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Predictive value of cerebrospinal fluid visinin-like protein-1 levels for Alzheimer's disease early detection and differential diagnosis in patients with mild cognitive impairment

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Running title:

Early diagnosis of AD using VILIP-1

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Abstract

Visinin-like protein 1 (VILIP-1) recently emerged as a potential biomarker of Alzheimer's disease (AD). This neuronal calcium sensor protein previously used as a marker of acute ischemic stroke is elevated in the cerebrospinal fluid (CSF) of AD patients. The goal of this study was to assess CSF VILIP-1 potential in early AD diagnosis and in differentiating MCI patients with and without risk of AD. Additionally, we tested VILIP-1 ability to differentiate AD from other primary causes of dementia, and predict the progression of AD-related cognitive decline. VILIP-1 levels were compared with five CSF AD biomarkers (t-tau, $A\beta_{1-42}$,p-tau_{181}, p-tau_{199}, and p-tau_{231}). VILIP-1 successfully differentiated two MCI patient groups characterized by absence or presence of pathological levels of these CSF biomarkers, except for t-tau.VILIP-1/$A\beta_{1-42}$and VILIP-1/p-tau_{181}ratios also differentiated MCI patients with pathological CSF biomarker levels. However, there was no difference in VILIP-1 levels

between AD and MCI patients. VILIP-1/Aβ_{1-42} and VILIP-1/p-tau$_{231}$ ratios reached high sensitivities (above 70%) and very high specificities (above 85%) in differentiating AD patients from HC. Additionally, VILIP-1 differentiated AD from patients with Lewy body disease with 77.1% sensitivity and 100% specificity. VILIP-1 potential as a prognostic biomarker of cognitive decline in AD was also proved since VILIP-1/t-tau, VILIP-1/p-tau$_{181}$and VILIP-1/p-tau$_{231}$ratios correlated with MMSE scores. These data indicate that VILIP-1 alone or in combination with other AD CSF biomarkers represent a valuable marker for the early diagnosis of AD, recognition of MCI patients at higher risk to develop dementia, and in differentiating AD from LBD.

Keywords: Visinin-like protein 1, dementia, biomarker, mild cognitive impairment, Alzheimer's disease, cerebrospinal fluid, early diagnosis.

Abbreviations

A$\beta_{1-42,}$ amyloid beta$_{1-42}$; AD, Alzheimer's disease; ApoE, apolipoprotein E; AUC, area under the curve; CDR, Clinical Dementia Rating; CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FTD, frontotemporal dementia; GWAS, genome-wide association studies; HC, healthy control; LBD, Lewy body disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NCS, neuronal calcium sensor; NFT, neurofibrillary tangles; PiB-PET, positron-emission tomography with [^{11}C]-labeled Pittsburgh Compound-B; PSP, progressive supranuclear palsy; p-tau$_{181}$, tau protein phosphorylated at threonine 181; p-tau$_{199}$, tau protein phosphorylated at serine 199; p-tau$_{231}$, tau protein phosphorylated at threonine 231; ROC, Receiver Operator Characteristic; SP, senile plaques; t-tau, total tau; VaD, vascular dementia; VILIP-1, visinin-like protein-1.

Introduction

A cure for Alzheimer's disease (AD), the most common cause of dementia, is still not available. Numerous potential disease-modifying therapies are currently in preclinical and clinical trials. It is crucial to initiate the treatment while the disease is in its early phase. The issue is to diagnose AD in asymptomatic individuals, in whom neurodegeneration is not yet advanced. This is complicated by the fact that the first AD symptoms occur in patients with mild cognitive impairment (MCI) of whom only 12% annually convert to AD [1]. For the proper administration of potential therapeutics, it is also important to differentiate AD from other primary causes of dementia like vascular dementia (VaD), Lewy body disease (LBD),

and frontotemporal dementia (FTD) [2-8]. Promising AD biomarkers, such as neuroimaging and cerebrospinal fluid (CSF) biomarkers, still cannot reliably detect the disease at preclinical stages [9-11]. However, the results of recent longitudinal studies on cognitively normal individuals have been encouraging: AD biomarkers that represent candidates for therapeutic trials were detected in asymptomatic individuals [12, 13].

Senile plaques (SP) and neurofibrillary tangles (NFT) are major neuropathological hallmarks of AD [14, 15]. SP and NFT are composed of amyloid β protein and hyperphosphorylated tau protein, respectively. Pathological processes in the brain are reflected in the CSF because of the molecular exchange that occurs at the brain/CSF barrier [16]. Three CSF biomarkers of AD - amyloid $\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$), total tau (t-tau), and tau protein phosphorylated at threonine 181 ($p\text{-}tau_{181}$) - are considered to reflect underlying pathological processes in the AD brain. Recently it was proposed that biomarkers of neuronal death would reflect disease progression better than markers of disease pathology ($A\beta_{1\text{-}42}$, t-tau, and p-tau) [17]. One of the proposed neuronal death biomarkers is visinin-like protein-1 (VILIP-1), a neuronal calcium sensor (NCS) protein.VILIP-1, VILIP-2, VILIP-3, neurocalcin δ, and hippocalcin constitute one of the five NCS proteins subfamilies, the visinin-like proteins [18, 19]. Braunewell proposed a mechanism by which Aβ deposition leads to dysregulation of calcium homeostasis in AD [20]. In this model, Aβ modulate the expression of NCS proteins so that calsenilin is upregulated, while VILIP-1 is downregulated [20]. Additionally, the neuroprotective calcium-buffer proteins calretinin and calbindin-D28K are downregulated in AD [21]. This imbalance of calcium sensor and buffer proteins ratio makes neurons more vulnerable to Aβ-induced calcium-mediated neurotoxicity, as Aβ induces the release of calcium from internal stores and enhances external calcium influx [20]. VILIP-1 overexpression in cellular models induces tau hyperphosphorylation [22]. Understanding calcium homeostasis disturbance in AD is important because changes in calcium signaling in AD precede neuronal loss, and may serve as a potential therapeutic target in AD [23]. In the brains of AD patients, VILIP-1 was detected near NFT and neuritic plaques in the temporal cortex and anterior cingulated cortex, and in dystrophic neuritis in the angular gyrus [22, 24]. VILIP-3 immunoreactivity was detected near NFT in the entorhinal cortex [24]. Also, recent genome-wide association studies (GWAS) showed an association of polymorphisms in VILIP-1 and VILIP-3 genes with the occurrence of AD [25, 26]. Altogether, these factors may contribute to neuronal cell death and to the increase of VILIP-1 in CSF of AD patients [12, 17, 27-30]. VILIP-1 levels were also increased in plasma of AD patients [27]. Recent studies reported the diagnostic potential of VILIP-1 in AD detection at early stages and in

monitoring of disease progression [17, 28]. However, whether VILIP-1 levels could discriminate MCI patients with higher risk of developing AD has not been investigated.

The scope of this cross-sectional study was to assess 1) CSF VILIP-1 diagnostic potential (detection of early AD in MCI patients); 2) VILIP-1 potential in differentiating MCI patients into two separate groups (with and without risk of AD); 3) the ability of VILIP-1 to differentiate AD from other primary causes of dementia; and 4) VILIP-1 potential as a prognostic marker of cognitive decline in AD.

Materials and methods

The patients included in this study were recruited at the University Hospital Centre, Zagreb and the General Hospital Vinkovci, Croatia. All procedures involving experiments on human subjects were done in accord 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 signed on 19th May 2011) and in accord with the Helsinki Declaration (World Medical Association, 2013). Demographic data of all patients and Mini-Mental State Examination (MMSE) scores are listed in **Table 1**. This study included altogether 195 patients of whom109 fulfilled NINCDS-ADRDA criteria for AD, 9 for VaD, 18 for FTD, 5 for LBD, and 45 for MCI [1, 31]. Nine healthy controls (HC) were also included in this study. Patients underwent MMSE, and neurological examination [32], complete blood tests including albumin, thyroid function, vitamin B_{12}, electrolytes, and VDRL test for syphilis. CSF was obtained by lumbar puncture between intervertebral spacesL3/L4 or L4/L5, always between 9 a.m. and 11 a.m. Samples were centrifuged for 10 minutes at 2,000 g, aliquoted, and stored at -80°C.

