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Cerebrospinal fluid biomarkers for the differential diagnosis of Alzheimer's disease

# Cerebrospinal fluid biomarkers for the differential diagnosis of Alzheimer's disease

*Aplicação de biomarcadores do fluido cerebrospinal no diagnóstico diferencial da doença de Alzheimer*

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## ABSTRACT

**Introduction:** Several studies have been conducted in order to validate cerebrospinal fluid biomarkers for the diagnosis of Alzheimer's disease (AD), aiming primarily to facilitate the early diagnosis. **Objective:** To evaluate CSF biomarkers on patients with probable AD and the applicability of the international references values in this population. **Methods:** 46 individuals were recruited and classified as probable AD ($n = 19$), mild cognitive impairment (MCI) ($n = 5$) and other dementias ($n = 22$). The cerebrospinal fluid (CSF) biomarkers were measured using the INNOTEST kits for enzyme-linked immunosorbent assay (ELISA). Higher Tau protein values and lower Aβ and Innotest Amyloid Tau Index (IATI) values were observed in AD group when compared with MCI; higher levels of Tau and phosphorylated Tau (P-Tau), and lower Aβ and IATI values were observed in AD group when compared to patients with other dementias. No biomarker or IATI was able to distinguish between MCI and other dementias. The kappa index between biomarkers and the clinical diagnosis was regular to Tau and IATI, and weak to Aβ and P-tau. **Conclusion:** The cut-off values for each biomarker that showed better combined sensibility and specificity differ from the reference values suggested by the manufacturer. The CSF biomarkers represent important resources that can help with the AD diagnosis, although the results interpretation must be made based on the analysis of the three analytes together. The cut-off values must be established to address the specificities and characteristics of each population.

Key words: amyloid; tau proteins; Alzheimer's disease; cerebrospinal fluid.

## INTRODUCTION

Dementia is a general term representing a number of neurodegenerative impairments, whose common characteristic is limitation on autonomy and poor quality of life. Intrinsically age-related, these clinical conditions have been increasing in social and public health contexts worldwide. According to the World Health Organization (2011)[1], developing countries such as Brazil, currently have the most pronounced aging population rates that reflects a rapid growth, especially among age groups over 65 years. A systematic review of articles published in our country found a prevalence of dementia between 5.1% and 19% in the elderly aged 60 years or over[2].

Dementia includes memory impairment with loss of ability to learn new information or remember information previously learned, as well as the presence of at least one of the following alterations that may compromise the social and occupational activities of the individual: aphasia (language impairment); apraxia (impaired ability to carry out motor activities despite intact motor function); agnosia (failure to recognize or identify objects despite intact sensory function), and executive functions deficits (ie, planning, organizing, sequencing and abstraction). Among possible causes of dementia, Alzheimer's disease (DA) is the most common, accounting for 50%-70% of cases[3, 4].

In the last decade, several studies have been conducted to validate biomarkers in cerebrospinal fluid (CSF) for the diagnosis of AD, aiming primarily to facilitate the early diagnosis of this dementia syndrome. The studies are dedicated primarily for measuring, in the cerebrospinal fluid, the two key proteins involved in the disease: a) beta-amyloid protein ($Aβ_{1-42}$ or simply Aβ), the main component

of amyloid plaques, which is decreased in the CSF of patients with AD due to its deposition in brain parenchyma, and b) tau protein and phosphorylated Tau (P-tau), which are increased in the CSF of patients due to neuronal degeneration associated with the intracellular accumulation of neurofibrillary tangles[5-10].

The examination of these biomarkers in the CSF may increase the accuracy of diagnosis in clinical practice, both in dementia phase as in mild cognitive impairment (MCI), which is described as the intermediate state between normal cognitive aging and dementia, or more specifically, AD[11]. Moreover, these biomarkers have been correlated with the intensity of neuropathological lesions and have demonstrated sensitivity and specificity of approximately 85%-90% for AD diagnosis[6, 12]. CSF biomarker analysis may be applied for identifying asymptomatic individuals at risk of developing and preclinical stages of AD, elucidation of atypical AD cases, monitoring disease progression, and evaluation of response to treatment[13].

