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Evaluation of cerebrospinal fluid phosphorylated tau₂₃₁ as a biomarker in the differential diagnosis of Alzheimer's disease and vascular dementia

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Summary

Background: The diagnosis of either Alzheimer's disease (AD) or vascular dementia (VaD) is still largely based on clinical guidelines and exclusion of other diseases that may lead to dementia.

Aims: In this study, we assessed whether the use of sensitive and specific biomarkers such as phosphorylated tau proteins could contribute to an earlier and more accurate diagnosis of AD and VaD, as well as to their differentiation.

Material and methods: A total of 198 patients, of which 152 had AD, 28 VaD, and 18 were healthy controls (HC), were included in the analyses. We analyzed cerebrospinal fluid (CSF) levels of total tau protein (t-tau), tau protein phosphorylated at threonine 231 (p-tau₂₃₁), and factor score (FS) determined by combination of p-tau₂₃₁ and Mini-Mental State Examination (MMSE) in patients with AD and VaD, as well as in HC. We tested the diagnostic accuracy of these biomarkers in the CSF and FS (p-tau₂₃₁, MMSE) in differentiating AD from VaD and HC.

Results: Total tau levels were significantly elevated in subjects with AD compared to HC, as well as in VaD subjects compared to HC.

Discussion: p-tau₂₃₁ levels were significantly higher in patients with AD vs HC as well in patients with VaD vs HC. p-tau₂₃₁ levels did not distinguish AD from VaD patients. Importantly, FS(p-tau₂₃₁ and MMSE) showed statistically significant differences in the distribution of subjects with AD and VaD.

Conclusion: These results indicate that FS (p-tau₂₃₁ and MMSE) has a strong potential to provide an early distinction between AD and VaD.

KEYWORDS

Alzheimer's disease, biomarkers, cerebrospinal fluid, tau proteins, vascular dementia

1 | INTRODUCTION

Dementia is a major public health problem worldwide, partly due to the increasing life expectancy of the population. Epidemiological data for developed countries show that the prevalence of Alzheimer's disease (AD) in adults at age 60-64 is less than 1% in Europe, but after age 65, the incidence doubles every 5 years of life.^{1,2} Overall, neurological diseases that cause dementia are the third leading cause of life with disabilities globally.³ The most common primary cause of dementia is AD,⁴ which represents over 65% of all dementia cases, followed by vascular dementia (VaD), which is responsible for most of the remainder.⁴ AD is characterized by an insidious onset with a slow progressive course with impairment of memory, language, and visuospatial functions ultimately resulting in global cognitive impairment.⁵ VaD represents a large group of clinical entities associated with vascular changes⁶ and heterogeneous clinical presentation described as less insidious in onset compared to AD, which shows a stepwise decline in cognitive abilities.⁷ The most important feature of VaD is that it can be prevented by acting on risk factors, stressing the need for an early diagnosis of VaD.⁶

Diagnosis of either AD or VaD is still largely based on clinical guidelines and exclusion of other diseases that may lead to dementia. For AD, we used diagnostic criteria of McKhann and collaborators from 2011.⁷ Compared to the older AD criteria from 1984,⁸ which addressed only one final stage of the disease and memory loss as the first and only major symptom, these new criteria recognize preclinical stage, middle stage of mild cognitive impairment (MCI), and final stage of AD marked by symptoms of dementia, and also take into account that other aspects of cognition may become impaired first. They also recognize the potential use of both imaging and biochemical biomarkers to help diagnose AD. For VaD, we used the National Institute for Neurological Disorders and Stroke-Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINCDS-AIREN) criteria for probable VaD,⁹ as well as the Hachinski Ischemic Score (HIS).¹⁰ The most important clinical discriminative factors for VaD vs AD are stepwise progression, prominent impairment of the executive functions, HIS > 4, and focal neurological signs implying cortical or subcortical lesions.¹¹

The use of sensitive and specific biomarkers such as tau proteins, especially phosphorylated tau proteins, could contribute to the earlier and more accurate diagnosis of both AD and VaD.¹² In this study, we analyzed cerebrospinal fluid (CSF) levels of total tau protein (t-tau) and tau protein phosphorylated at threonine 231 (p-tau₂₃₁) in patients with AD and VaD, as well as in healthy controls (HC), to assess the diagnostic accuracy of these biomarkers in the CSF in differentiation of AD from VaD and HC.