CSF $A\beta_{1-42}$, t-tau, and p-tau_{181} levels were determined using Innotest β-$amyloid_{1-42}$, Innotest hTau Ag, and Innotest Phospho-$Tau_{(181P)}$ ELISA (enzyme-linked immunosorbent assay) kits (Fujirebio, Gent, Belgium), respectively. P-tau_{199}, p-tau_{231}, and VILIP-1 CSF levels were measured using TAU [pS199] Phospho-ELISA Kit, Human (Life Technologies, Carlsbad, CA, USA), Tau [pT231] Phospho-ELISA Kit, Human (Life Technologies, Carlsbad, CA, USA), and VILIP-1 Human ELISA (BioVendor, Brno, Czech Republic), respectively.Each sample was analyzed in duplicate using all aforementioned ELISA kits. Measurements of each biomarker were performed on the same day using the same batch of reagents. Protein concentrations were calculated in GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) using a 4-parameter algorithm.The limit of detection of VILIP-1 Human ELISA (BioVendor, Brno, Czech Republic) kit is 27 pg/ml (as indicated in the manufacturer's protocol). All samples with VILIP-1 levels lower than blank, were assigned the value of 27 pg/ml since the limit of detection for BioVendor ELISA kit was set at 27 pg/ml. As the

BioVendor ELISA kit includes quality controls (including samples with VILIP-1 in high and low concentrations), it ensures that the kit is working properly.

CSF biomarker levels were compared using a Student's t-test between groups and were mutually correlated using Pearson's correlation. Before statistical analyses we tested data for normality using the Kolmogorov-*Smirnov test. If the* Kolmogorov-*Smirnov test did not support normality, we used* the Mann-Whitney U test and Spearman's correlation for pairwise comparisons and for correlations, respectively. Statistical analyses were performed using SPSS 19.0.1 (SPSS, Chicago, IL, USA), with the statistical significance set at □ = 0.05.

Results

VILIP-1 in early AD diagnosis and differentiation of AD from other primary causes of dementia

The levels of VILIP-1 measured in AD, MCI, VaD, FTD, LBD patients, and HC, as well as demographic data and MMSE scores are summarized in **Table 1**. **Figure 1** shows the CSF levels of VILIP-1 in these groups. CSF levels of $A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231}measured in AD, MCI, VaD, FTD, LBD patients, and HC are listed in **Table 2**.

VILIP-1 levels were significantly higher in AD patients compared to HC (t = 2.871, df = 116, p = 0.005). Also, CSF levels of VILIP-1 were higher in VaD (t = 2.757, df = 16, p = 0.014). No significant difference in VILIP-1 levels was found between LBD patients and HC (U = 20, Z = -0.341, p = 0.733) and FTD and HC patients (t = 1.958, df = 25, p=0.062).When patients with FTD, VaD, and LBD were grouped together as “other dementias” (**Figure 1**), no significant difference in CSF levels of VILIP-1 between HC and “other dementias” group was obtained (t = 1.918, df = 39, p = 0.062).

There was no difference in VILIP-1 CSF levels between AD patients and either VaD (t= -0.337, df = 116, p = 0.737) or FTD (t = 1.449, df = 125, p = 0.150) patients. VILIP-1 levels were lower in LBD than in AD patients (t = 2.869, df = 112, p = 0.005; **Figure 1**). No significant difference in VILIP-1 levels was observed when comparing AD patients to the other dementias group (t = 1.736, df = 139, p = 0.085; **Figure 1**). Using VILIP-1 as a biomarker, AD patients were differentiated from VaD patients with 86.2% sensitivity and 22.2% specificity (area under the curve - AUC = 0.456, p = 0.659), from FTD patients with 66.1% sensitivity and 55.6% specificity (AUC = 0.592, p = 0.213), and from LBD patients with 77.1% sensitivity and 100% specificity (AUC = 0.839, p = 0.01).

The CSF concentration of VILIP-1 was significantly higher in AD patients than in MCI patients ($t = 3.271$, $df = 150$, $p = 0.001$), while there was no difference in VILIP-1 levels between MCI and HC cases ($t = 1.190$, $df = 50$, $p = 0.240$; **Figure 1**). No difference in VILIP-1 levels was found when comparing MCI to FTD patients ($t = -0.853$, $df = 59$, $p = 0.397$), MCI and LBD patients ($U = 86.5$, $Z = -0.718$, $p = 0.473$), and MCI patients with FTD, VaD, and LBD patients grouped together ($t = -1.046$, $df = 73$, $p = 0.299$). VILIP-1 concentration was higher in VaD than in MCI patients ($t = -2.063$, $df = 50$, $p = 0.044$; **Figure 1**).

A cut-off level of 116.3 pg/ml for VILIP-1 was determined by Receiver Operator Characteristic (ROC) curve analysis. AD patients were differentiated from HC by 62.4% sensitivity and 88.9% specificity (AUC = 0.811, $p = 0.002$; **Figure 2A**). Fifteen of 43 MCI patients had VILIP-1 levels higher than the cut-off value. The VILIP-1/$A\beta_{1-42}$ ratio differentiated AD patients from HC with high sensitivity and specificity (82% sensitivity and 87.5% specificity, AUC = 0.816, $p = 0.004$, with VILIP-1/$A\beta_{1-42}$ cut-off set at 0.0877), as well as VILIP-1/p-tau_{199} ratio (96% sensitivity and 40% specificity, AUC = 0.600, $p = 0.464$, with VILIP-1/p-tau_{199} cut-off set at 12.71) and VILIP-1/p-tau_{231} ratio (72.4% sensitivity and 100% specificity, AUC = 0.772, $p = 0.055$, with VILIP-1/p-tau_{231} cut-off set at 63.53). VILIP-1/p-tau_{181} and VILIP-1/t-tau ratios showed lower efficiency in differentiating AD patients and HC. VILIP-1/p-tau_{181} ratio differentiated AD patients and HC with 46.6% sensitivity and 66.7% specificity (AUC = 0.444, $p = 0.583$) with cut-off set at 1.8022, while VILIP-1/t-tau ratio showed 76% sensitivity and 22.2% specificity (AUC = 0.424, $p = 0.456$) in differentiating AD patients from HC with cut-off set at 0.1743 (**Figure 2B-F**).

VILIP-1 as an index of cognitive decline progression in AD

AD patients were divided into three groups according to MMSE scores:1) mild AD (MMSE 20 - 25), 2) moderate AD (MMSE 10 - 19), and 3) severe AD (MMSE < 10). As only one AD patient had a MMSE score below 10, only patients with mild and moderate AD were analyzed. There was no significant difference ($t = 1.890$, $df = 106$, $p = 0.061$) in VILIP-1 levels between patients with mild (161.5 ± 85.3 pg/ml) and moderate AD (130.6± 81 pg/ml; **Figure 3**). Levels of VILIP-1 did not correlate with MMSE scores in the mixed group of AD and MCI patients and HC ($r_S = -0.053$, $df = 159$, $p = 0.502$). There was a negative correlation between VILIP-1/$A\beta_{1-42}$ ratio and MMSE scores in the mixed group of AD, MCI, and HC cases ($r_S = -0.279$, $df = 97$, $p = 0.005$; **Figure 4A**). There was a positive correlation between MMSE scores and VILIP-1/t-tau ($r_S = 0.285$, $df = 116$, $p = 0.002$; **Figure 4B**), VILIP-1/p-tau_{181} ($r_S = 0.185$, $df = 112$, $p = 0.049$; **Figure 4C**), and VILIP-1/p-tau_{231} ($r_S = 0.278$, $df = 53$,

p = 0.040; **Figure 4D**) ratios in the mixed group of AD and MCI patients and HC. There was no significant correlation between MMSE scores and VILIP-1/p-tau$_{199}$ ratio (r_S= 0.148, df = 78, p = 0.189)

VILIP-1 in differentiation of MCI patients with increased risk of AD

MCI patients were divided into two groups according to the levels of CSF biomarkers of AD (t-tau, Aβ$_{1-42}$, p-tau$_{181}$,p-tau$_{199}$, and p-tau$_{231}$). Cut-off levels were set at 450 pg/ml (t-tau), 500 pg/ml (Aβ$_{1-42}$) and 60 pg/ml (p-tau$_{181}$) according to Humpel [33]. A cut-off level of 2.58 pg/ml for p-tau$_{199}$ biomarker was determined on a cohort of 36 AD patients and 15 HC, while p-tau$_{231}$ cut-off level of 1.281 U/ml was determined on a cohort of 34 AD patients and 17 HC. In order to establish the best possible cut-off values, we wanted to collect as much samples as possible. Therefore, the number of AD and HC samples with determined p-tau$_{199}$ or p-tau$_{231}$ and VILIP-1 levels slightly differed (**Table 2**). Because not all MCI patients with determined VILIP-1 levels had all five biomarkers measured, 14, 10, 12, 19 and 22 MCI patients in the case Aβ$_{1-42}$, t-tau, p-tau$_{181}$,p-tau$_{199}$, and p-tau$_{231}$were excluded, respectively. MMSE scores and demographic data of MCI patients included in this part of analysis are listed in **Table 3**.