Studies show that the results of biomarkers should always be evaluated as a whole to characterize the pathological signature in AD[5-10], which gives strength to the diagnostic laboratory finding. Therefore, Innotest Amyloid Tau Index (IATI) obtained by the relation between Aβ and Tau values: $(A\beta_{1-42}/(240 + 1.18 \times Tau)$ shows better performance diagnosis for AD than the isolated result of each biomarker[14, 15].

Despite the advantages of using biomarkers in CSF to aid in the diagnosis of AD, there some are still challenges in the implementation of these tools in the laboratory routine, especially regarding the reproducibility of results and the establishment of optimal cut-off values. This difficulty is exemplified by the fact that in multicenter studies, including different laboratories using the same biochemical assay, a large variability of these biomarkers levels have been found[12]. A number of reasons may explain the interlaboratory variations of these measurements; among them: demographic characteristics of patients, source of recruitment, severity of disease, and selection and diagnosis criteria. Other factors may also be involved, such as the process of obtaining the CSF, collection and sample transportation, besides the storage conditions. Other interfering include variations of the kits used in the assay, laboratory equipment and variations in test procedure[12]. Therefore, it is essential that each laboratory establish its own reference values or a reproducible methodology used in other laboratories[14].

## OBJECTIVE

This study aimed at assessing biomarkers from the cerebrospinal fluid, Aβ, Tau and P-tau proteins, in a sample of the

Brazilian population, since there are no validation studies of these biomarkers in this population. Thus, our results may contribute to the suitability of using these CSF biomarkers and determining reference values in clinical laboratories in Brazil.

## METHODS

### Participants

We recruited 46 patients seen in the Neurology clinic at the Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte (MG), from November 2012 to March 2014. The average age was 65.89 ± 9.65, of which 29 female patients and 17 male.

The participants underwent clinical and neurological exams, including cognitive and functional assessment. Diagnoses were based on the criteria of the National Institute on Aging and the Alzheimer's Association workgroup[16] and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[17]. Based on the clinical diagnosis, the participants were divided into the following groups: probable AD ($n = 19$), MCI ($n = 5$), other dementias/clinical conditions: behavioral variant frontotemporal dementia (bvFTD) ($n = 19$), primary progressive aphasia (PPA) ($n = 2$), and antibodies anti-N-methyl-D-aspartate (NMDA) receptor encephalitis ($n = 1$), these conditions' symptoms overlap with MCI or AD.

This study was approved by the Research Ethics Committee of the UFMG, and all participants or their caregivers signed a consent form prior to collection.

### CSF analysis

Lumbar puncture was performed to collect the cerebrospinal fluid with participants fasted for eight hours. The samples were centrifuged at 3,000 revolutions per minute (rpm) for 10 minutes, at 4ºC, maximum 4 hors after collection. Then, they were frozen in polypropylene tubes at -80ºC until the time of analysis. The biomarkers were measured using the INNOTEST hTAU Ag, INNOTEST PHOSPHO-TAU (181P), and INNOTEST β-Amyloid (1-42) (Innogenetics, Bélgica) kits by enzyme-linked immunosorbent assay (ELISA) technique, the manufacturer's instructions were strictly followed. The reference values indicated by the manufacturer are: IATI < 0.8 pg/ml – indicative of AD; > 1.2 pg/ml – normal; Aβ < 500 pg/ml; Tau total > 375 pg/ml and P-tau > 60 pg/ml. The samples were analyzed simultaneously

for the three biomarkers using kits of the same lot, and intra-assay variations based on replicates was < 5%. In all assays was used an internal quality control.

## Statistical analysis

Statistical analyzes were performed using SPSS v. 13.0 software. Shapiro-Wilk test was used to evaluate the normality of the variables. The comparison for two groups was performed by the Mann-Whitney or *t*-test for non-parametric and parametric variables, respectively. The comparison between the three groups was performed by the Analysis of Variance (ANOVA) post-hoc least-significance difference (LSD) or Kruskall-Wallis, followed by Mann-Whitney test with Bonferroni correction, for parametric and nonparametric variables, respectively. Correlations were evaluated by Pearson, for parametric variables, or Spearman, for nonparametric. The agreement between the variables was evaluated by the kappa index. Receiver operating characteristic (ROC) curves were used to represent the sensitivity and specificity to AD diagnose in different cut-off values. The best cut-off for each marker has been established by the highest value of the Youden index based on ROC curve. We considered $p < 0.05$ value significant.