2 | PATIENTS AND METHODS

2.1 | Patients

Patients who met all inclusion criteria were invited to participate in the study. The patients were examined at two study sites, General Hospital Varaždin and Clinical Hospital Center Rebro Zagreb from August 2011 until May 2015.

At the initial visit, the patients signed an informed consent form. Consent form was explained in details to patients and caregiver. Patient and caregiver gave consent to participate in the study as well as to perform lumbar puncture. The possible complications of the procedure were explained in detail. All procedures involving human subjects were performed with the approval of the Central Ethical Committee of the University of Zagreb Medical School, case no. 380-59/11-500-77/90, class 641-01/11-02. This research study had exclusively a diagnostic purpose and was not evaluating any health-related interventions to evaluate possible effects on health outcomes. As such, it has not been registered as a clinical trial. Two hundred patients were assessed. Two patients were not included in the study because of pathological findings on magnetic resonance (MR) scan of the brain. The study included 198 patients, of which 152 patients with AD, 28 patients with VaD, and 18 HC. For AD, we used diagnostic criteria of McKhann and collaborators from 2011.⁷ For VaD, the patients fulfilled NINCDS-AIREN⁹ and HIS criteria.¹⁰ The following inclusion criteria were used: age between 50 and 90 years, anamnestic data suspicious of developing dementia, normal neurological status or the presence of focal pyramidal or cerebellar lesion, Mini-Mental State Examination (MMSE) 27-15, neuropsychological testing suspected to dementia syndrome (Montreal Cognitive Assessment, MoCA, Alzheimer's Disease Assessment Scale-cognitive subscale, ADAS-Cog), complete blood tests including levels of vitamin B₁₂, folic acid (B₉) and thyroid function test, a negative serology for syphilis or Lyme's disease, and absence of other pathologies on brain MR imaging. Patients who did not fulfill these criteria were excluded from the study. It should be noted that HIS only suggests the likelihood of VaD, but does not confirm the presence or absence of VaD and that neuroradiologic diagnosis remains unreliable in the early phase of the disease.

2.2 | Cerebrospinal fluid (CSF) sampling and analysis

Lumbar puncture was performed between 8 AM and 10 AM, and CSF was obtained by a routine protocol from the L3/L4 or L4/L5 intervertebral space. After the first few drops were discharged, 2 mL of the CSF was collected in polypropylene tubes. In case of a traumatic puncture with hemorrhagic CSF, these samples were excluded from analysis. CSF samples were stored at 4°C, wrapped in aluminum foil, and transported on ice to the laboratory. All enzyme-linked immunosorbent assay (ELISA) analyses of CSF samples were performed at the Laboratory for Developmental Neuropathology of the Croatian Institute for Brain Research, University of Zagreb School of Medicine. Each sample was first centrifuged for 10 minutes at 2000 g at 4°C to exclude cells and other insoluble materials. The remaining amount of CSF was stored at -80°C for later analysis of CSF biomarkers. All CSF analyses were performed according to the manufacturers' detailed instructions, with at least two internal sample controls used to verify the consistency of the results. The total tau protein concentration (all 6 isoforms) was determined using human Tau Ag Fujirebio (Ghent, Belgium) ELISA kit. To determine the concentration of tau protein phosphorylated at threonine 231

(p-tau₂₃₁), the Tau [pT231] (Phospho) Human ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. Results were read using an ELISA Reader Model Bio-Rad 680 (Bio-Labor Laboratories, Hercules, CA, USA). There were no significant differences in the levels of total tau and p-tau₂₃₁ between the samples from the two different hospitals (Zagreb and Varaždin).

