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Salivary Biomarkers in Alzheimer´s Disease

Biomarcadores Salivales en la Enfermedad de Alzheimer

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SUMMARY: The hypotheses currently considered the most likely causes of Alzheimer's disease (AD) are amyloid beta peptide deposition in the cerebral cortex and hyperphosphorylation of the Tau protein, with the consequent formation of neurofibrillary tangles. In clinical practice, although not accurate, AD diagnosis is based on the exclusion of other diseases, behavioural assessments and complementary examinations, such as imaging and blood tests. Advances in the field of biotechnology have created exciting prospects for the early detection of AD via biomarker assessment, which is considered a safer and more efficient procedure. Molecules recognised as biomarkers can be expressed in some body fluids, including cerebrospinal fluid, saliva and blood. The presence of amyloid beta peptide and Tau can be confirmed in saliva, which is also an easily and non-invasively collectable material with an accessible cost. The objective was evaluate the concentrations of the t-Tau protein and Ab42 peptide in the saliva of elderly individuals with and without dementia of the AD type Method: The objective of this case-control study, involving a total of 120 individuals, was to analyse whether a correlation exists between variations in the concentrations of the t-Tau and Ab42 biomarkers in the saliva of patients with confirmed AD and individuals in the inclusion group but without AD . We found that t-Tau expression in AD patients is significantly lower than that in individuals without AD, whereas the salivary concentration of Ab42 is higher in patients with AD but not significantly different from that of the group without AD . Conclusion: Thus, we demonstrate the feasibility of using salivary biomarkers as predictive markers for diagnosis of Alzheimer's disease.

KEY WORDS: Alzheimer's disease; Biomarkers; Amyloid beta-peptide; Saliva; Tau protein; Dementia.

INTRODUCTION

The incidence of Alzheimer's disease (AD) has significantly increased in recent decades, placing this dementia among the most common neurodegenerative diseases worldwide (Arevalo-Rodriguez *et al*., 2013). It is estimated that currently, 35.6 million people live with AD, and this number is expected to double in the next 20 years, reaching 65.7 million by 2030 (Wimo *et al*., 2010). Researchers have sought more accurate techniques for AD detection, with special emphasis on biomarkers that may indicate the onset of this disease. The investigated biomarkers include the Tau protein and amyloid beta (Ab) peptide, which are both found in cerebrospinal fluid (CSF) (Tapiola *et al*., 2009).

According to the Alzheimer's Association (2019), the most common symptoms of AD are as follows: Early clinical symptoms - Difficulty remembering recent conversations, places, names or events, apathy and depression and Late symptoms - impaired communication, disorientation, mental confusion, altered judgement, mood and personality swings and, ultimately, difficulty speaking, swallowing and walking.

Grossman & Irwin (2016) argue that the diagnosis of AD is a matter of clinical judgement, obtained from a careful history and a judicious examination of the mental state, with other causes leading to dementia being excluded.

In addition, the diagnosis of dementia is based on the presence of deficits in memory and other functions, such as language, the ability to recognise objects and organisation and planning skills (Nitrini *et al*., 2005). AD is a complex neurodegenerative disorder with typical histological features, including neuritic plaques, neurofibrillary tangles and a variety of neurochemical deficits affecting the serotonergic, noradrenergic and cholinergic systems (Cummings, 2000). These plaques consist of amyloid peptide aggregates, acid residues derived from amyloid precursor proteins (APPs) and intracellular clusters of Tau proteins, forming neurofibrillary tangles. Synaptic destruction and neuronal death are consequences of the formation of these plaques (Sereniki & Vital, 2008).

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In AD, from the histopathological point of view, massive synaptic loss occurs, and neuronal death is observed mainly in the brain regions responsible for cognitive functions, especially the cerebral cortex, hippocampus, ventral striatum and entorhinal cortex. In addition to these characteristics, AD patients exhibit some remarkable organic changes, including the following (Selkoe, 2001).

The Tau protein belongs to the family of microtubule-associated proteins (MAPs); therefore, its main function is precisely to stabilise microtubules through tubulin aggregation. The gene encoding the microtubule-associated Tau protein has 16 exons, and its transcription is complete, yielding 12 mRNA copies by alternative splicing and six isoforms of the protein. The protein is composed of 352 to 441 amino acid residues (Maurer *et al*., 1997).

Tau protein accumulation in nerve or glial cells is seen as an important biological marker of tauopathies. Neurodegenerative mechanisms are directly related to changes in the isoforms of this protein (de Paula *et al*., 2009).