VILIP-1 CSF levels were significantly higher in MCI patients with Aβ$_{1-42}$ CSF levels lower than the 500 pg/ml cut-off value (8 of 29 MCI patients; t = 2.355, df = 27, p = 0.026; **Figure 5A**). There was no difference in VILIP-1 levels between the two groups of MCI patients with normal and pathological levels of t-tau (above 450 pg/ml; t = 1.577, df = 32, p = 0.125; **Figure 5B**). By taking into account that t-tau levels differ according to the age of patients, a cut-off level of 450 pg/ml was taken in view of the average age of the MCI patients (67 years), as based on Humpel's recommendations [33]. VILIP-1 levels were significantly higher in MCI patients (13 of 32 MCI patients) with pathological levels of p-tau$_{181}$ (above 60 pg/ml, U = 41, Z = -3.252, p =0.001; **Figure 5C**). VILIP-1 concentration was also significantly higher in MCI patients (8 of 21 MCI patients) with pathological levels of p-tau$_{231}$ (above 1.281 U/ml; t = 5.839, df = 19, p < 0.001; **Figure 5E**). Although statistical significance in the levels of VILIP-1between MCI patients with normal and pathological levels of p-tau$_{199}$ was not proved, a trend of VILIP-1 increase in the MCI group with pathological levels of p-tau$_{199}$was observed (above 2.58 pg/ml, U = 45, Z = -1.838, p = 0.066; **Figure 5D**).

A negative correlation was observed between VILIP-1 and Aβ$_{1-42}$ CSF levels (**Figure 6A; Table 4**). The CSF VILIP-1 levels positively correlated with CSF t-tau (**Figure 6B; Table 4**), p-tau$_{181}$ (**Figure 6C; Table 4**), p-tau$_{199}$ (**Figure 6D; Table 4**) and p-tau$_{231}$ (**Figure**

6E; Table 4). A correlation between VILIP-1 and AD protein biomarkers was determined across the mixed group of AD, MCI and HC cases (**Figure 6A-E; Table 4**). Additionally, a positive correlation between VILIP-1 and t-tau, p-tau$_{181}$, p-tau$_{199}$, and p-tau$_{231}$levels was also observed in AD and MCI patients (**Table 4**).

The ratios of VILIP-1 to all five biomarkers were also compared between the two groups of MCI patients. VILIP-1/Aβ_{1-42} ratio was significantly higher in MCI patients with pathological levels of p-tau$_{231}$ (**Figure 7A; Table 5).**The MCI group with pathological levels of p-tau$_{231}$ had significantly higher VILIP-1/p-tau$_{181}$ ratio (**Figure 7D; Table 5**). Although statistical significance in the VILIP-1/Aβ_{1-42} ratio between MCI groups with pathological levels of p-tau$_{199}$ and p-tau$_{181}$ was not proved (**Table 5**), there was a trend of VILIP-1/Aβ_{1-42} ratio increase in the MCI groups with pathological levels of these biomarkers (**Figure 7B, C**). The same trend was observed for the VILIP-1/p-tau$_{181}$ ratio in MCI group with pathological p-tau$_{199}$ levels (**Figure 7E**) and VILIP-1/p-tau$_{199}$ ratio in MCI group with pathological p-tau$_{231}$ levels (**Figure 7F**; **Table 5**). On the contrary, VILIP-1/p-tau$_{231}$ ratio increase in MCI group with normal levels of A$\beta_{1-42}$was also observed (**Table 5**).

Discussion

In this study we used CSF VILIP-1 levels to differentiate MCI patients into two groups: with and without the risk of AD. AD risk groups within the MCI groups of patients were defined by the levels of five CSF AD biomarkers ($A\beta_{1\text{-}42}$, t-tau, p-tau_{181},p-tau_{199}, and p-tau_{231}). We tested VILIP-1 effectiveness in early detection of AD in MCI patients, and confirmed previous observations that VILIP-1 is a useful biomarker in early AD diagnosis [12, 17, 27-30]. Additionally, we tested VILIP-1 potential in differentiating AD from other primary causes of dementia, and its ability in predicting disease progression. Our results show that the levels of VILIP-1 were higher in AD compared to HC and MCI patients. No difference in VILIP-1 levels was seen between AD and patients with other dementias. This contrasts with the results of Tarawneh et al. [27] who reportedVILIP-1 levels to be prognostic of future cognitive decline in early symptomatic AD (Clinical Dementia Rating, CDR 0.5) or mild AD (CDR 1) in comparison to non-AD dementia cases. The non-AD dementia group from that study was a small cohort of 19 individuals either with FTD, PSP, or LBD [27]. Our "other dementia" group consisted of 32 individuals, of whom 9 presented with VaD, 18 with FTD, and 5 with LBD. There was no significant difference in VILIP-1 levels between AD and either VaD or FTD. VILIP-1 levels were significantly lower in LBD compared to AD. This finding agrees with the study of Luo et al. (2013) in whichVILIP-1 levels and VILIP-1/$A\beta_{1\text{–}42}$ ratios were higher in AD compared to LBD [29]. Altogether, these results indicate that VILIP-1 specificity in differentiating AD from other primary causes of dementia is low for VaD and marginal for FTD, whereas it does differentiate AD from LBD with 77.1% sensitivity and 100% specificity.

We tested whether there was a correlation between VILIP-1 and other CSF AD biomarkers. VILIP-1 levels negatively correlated with $A\beta_{1\text{–}42}$ levels in a mixed group of AD, MCI, and HC cases. This was not observed in two previous studies [17, 29]. However, the neuroimaging study of Tarawneh et al. [28] showed that VILIP-1 levels and VILIP-1/$A\beta_{1\text{–}42}$ ratios correlate with amyloid load in the brain as detected by Pittsburgh Compound-B [28]. We did not observe VILIP-1 and $A\beta_{1\text{–}42}$ correlation in both AD and MCI patients. Four biomarkers (t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231}) positively correlated with VILIP-1 across AD, MCI, and HC cases and also in separate groups of AD and MCI patients. Because the HC group was significantly younger than the AD and MCI patients, it was also important to demonstrate a correlation of VILIP-1 with AD biomarkers within AD and MCI patients

separately. The VILIP-1 correlation with $A\beta_{1–42}$, t-tau and p-tau_{181} agrees with previous data [17, 29, 30]. Surprisingly, there was no significant difference in VILIP-1 levels between patients with mild and moderate AD. Other authors however reported that VILIP-1 levels and VILIP-1/$A\beta_{1-42}$ratio predict cognitive decline in cognitively healthy individuals and AD patients [27, 28]. Both parameters predicted conversion of CDR 0 HC group to CDR 0.5 or higher [27]. Also, VILIP-1 levels and VILIP-1/$A\beta_{1-42}$ ratio predicted AD patients likely to cognitively decline more rapidly over the follow-up period of 2.6 years [28]. It should be taken into account that different neuropsychological tests were used in our and the studies by Tarawaneh and colleagues [27, 28]. VILIP-1 levels did not correlate with MMSE scores, but the VILIP-1/$A\beta_{1-42}$ratio negatively correlated with MMSE scores. Additionally, VILIP-1/t-tau, VILIP-1/p-tau_{181} and VILIP-1/p-tau_{231} ratios positively correlated with MMSE scores. Thus, our results indicate that possibly in the combination with other biomarkers, VILIP-1 could be used as a prognostic biomarker of cognitive decline in AD. A recent longitudinal study strengthened the notion of VILIP-1 being a prognostic biomarker of AD, and reported that VILIP-1 levels increase over time in MCI patients by as much as 10.7 pg/ml per year [34].