2.3 | Statistical analysis

For comparison of demographic, clinical, and laboratory parameters, the nonparametric Kruskal-Wallis test was used. Due to the relatively small sample sizes and because it does not require assumption of normal distributions, the nonparametric Mann-Whitney *U* test was used for some comparisons between the groups, in addition to the *t* test. Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic value of the parameters from the variables in the differentiation of the examined groups. Cut-off levels of both markers used in the study were derived from the ROC curve analysis, with the sum of sensitivity and specificity maximized. In addition, the specificity of both markers in differentiating the groups, with sensitivity set to 85%, was determined. Sensitivity and specificity were visualized with confidence interval (CI) of 95%. In addition, the area under the ROC curve (AUC) was shown as an accuracy indicator. The correlation between CSF biomarkers was tested using the Spearman test (r_s) and the Pearson coefficient of correlation (r). The factor score (FS) was used as a multidimensional analysis method that describes relationship of a large number of original variables by means of a smaller number of common factors or by a variable that better describes them, or explain their relationship more clearly. The factor score of p-tau₂₃₁ and MMSE was determined using the dimension reduction option in SPSS. With this function, two or more variables (so-called manifest variables) are unified into one (factor score or so-called latent variable).

3 | RESULTS

The demographic data of the subjects included in the study, their MMSE scores, and CSF levels of t-tau and p-tau₂₃₁ are shown in Table 1. On average, patients with AD ($U = 635, Z = -3.437, P = 0.001$) and VaD patients ($U = 132, Z = -2.484, P = 0.013$) were significantly

older than HC. However, because there was no significant correlation between age and CSF biomarker levels, the lower mean age of the HC was not considered as a significant factor in the interpretation of the results. Patients with AD ($U = 57.5, Z = -6.065, P < 0.001$) and VaD patients ($U = 41, Z = -4.334, P < 0.001$) had significantly lower MMSE scores in comparison to HC. Levels of t-tau were significantly higher in AD patients in comparison to HC ($U = 654, Z = -3.616, P < 0.001$) and VaD patients in comparison to HC ($U = 124, Z = -2.881, P = 0.004$).

The levels of p-tau₂₃₁ were significantly higher in AD ($U = 548, Z = -4.030, P < 0.001$) and VaD patients ($U = 79.5, Z = -2.940, P = 0.003$) in comparison to HC (Figure 1).

The factor score values for p-tau₂₃₁ and MMSE, FS (p-tau₂₃₁, MMSE), were significantly lower in AD ($U = 71, Z = -5.946, P < 0.001$) and VaD patients ($U = 39, Z = -3.700, P < 0.001$) in comparison to HC. The FS (p-tau₂₃₁, MMSE) was significantly lower in AD ($U = 760, Z = -3.417, P = 0.001$) in comparison to VaD patients (Figure 2).

Also, p-tau₂₃₁ levels positively and significantly correlated with the levels of t-tau (Table 2, Figure 3). ROC curve analyses of AD vs HC and VaD vs HC were significant for p-tau₂₃₁, as well as when comparing FS (p-tau₂₃₁, MMSE) in AD vs HC, VaD vs HC, and AD vs VaD (Figure 4). FS (p-tau₂₃₁ and MMSE) additionally increased the sensitivity of differentiation between AD vs HC and AD vs VaD, but not between VaD vs HC (Table 3).

4 | DISCUSSION

We analyzed the diagnostic value of specific biomarkers of the t-tau protein, p-tau₂₃₁, and FS (made from p-tau₂₃₁ and MMSE) in the differentiation of patients with AD vs VaD group. The t-tau levels were significantly elevated in subjects with AD compared to HC. P-tau₂₃₁ levels were significantly higher in patients with AD vs HC as well in patients with VaD vs HC. There was no statistical significance of p-tau₂₃₁ in distinguishing patients with AD vs VaD. When we examined FS (p-tau₂₃₁ and MMSE), we received statistical significance for distinguishing patients with AD vs HC and patients with VaD vs HC. The most significant was the fact that FS (p-tau₂₃₁ and MMSE) showed significant differences in the distribution of subjects with AD and VaD. Based on the *Recommendations of a Consensus Report for Useful Biomarkers of AD*, setting specificity and sensitivity to 85% or higher,¹³ our results indicate that p-tau₂₃₁ shows specificity and