## RESULTS

The median values and average concentration of each biomarker measured in the CSF and the IATI calculated are shown in **Tables 1** and **2**. In Table 1, individuals with probable AD, MCI and other dementia/other medical conditions; in Table 2, individuals with AD or MCI in relation to other dementias.

We observed higher values of Tau protein and lower values of Aβ and IATI in the AD group when compared with MCI group. We was also observed increased levels of Tau e P-tau, as well as lower levels of Aβ and IATI in AD group when compared with patients with other dementias. However, no biomarker or IATI was able to distinguish between MCI and other dementias (Table 1). In addition, significant differences were found between AD/MCI groups and other dementias with higher levels of Tau, P-tau and lower levels of Aβ and IATI in the first group.

Considering the diagnosis of AD based on IATI reference values provided by the manufacturer (< 0.8) compared with the clinical diagnosis (in this case considered the gold standard), sensitivity 63.6%, specificity 89.6%, positive predictive value (PPV) 87.5%, negative predictive value (NPV) 68.4%, and accuracy 75.8% was

**TABLE 1** – Comparison of Tau, P-tau and Aβ values and IATI in groups with AD, MCI and other dementia

| Biomarkers | AD ($n$ = 19) | MCI ($n$ = 5) | Other dementia ($n$ = 22) | $p$ value |
|---|---|---|---|---|
| Tau (pg/ml) | 541 (502) | 197 (208) | 183 (119) | < 0.001*[a, b] |
| P-tau (pg/ml) | 65 (32) | 51 (22) | 40 (16) | < 0.001*[b] |
| Aβ (pg/ml) | 624 ± 212 | 959 ± 250 | 852 ± 293 | < 0.001*[c, d] |
| IATI | 0.5 (0.4) | 2.1 (1.3) | 2.1 (1.2) | < 0.001*[a, b] |

*Variable expressed as median (interquartile range) or mean ± standard deviation.*

*P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index; AD: Alzheimer's disease; MCI: mild cognitive impairment; a: p = 0.007 between AD × MCI to Tau and IATI; b: p < 0.001 between AD × other dementia to Tau, P-tau and IATI; c: p = 0.005 between AD × MCI to Aβ; d: p = 0.002 between AD × other dementia to Aβ; * significant: p < 0.05.*

**TABLE 2** – Comparison of Tau, P-tau and Aβ values and IATI in groups with AD/MCI e other dementia

| Biomarkers | AD/MCI ($n$ = 24) | Other dementia ($n$ = 22) | $p$ value |
|---|---|---|---|
| Tau (pg/ml) | 488 (603) | 183 (119) | < 0.001* |
| P-tau (pg/ml) | 60 (26) | 40 (16) | < 0.001* |
| Aβ (pg/ml) | 685 (252) | 852 (293) | 0.019* |
| IATI | 0.6 (0.9) | 2.1 (1.2) | < 0.001* |

*Variable expressed as median (interquartile range) or mean ± standard deviation.*

*P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index; AD: Alzheimer's disease; MCI: mild cognitive impairment; * significant: p < 0.05.*

obtained for the diagnosis of AD. The result of the kappa index among the variables and the clinical diagnosis was regular for Tau and IATI (0.568 for both), and weak to Aβ (0.111) and P-tau (0.371).

The ROC curve was determined for the variables Tau, P-tau, Aβ and IATI, considering the AD classification according to the clinical diagnosis (**Figure**). It was observed an area under the curve 0.821 for Tau and 0.817 for p-tau, and good the relationship between these biomarkers and clinical diagnosis is considered[18]. As for Aβ and IATI, the area under the curve was 0.701, and 0.777, this relationship is considered poor and regular, respectively[18].