The cut-off level for VILIP-1 of 116.3 pg/ml set in this study was lower than the cut-off values of other studies (535 pg/ml [27], 560 pg/ml ([28] and 365 pg/ml [17]). Sutphen et al. also reported the mean value for VILIP-1 to be around 150 pg/ml in healthy middle-aged individuals [12]. The cut-off value of biomarker depends on the ELISA kit used. For example, in our previous study we reported that ELISA kits for $A\beta_{1–42}$ and t-tau from two different vendors cannot be used interchangeably [35]. A microplate-based immunoassay (Erenna, Singulex, CA) was used in three studies [12, 27, 28], while one study [17] used an in-house VILIP-1 ELISA [36]. In the present study we used a VILIP-1 Human ELISA kit (BioVendor). Two recent studies in which this kit was also used reported mean VILIP-1 values of 105 pg/ml, 69 pg/ml, and 42 pg/ml in AD, MCI, and HC, respectively [30], and 72.1 pg/ml and 43 pg/ml in AD and HC, respectively [29], which corresponds to our results.

We compared VILIP-1 levels between two groups of MCI patients with and without pathological levels of five CSF biomarkers ($A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231}). VILIP-1 levels were significantly higher in MCI patients with pathological levels of $A\beta_{1-42}$, p-tau_{181}, and p-tau_{231}. There was no difference in VILIP-1 levels between MCI groups based on levels of p-tau_{199} and t-tau, although there was a trend of VILIP-1 increase in the MCI group with pathological p-tau_{199} levels. Additionally, we compared VILIP-1/$A\beta_{1-42}$, VILIP-1/t-tau, VILIP-1/p-tau_{181}, VILIP-1/p-tau_{199}, and VILIP-1/p-tau_{231} ratios between the MCI groups.

VILIP-1/Aβ_{1-42} and VILIP-1/p-tau$_{181}$ ratios were significantly higher in the MCI groups with pathological levels of p-tau$_{231}$. Finally, there was a trend of increase in the VILIP-1/Aβ_{1-42} ratio between MCI groups with pathological levels of p-tau$_{199}$ and p-tau$_{181}$ and VILIP-1/p-tau$_{181}$ ratio in MCI group with pathological p-tau$_{199}$ levels. Our results indicate that VILIP-1 levels alone or in combination with Aβ_{1-42}, t-tau, and p-tau$_{199}$ (VILIP-1/Aβ_{1-42}, VILIP-1/t-tau, VILIP-1/p-tau$_{199}$) could be used as early biomarkers of AD. However, we recommend combination of VILIP-1 with other CSF biomarkers rather than use of VILIP-1 alone.We could discriminate two groups of MCI patients by CSF VILIP-1 levels, whose risk for AD was defined by their levels of Aβ_{1-42}, t-tau, p-tau$_{181}$, p-tau$_{199}$, and p-tau$_{231}$. It must be kept in mind that MCI patients whose CSF samples were analyzed in this cross-sectional study should be monitored and these results should be further validated on a larger cohort of MCI patients with regular follow-up every 6 months.

Besides the fact that this study is cross-sectional, the other limitation of this study is that our HC group is significantly younger than the AD group. The longitudinal study of Sutphen et al. showed that VILIP-1 levels increase with age in healthy individuals, with significantly higher levels in late middle age (65-74 years of age) compared with early (45-54) and mid middle age (55-64 years) in the ε4 allele of apolipoprotein E (APOE) gene non-carriers and within-person increases over time in late middle age [12]. While baseline levels of VILIP-1 in the at-risk ε4 carriers at baseline were not significantly different among those age groups, they significantly increased longitudinally within individuals in mid middle age compared with the ε4 non-carriers in late middle age [12]. At the same time, the annual mean increase in VILIP-1 levels in mid middle age was greater in ε4 carriers compared with ε4 non-carriers [12]. These and previous results suggest that VILIP-1 should be considered as marker of neurodegeneration or neuronal death, comparably to t-tau. For example, major increases in t-tau levels have been detected in diseases characterized by extensive neuronal damage, such as Creutzfeldt-Jakob disease [37, 38] or stroke [39]. Similarly to t-tau, before being considered an AD biomarker, VILIP-1 was used as a marker of acute ischemic stroke [36]. Thus, in this study VILIP-1 levels were normalized as a ratio to CSF biomarkers that do not fluctuate with the age of a patient (Aβ_{1-42}, p-tau$_{181}$, p-tau$_{199}$, and p-tau$_{231}$) [33]. The VILIP-1/t-tau ratio was also determined. ROC curve analysis showed that VILIP-1/Aβ_{1-42} and VILIP-1/p-tau$_{231}$ ratios reached the highest values of sensitivity and specificity in differentiating AD patients from HC followed by VILIP-1/p-tau$_{199}$ratio that had higher sensitivity, but lower specificity thanVILIP-1 alone. Sensitivities and specificities of VILIP-1 and VILIP-1/Aβ_{1-42} ratio observed in this study are high, yet Luo et al. reported even higher values of sensitivity

and specificity (78.7% and 87.5% for VILIP-1, 98.4% and 97.5% for VILIP-1/$A\beta_{1-42}$) [29]. In this respect, our results are more similar to those obtained by Lee et al. where 67.9% sensitivity and 90.5% specificity was reached in distinguishing AD patients from HC [17]. Tarawneh et al. showed that VILIP-1 and VILIP-1/$A\beta_{1-42}$ ratio differentiated AD group from HC with 0.75 AUC (our study 0.832 AUC) and 0.87 AUC (our study 0.820 AUC), respectively [27].

The results of the present study indicate that VILIP-1 may represent a valuable biomarker in addition to $A\beta_{1–42}$, t-tau and p-tau_{181}in the early diagnosis of AD and in differentiating MCI patients with higher risk of progression to AD. In spite of a relatively small cohort of patients, comparison of VILIP-1 levels to two other potential AD biomarkers (p-tau_{199} and p-tau_{231}) further supports the use of VILIP-1 as a reliable early AD biomarker. We also compared VILIP-1 levels between two MCI groups of patients with and without pathological CSF levels of $A\beta_{1–42}$, t-tau, p-tau_{181}, p-tau_{199} and p-tau_{231}. CSF biomarker levels were used in the definition of AD risk groups among these MCI patients. VILIP-1 levels successfully differentiated two MCI groups of patients with normal and pathological levels of all CSF biomarkers, except for p-tau_{199} and t-tau (although there was a trend of VILIP-1 increase in the MCI group with pathological p-tau_{199} levels). VILIP-1 in combination with $A\beta_{1-42}$ and p-tau_{181} (VILIP-1/$A\beta_{1-42}$, VILIP-1/p-tau_{181} ratios) also differentiates MCI patients with normal and pathological levels of CSF biomarkers. Future studies will provide further validation of VILIP-1 diagnostic potential by longitudinal follow-up of MCI patients and development of prediction models for MCI progression in AD based on VILIP-1, $A\beta_{1–42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231}.

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Table 1. CSF levels of VILIP-1 measured by ELISA, demographic data, and MMSE scores of patient groups. Data are presented as mean ± SD and for VILIP-1 levels also as median (25th-75th percentile). AD – Alzheimer's disease, MCI – mild cognitive impairment, HC – healthy control, VaD – vascular dementia, FTD – frontotemporal dementia, LBD –Lewy body disease, MMSE – Mini-Mental State Examination.

