkinsonism and/or motor dysfunction [4-9]. Currently, the definite diagnosis can only be done either demonstrating misfolded proteins in brain tissue or with genetic testing. Nevertheless, these proteins are also expressed outside the brain, not only in the peripheral nervous system but in the case of α -synuclein, also in other tissues such as the heart, gut and skin [10,11]. Tau is also expressed in skeletal muscle, lungs and kidneys. This opens the possibility of detecting proteinopathies in extra-cerebral tissues [12].

The brain and skin both share an ectodermal origin and therefore it is plausible to find molecular pathological alterations taking place in the skin as in the central nervous system. Since α -synuclein aggregates have been recently demonstrated in peripheral nerve terminals,

epidermis and skin appendages of Parkinson's disease (PD) patients [13-15], our objective was to demonstrate the usefulness of demonstrating protein aggregates in the skin cells as a potential biomarker for AD living patients. For this purpose, phosphorylated Tau aggregates were analyzed by immunohistochemistry in the skin of patients with Alzheimer (AD) and non-degenerative dementia (NDD). These groups were compared with a control group with similar demographic characteristics.

Materials and Methods

Reagents and antibodies

Reagents were analytical grade and the solutions were prepared in molecular grade water (18.2 Ω /cm). The following primary antibodies were employed: 1) rabbit monoclonal anti-tau (pSer396) [E178] (ab32057, Abcam, Cambridge, MA), which according to the supplier's instructions detects both phosphorylated and non-phosphorylated tau and 2) Mouse monoclonal anti-tau (pSer202+pThr205) [AT8] (MN1020, Thermo Scientific, Rockford, IL). A biotinylated secondary antibody was obtained from DAKO (Carpinteria, CA) and for immunofluorescence we employed a goat anti-mouse IgG antibody

marked with Alexa Fluor 488 and a goat anti-rabbit IgG antibody marked with Cy5 (A1101 and A10523, Molecular Probes, Eugene, OR). Cellular nuclei were evidenced with an orange nucleic acid stain, Sytox (S-11368, Molecular Probes, Eugene, OR). Western blot reagents were molecular biology grade and obtained from IBI Scientific (Peosta, IA, USA); except for bis-acrylamide and molecular weight markers, which were obtained from BioRad (Hercules, CA, USA).

Patient selection

The patients were selected from the Neurology Department external practice in the Central Hospital in San Luis Potosi, Mexico, and all the participants or their tutors signed an informed consent letter. The Institutional Ethics and Research Committee previously authorized the protocol. The inclusion criteria were being diagnosed with AD, a non-neurodegenerative dementia or to be an apparently healthy asymptomatic subject who accepted to participate in this study. Three groups of patients were studied 1) Healthy subjects with similar age and demographic characteristics to the rest of the participants 2) Patients with a probable AD diagnosis according to the NINCDS-ARDA criteria [16] and 3) Subjects with a diagnosis of a non-degenerative dementia (NDD) (normotensive hydrocephalous, subdural hematoma post-traumatic dementia, vascular dementia and epilepsy). The exclusion criteria were 1) patients with a local or systemic infectious process; 2) Severely impaired coagulation and 3) not accepting to be part of the study.

All AD and NDD subjects underwent cranial CT or MRI to aid their diagnosis; it was not a requisite for the control group.