When hyperphosphorylation of the Tau protein occurs, the microtubule dynamics are compromised, affecting intraneuronal transport, which causes deleterious effects on several cellular processes. These effects include a lack of preservation of the morphology and functions of nerve cells, which affects cell viability (Stoothoff & Johnson, 2005; Jayapalan & Natarajan, 2013). Senile plaques are formed by Ab and are the first features that arise in AD. The APP has a large extracellular amino-terminal sequence and a short carboxyl-terminal sequence. Studies indicate that one of the functions of the APP is to act as a G-protein receptor in the membranes. The receptors function as channels of communication between cells. The expression of APP is also increased during cellular stress (Menéndez *et al*., 2002).

Changes in the ApoE4 gene sequence are a risk factor for AD, suggesting that cholesterol plays an important role in this pathology (Tanzi & Bertram, 2001; Rocchi *et al*., 2003). The key challenge in the current clinical management of AD is the lack of an accurate biomarker for reliable diagnosis of the disease.

The biomarkers currently studied include the Ab and Tau proteins. Based on analysis of CSF, researchers have concluded that when Ab plaques appear in the brain, Ab42 levels are decreased compared to those in the CSF of healthy individuals, whereas an increase in Tau occurs. In molecular imaging examinations, such as positron emission tomography (PET), which allows visualisation of the glucose metabolism in the brain, Ab plaques are observed in topographic areas involving the temporoparietal cortex. Nuclear magnetic resonance (NMR) has also shown atrophy of the medial, basal and lateral areas of the temporal lobe cortex and basal medial parietal regions (Hu *et al*., 2010; de Souza *et al*., 2014).

Currently, several tests are available for the early diagnosis of AD through saliva that seek to identify the presence of substances, such as the Ab peptide and phosphorylated Tau (p-Tau) protein, as biomarkers. These tests are non-invasive, and further analysis is performed by the ELISA method, which is highly sensitive (Starling, 2012).

Salivary levels of Ab42 may be considered a potential marker of AD, aiding in the exclusion of other types of degenerative disorders. The levels of Ab42 differ in control subjects without mild dementia and are markers of risk for development of AD (Bermejo-Pareja *et al*., 2010).

The aim of this study is to detect and evaluate the concentrations of the total-Tau (t-Tau) protein and the Ab42 peptide in the saliva of elderly with and without AD-type dementia, in the search for differences that could be treated as a biomarker for Alzheimer's dementia.

The concentrations of Tau and Ab in the saliva of patients with a confirmed diagnosis of AD compared to individuals in the inclusion group without the disease.

This case-control study was conducted on patients with a diagnosis of probable AD and cognitively healthy individuals without AD. The research centres were in the cities of São Paulo and Cuiabá, the capital cities of the states of São Paulo and Mato Grosso, Brazil, respectively.

MATERIAL AND METHOD

A total of 120 elderly individuals were invited to participate in this experiment and were organised as follows:

· Group without AD: 60 cognitively healthy individuals with no diagnosis of AD, aged over 60 years, recruited through the Vestibular Rehabilitation Laboratory of the Master's Programme in Vestibular Rehabilitation and Social Inclusion, Anhanguera University, located in the city of São Paulo - São Paulo State, Brazil.

· Group with AD: 60 patients with a diagnosis of probable AD, recruited through the Centre of Geriatrics in the city of Cuiabá - Mato Grosso, Brazil.

Important considerations regarding the eligibility of the participating volunteers included the following:

· Individuals of both sexes were randomly selected, with no numerical limit according to sex.
· The level of literacy and socioeconomic status of the volunteers were not taken into account.
· There was no distinction as to the disease stage in patients with probable AD.
· None of the evaluated individuals presented any signs of mouth injury at the time of saliva collection.
· Individuals in the group without AD were recruited after lectures on AD given by members of the Vestibular Rehabilitation Laboratory in São Paulo. The group with AD was selected by the team of geriatricians of the Centre of Geriatrics of Cuiabá.

Saliva collections in São Paulo were performed between 1 December 2014 and 8 May 2015, while saliva collections in Cuiabá occurred during 1-30 April 2015.

Saliva analysis. Saliva was collected using Salivette® tubes, which consist of a plastic tube containing a cotton swab and a plastic filter.

For the correct collection procedure the following information was provided to the research volunteers:

* Place the cotton present inside the Salivette® inside the mouth, near the tongue and wait for a 3 minute period (times by our team), preferably without chewing the cotton

* During the collection, the ingestion of water, coffee or any other liquid, as well as any food, wasn't allowed

* Patients were advised about the need of not having ingested any food or liquids prior to one hour before de collection, as well as not having brushed their teeth within the last three hours prior to collection

* After three minutes, the volunteer removed the cotton of their mouth and put it back into the Salivette®, returning the tube to a member of our team

In patients with probable AD, at an advanced stage of the disease, when needed, the assistance of a companion or a health professional present was requested. Immediately after the collection the patients saliva were conditioned in a cooler at -20 °C until the time they were processed for results reading. After being removed from the freezing temperature, the saliva was subjected to a 3000 rpm (rotation per minute) centrifugation for five minutes. In this stage, called sample processing, the Salivette® cotton was discarded and the saliva separated in 200 µL aliquots into Eppendorf® type tubes for quantification of Ab peptide and Tau protein by the ELISA method.