Group	**VILIP-1 (pg/ml)**		**Age**	**Gender**	**MMSE**
(number of patients)	Mean ± SD	Median (25-75th percentile)	Mean ± SD	Women vs. Men	Mean ± SD
AD (109)	147.9 ± 84.8	145.5 (86.4 – 200.7)	72 ± 8.2[a]	60 vs. 49	20.1 ± 4.6
MCI (43)	98.7 ± 80.3	77.3 (27 – 137.5)	68 ± 9.8[b]	21 vs. 22	24.9 ± 2.9
HC (9)	65.5 ± 49.3	27 (27– 112.4)	50 ± 9.8	6 vs. 3	27.7 ± 1.7
Other dementias (32)	118.1 ± 77.5	99.9 (48.5 – 181.2)	65 ± 10.1[c]	11 vs. 21	19.0 ± 5.8
VaD (9)	157.9± 87.6	167.3 (70.1 – 221.8)	73 ± 8.6	2 vs. 7	22.4 ± 6.2
FTD (18)	117.4 ± 71.0	106.5 (57.7 – 177)	60 ± 10.6[d]	8 vs. 10	17.2 ± 5.3
LBD (5)	49 ± 16.2	48 (33.3 – 65.2)	71 ± 4.4	1 vs. 4	19.6 ± 4.7

[a]Age significantly different from HC group ($t = 7.857$, $df = 116$, $p < 0.001$).
[b]Age significantly different from HC group ($t = -5.002$, $df = 50$, $p < 0.001$).
[c]Age significantly different from HC group ($t = -3.820$, $df = 39$, $p < 0.001$).
[d]Age significantly different from HC group ($t = -2.410$, $df = 25$, $p = 0.024$).

Table 2. CSF levels of $A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231} measured by ELISA. Data are presented as mean ± SD and as median (25th-75th percentile). AD – Alzheimer's disease, MCI – mild cognitive impairment, HC – healthy control, VaD – vascular dementia, FTD – frontotemporal dementia, LBD –Lewy body disease.
*Not all patients with determined VILIP-1 levels had determined all other CSF biomarkers ($A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199} and p-tau_{231}).

Group (number of patients with determined VILIP-1 levels)*	**$A\beta_{1-42}$ (pg/ml)**		**t-tau (pg/ml)**		**p-tau_{181} (pg/ml)**		**p-tau_{199} (pg/ml)**		**p-tau_{231} (U/ml)**	
	Mean ± SD (number of patients)	Median (25-75th percentile)	Mean ± SD (number of patients)	Median (25-75th percentile)	Mean ± SD (number of patients)	Median (25-75th percentile)	Mean ± SD (number of patients)	Median (25-75th percentile)	Mean ± SD (number of patients)	Median (25-75th percentile)
AD (109)	601.5 ± 308.1 (**61**)	591 (353.5 – 792.5)	482.2 ± 422.8 (**75**)	399 (232 – 604)	79.2 ± 42.6 (**73**)	69 (56 – 95.5)	3.860 ± 3.129 (**50**)	3.080 (1.295 – 5.625)	6.955 ± 7.324 (**29**)	3.347 (1.346 – 12.825)
MCI (43)	761 ± 365.9 (**30**)	721.5 (478.8 – 1046.5)	255.4 ± 174.4 (**34**)	210.5 (145.5 – 332.3)	61.6 ± 35.1 (**32**)	52.1 (38.1 – 71.9)	2.695 ± 1.753 (**25**)	2.500 (1.245 – 4.600)	3.070 ± 4.661 (**21**)	0.910 (0.431 – 2.760)
HC (9)	922.6 ± 397 (**8**)	963.5 (616.3 – 1277.5)	160.4 ± 98.1 (**9**)	157 (72.5 – 264.5)	33.5 ± 23.2 (**9**)	18 (15.9 – 57.3)	1.698 ± 1.002 (**5**)	1.660 (0.750 – 2.665)	0.606 ± 0.539 (**5**)	0.360 (0.344 – 0.992)
VaD (9)	NA	NA	590 ± 333.3 (**4**)	630.5 (263.8 – 876.7)	86.4 ± 33.8 (**4**)	86.1 (53.8 – 119.3)	NA	NA	NA	NA
FTD (18)	598 ± 243.2 (**2**)	598 (426 – 770)	526.8 ± 311.7 (**14**)	463.7 (234.7 – 790.8)	77.9 ± 33.2 (**15**)	80.3 (42.6 – 109.4)	3.151 ± 3.062 (**6**)	2.745 (0.157 – 6.277)	4.788 ± 4.067 (**3**)	3.090 (1.846 – 9.43)
LBD (5)	NA	NA	114.1 ± 28.3 (**4**)	115.7 (86.1 – 140.5)	49.4 ± 37.7 (**4**)	33.1 (27.3 – 87.9)	NA	NA	NA	NA

Table 3. MMSE scores and demographic data of AD, MCI and HC patients with determined levels of VILIP-1 and one of the five biomarkers ($A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199} or p-tau_{231}). Data are presented as mean ± SD.

		Age	**Gender**	**MMSE**
		Mean ± SD	**Women vs. Men**	**Mean ± SD**
$A\beta_{1-42}$ and VILIP-1	AD (61)	72 ± 7.2	32 vs. 29	19.2 ± 4.5
	MCI (29)	67 ± 11.1	14 vs. 15	24.4 ± 3.2
	HC (8)	48 ± 9.2	5 vs. 3	27.4 ± 1.6
t-tau and VILIP-1	AD (75)	73 ± 7.7	37 vs. 38	19.3 ± 4.4
	MCI (34)	68 ± 10.3	16 vs. 18	24.5 ± 3.0
	HC (9)	50 ± 9.8	6 vs. 3	27.7 ± 1.7
p-tau_{181} and VILIP-1	AD (73)	72 ± 8.1	37 vs. 36	19.3 ± 4.8
	MCI (32)	67 ± 10.5	15 vs. 17	24.7 ± 2.9
	HC (9)	50 ± 9.8	6 vs. 3	27.7 ± 1.7
p-tau_{199} and VILIP-1	AD (50)	73 ± 6.5	27 vs. 23	19.2 ± 4.6
	MCI (25)	67 ± 11.5	14 vs. 11	24.5 ± 3.1
	HC (5)	44 ± 19.1	4 vs. 1	27 ± 1.9
p-tau_{231} and VILIP-1	AD (29)	72 ± 7.3	18 vs. 11	18.7 ± 4.2
	MCI (21)	69 ± 11.5	11 vs. 10	24.4 ± 3.0
	HC (5)	44 ± 10.1	4 vs. 1	27.0 ± 1.9

Table 4. Correlations between VILIP-1 and five biomarkers across the mixed group of AD, MCI, and HC cases, and in AD and MCI patients.

	Correlation between VILIP-1 and CSF biomarkers	
$A\beta_{1-42}$ and VILIP-1	AD (61), MCI (29) and HC (8) AD (61) MCI (29)	(r=-0.225,df=96, p=0.026) (r=-0.086,df=59, p=0.510) (r=-0.317,df=27, p=0.093)
t-tau and VILIP-1	AD (75), MCI (34) and HC (9) AD (75) MCI (34)	(r_S=0.419,df=116, p<0.001)* (r_S =0.363,df=73, p=0.001)* (r=0.338,df=32, p=0.051)
p-tau_{181} and VILIP-1	AD (73), MCI (32) and HC (9) AD (73) MCI (32)	(r_S=0.679,df=112, p<0.001)* (r=0.554,df=71, p<0.001) (r=0.688,df=30, p<0.001)
p-tau_{199} and VILIP-1	AD (50), MCI (25) and HC (5) AD (50) MCI (25)	(r_S =0.514,df=78, p<0.001)* (r=0.683,df=48, p<0.001) (r=0.489,df=23, p=0.013)
p-tau_{231} and VILIP-1	AD (29), MCI (21) and HC (5) AD (29) MCI (21)	(r_S =0.712,df=53, p<0.001)* (r_S =0.492,df=27, p=0.007)* (r_S=0.788,df=19, p<0.001)*

*Groups without a normal distribution were correlated by Spearman's correlation.

