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RESEARCH ARTICLE

Combined measurement of PEDF, haptoglobin and tau in cerebrospinal fluid improves the diagnostic discrimination between alzheimer's disease and other dementias

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ABSTRACT

Using proteomic approach in cerebrospinal fluid (CSF) we identified pigment epithelium-derived factor (PEDF) and Haptoglobin (Hp) as putative markers that could discriminate between AD and other dementias. ELISA assays were developed to measure the levels of PEDF and Hp in CSF from patients with AD (AD, n = 27), non-AD (NAD, n = 30) and in non-demented patients (ND, n = 27). The combined assessment of PEDF, Hp and Tau levels, using Iterative Marginal Optimization, improved the differential diagnosis of AD, especially in patients with moderate to severe dementia ($p<0.002$). This pilot study highlights the probable different contribution of oxidative mechanisms in dementia.

Keywords: *Neurological disease; Biomarker; ROC; Cognitive Test*

Introduction

Alzheimer's Disease (AD), the most common cause of dementia in Western countries, is a progressive neurodegenerative disorder which usually appears sporadically. AD neuropathological hallmarks are senile plaques (SP), which contain deposits of β–amyloid peptide (Aβ), and neurofibrillary tangles (NFT), which result from the aggregation of hyperphosphorylated Tau protein (Wisniewski and Wegiel, 1995). AD diagnosis is based on clinical exclusion criteria, resulting at the very best in a diagnosis of "probable AD", which reflects common clinical features between AD and non-AD dementias (NAD), such as Vascular Dementia (VaD) or Fronto-Temporal Dementia (FTD). Thus, there is evidence of cerebrovascular pathology in AD suggesting a substantial overlap between AD and VaD, a neurologic disease which may be caused by brain oxygen deficiency. Vascular risk factors such as hypertension, arterial disease or atherosclerosis, ischemic heart disease, smoking and diabetes mellitus are risk factors for AD as well, and markers of systemic vascular disease (e.g., hypercholesterolemia) are associated with AD aetiopathogenesis. To date, extensive studies have confirmed the relevance of total Tau protein (T-Tau), hyperphosphorylated Tau (P-Tau) and Aβ 1-42 (Aβ42) in AD physiopathology and have shown that the combined measurement of these markers in cerebrospinal fluid (CSF) has a high diagnostic value (Diniz et al., 2008; Hansson et al., 2006; Vanderstichele et al., 2006). In AD patients, CSF Aβ42 concentration is reduced while it remains normal in patients with depression and is decreased in dementia with Lewy bodies (DLB), frontotemporal dementia (FTD) and vascular dementia (VaD) (Motter et al., 1995; Vandermeeren et al., 1993; Hampel

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et al., 2004; Buerger et al., 2005). CSF T-Tau concentration is increased in AD, normal in patients with depression, slightly increased in DLB and FTD and is very high in Creutzfeldt-Jakob disease (Blennow and Hampel, 2003; Andreasen et al., 2001). In VaD, T-Tau concentration increases and Aβ42 decreases in CSF as in AD, partly because of the presence of similar neuropathological alterations (Andreasen et al., 2001; O'brien, 1988). Thus, combined decrease in Aβ42 and increase in T-Tau concentrations can be found in different types of dementia. This lack of specificity could be explained by the absence of histopathological verification of the diagnosis or by the misevaluation of dementia of mixed origin.

Thus, there is no reliable ante mortem biological method to differentiate AD from NAD. To improve AD diagnosis, many proteomic studies have been carried out to identify potential specific biomarkers that are involved in general physiological processes such as inflammation (Gu et al., 2008; Bonifati and Kishore, 2007; Tan et al., 2007; Griffin et al., 2006; Franciosi et al., 2005), neuroprotection (Chuu et al., 2006; Riemenschneider et al., 2002; Nakamura et al., 1998; Wyss-Coray et al., 2001), vascularisation (Zhang et al., 2005; Thirumangalakudi et al., 2006) or oxidative stress (Biroccio et al., 2006; Castano et al., 2006; Puchades et al., 2003; Simonsen et al., 2008). Whatever the technology used in these studies, only in few cases the proteomic data were validated by immunoassays in CSF samples, thus limiting their potential clinical application (Abdi et al., 2006).

In a previous study, we analysed CSF samples of a large group of patients suffering from different types of dementia (AD, VaD, FTD, Pick's disease) by two-dimensional gel electrophoresis in order to find biomarkers that are differentially expressed in AD and NAD. We identified two potential biomarkers, Pigment Epithelium-Derived Factor (PEDF) and Haptoglobin (Hp), that showed specific proteomic profiles in the different types of dementia (Verdier, 2007).

PEDF, a glycoprotein that belongs to the superfamily of serine protease inhibitors (SERPIN), can be found in many tissues and fluids including central nervous system, skeletal muscle, liver, heart, placenta, CSF and blood (Castano et al., 2006; Tombran-Tink and Barnstable, 2003; Petersen et al., 2003). Although it belongs to the SERPIN family, PEDF has no anti-protease activity but displays anti-oxidative (Amano et al., 2005) and neuroprotective properties (Cao et al., 1999; Bilak et al., 1999). Importantly, according to the site and the number of phosphorylations, PEDF switches from anti-angiogenic to neurotrophic activities (Maik-Rachline et al., 2005; Maik-Rachline and Seger, 2006). Thus, increased expression of PEDF could be correlated either to neuronal degeneration or regeneration, depending on its phosphorylation status, itself associated to vascular events. While it was demonstrated by immunohistochemistry the specific detection of PEDF in AD patient brains (Yamagishi et al., 2004), other studies led to the identification of two isoforms whose concentrations increase in CSF from AD patients, but which were not further described (Castano et al., 2006). The exact role of PEDF in AD physiopathology remains unknown, but recent work highlights its implication in the regulation of proliferation in hippocampal progenitor cells and thus in memory consolidation (Namba et al., 2010).