Tissue sampling and processing

The participants were biopsied by a punch of 4 mm, taking a skin sample 3 cm behind the ear insertion, with previous local anesthesia and aseptic cleaning of the region, followed by compression for hemostasis. The biopsies were immersed in 0.1 M phosphate buffer containing 4% paraformaldehyde during 24 h and embedded in paraffin. Coronal 5 μ m sections were collected in electro-charged slides (Biocare Medical LLC, Concord, USA). Next, sections were dewaxed by heating (60°C, 10 min), followed by xylene and ethanol rinses, and rehydrated. For epitope recovery, slides with 3 tissue sections each were submerged in DIVA decloacker solution (Biocare Medical, LLC, Concord, CA) and placed in a pressure cooker during 30 min followed by a 20 min cooling time at room temperature. After 3 rinses with distilled water, endogenous peroxidase was depleted incubating the sections 15 minutes with 3% hydrogen peroxide. The sections then underwent incubation steps (15 min each) in a humidity chamber at room temperature to block unspecific background staining (Background sniper, Biocare Medical, LLC, Concord, CA) and endogenous biotin and biotin binding proteins (avidin/biotin blocking kit, Vector Laboratories Inc, Burlingame, CA) followed always by rinses with TBS-tween. Monoclonal antibodies were incubated during 1 hour, followed by the streptavidin-biotin marked secondary antibody for 15 min. Peroxidase activity was visualized by incubating the sections with either diaminobenzidine, obtaining a brownish coloration, or with amine-ethylcarbazol to obtain a red coloration, and counterstained with Harris hematoxyline. Negative controls consisted of tissue sections treated without the primary antibody. The sections were viewed with a Nikon microscope (NikonLabophot-2, Japan) equipped with a digital camera. For immunofluorescence, the primary antibodies were incubated overnight at 4°C, and analyzed with a

confocal microscope (LEICA TCS SP2, Leica Microsystems GmbH, Heidelberg, Ger).

Western blot

Fifty mg of rat brain or human skin were processed obtaining 150-500 μ g of total protein by homogenization with a lysis buffer (0.025 M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40 and 5% glycerol) at pH 7.4 and supplemented with a protease and phosphatase inhibitor cocktail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The supernatant was recovered by centrifugation at 12000 x g 10 min. Total protein was quantified by BCA assay. 50-80 μ g were suspended in Laemli buffer 2X supplemented with 10% β -mercaptoethanol, boiled 10 min at 95°C and run on SDS-PAGE 10-15% gel. The proteins were electrophoretically transferred to 0.2 μ m PVDF membranes (BioRad Laboratories, Hercules, CA) and blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (pH 7.6) containing 0.1% Tween (TBST) and probed overnight at 4°C with PHF-Tau (1:500) and AT8 (1:250). In addition, all membranes were probed with an antibody directed against β -actin as a control protein in whole lysates. After washing with TBST, the membranes were incubated with secondary antibodies HRP-conjugated (1:5000) in TBST+3% BSA during 1h 30 min. After subsequent washes, bands were visualized by chemiluminescence (Pierce, Rockford, IL USA) followed by autoradiography.

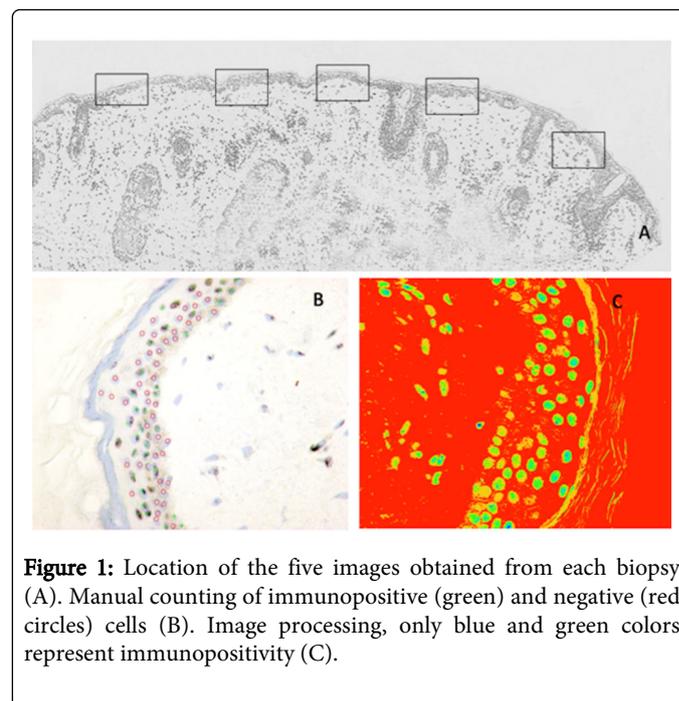


Figure 1: Location of the five images obtained from each biopsy (A). Manual counting of immunopositive (green) and negative (red circles) cells (B). Image processing, only blue and green colors represent immunopositivity (C).