Analyzing the same ROC curves, we evaluated the sensitivity and specificity criteria based on different concentrations of the markers, which were simulated as possible reference value or cut-off (**Table 3**). Only the concentrations which resulted in sensitivity and specificity values > 60% were considered. For Tau variable, concentrations from 244.5 to 327.5 pg/ml showed sensitivity values around 70%-80% with specificity between 84%-70%, respectively, for the diagnosis of AD, and the cut-off 265.7 pg/ml, the value that best showed balance between these parameters. For P-tau protein, the cut-off 47.7 pg/ml showed both variables around 80% and 75%, and the cut-off 680 pg/ml value for Aβ the one that presented
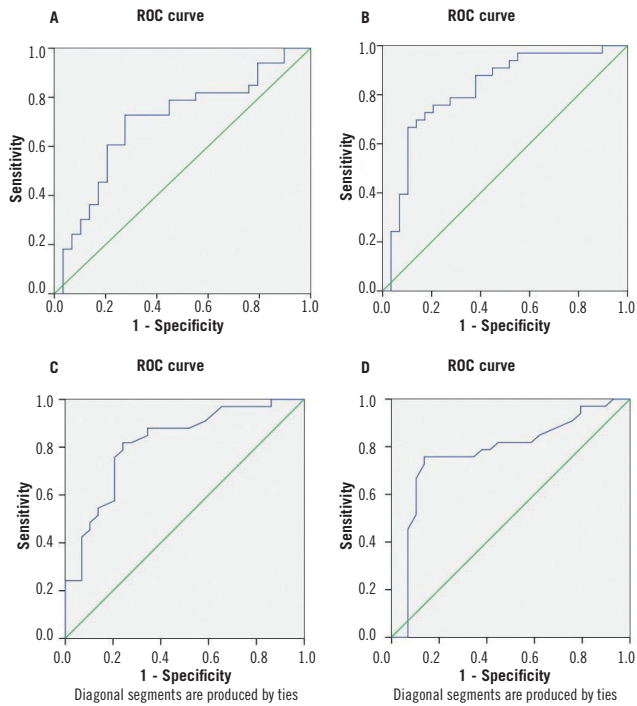
Carolina A. Magalhães; Micheli Figueiró; Vanessa G. Fraga; Elvis C. Mateo; André A. S. F. Toledo; Maria das Graças Carvalho; Paulo Caramelli; Karina B. Gomes

FIGURE – *ROC curve for Aβ (A), Tau (B), P-tau (C) and IATI (D) biomarkers considering the clinical diagnosis as reference*

*ROC: receiver operating characteristic; Aβ: amyloid beta-protein; P-tau: phosphorylated Tau protein; IATI: Innotest Amyloid Tau Index.*

better sensitivity and specificity combined. For IATI the 1.05 cut-off showed best combined values of sensitivity and specificity, around 75% and 84%, respectively.

When Tau, P-tau and Aβ variables were correlated, there was a strong positive correlation between Tau and P-tau levels ($r = 0.917, p < 0.001$) and strong negative correlation between Aβ and Tau levels ($r = -0.659, p < 0.001$), and Aβ and P-tau ($r = -0.612, p < 0.001$).

## DISCUSSION

To our knowledge, this is the first study to assess the concentration of CSF markers for the diagnosis of AD in Brazil. Although this is a preliminary study that requires a larger sample size to validate the findings, the obtained data demonstrate the need for the laboratory to establish the reference values, according to the characteristics of each population, for the diagnosis of the disease.

Currently, the majority of AD patients are diagnosed based on clinical criteria and exclusion of other diseases that cause dementia. While these criteria are widely used, their sensitivity varies around

**TABLE 3** – Coordinates of each ROC curves in the diagnosis of probable AD for Tau, P-tau and Aβ variables and IATI in the group with clinical AD