Hp is a tetrameric glycoprotein which is mainly synthesized in the liver with two α and two β chains that functions as a haemoglobin (Hb) scavenger, preventing kidney damage, iron loss, and tissue destruction (Kazim and Atassi, 1981). Hp presence in CSF of AD patients may be associated with altered blood-brain barrier (BBB) function (Mattila et al., 1994), or with in situ production, as demonstrated by studies showing Hp expression in human glioblastoma cell lines or in brain (Tseng et al., 2004; Sanchez et al., 2001). Thus, we can assume that Hp could exert in brain a protective function against Hb-induced oxidative damage. In humans, two Hp α alleles (1 and 2) exist, leading to the production of two different alpha chains, α1 and α2. Hp biophysical and biochemical properties and hence its scavenging functions depend on its phenotype: Hp affinity for Hb decreases with the number of α2 alleles and α2/α2 individuals synthesize less Hp than α1/α1 subjects. Thus, α2/α2 individuals are more susceptible to oxidative stress-induced pathologies such as haemochromatosis (Van Vlierberghe et al., 2001) or AD (Johnson et al., 1992). Clearance of Hb by Hp may play an important role in AD physiopathology as it has been shown that Hb tightly associates with Aβ, leading to induction of peroxidase activity (Atamna and Boyle, 2006) and can promote Aβ oligomer formation (Wu et al., 2004).

To validate our proteomic data on the differential expression of PEDF and Hp in CSF samples from AD and NAD patients (Verdier, 2007), we designed specific ELISA immunoassays to measure PEDF and Hp concentrations in CSF from AD and NAD patients to evaluate their real potential discriminative properties in AD diagnosis. To limit biological variation and to favour direct comparison, the immunological validation was performed using CSF samples previously used for the proteomic analysis. The diagnostic value of the relative changes in CSF concentration of PEDF or Hp alone and in combination with T-Tau was investigated. Our pilot study shows that the measurement of PEDF and Hp in combination with T-Tau improves the discrimination of AD patients from NAD patients in comparison to T-Tau alone, particularly in patients with moderate and severe dementia.

Material and Methods

Clinical population

The study was conducted in accordance with the provisions of the Helsinki Declaration. Patients followed in two university hospitals (CHU Sainte Marguerite, Marseille and CHU Gui de Chauliac, Montpellier, France) were included in this study. All included patients signed the informed consent for CSF punction and the use of human samples for research. CSF samples were stored in polypropylene tubes at -80°C until use. To certify sample quality for biomarker evaluation, we compared the level of T-Tau (Innotest, Innogenetics, Belgium) at inclusion time (Molina et al., 1999) as well as at the end of the inclusion process after several years storage. This tight sample quality control was performed by blind testing 40 random CSF samples (see figure 2). The target population included 27 patients with AD, 30 with NAD (15 FTD, 12 VaD and 3 with dementia of unidentified origin) and 27 non-demented (ND) subjects (normal pressure hydrocephalus, multiple sclerosis, Parkinson's disease or stroke) according to the DSM IV and the NINCDS-ADRDA criteria. The patients' age and gender as well as the Clinical Dementia Rating (CDR) score for AD and NAD patients (Hughes et al., 1982), are described in Table 1.

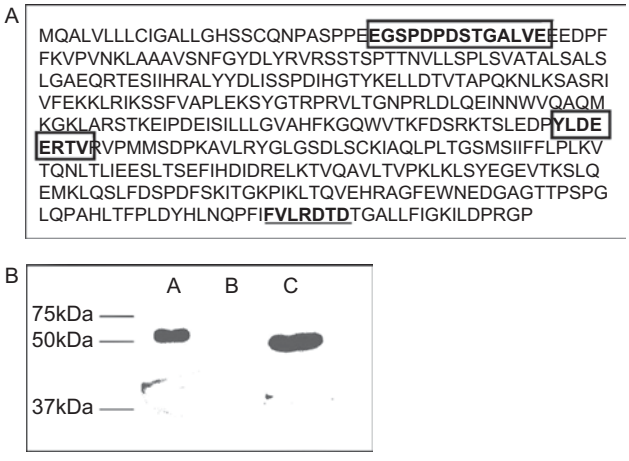


Figure 1. Characterization of the anti-PEDF antibodies: a) Epitope mapping of the anti-PEDF antibodies: the epitopes of the polyclonal (Pab-PEDF) and the monoclonal (Mab-PEDF) anti-PEDF antibodies were identified using overlapping pentadecapeptides that cover the entire PEDF sequence and that had been synthesized on cellulose membrane. Pab-PEDF recognized the ^{28}EGSPD^{32} and ^{232}YLDEERTV^{239} epitopes (framed in the figure), whereas Mab-PEDF the ^{396}FVLRDTD^{402} sequence (underlined). b) Pab-PEDF specificity was evaluated by SDS-PAGE and western blot analysis using supernatants from HEK cells that express either recombinant PEDF (A) or beta-galactosidase (B), and on collagen-purified plasma PEDF (C).