TABLE 1 CSF levels of t-tau and p-tau₂₃₁, demographic data, and MMSE scores in AD, VaD, and HC

| Group (number of patients) | Age Mean ± SD | Sex F vs M | MMSE Mean ± SD | Total tau (pg/ml) | | p-tau ₂₃₁ (U/ml) | |
|----------------------------|---------------|------------|----------------|-------------------|-----------------------------|-----------------------------|-----------------------------|
| | | | | Mean ± SD | Median (25-75th percentile) | Mean ± SD | Median (25-75th percentile) |
| AD (152) | 71 ± 8.0 | 83 vs 69 | 20.4 ± 4.4 | 532 ± 377 | 439 (257-666.6) | 3.501 ± 5.007 | 1.758 (0.805-3.493) |
| HC (18) | 59 ± 14.8 | 8 vs 10 | 28.5 ± 1.8 | 271 ± 265.9 | 185 (83.5-334) | 0.907 ± 1.481 | 0.587 (0.164-0.803) |
| VaD (28) | 70 ± 9.6 | 15 vs 13 | 22.6 ± 4.5 | 505 ± 314 | 429.8 (237.5-692) | 1.324 ± 0.704 | 1.098 (0.763-1.905) |

AD, Alzheimer's disease; F, female; HC, healthy controls; M, male; MMSE, Mini-Mental State Examination; SD, standard deviation; VaD, vascular dementia.

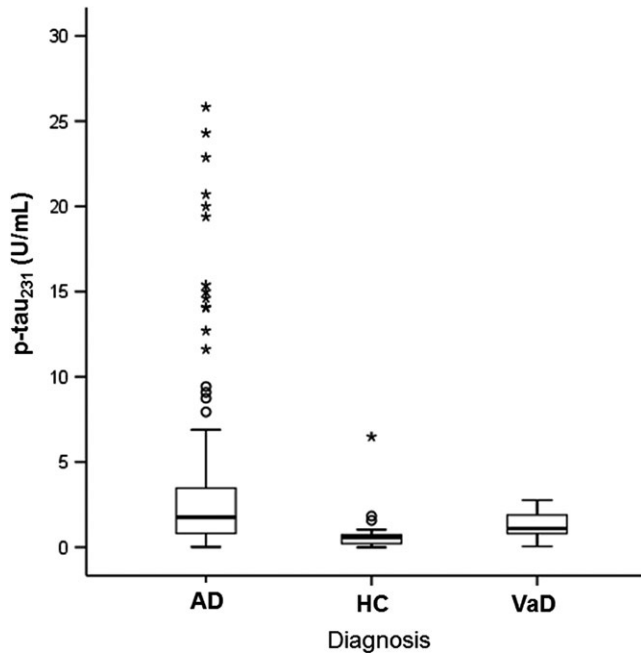


FIGURE 1 Levels of p-tau₂₃₁ in Alzheimer's disease and vascular dementia patients, and healthy controls. Boxes represent the median, the 25th and 75th percentiles, and bars indicate the range of data distribution. Circles represent outliers, asterisks represent extreme data points

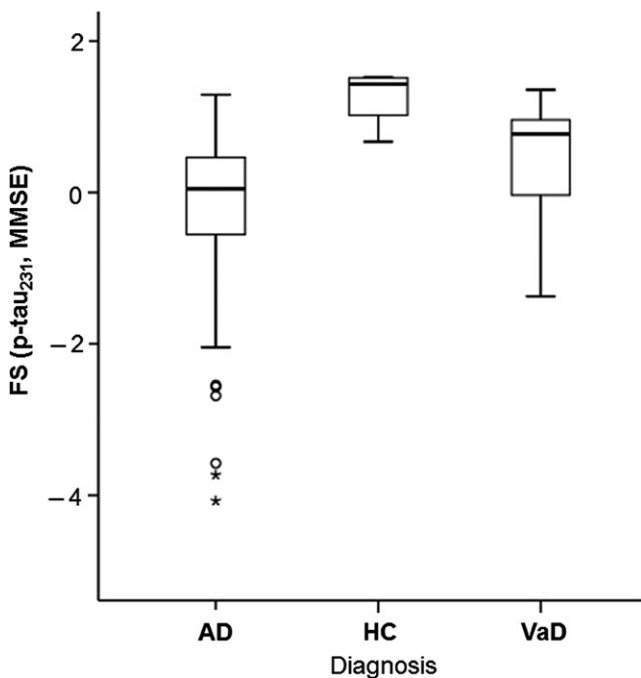


FIGURE 2 Levels of FS (p-tau₂₃₁, MMSE) in Alzheimer's disease and vascular dementia patients, and healthy controls. Boxes represent the median, the 25th, and 75th percentiles, and bars indicate the range of data distribution. Circles represent outliers, asterisks represent extreme data points

sensitivity greater than 75% in distinguishing subjects with AD vs HC (76.7% sensitivity and 77.8% specificity) while FS(p-tau₂₃₁ and MMSE) shows 90% sensitivity in differentiating subjects with AD vs HC, but