Evaluation and statistical analysis

The conformation of the three groups was made with the clinical presentation, MMSE, laboratory and image features. The pathologist was blind about the clinical diagnosis. In order to validate the evaluation procedure, 5 fields per section were captured at the same magnification (40X) and the immunopositive and immunonegative cells were manually counted using the Photoshop software (V 12.1 Adobe Systems Inc, San Jose CA) in the obtained color images; this quantification was finally expressed in percentage for each one of the participants (Figure 1A).

Condition	Number of Subjects	Male/ Female	Age (Years, Mean ± SD)	MMSE (Points, Mean ± SD)	Immunopositivity percentage (median, min -max)	
					PHF	AT8
Healthy	17	6/11	72 ± 14	29.4 ± 1.4	38 (9-58)	0.6 (0.4-1.7)
Alzheimer's disease	20	8/12	79 ± 9	14.5 ± 7.3 ^A	46 (9-67)	4.6 (2.7-5.4) ^A
Non-degenerative dementia	12	9/3	76 ± 13	17.2 ± 9.7 ^A	46 (18-58)	1.4 (0.3-1.7) ^B

Table 1: Significant differences among groups were found in MMSE scores and % immunopositivity for AT8. ^A p<0.001 as compared with the control group, ^B p<0.001 as compared with the AD group. ANOVA followed by Tuckey test for the variables age and MMSE. Kruskal-Wallis followed by Mann-Whitney U-test for immunopositivity percentages.

The quantification of immunopositivity, estimated by manual cell counting performed by two separated observers was compared, obtaining a 95% of correlation (Figure 1B). To corroborate these estimates and to obtain a confident and unbiased evaluation we also used a digital image processing. We employed the numerical principles from the model RGB (red, green, blue); 5 images from each sample of skin were evaluated, quantifying the total area and the expression of immunopositivity (Figure 1C). The images were processed by means of the commercial software Image Pro Plus 7 (MediaCybernetics, Bethesda, MD, USA). The kappa coefficient between the two employed methods was 0.8.

significant. Percentages of immunopositivity were subjected to a non-parametric analysis by means of Kruskal-Wallis test followed by U-Mann Whitney to make the comparison between groups.