Recombinant and purified proteins

Human PEDF was PCR-amplified using the oligonucleotides 5'-ATGCAGGCCCTGGTGCTACTC-3' and 5'-GGGGCCCCTGGGGTCCAGAATC-3', cloned in the pENTR/SD/D-TOPO vector (#K2420-20, Invitrogen, CA, USA) and then subcloned into pET-DEST42 Gateway (#12276-010, Invitrogen, CA, USA) and pcDNA-DEST40 Gateway (#12274-015, Invitrogen, CA, USA) in frame with the V5 epitope and the His-Tag at C-terminus for prokaryotic and eukaryotic expression, respectively. BL21-D3 E.coli bacteria were transformed with the pET-DEST42(PEDF) plasmid in order to express PEDF after isopropylthio-β-galactoside (IPTG, #15529-019, Invitrogen, CA, USA) induction. Recombinant PEDF was purified through Ni-sepharose columns (HisTrap FF crude, #17–5247-05, GE Healthcare, CO, USA) according to the manufacturer's protocol and used for animal immunization. HEK (human embryonic kidney, ATTC#CRL-1573) cells were transfected using the calcium phosphate method (Chen and Okayama, 1987)

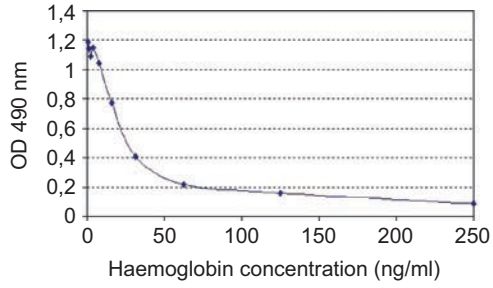


Figure 2. Haemoglobin-specific inhibition of Hp ELISA: Before measurement by Hp ELISA, 5ng/ml Hp was incubated with different concentrations of haemoglobin (Hb). Results show the inhibition of Hp detection according to the amount of Hb, possibly due to Hb-Hp complex formation.

Table 1. General description of the groups of patients included in this study.

	Gender distribution		Age (years)			CDR		
	Female	Male	(±SD)	Min	Max	0.5	1	>2
AD (n = 27)	11 (40%)	16 (60%)	73 (±7)	61	88	7 (26%)	11 (40%)	8 (30%)
NAD (n = 30)	17 (57%)	13 (43%)	71 (±8)	58	87	7 (23%)	9 (30%)	11 (37%)
D (n = 57)	28 (51%)	29 (49%)	72 (±7)	58	88	14 (25%)	20 (35%)	19 (33%)
ND (n = 27)	-	-	56 (±17)*	23	80	-	-	-

The target population is composed of 27 AD (Alzheimer's disease), 30 NAD (Non-Alzheimer's Dementia) and 27 ND (Non-Demented) patients. The different groups do not statistically differ between each other in term of gender, age, and CDR (clinical dementia rating), excepted for ND class which is younger than the others (*$p = 10^{-4}$).

with the pcDNA-DEST40(PEDF) plasmid, positive clones were selected with 250μg/ml geneticin (G418, #10131027, Invitrogen, CA, USA) and amplified. PEDF from stably transfected HEK cells and plasma PEDF, obtained by collagen-affinity chromatography purification as previously described (Petersen et al., 2003), were used for anti-PEDF antibodies characterization.

Hp used in this study was from commercial purified pools of human plasma (#H3536, Sigma-Aldrich, MI, USA)

Animal immunization

All animal experiments were performed according to the European Guidelines on animal experimentation (EC-86/609); animals were housed in controlled facilities (Agreement #A3417228) that are fully compliant with the European Policy on the use of laboratory animals. Adult (6 week/old females) Balb/c mice were purchased from Charles River (Lyon, France) and New Zealand rabbits (2 months old at immunization time, 2.5kg in weight) from Centre Lago (Vonnas, France).

Polyclonal antibody production and purification

Briefly, rabbits were immunized subcutaneously with 200μg recombinant purified PEDF or 200μg of Hp. Rabbits were primed with complete Freund's adjuvant (F5881, Sigma-Aldrich, MI, USA) and four immunizations were then performed with incomplete Freund's adjuvant (F5506, Sigma-Aldrich, MI, USA). Polyclonal sera were purified through protein A columns for immunoglobulin isolation and specific polyclonal antibody obtention (Pab-PEDF and Pab-Hp) essentially as previously described (Dreja et al., 2003).

Anti-Hp monoclonal antibody production and selection

Mice were primed with 100μg of Hp and complete Freund's adjuvant (F5881, Sigma-Aldrich, MI, USA), and boosted every two weeks with 100μg of Hp and incomplete Freund's adjuvant (F5506, Sigma-Aldrich, MI, USA). The anti-Hp antibody titre of serum samples was assessed using direct ELISA assays with immobilized Hp. The mouse with the highest serum anti-Hp antibody titre was selected for lymphocyte fusion. Mouse spleen cells were fused with Ag8x653 myeloma cells following a modified version of the protocol by Kohler and Milstein (Kohler et al., 1976). Hybridoma cells were seeded in 96-well plates and screened after 10 days using a direct ELISA assay with immobilized Hp. Cells secreting anti-Hp antibodies were subcloned and frozen in liquid nitrogen. The Ham2 hybridoma clone was selected after successful subcloning. Monoclonal Ham 2 anti-Hp antibodies were purified from serum-free cell supernatant using Protein-A Sepharose (Dreja et al., 2003) and tested for specificity with a sandwich ELISA.