TABLE 2 Correlation between t-tau and p-tau₂₃₁

| | Total tau vs p-tau ₂₃₁ | |
|--------------|------------------------------------|--------------------------------------|
| | Pearson's correlation | Spearman's correlation |
| All patients | $r = 0.251, df = 185, P = 0.001^*$ | $r_s = 0.609, df = 185, P < 0.001^*$ |
| AD (146) | $r = 0.211, df = 144, P = 0.011$ | $r_s = 0.543, df = 144, P < 0.001^*$ |
| HC (18) | $r = 0.815, df = 16, P < 0.001^*$ | $r_s = 0.272, df = 16, P = 0.275$ |
| VaD (20) | $r = 0.781, df = 18, P < 0.001^*$ | $r_s = 0.772, df = 18, P < 0.001^*$ |

AD, Alzheimer's disease; HC, healthy controls; MMSE, Mini-Mental State Examination; VaD, vascular dementia.

* $P < 0.05$.

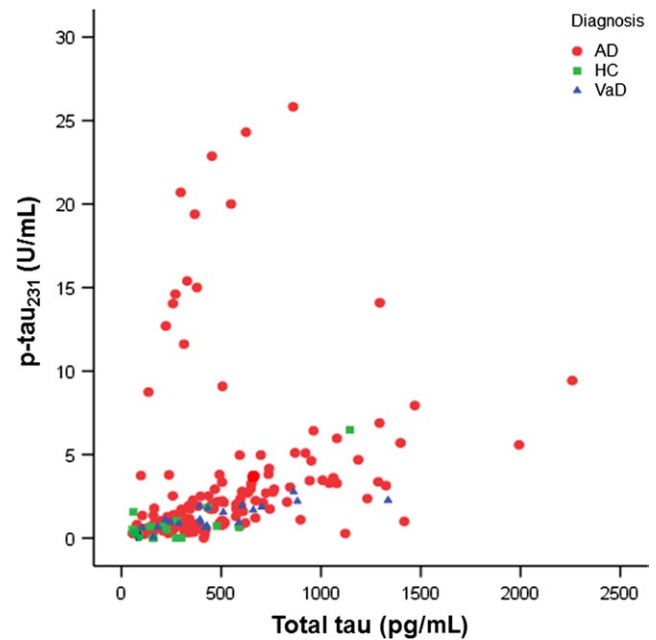


FIGURE 3 Correlation between t-tau and p-tau₂₃₁ levels

the specificity of this group is less than 75%. Similarly, p-tau₂₃₁ differentiates subjects for VaD vs HC with sensitivity 80% and specificity 77.8%, and FS(p-tau₂₃₁ and MMSE) shows sensitivity 80% and specificity 85%, which makes it an ideal candidate biomarker.

Vandermeeren and colleagues reported that t-tau was increased in patients with AD compared to HC, as confirmed by later studies.^{14,15} Levels of t-tau were shown to be a marker of neural damage, increased not only in AD, but also in other primary causes of dementia.¹⁶ In addition, subsequent investigations have confirmed that t-tau levels in CSF reflect the intensity of neuronal damage and degeneration. Therefore, t-tau levels can also be elevated in patients with vascular damage,¹⁷ whereas the highest values of the t-tau protein have been described