Table 5. Comparison of VILIP-1 and $A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231} ratios between MCI patients with normal and pathological levels of five biomarkers ($A\beta_{1-42}$, t-tau, p-tau_{181}, p-tau_{199}, and p-tau_{231}).

MCI group	**VILIP-1/$A\beta_{1-42}$**	**VILIP1/t-tau**	**VILIP-1/p-tau_{181}**	**VILIP-1/p-tau_{199}**	**VILIP-1/p-tau_{231}**
$A\beta_{1-42}$		(U=54, Z=-1.464, p=0.143)	(U=54, Z=-0.525, p=0.600)	(U = 35, Z=-0.889, p=0.374)	(U=9, Z=-2.803, p=0.005)*
t-tau	(U=20, Z=-0.385, p=0.700)		(U=58, Z=-0.493, p=0.622)	(U=26, Z=-0.585, p=0.558)	(U=3, Z=-1.156, p=0.248)
p-tau_{181}	(U=48, Z=-1.858, p=0.063)	(U=93, Z=-1.170, p=0.242)		(U=61, Z=-0.248, p= 0.804)	(U=26, Z=-1.486, p=0.137)
p-tau_{199}	(U=44, Z=-1.593, p=0.111)	(U=51, Z=-1.469, p=0.142)	(U=42, Z=-1.426, p=0.154)		(U=37, Z=-0.983, p=0.326)
p-tau_{231}	(U=3, Z=-3.549, p<0.001)*	(U=51, Z=-0.072, p=0.942	(U=12, Z=-2.368, p = 0.018)*	(U=21, Z=-1.941, p=0.052)	

*Statistically significant at $p<0.05$.

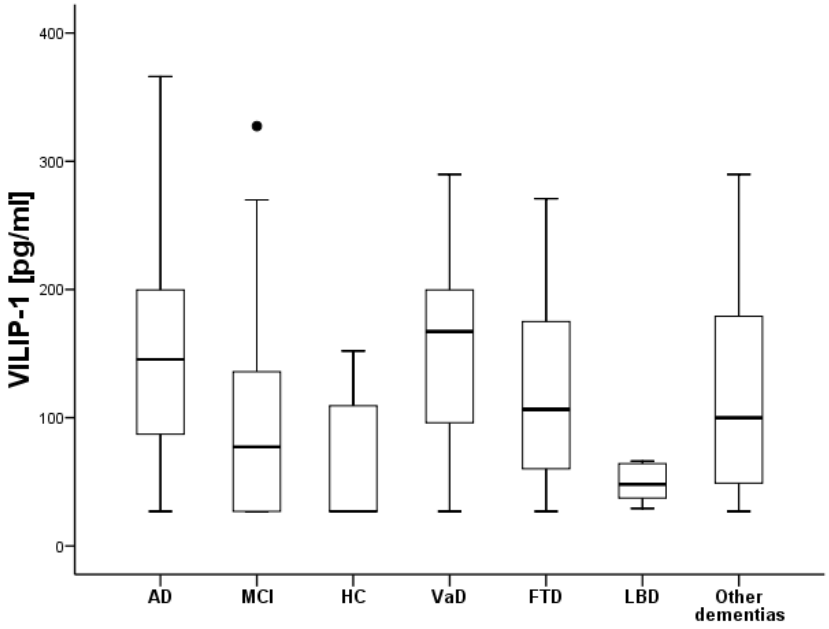

Figure1. CSF VILIP-1 levels in AD, MCI, HC, FTD, and LBD patients, measured by ELISA. Patients with FTD, VaD, and LBD are also grouped as other dementias. Boxes represent the median, the 25th and 75th percentiles, and bars indicate the range of data distribution.

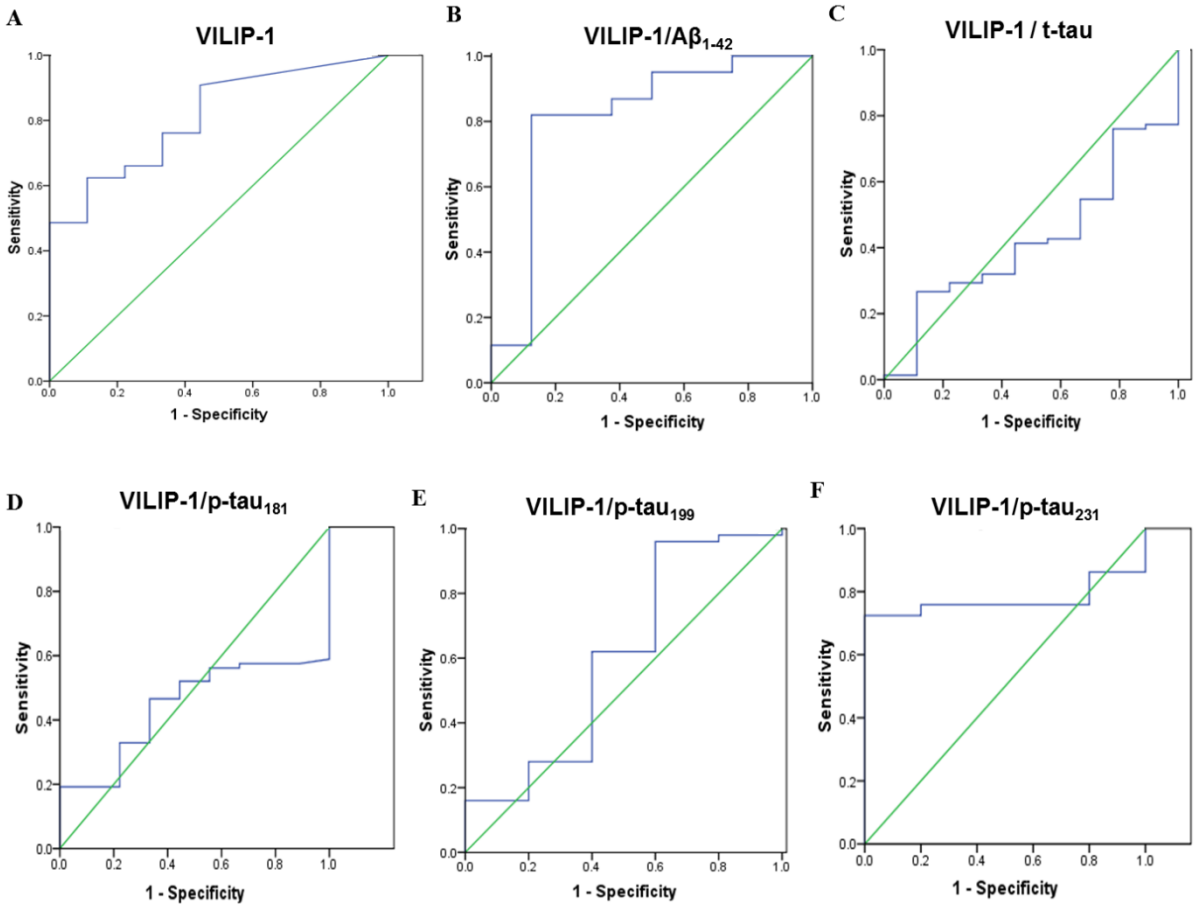

Figure 2. Receiver Operator Characteristic (ROC) curve analysis for A) VILIP-1, B) VILIP-1/$A\beta_{1-42}$ ratio, C) VILIP-1/t-tau ratio, D) VILIP-1/p-tau_{181} ratio, E) VILIP-1/p-tau_{199} ratio, and F) VILIP-1/p-tau_{231} ratio.