PEDF and Hp epitope mapping

The protocol for epitope mapping has been described in detail by Laune et al (Laune et al., 2002). Briefly, overlapping pentadecapeptides that cover the entire sequence of PEDF or Hp were prepared using the Spot method of multiple peptide synthesis on cellulose membrane (Maria et al., 2005, Laune et al., 2002). Membranes were rehydrated through three TBS washes (Tris-Buffered Saline, pH 7.0) and saturated with 15 ml of 10 % saturation buffer ("blocking buffer", Roche) and 5 % sucrose in 0.1 % Tween® 20/TBS (TBS-T) by gentle stirring at room temperature overnight. Membranes were then washed three times with 0.1 % TBS-T for 10 minutes and incubated with the antibody to be tested at 37°C for 90 minutes. Antibodies were diluted in saturation buffer. After three washes with 0.1 % TBS-T for 10 minutes, membranes were incubated with secondary anti-rabbit and anti-mouse IgGs coupled to alkaline phosphatase and diluted in saturation buffer. After two washes with 0.1% TBS-T and two with citrate buffered saline (each for 10 minutes), the alkaline phosphatase substrate was added and membranes incubated at room temperature for 1 to 30 minutes, depending on the speed at which the signal appeared.

ELISA assays

T-Tau determination was performed using the ELISA INNOTEST (hTau, Innogenetics Corp., Belgium), according to the manufacturer's protocol.

The PEDF ELISA test was based on the capture of the antigen by our rabbit polyclonal antibody (Pab-PEDF) and its indirect detection by a monoclonal anti-PEDF antibody (Mab-PEDF Mab1059, Chemicon, Millipore, USA) and horseradish peroxidase–labelled goat anti-mouse IgGs (Sigma-Aldrich, MI, USA). ELISA plates (Nunc Maxisorb) were coated with the rabbit polyclonal anti-PEDF antibody diluted to 2μg/ml in PBS overnight at 4°C. After three washes in PBS/0.1% Tween20, wells were blocked at 37°C with blocking buffer (PIERCE) for at least 1 hour. Standards (recombinant PEDF) and CSF samples were added to the wells and incubated at room temperature for 2 hours. After washing, the monoclonal anti-PEDF antibody, diluted in PBS, was added to the wells at 37°C for 2 hours. After washing, the horseradish peroxidase–labelled goat anti-mouse secondary antibody, diluted 1:10.000 in PBS, was added to the wells and incubated at 37°C for 1 hour. Plates were washed and each well was treated with developing solution (TMB, Sigma-Aldrich) at room temperature for 20 minutes. The

reaction was stopped with 1 M H2SO4, and the colour reaction was quantified using a microplate reader (Bio-Rad) at 492 nm.

The Hp ELISA test was based on the capture of the antigen by the monoclonal antibody Ham2 and its direct detection by the peroxidase-coupled rabbit polyclonal anti-Hp (Pab-Hp) antibody we produced. ELISA plates (Nunc Maxisorb) were coated with Ham2 diluted to 2µg/ml in PBS at 4°C overnight. After three washes in PBS/0.1% Tween20, wells were blocked at 37°C with blocking buffer (PIERCE) for at least 1 hour. Standards (commercial Hp #H3536, Sigma-Aldrich, MI, USA) and CSF samples were added to the wells and incubated at room temperature for 2 hours. After washing, Pab-Hp, diluted 1:10.000 in PBS, was added to the wells for 2h at 37°C. Wells were washed with PBS/0.1% Tween20 three times, and developing solution (TMB, Sigma-Aldrich) was added to each well at room temperature for 20 minutes. The reaction was stopped with 1 M H2SO4, and the colour reaction was quantified using a microplate reader (Bio-Rad) at 492 nm.

The analytical sensitivities of the PEDF and Hp immunoassays were 600 pg/ml and 200 pg/ml respectively, and the inter-plate and intra-plate variation coefficients were less than 15%.

Statistical analysis

All statistics and figures were computed with the "R/Bioconductor" statistical open source software (Ge et al., 2003; Gentleman et al., 2004). The differences between groups were analyzed by the nonparametric Wilcoxon rank sum test (also called the Mann–Whitney test). With the multiple testing methodologies, it is important to adjust the p-value of each marker to control the False Discovery Rate (FDR). The Benjamini and Hochberg (BH) procedure (Benjamini and Hochberg, 1995) was applied on all statistical tests with the "multtest package" and an adjusted p-value below 0.05 was considered as statistically significant. The accuracy of each marker and its discriminatory power was evaluated using a Receiving Operating Characteristics (ROC) analysis. ROC curves are the graphical visualization of the reciprocal relation between the sensitivity (Se) and the specificity (Sp) of a test for various values. In addition, PEDF and Hp values were combined with T-Tau values to evaluate the potential increase in sensibility and specificity using the Iterative Marginal Optimization (IMO) algorithm described by Wang (Wang, 2007). The IMO algorithm calculates the MRC (Maximum Rank Correlation) estimator which maximizes the area under the ROC curve for selected markers. The linear equation for the respective combinations is provided and can be used as a new virtual marker (Z): $Z = a \times Marker1 + b \times Marker2 + c \times Marker3$. The values of a, b and c depend on the considered comparison and are given by IMO method. Marker 1, 2 and 3 correspond, respectively, to the Hp, T-Tau and PEDF concentrations in CSF. Thus, to compare the combination of PEDF, Hp and T-Tau in NAD and AD patients $Z = (a \times Hp) + (b \times T - Tau) + (c \times PEDF)$ in which a = 0.0001, b = 1 and c = -0.0147.