| Variable | Positive if greater or less than | Sensitivity | 1-Specificity |
|---|---|---|---|
| Tau | 190 | 0.879 | 0.379 |
| | 194.5 | 0.848 | 0.379 |
| | 197.5 | 0.818 | 0.379 |
| | 206.5 | 0.788 | 0.379 |
| | 227 | 0.788 | 0.345 |
| | 241.5 | 0.788 | 0.310 |
| | 244.5 | 0.788 | 0.276 |
| | 251 | 0.758 | 0.276 |
| | 257.7 | 0.758 | 0.241 |
| | 265.7 | 0.758 | 0.207 |
| | 277.5 | 0.727 | 0.207 |
| | 292.5 | 0.727 | 0.172 |
| | 304 | 0.697 | 0.172 |
| | 327.5 | 0.697 | 0.138 |
| | 353.5 | 0.667 | 0.138 |
| | 384 | 0.667 | 0.103 |
| | 418.5 | 0.636 | 0.103 |
| | 435.5 | 0.606 | 0.103 |
| P-tau | 42.2 | 0.879 | 0.379 |
| | 44 | 0.879 | 0.345 |
| | 45.5 | 0.848 | 0.345 |
| | 46.7 | 0.818 | 0.276 |
| | 47.7 | 0.818 | 0.241 |
| | 50 | 0.788 | 0.241 |
| | 53 | 0.758 | 0.207 |
| | 54.5 | 0.606 | 0.207 |
| Aβ | 648.5 | 0.606 | 0.207 |
| | 656.5 | 0.606 | 0.241 |
| | 661.5 | 0.606 | 0.276 |
| | 663 | 0.636 | 0.276 |
| | 664.5 | 0.667 | 0.276 |
| | 665.5 | 0.697 | 0.276 |
| | 680 | 0.727 | 0.276 |
| | 706 | 0.727 | 0.310 |
| | 728 | 0.727 | 0.345 |
| | 745 | 0.727 | 0.379 |
| IATI | 0.75 | 0.606 | 0.103 |
| | 0.85 | 0.667 | 0.103 |
| | 0.95 | 0.727 | 0.138 |
| | 1.05 | 0.758 | 0.138 |
| | 1.15 | 0.758 | 0.207 |
| | 1.30 | 0.758 | 0.241 |
| | 1.55 | 0.758 | 0.345 |
| | 1.75 | 0.788 | 0.379 |

*ROC: receiver operating characteristic; AD: Alzheimer's disease; P-tau: phosphorylated Tau protein; Aβ: amyloid beta-protein; IATI: Innotest Amyloid Tau Index.*

70.9%-87.3%[19]. Thus, a large percentage of patients are misclassified, particularly the cases of early AD, atypical AD or with multiple etiologies[20]. CSF

biomarkers are important to optimize the diagnosis, especially in differentiating AD from other neurodegenerative disorders such as frontotemporal, vascular, psychiatric dementia or neuroinfectious diseases.

According to members of Alzheimer's Biomarkers Standardization Initiative (ABSI)[21], the measurement of CSF biomarkers should be considered for patients with early AD, MCI or with atypical presentations of dementia with complex differential diagnosis. Furthermore, they must be evaluated in together with Tau, P-tau and Aβ values for greater accuracy in the AD diagnosis, which can be interpreted using in the IATI calculation.

In this study, the use of Tau, Aβ and IATI markers calculated was useful for the differential diagnosis of MCI or other dementia and AD, while the p-tau values differentiated only AD and other dementia groups, which confirms the usefulness of these markers in the differential diagnosis and the need for the analysis to be performed together. Furthermore, data suggest the use of markers for checking the conversion of prodromal MCI stage to AD. However, it should be noted that the CSF markers were unable to differentiate the individual with MCI from those with other types of dementia, and, therefore, it is not suitable for cases where the AD is unlikely.

When the AD and MCI groups were collected and the values of variables compared to the group with other dementia, all biomarkers and IATI showed differences between groups, suggesting its usefulness in the diagnosis of pre-clinical and clinical stages of AD compared with other dementias.

Although INNOTEST® kit is not the only kit commercially available for the quantification of these markers, it is known that its use is widespread, and in Brazil it is already being applied in the routine laboratory. Considering the clinical diagnosis of AD and diagnosis performed in our patients based on IATI provided by the manufacturer, were observed low sensitivity and NPV, as well as regular to weak relationship with the clinical diagnosis measured by the kappa index, which suggests that the cut-off value of IATI suggested by the manufacturer is not suitable for the population studied.

In fact, the variability of the cut-off between different laboratories, using the same, can reach 20%-30%[14, 22]. Since these values are calculated using the results from patients with clinical diagnosis of AD, patients with other types of dementia and healthy individuals may exhibit altered biomarker values without necessarily demonstrate the clinical features of the disease, since AD may be present up to 20 years before the onset of symptoms[23].

There was variation in sensitivity and specificity according to different cut-off values. The values that showed better balance

between the two criteria were suggested (**Table 4**), which differed from the values suggested by the kit manufacturer. However, choosing the best cut-off should be discussed with clinicians, since the impact of false positive or false negative results should be evaluated selectively. According to ABSI, because there is no yet an effective therapy for AD, it is better to incorrectly diagnose some patients who actually have the disease than patients classified as healthy individuals[24]. It will also be necessary to establish, based on Brazilian large cohort studies, the "gray zone" or each biomarker and IATI value. Although commonly it has been used values 10% higher or lower than the reference value for determining test positivity or negativity[21], this value is arbitrary and its interpretation must be done with caution – an um "unlikely AD" does not mean an "excluded AD"[24].