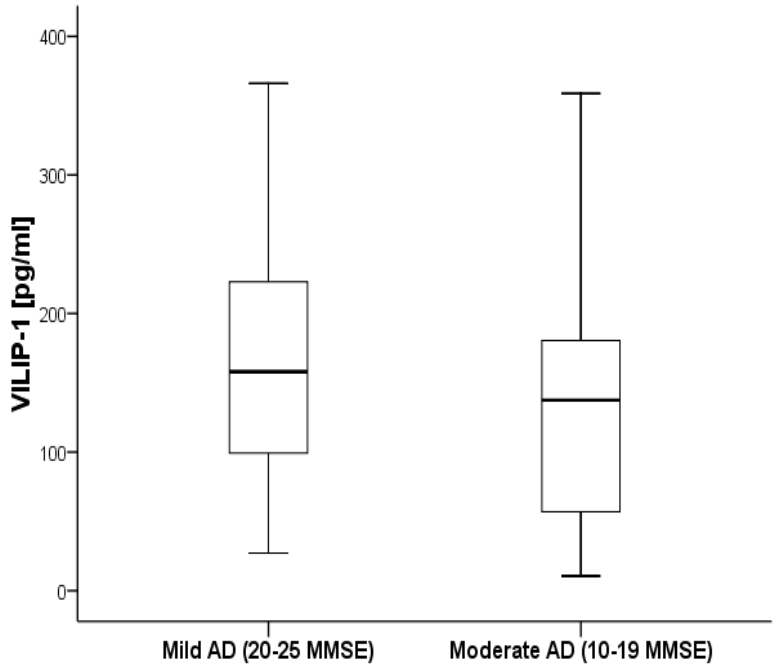

Figure 3. CSF VILIP-1 levels in patients with mild (161.5 ± 85.3pg/ml) and moderate (130.6± 81pg/ml) AD. There were 64 patients with mild AD and 44 patients with moderate AD. Boxes represent the median, the 25th and 75th percentiles, and bars indicate the range of data distribution.

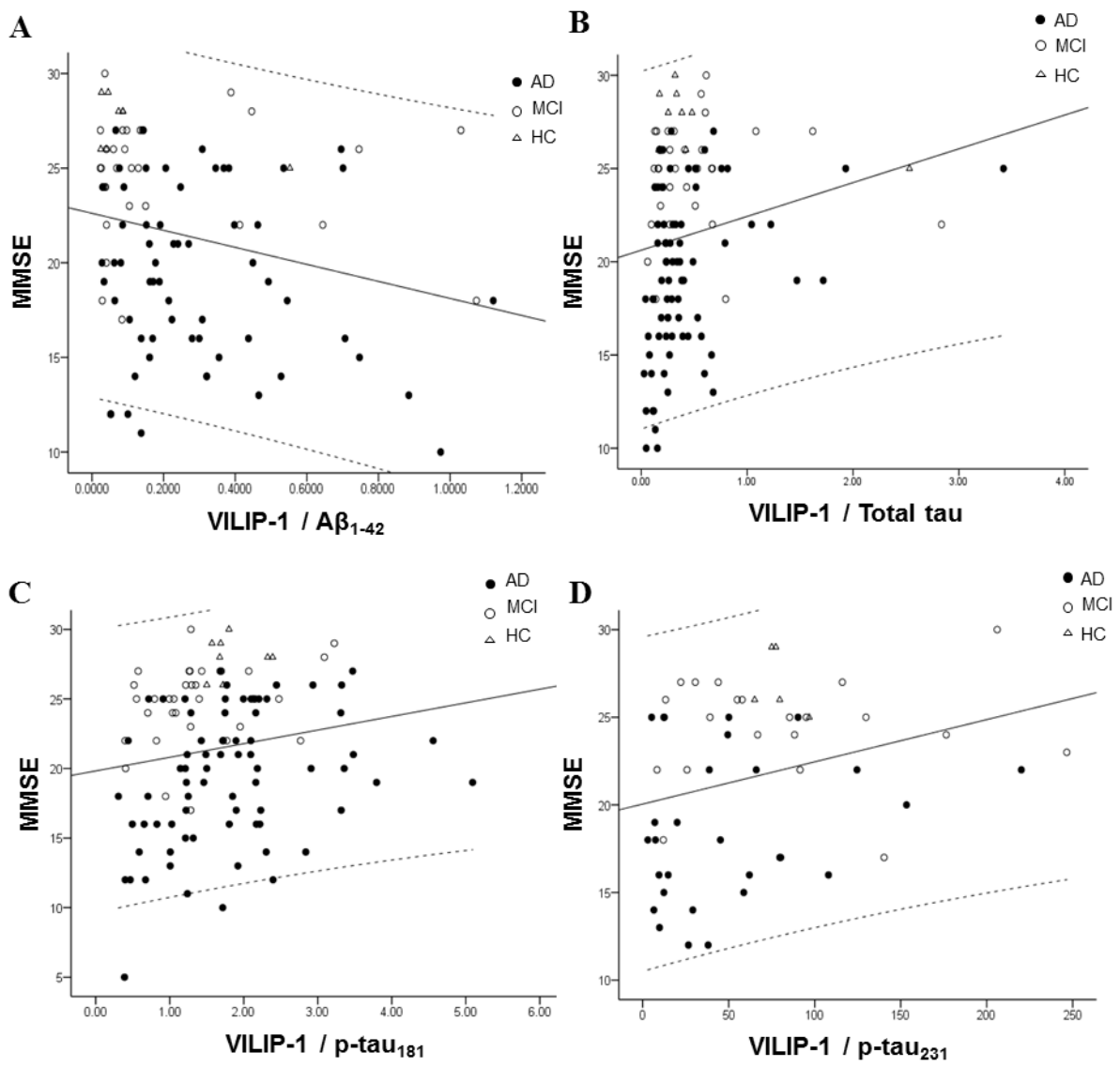

Figure 4. Correlation of MMSE scores and CSF VILIP-1/$A\beta_{1-42}$ ratio (r_S= -0.279, p = 0.005), VILIP-1/t-tau ratio (r_S= 0.285, p = 0.002), VILIP-1/p-tau_{181} ratio (r_S= 0.185, p = 0.049) and VILIP-1/p-tau_{231} ratio (r_S= 0.278, p = 0.040). Dotted lines represent 95% confidence intervals (CI) for r value.

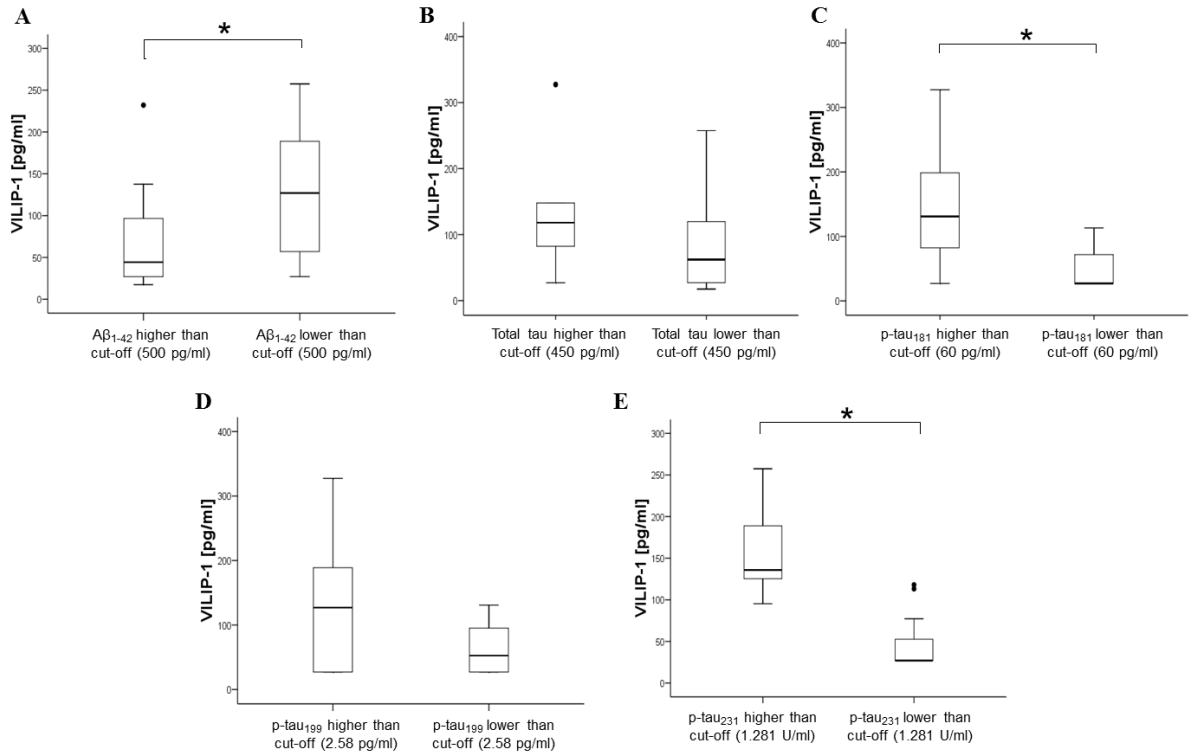

Figure 5. CSF VILIP-1 levels between MCI patients with pathological and normal levels of A) $A\beta_{1\text{-}42}$ (cut-off = 500 pg/ml), B) t-tau (cut-off = 450 pg/ml), C) p-tau_{181} (cut-off = 60 pg/ml), D) p-tau_{199} (cut-off = 2.58 pg/ml), and E) p-tau_{231} (cut-off = 1.281U/ml). Boxes represent the median, the 25th and 75th percentiles, and bars indicate the range of data distribution. The black dot in B represents an outlier.
*Statistically significant at $p<0.05$.