An important problem concerns the comparison of 2 AUCs (Area Under the Curve) derived from 2 diagnostic tests administered on the same set of patients. So, we tested the null hypothesis versus using statistical tests derived either from correlated AUCs by Hanley and McNeil (Hanley and McNeil, 1983), from the bootstrap approach (Efron, 1979) or from DeLong's test (Delong et al., 1988) for two correlated ROC curves with the R package pROC.

Results

Antibody and ELISA characterization

To validate our proteomic data on the differential expression of PEDF and Hp in CSF samples from AD and NAD patients (Verdier, 2007), we developed specific ELISA assays using, on one hand, the polyclonal anti-PEDF (Pab-PEDF) for PEDF capture and monoclonal anti-PEDF (Mab-PEDF) for its detection, and, on the other hand, the monoclonal anti-Hp (Ham-2) for Hp capture and polyclonal anti-Hp (Pab-Hp) for its detection. The epitopes of Pab-PEDF and of Mab-PEDF antibodies used for the PEDF ELISA assay were characterized using the SPOT technique (Laune et al., 2002). Pab-PEDF recognized two epitopes in the PEDF sequence ^{28}EGSPD^{32} and ^{232}YLDEERTV^{239}, while Mab-PEDF was specific for the ^{396}FVLRDTD^{402} sequence (Figure 1a). According to previous published results, these epitopic sequences do not contain critical phosphorylation site for PEDF activity, which are mainly S24, S114 and S227 (Maik-Rachline and Seger, 2006, Maik-Rachline et al., 2005). In western blot experiments, Pab-PEDF could detect recombinant PEDF expressed by HEK cells, and PEDF purified from plasma (Figure 1b). These results show that produced antibodies do not depend on phosphorylation status of the protein. Pab-PEDF and Mab-PEDF were used to engineer a specific ELISA test with a sensitivity of 600pg/ml (signal/background ratio = 2) for purified recombinant PEDF expressed in E.coli.

Similar tests also were performed to characterize Pab-Hp and the Mab-Hp Ham-2. No signal could be obtained using the SPOT method or denaturating techniques such as SDS-PAGE followed by western blotting (not shown) when using Ham2. Thus we concluded that the activity of this antibody was conformational-dependent. We set up an Hp ELISA test using Ham-2 for Hp capture and Pab-Hp for its detection. This test showed

a sensitivity of about 200pg/ml with a signal/background ratio = 2. Hp ELISA specificity was validated as no cross-reactivity could be observed towards other non relevant antigens, such as bovine or human serum albumin, or Tau (data not shown). Finally, we demonstrated the Hp ELISA was specifically inhibited by pre-incubation of Hp with Haemoglobin (Hb) due to the formation of Hb-Hp complexes as already described (Kazim and Atassi, 1981), further demonstrating the specificity of this test (Figure 2).

Quality assessment of CSF samples

Before measuring the concentration of PEDF and Hp in our CSF samples from AD, NAD and ND patients using the new PEDF and Hp ELISA assays, we ascertained that the CSF samples did not undergo degradation during the long storage period (in certain cases about 10 years). To this aim we tested 40 random CSF samples by evaluating T-Tau concentration at the time of inclusion (Molina et al., 1999) and then again at the moment of the experiment (storage at - 80°C). These measurements demonstrated that T-Tau level in CSF remained fairly constant during the storage period (Figure 3). Such stability over a decade should add confidence in the use of CSF samples that have been stored for a long period of time when storage conditions are well controlled.

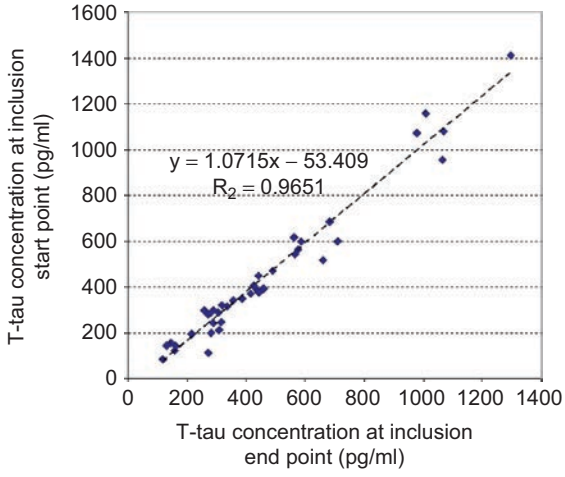


Figure 3. Quality control of the CSF samples. CSF T-Tau levels were evaluated in 40 random CSF samples and compared to their value at time of inclusion in our bio-bank. The very high correlation between the two values indicates good sample conservation at -80°C over a long period.