It should also be considered that the AD develops a dynamically and progressively, which means that the cut-off values can be modified depending on the course of the disease (preclinical and clinical phase) and age group. The final diagnosis should be complemented, whenever possible, with imaging tests such as magnetic resonance imaging (MRI) and positron emission tomography (PET)[25-27].

It is noteworthy that no biomarker provided sufficient sensitivity and specificity for the diagnosis of AD when assessed alone, which justifies the recommendation of a joint assessment of the three biomarkers. The mathematical calculation of the IATI, based on these markers, that although has demonstrated a strong correlation, meaning that they have the same tendency for the diagnosis, did not show sufficient accuracy to be used alone for the diagnosis of AD. This fact confirms the need to include other parameters along with the cognitive tests and clinical features for the diagnosis to be completed.

TABLE 4 – Comparative description of cut-off values for diagnosis of AD according to the kit used and the clinical diagnosis

| Biomarkers | INNOTEST® | Clinical diagnosis |
| --- | --- | --- |
| Aβ | < 500 pg/ml | < 680 pg/ml |
| Tau | > 375 pg/ml | > 268 pg/ml |
| P-tau | > 60 pg/ml | > 48 pg/ml |
| IATI | < 0.8 | < 1.05 |

*AD: Alzheimer's disease; Aβ: amyloid beta-protein; P-tau: phosphorylated Tau protein; IATI: Innotest Amyloid Tau Index.*

## CONCLUSION

Although insufficient to complete the diagnosis of AD alone, CSF biomarkers represent important resources that can

Carolina A. Magalhães; Micheli Figueiró; Vanessa G. Fraga; Elvis C. Mateo; André A. S. F. Toledo; Maria das Graças Carvalho; Paulo Caramelli; Karina B. Gomes

assist the medical team. It is true that the search for predictive biomarkers for AD is a high priority on research related to neurodegenerative diseases and, in the near future, it is expected to establish cut-off values that meet specificities of each population, enabling the use of these tools in the search of early diagnosis of AD and modifying perspective of the course of the disease.

## RESUMO

*Introdução: Estudos têm sido conduzidos no sentido de validar biomarcadores no liquor para o diagnóstico da doença de Alzheimer (DA), objetivando, sobretudo, facilitar o diagnóstico precoce. **Objetivo:** Avaliar os biomarcadores do liquor em indivíduos com provável DA, bem como a aplicabilidade dos valores de referência internacionais nesta população. **Métodos:** Foram recrutados 46 indivíduos, sendo classificados como provável DA (n = 19), comprometimento cognitivo leve (CCL) (n = 5) e outras demências (n = 22). Os biomarcadores foram dosados no liquor utilizando-se os kits INNOTEST por ensaio imunossorvente ligado à enzima (ELISA). Maiores valores de proteína Tau e menores valores de Aβ e índice Innotest Amiloide Tau Index (IATI) foram observados no grupo de DA quando comparados com o de CCL; maiores níveis de Tau e Tau fosforilada (Tau-P) e menores valores de Aβ e IATI foram observados no grupo de DA quando comparados com os pacientes que apresentavam outras demências. Nenhum biomarcador ou o IATI foi capaz de discernir entre CCL e outras demências. O índice* kappa *entre os biomarcadores e o diagnóstico clínico foi regular para a Tau e IATI, e fraco para Aβ e Tau-P. **Conclusão:** Os valores de* cut-off *para cada biomarcador que apresentou melhor sensibilidade e especificidade conjugadas diferiram dos valores de referência sugeridos pelo fabricante. Os biomarcadores do liquor representam importantes recursos que podem auxiliar no diagnóstico da DA, mas a interpretação dos resultados deve ser feita com base na análise dos três analitos em conjunto. Os valores de* cut-off *devem ser estabelecidos de modo a atender as especificidades e as características de cada população.*

*Unitermos: amiloide; proteínas tau; doença de Alzheimer; líquido cefalorraquidiano.*

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