RESULTS

All data from this experiment were statistically treated with the aid of Software graph pad prism 5.0. We applied the non-parametric Mann-Whitney test to compare the different groups (concentrations). The kruskal-wallis test was also applied for the comparison between the times experiments. The level of significance of the null hypothesis was 5 % ($p \leq 0.05$). After performing the triplicate tests for the quantification of Ab40 and t-Tau by the saliva ELISA method, using high specificity kits we can observe that, the Figure 1 shows the concentration of t-Tau expressed in the saliva of healthy individuals and AD patients. The expression of t-Tau in AD patients is significantly lower than that in individuals without AD, which confirms the hypothesis that the t-Tau and p-Tau concentrations in AD patients are antagonistic; i.e., when the concentration of t-Tau in saliva is reduced, that of salivary p-Tau is increased.

In the evaluation of the concentration of Ab42 in the saliva of patients without AD and AD, we can observe that there is a small difference although a reduced one concentration of Ab42 in patients with AD, as shown in Figure 2.

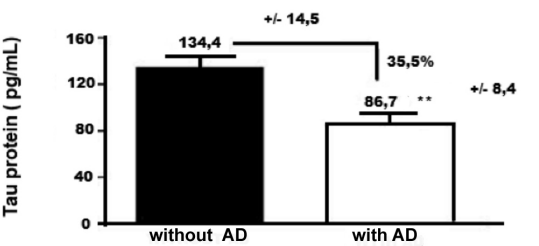

Fig. 1. Evaluation of the t-Tau protein concentration in the saliva of patients without AD and patients with AD, showing significant difference in healthy patients for the groups evaluated by (ANOVA), followed by the Student's t test for unpaired samples for a n = 120

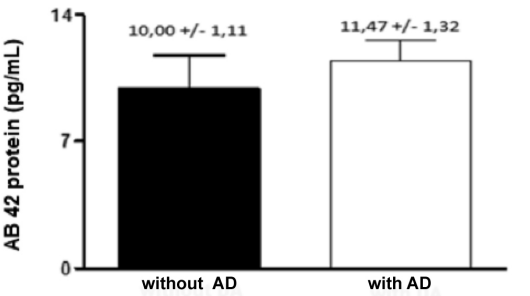

Fig. 2. Concentration of Ab42 in the saliva of patients with and without AD. The difference, although small, indicates a higher concentration of Ab42 in patients with AD.

DISCUSSION

Aging is the main risk factor involved in some diseases, including cancer, diabetes,cardiovascular disease and neurodegenerative diseases, such as Alzheimer's disease. Although many studies are being conducted to find an accurate diagnosis for Alzheimer's disease, no simple and inexpensive method has been devised for early detection of Alzheimer's disease.

Another issue is noninvasiveness, a key feature of a new diagnostic method, represented by saliva. It is now recognized that many aging-related diseases, including AD, are associated with peripheral biomarkers, not just restricted to brain pathologies and biomarkers. The use of innovative platforms, biosensors and other devices capable of extracting real-time information about an individual's health status is largely linked to healthy aging (François *et al*., 2019).

The search for accurate diagnostic methods capable of predicting the onset of AD has been the subject of continued research. In recent years, several biomarkers for AD have been developed, including assays for Ab42 and t-Tau. Saliva is an easily obtainable body fluid. In addition, studies have reported that central nervous system proteins (CNS) are excreted in this fluid. A large PubMed survey was conducted to identify 63 studies related to the diagnosis of saliva AD (Spielmann & Wong, 2011).

According to Gleerup *et al*. (2019), the most commonly used salivary biomarkers can be divided into the following categories: b-amyloid, tau, acetylcholinesterase and other biomarkers.

Seven studies investigated Ab42 and Ab40 proteins in the saliva of 187 individuals with AD; 72 individuals with Parkinson's disease (PD) and 195 healthy controls. Increased Ab42 levels were detected in four AD patients by ELISA (Lee *et al*., 2008; Bermejo-Pareja *et al*.; McGeer *et al*., 2018; Sabbagh *et al*., 2018).

Our results demonstrated an increase in Ab in the saliva of AD patients compared to healthy controls, but this difference was not significant. Nevertheless, considering that elevation in Ab protein is related to ageing and neurodegeneration, this finding may indicate that even in the healthy group, indications of disease onset may be present, as the first inclusion criteria were age 60 years or above and diagnosis of probable AD.