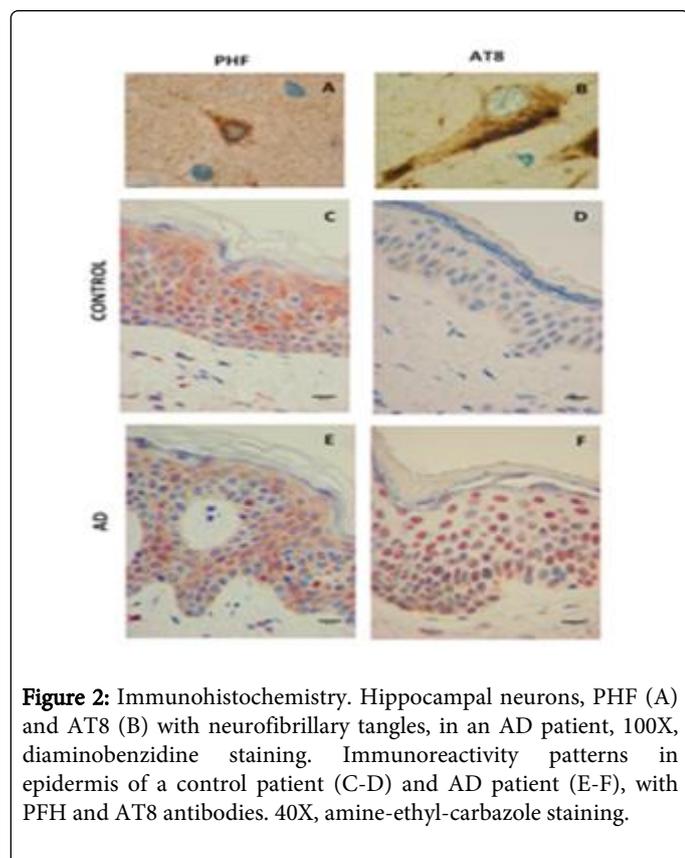


Figure 2: Immunohistochemistry. Hippocampal neurons, PHF (A) and AT8 (B) with neurofibrillary tangles, in an AD patient, 100X, diaminobenzidine staining. Immunoreactivity patterns in epidermis of a control patient (C-D) and AD patient (E-F), with PHF and AT8 antibodies. 40X, amine-ethyl-carbazole staining.

The analyzed structure was the epidermis. A parametrical statistical test was applied in order to compare age and MMSE among the 3 analyzed groups, considering values of p less than 0.05 as being

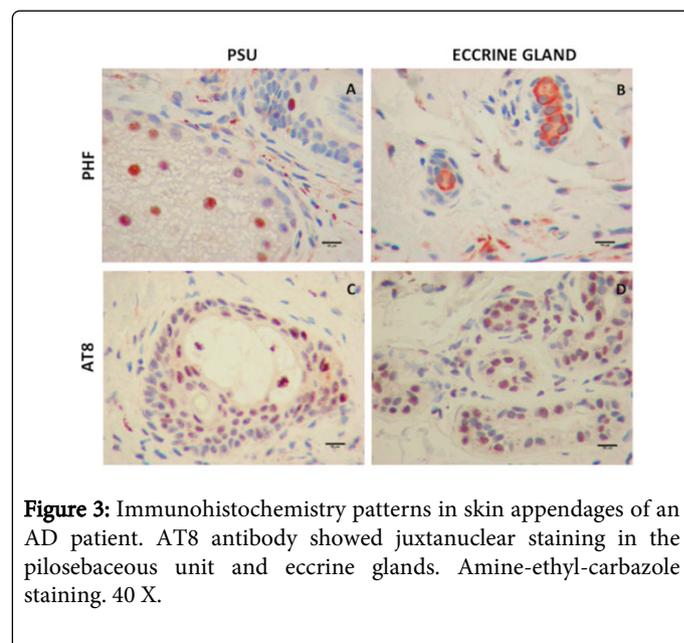


Figure 3: Immunohistochemistry patterns in skin appendages of an AD patient. AT8 antibody showed juxtannuclear staining in the pilosebaceous unit and eccrine glands. Amine-ethyl-carbazole staining. 40 X.

Results

Control subjects and patient were in the same age range (p=0.7). The control group was conformed by 17 subjects without neurological disease (normal MMSE); 20 patients with AD (mean MMSE 14) constituted the second group and individuals that presented non degenerative dementias (mean MMSE 17) constituted the third group of 12 subjects (Table 1). Significant differences among groups were found in MMSE scores between AD and control (p<0.001) and the NDD and control group (p<0.001).

The employed antibodies were previously tested in autopsied brain tissue from a confirmed AD cadaver. Both anti-tau antibodies reacted positively with cortical and hippocampal neurons presenting neurofibrillary tangles (Figure 2A and 2B). Both Anti-AT8 and anti - PHF antibodies showed immunoreactivity localized to the cytoplasm of the affected neurons, and in the neuropil of the cortical and hippocampal regions. Next, the immunoreactivity pattern in control skin biopsies was analyzed. Abundant cytoplasmic positivity and

scarce positive nuclear staining was observed in epidermal cells with PHF antibody (Figure 2C), nerve twigs were also stained. Null to scarce immunopositivity was detected to the AT8 epitope in control subjects (Figure 2D). In the skin from AD (Figure 2E) a clear PHF-positivity was demonstrated.