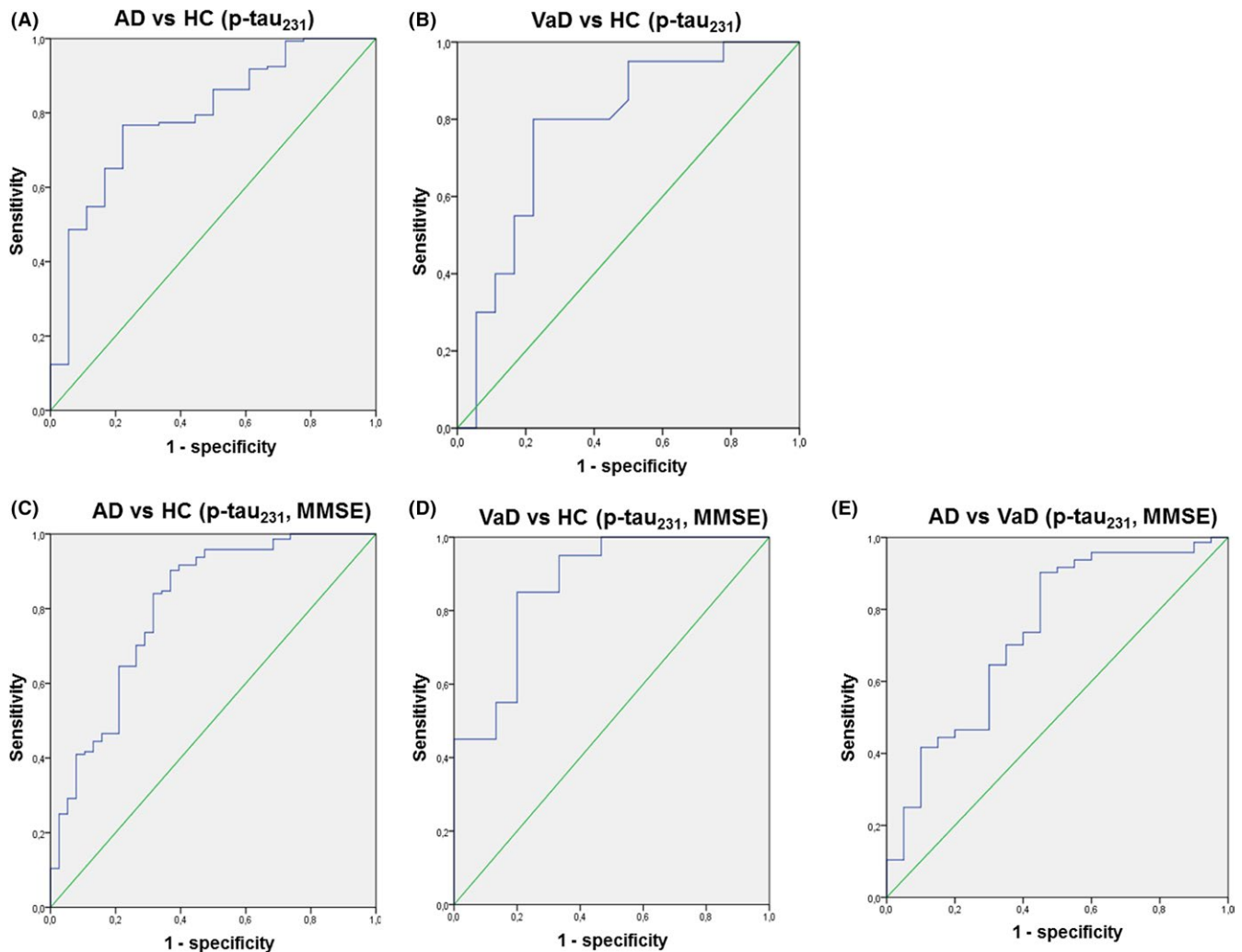


FIGURE 4 ROC curve analysis for p-tau₂₃₁ between A) Alzheimer's disease (AD) and healthy controls (HC), B) vascular dementia (VaD) and HC, and for FS (p-tau₂₃₁, MMSE) between C) AD and HC, D) VaD and HC, and E) AD vs VaD

| | AD vs HC | | VaD vs HC | | AD vs VaD |
|-----------------|-----------------------------|----------------------------------|-----------------------------|----------------------------------|----------------------------------|
| | p-tau ₂₃₁ (U/ml) | FS (p-tau ₂₃₁ , MMSE) | p-tau ₂₃₁ (U/ml) | FS (p-tau ₂₃₁ , MMSE) | FS (p-tau ₂₃₁ , MMSE) |
| Sensitivity (%) | 76.7 | 90.3 | 80 | 80 | 90.3 |
| Specificity (%) | 77.8 | 63.2 | 77.8 | 85 | 55 |
| Cut-off | 0.734 | 0.748 | 0.728 | 0.870 | 0.748 |
| P | <0.001* | <0.001* | <0.001* | <0.001* | 0.001* |

TABLE 3 Sensitivity, specificity, cut-off levels, and P-values for p-tau₂₃₁ and FS (p-tau₂₃₁, MMSE) among examined groups

AD, Alzheimer's disease; FS, factor score; HC, healthy controls; MMSE, Mini-Mental State Examination; VaD, vascular dementia.