Sabbagh *et al*. state that after saliva levels were stabilized and mixed with an antibacterial agent, it was possible to quantify Ab42 concentration in a series of samples using ELISA-type assays. Saliva Ab42 levels were significantly higher in AD patients than in controls (51.7 ± 1.6 pg / ml for AD patients and 21.1 ± 0.3 pg / ml for controls ($p < 0.05$).

The phosphorylated Tau (p-tau) and total tau (t-tau) proteins present in saliva have been investigated in studies that included 181 individuals with AD, 123 individuals with amnesia mild cognitive impairment (aMCI), twenty individuals with Parkinson's disease, sixteen individuals with frontotemporal dementia (FTD) and 317 healthy controls. Elevation of p-tau / t-tau ratio was identified in AD patients (Shi *et al*., 2011), moreover, one of the studies reported a increasing the p-tau / t-tau ratio using Western blot analysis for the S396, S404, T404 phosphorylation sites and a combination S400 and T403 ($p < 0.05$) and increase of the median p-tau / t-tau ratio at the S396 phosphorylation site ($p < 0.05$) (Pekeles *et al*., 2018). Although p-tau and t-tau levels have been described as increased in both studies, there was no statistical significance reported (Gleerup *et al*.).

In addition, Pekeles *et al*. claim that salivary Tau dosing can be an easy and reliable method, used as a biomarker for Alzheimer's disease through cheaper tests, for example, when compared to spectrometry.

The Tau protein, found in saliva, is closely related to the pathogenesis of AD. The preliminary finding that Tau levels are increased in individuals with AD may indicate its excellent potential for diagnosis of AD (Shi *et al*.). The t-Tau expression in AD patients was found to be significantly lower, with a reduction of 35.5 %, than that in individuals without AD. This finding confirms the hypothesis that t-Tau can be used as a biomarker for AD.

Our results are consistent with those of Shi *et al*., who reported a reduction in salivary t-Tau and an increase in p-Tau in AD patients compared to healthy subjects. These authors suggest that an increased salivary p-Tau level is consistent with the changes in the brain and CSF of AD patients. This finding indicates that the concentrations of the Tau protein in the total and phosphorylated forms are antagonistic in the saliva of AD patients.

The mean age of the study volunteers was within the expected range for AD, with 48 % and 52 % of the sample composed of individuals without and with AD, respectively. The mean age of AD patients was 77 years, which is within the age range at which the prevalence of AD begins to grow exponentially (Alzheimer´s Association).

The experimental results and their validation by the literature survey indicate that a reduction in t-Tau expression

and an increase in salivary p-Tau occur in patients with probable AD, demonstrating that the use of these substances as biomarkers is feasible. Further experiments involving evaluation of the salivary expression of substances such as Ab peptide and Tau protein are required to confirm the involvement of these components in patients with AD.

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RESUMEN: Las hipótesis consideradas actualmente como las causas más probables de la enfermedad de Alzheimer (EA) son la deposición de péptido beta amiloide en la corteza cerebral y la hiperfosforilación de la proteína Tau, con la consiguiente formación de ovillos neurofibrilares. En la práctica clínica, aunque no es precisa, el diagnóstico de la EA se basa en la exclusión de otras enfermedades, evaluaciones de comportamiento y exámenes complementarios, como imágenes y análisis de sangre. Los avances en el campo de la biotecnología han creado interesantes perspectivas para la detección temprana de la EA a través de la evaluación de biomarcadores, que se considera un procedimiento más seguro y más eficiente. Las moléculas reconocidas como biomarcadores se pueden expresar en algunos fluidos corporales, incluidos el líquido cerebroespinal, la saliva y la sangre. La presencia del péptido beta amiloide (AB) y la proteína Tau (t-Tau) se puede confirmar en la saliva, que también es un material fácil y no invasivo de recolección con un costo accesible. El objetivo fue evaluar las concentraciones de la proteína t-Tau y el péptido Ab42 en la saliva de las personas de edad avanzada con y sin demencia del tipo de tipo EA. El estudio de casos y controles, se realizó en un total de 120 personas, para analizar si existe una correlación entre las variaciones en las concentraciones de los biomarcadores t-Tau y Ab42 en la saliva de pacientes con EA confirmada e individuos en el grupo de inclusión pero sin AD. Encontramos que la expresión de t-Tau en pacientes con EA es significativamente menor que en individuos sin EA, mientras que la concentración salival de Ab42 es mayor en pacientes con EA pero no significativamente diferente de la del grupo sin la enfermedad . Por lo tanto, se demuestra la viabilidad del uso de biomarcadores salivales como marcadores predictivos para el diagnóstico de la enfermedad de Alzheimer.

PALABRAS CLAVE:Enfermedad de Alzheimer; Biomarcadores; Péptido beta amiloide; Saliva; Proteína Tau; Demencia.

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