Also, in AD patients (Figure 2F) we found juxtannuclear red expression, with granular staining involving keratinocyte nuclei throughout the thickness of the epidermis with AT8 antibody. When the skin appendages were evaluated (Figure 3), the pilosebaceous unit (PSU) of AD patients showed a strong red juxtannuclear immunopositivity to PHF and AT8 in both, PSU (A,C) and eccrine gland (B,D). PHF antibody stained the cytoplasm of eccrine gland cells intensely, while cell nuclei were immunopositive to AT8 [17]. The nuclear and juxtannuclear localization of abundant AT8 immunopositivity in AD patients was confirmed by

immunofluorescence (Figure 4F), while the PHF epitope showed immunopositivity both in control (4A) and AD patients (4E), with a predominant cytoplasmic localization (Figure 4A and 4E).

The localization of the granules was confirmed with the staining to delineate nuclei in red (Sytox, Figure 4C and 4G) and by merging the images from both antibodies. A clearly different pattern of immunofluorescence between controls and patients is observed in these images, marked by a difference in AT8 immunostaining (Figures 4D and 4H). The comparative analysis of the control group with AD patients showed that controls presented sparse, green, juxtannuclear granules in few keratinocytes with AT8 antibody (Figure 4B); versus frequent juxtannuclear green granules in AD patients (Figure 4F). The presence of positivity to AT8 in keratinocytes was clear in the merged image (Figure 4H). The identity of p-Tau in human skin was confirmed through Western blots (Figure 5).