PEDF, Hp and T-Tau levels in CSF samples from AD, NAD and ND patients

In order to validate the use of PEDF and Hp measurement in CSF for the differential diagnosis of dementia, we chose CSF samples obtained from AD, NAD and ND patients. Sex, age and CDR were not significantly different in AD and NAD groups (Table 1). Conversely, patients of the ND group were younger than those in the AD and NAD groups ($p = 10^{-4}$), as this group included pathologies such as multiple sclerosis, normal pressure hydrocephalus or stroke.

PEDF and Hp values, assessed using the ELISA assays we developed, did not significantly vary in the three groups (Table 2). Indeed PEDF and Hp mean concentrations were, respectively, about 50 ng/ml and 1µg/ml whatever the pathology. Conversely, T-Tau concentrations significantly discriminated ND from AD patients ($p = 10^{-4}$), NAD from AD ($p = 10^{-2}$) and also all patients with dementia (AD and NAD) from ND patients ($p = 10^{-3}$) (Table 2).

Combined assessment of PEDF, Hp and T-Tau discriminates more efficiently NAD and AD than T-Tau alone

Since PEDF and Hp concentrations alone were not discriminative, we asked whether their assessment in combination with T-Tau could improve Tau diagnostic value. The accuracy of the three markers to discriminate the three groups of patients was evaluated using ROC curve analysis as it allowed comparing the performance of T-Tau alone and in combination with PEDF and Hp. To this aim, the three markers T-Tau, PEDF and Hp were combined to create the virtual biomarker Z, which was calculated for each patient according to the equation described in Material and Methods. The Z biomarker did not increase the diagnostic power of T-Tau in discriminating ND from AD (Figure 4a). This result confirmed the

Table 2. PEDF, Hp and T-Tau concentration in CSF samples from the different groups of patients.

	PEDF (ng/ml)			Hp (ng/ml)			Tau (pg/ml)		
	Mean (±SD)	Min	Max	Mean (±SD)	Min	Max	Mean (±SD)	Min	Max
AD (n = 27)	46 (±14)	21	77	941 (±587)	166	2526	635 (±336)**	115	1300
NAD (n = 30)	50 (±9)	30	74	728 (±553)	132	2312	414 (±245)	118	1045
D (n = 57)	48 (±12)	21	77	830 (±575)	132	2526	519 (±310)*	115	1300
ND (n = 27)	47 (±20)	17	91	1736 (±1823)	148	6740	347 (±399)	107	2204

PEDF and Hp CSF levels were evaluated with home-made immunoassays, while T-Tau concentration was by Innotest assay (Innogenetics). Individual protein evaluation shows no significant difference in Hp and PEDF concentration according to the pathology, while T-Tau levels significantly discriminate ND (Non-Demented) from D (Demented) (*$p = 10^{-3}$) and ND from AD (Alzheimer's Disease) (**$p = 10^{-4}$).

significant discrimination power of T-Tau alone in case of ND and AD ($p = 10^{-4}$). Conversely, the use of the Z biomarker improved the discrimination accuracy between NAD and AD patients compared to T-Tau alone. Indeed, the combination test increased the sensitivity (from 74.1% to 85.2%), the specificity (from 60% to 76.7%), the negative predictive value (NPV, from 72% to 85.2%) and the positive predictive value (PPV, from 62.5% to 76.7%) of the discrimination between NAD and AD, resulting in an AUC increase 0.694 to 0.802 (Figure 4b). Three different statistical tests were performed to validate the significance of this difference (DeLong's, Hanley's and bootstrap tests) and all three led to a significant p-value (<0.05) (Figure 4b).

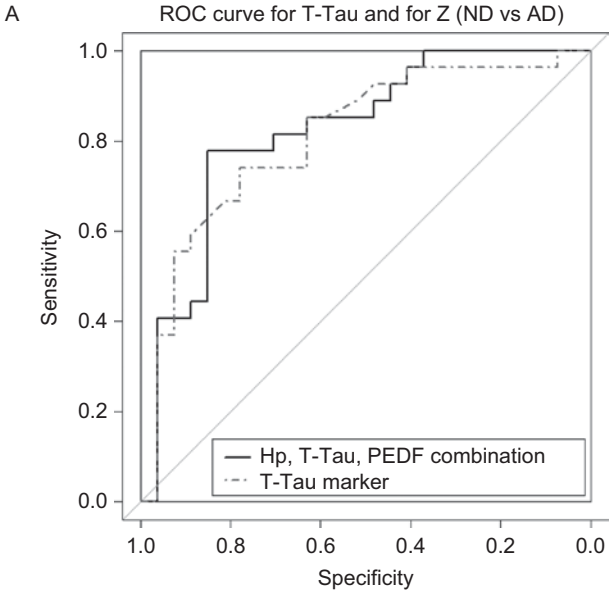
We then compared the diagnostic power of the biomarker Z and of T-Tau alone in NAD and AD patients who were divided in sub-groups based on their CDR score (Figure 5). Again Z could significantly discriminate between NAD and AD, independently from their CDR score. However, as shown also in Table 3, the combination test seemed more accurate in case of moderate and severe dementia (CDR score between 2 and 3: $p<0.002$) than for mild dementia (CDR score 0.5 and 1; $p>0.1$).