*P < 0.05.

in Creutzfeldt-Jakob's disease due to a very intensive neuronal degeneration.¹⁸ In one study, the transient increase in CSF t-tau concentration in acute stroke positively correlated with the area of brain infarction, measured by computerized tomography.¹⁷ Schönknecht et al¹⁹ showed that t-tau levels were significantly higher in patients

with AD compared to VaD, as well as HC and patients suffering from major depression. Several other authors confirmed these results,²⁰⁻²² although other authors reported divergent data.^{23,24} Some factors may explain these differences. First, in acute stroke t-tau levels are increased in CSF, but the highest values have been observed only

3 weeks after stroke after which they gradually decreased over the next 3-5 months.¹⁷ Second, elevated levels of t-tau in VaD observed in some studies may mean that some patients may have experienced repeated strokes during the follow-up period, but without any clinical signs, especially if there had been a preexisting history of neurological signs without significant changes in HIS score. Because neuroimaging is more frequently performed than lumbar puncture for diagnosis of VaD, clinicians should consider repeating neuroimaging before lumbar puncture in VaD patients.

According to several previous investigations, p-tau₂₃₁ is a marker highly specific of AD.²⁵⁻²⁷ Thus, it can be detected very early in the CSF of patients with AD.²⁵ The p-tau₂₃₁ concentration alone has also shown a good correlation with the progression of the disease in a single patient with AD.^{25,26} Bürger and colleagues showed that p-tau₂₃₁ levels were elevated in subjects with MCI and also inversely correlated with their MMSE score.²⁷ Also, the data from Hampel and collaborators agree with the notion that variations in p-tau₂₃₁ levels reflect differences in the degree of neuronal damage across AD patients. Although the strength of the correlations presently suggests no sufficient clinical utility to individual patients, p-tau₂₃₁ levels may be used to predict progression of brain atrophy in AD.²⁸ Hampel and collaborators demonstrated the discriminatory power of p-tau (p-tau₂₃₁, p-tau₁₈₁, p-tau₁₉₉) in differentiating patients with AD from HC as well as other types of dementia including VaD.²⁹ Unfortunately, due to insufficient number of patients with VaD, the discriminatory (cut-off) levels of p-tau₂₃₁ for VaD could not be demonstrated in that study.²⁹ A longitudinal study of CSF tau protein in AD and VaD showed a statistically significant difference in the concentrations of t-tau and p-tau in AD (moderate to severe AD) compared to VaD, especially in the case of severe AD, but no significant differences in the levels of t-tau and p-tau₂₃₁ as MMSE scores after 6 months of monitoring compared to initial measurements.³⁰ It should be noted that our study revealed similar results, whereas our patients generally had milder dementia, as measured by MMSE scores, than the study of Wang and collaborators.³⁰

The present study took care to match subject groups as closely as possible for age, sex, and other demographic characteristics, so that confounding biases could be minimized. Nevertheless, HC were on average younger than subjects from the other groups, which could have influenced the outcome to some level. We also had a relatively fewer HC. The possible discrepancies could also result from differences in study design. To verify the reliability of our results, we therefore performed all CSF analyses with at least two internal sample controls.

Previous studies have shown that p-tau₂₃₁ CSF levels reliably differentiate patients with AD and HC.³¹ Our results confirm and extend these observations, as they indicate that p-tau₂₃₁ CSF levels can also be used for differentiating subjects with VaD from HC. Additionally, in distinguishing between AD and VaD, we established that FS (p-tau₂₃₁ and MMSE) has a strong potential to offer earlier distinction between these conditions. This finding has not only clinical significance, but also potential to prevent development and complications of VaD at an early stage.

ETHICAL APPROVAL

All procedures involving human subjects were in accord with the ethical approval from the Central Ethical Committee of the General Hospital Varaždin and with the approval of the Central Ethical Committee of the University of Zagreb Medical School, case no. 380-59/11-500-77/90.

CONFLICT OF INTERESTS

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; and no other relationships or activities that could appear to have influenced the submitted work.

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