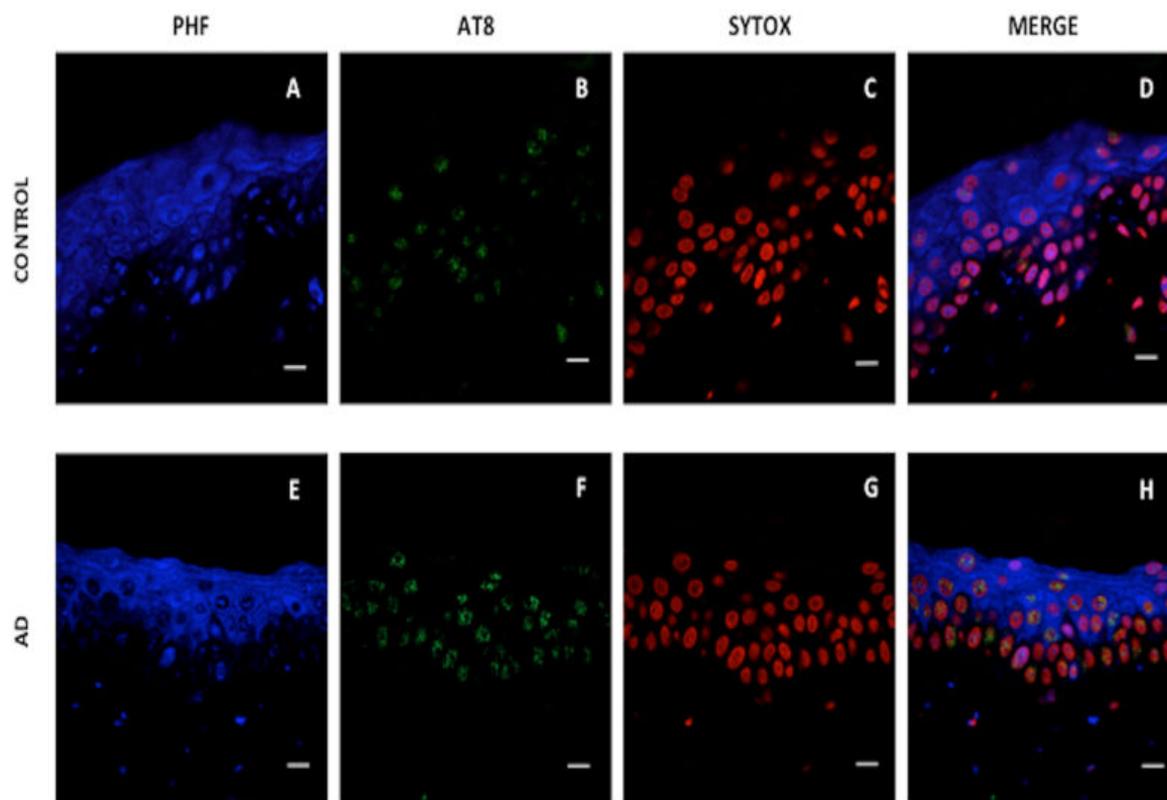


Figure 4: Skin immunofluorescence. Confocal microscopy. PHF (Cy5) and AT8 (Alexa Fluor 488) antibodies. Control subject (A-D) and AD patient (E-H). Cell nuclei stained with Sytox (C-G). Positive juxtannuclear staining is observed in AD patient with AT8 antibody. Scale bar 10 μ m.

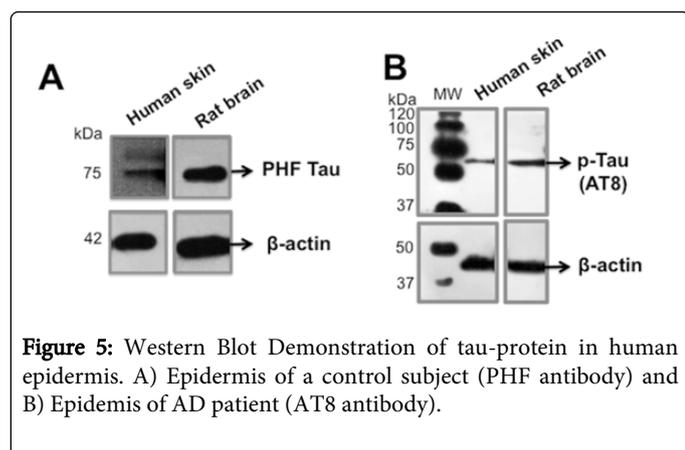
Although protein aggregates were detected in the different epidermis strata and skin appendages, the structure that allowed an in depth analysis and comparison of protein immunoreactivity was the epidermis (Figure 6). Immunoreactivity to PHF antibodies was similar in the 3 groups of patients showing not statistically significant difference [H(2, N=49)=5.1, p=0.08]. By contrast, immunoreactivity to AT8 antibodies was significantly different among groups [H(2, N=

49)=35.8, p<0.001]. The analysis between groups showed that AD patients presented significantly higher AT8 immunoreactivity than controls and patients suffering from non-degenerative dementias (p<0.001).

Discussion

A skin biopsy was taken from a sample of 49 subjects with similar demographic characteristics. When we compared the group of AD, this presented significantly higher levels of p-Tau (AT8: hyperphosphorylated at Ser 202) than both control and non-degenerative dementia group. These antibodies were chosen because in human studies, Ser396 phosphorylation (PHF) has been reported as an event that starts early in neurodegeneration and increases with aging and with the disease progress. Phosphorylation at Ser202 on the other hand is always present and significantly increased in diseased brains when compared to controls [18].