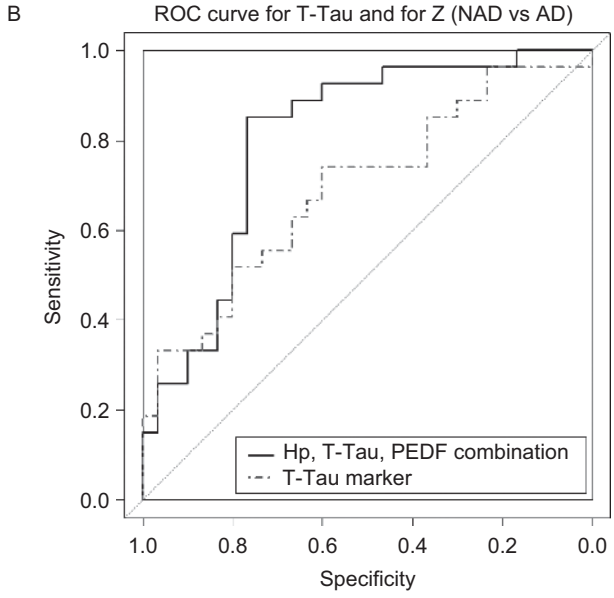
Discussion

Regarding the numerous proteomic analyses of CSF, it was relevant to evaluate the correlation between 2D-identified biomarkers with their CSF concentration. In this study, we determined the discriminative power in AD diagnosis of CSF Hp and PEDF, two potential markers we previously identified by proteomic analysis of CSF samples from AD, NAD and ND patients (Verdier, 2007). To this aim, we produced antibodies that target those two proteins and developed specific ELISA sandwich immunoassays. These ELISA are sensitive enough to detect PEDF and Hp in CSF whose concentrations are respectively about 50ng/ml and 1µg/ml whatever the pathology. Using this method, we did not confirm their potential diagnostic interest, as they could not discriminate AD from NAD or ND patients, although both ELISA assays were performed using a set of CSF samples that belonged to the same cohort used for the proteomic study. Different hypotheses could explain this discrepancy.

First, CSF sample conservation and protein stability could have been impaired by the long storage period at -80°C. However, in our study, the quality of the samples was confirmed by measuring T-Tau levels at the time of the inclusion of the sample and then at the time of the study (up to ten years) (Fig. 3). Thus, although we can not exclude that other proteins, such as PEDF or Hp, might have been degraded, T-Tau stability is a strong argument in favour of the overall integrity of our CSF samples. In the present study, the values of sensitivity (74.1%) and specificity (60%) of T-Tau alone in discriminating between NAD and AD patients fits with the available data in the literature and confirm the difficulty to segregate AD from other dementias using a simple biological test (Clark et al., 2003). However, it is noticeable that Tau discrimination power in ND vs AD comparison in our study is lower than values usually described (74.1% vs 81% of sensitivity and 77.8% vs up to 90% of specificity (Blennow and Hampel, 2003)). This could be explained by our ND group population, which may differ in age and composition from those of other studies.

A second hypothesis concerns the presence of different protein isoforms. PEDF has been reported to be present in CSF at least in two different phosphorylated isoforms (Castano et al., 2006) and Hp, which is a multimeric proteic complex, can adopt many different conformations according to the haplotypes (Van Vlierberghe et al., 2004; Van Vlierberghe et al., 2001). Thus, the lack of correlation between proteomic and immunological validation could result in a low identification of the very specific isoform of PEDF (phosphorylation status, glycosylation status) and Hp (haplotype, multimerisation). PEDF isoforms and Hp conformation variability could be reinforced by the technology used: the sandwich ELISA is more sensitive to protein conformation integrity as compared to two-dimensional electrophoresis (denaturating conditions). Finally, we can not completely exclude the occurrence of micro-haemorrhages during CSF sampling by lumbar puncture that could have led to haemoglobin release in CSF. Indeed, Hp ELISA is impaired by the presence of free Hb that can decrease or even inhibit Hp detection (Figure 2). This lack of correlation between proteomic data and immunological validation has been also described in a recent publication by Roher et al., who used commercially available ELISA to validate in AD and NAD patients some potential biomarkers identified by 2D-gels in pooled CSFs (among which PEDF (Castano et al., 2006; Roher et al., 2009b)). No difference in PEDF levels was shown between the groups in this study, what confirms our results. Interestingly, Hp and PEDF concentrations (2 µg/ml and 500 ng/ml, respectively) were much different from the ones obtained with our ELISA assays (Hp: 1 µg/ml and PEDF: 50 ng/ml). This discrepancy can be explained by the specificities of the different immunoassays used that probably lead to the detection of different isoforms according to the antibodies used. However, it also confirms the need to accurately identify the putative markers discovered by proteomic analysis and to better characterize the specificity of the antibodies used for immunoassays. As described above, we know that, on one hand, PEDF activity (anti-angiogenic or neurotrophic properties) depends on the site and the number of phosphorylations (Maik-Rachline et al., 2005, Maik-Rachline and Seger, 2006), and on the other hand, Hp α2/α2 individuals are more susceptible to AD (Johnson





	ND vs AD	
	T-Tau	Z (IMO)
Cut-off	0.407 ng/ml	–0,001
Sensitivity (%)	74,1	77,8
Specificity (%)	77,8	85,2
NPV	70,5	79,3
PPV	79,6	84
AUC	0,809	0,822
95% Confidence Interval	[0.689 ; 0.928]	[0.706 ; 0.937]
p-value (FDR)	1,0E–04	1,0E–04