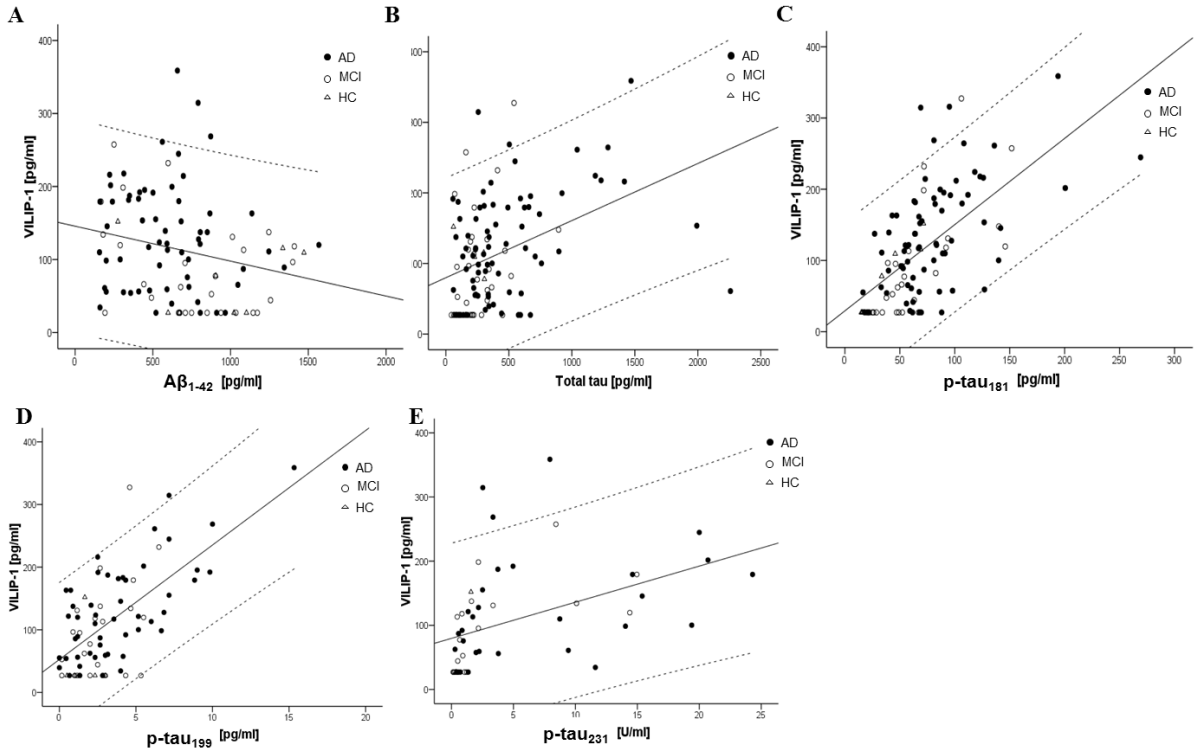

Figure 6. Correlation of CSF VILIP-1 and CSF A) $A\beta_{1\text{-}42}$ (r = -0.225, p = 0.026), B) t-tau (r_S = 0.419, p < 0.001), C) p-tau_{181} (r_S = 0.679, p < 0.001), D) p-tau_{199} (r_S = 0.514, p < 0.001) and E) p-tau_{231} (r_S = 0.712, p < 0.001). Dotted lines represent 95% confidence intervals (CI) for r value.

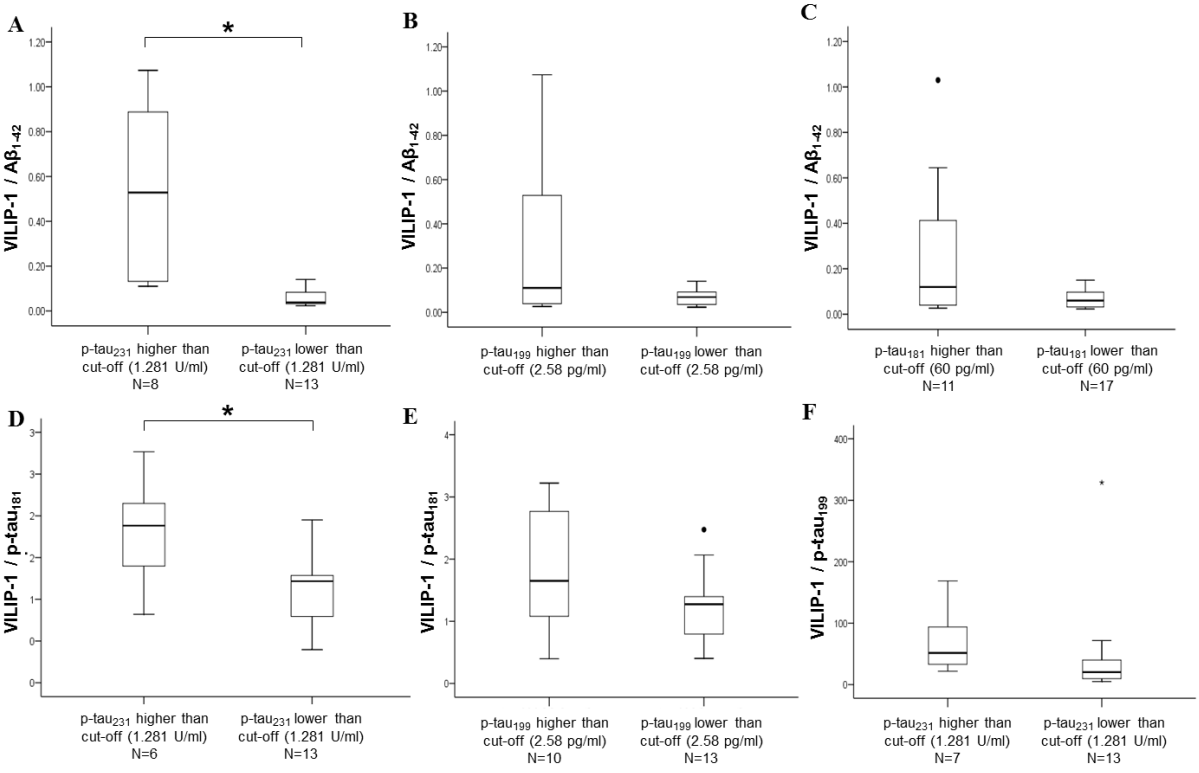

Figure 7. VILIP-1/$A\beta_{1\text{-}42}$ ratio between MCI patients with pathological and normal levels of A) p-tau_{231} (cut-off = 1.281 U/ml), B) p-tau_{199} (cut-off = 2.58 pg/ml), C)p-tau_{181} (cut-off = 60 pg/ml). VILIP-1/p-tau_{181} ratio between MCI patients with pathological and normal levels of D) p-tau_{231} and E) p-tau_{199}. VILIP-1/p-tau_{199} ratio between MCI patients with pathological and

normal levels of E) p-tau$_{231}$. Boxes represent the median, the 25th and 75th percentiles, and bars indicate the range of data distribution.
*Statistically significant at $p<0.05$.