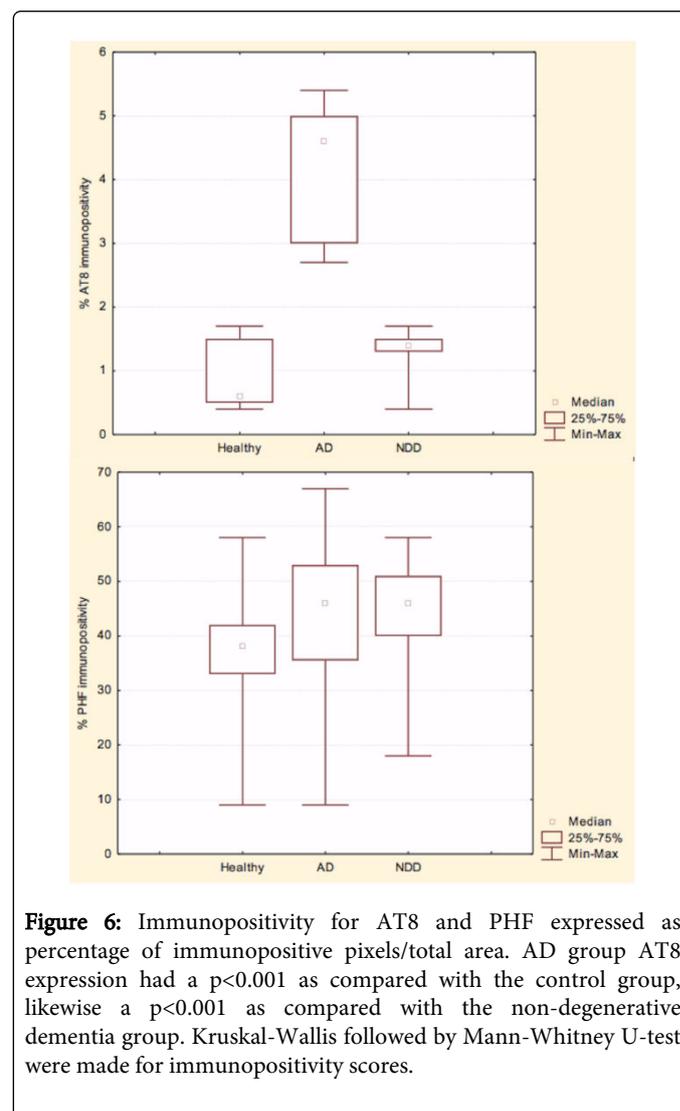
These phosphorylation sites have also been reported in the reversible tau hyperphosphorylation that takes place in hibernating animals proposed as a model for the study of tauopathies. In this model, both sites (S396 and S202) present an important increase in phosphorylation during that physiological period [18,19]. We confirm here by means of Western blot the presence of p-Tau in the skin showing a similar molecular weight as in other organs [20-22]. The presence of p-Tau in the skin provides thus relevant information, and in our model AT8 (S202) immunoreactivity was significantly associated with the presence of AD.



The skin opens interesting possibilities for the study of neurodegenerative diseases because it is highly innervated and has the capacity of producing and releasing several neuropeptides [23]. It also expresses genes involved in neurological diseases like APP, Tau, PSEN1 and PARK2 among others [24]. According to this, several diseases from the nervous system have dermal manifestations, as it is the case of PD, where there is an increased risk of melanoma and patients present seborrhea and hyperhidrosis besides the classical motor manifestations of the disease [25].

In addition to becoming aggregated, Tau and other proteins that participate in neurodegenerative diseases translocate inside the cell nucleus [26,27] and interact with DNA [28, 29]. Current evidence supports that Tau participates in stress response protecting DNA [30,31], promotes chromosome stability by means of its interaction with both microtubules and chromatin [32], and upon DNA binding allows expression of silent genes immersed in heterochromatin [33]. These epigenetic changes however, are regarded as a source of neurodegeneration promoted by Tau, since it leads to the expression of aberrant genes in neurons. It is then of great interest that skin biopsies provide the possibility to explore Tau behavior in relation with epigenetic modifications.

Biomarkers of neurodegenerative diseases have been developed in recent years, mainly based on advanced molecular neuroimaging. Most health systems in the world, however, cannot afford these studies for the clinical practice. Therefore, skin biopsies represent an alternative to support the diagnosis of the two most important neurodegenerative diseases, by applying immunohistochemistry with commercially available antibodies. This can be done in a standard pathology lab of worldwide hospitals and clinical laboratories.



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