	Comparison test of ROC curves	
	z-score	p-value
DeLong's Test	–0,319	0,749
Hanley's Test	–0,391	0,696
Bootstrap Test (n=1000 permutations)	–0,313	0,754

	NAD vs AD	
	T-Tau	Z (IMO)
Cut-off	0.407 ng/ml	–0,148
Sensitivity (%)	74,1	85,2
Specificity (%)	60	76,7
NPV	72,0	85,2
PPV	62,5	76,7
AUC	0,694	0,802
95% Confidence Interval	[0.555 ; 0.833]	[0.684 ; 0.921]
p-value (FDR)	0,012	6,0E–04

	Comparison test of ROC curves	
	z-score	p-value
DeLong's Test	–1,985	0,047
Hanley's Test	–2,340	0,019
Bootstrap Test (n=1000 permutations)	–1,989	0,046

Figure 4. Comparison of the diagnostic value of T-Tau alone and in combination with Hp and PEDF. ROC curves allow the visualization of the discriminatory power of T-Tau alone and of the combined markers (Hp, T-Tau and PEDF); a) ND (Non-Demented) and AD (Alzheimer's Dementia) ROC curves overlay, showing no diagnostic gain by using the combined markers; b) NAD (Non-Alzheimer's Dementia) and AD (Alzheimer's Dementia) ROC curves show an improvement of sensitivity, specificity, and AUC (Area Under the Curve), corresponding to an higher discriminatory power of the combined markers ($p<0.05$), which are represented by the virtual biomarker Z.

et al., 1992). Thus, in our study, maybe it would have been interesting to produce on one hand antibodies recognizing specific phosphorylation status of PEDF, and on the other hand antibodies specific from Hp α2 chain.

Nevertheless, despite we could not highlight significant difference in PEDF and Hp concentrations between the different groups studied, statistical analyses showed the benefit of adding Hp and PEDF measurements to Tau determination in term of specificity, sensitivity and AUC for the discrimination of NAD from AD ($p<0.05$). Interestingly, no improvement was obtained with the combination in ND *vs* AD comparison, showing that Tau measurement by itself allows a significant discrimination. As mentioned in M&M section, the NAD group (30 samples) included 15 FTD, 12 VaD and 3 patients with unidentified dementia. Statistical analyses showed that, when FTD or VaD and AD patients were compared, the Z biomarker increased sensitivity (from 74% to 85%), specificity (from 60% to 75%), NPV (from 55% to 70%) and PPV (from 75% to 85%) compared to T-Tau alone. However, when we compared FTD and VaD, we could not differentiate the two populations either with T-Tau alone or with the Z biomarker (data not shown). In a mechanistic point of view, these results show that PEDF and Hp may be involved in AD physiopathology, in a different way that they may contribute to FTD or VaD.

These results may be surprising regarding the weakness of diagnostic value of PEDF and Hp biomarker between the different groups. To our knowledge, no previous study described the use of potentially "irrelevant"

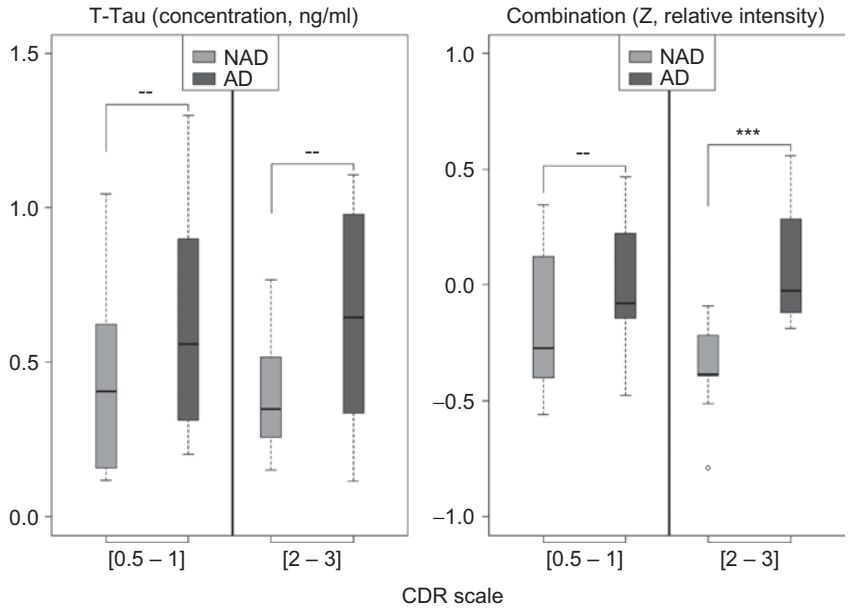


Figure 5. The Z biomarker is more accurate in the diagnosis of moderate to severe dementia. Box-plot distribution of T-Tau concentration and Z value in AD and NAD sub-groups relative to their CDR score. The mild dementia (CDR: 0.5-1) sub-groups included 18 AD and 16 NAD, while the moderate to severe dementia (CDR: 2-3) sub-groups were made up of 8 AD and 11 NAD, respectively. Virtual biomarker Z significantly discriminates NAD vs AD in moderate to severe dementia (CDR: 2-3; p-value<0.002).

Table 3. Sensitivity, specificity and accuracy of the combination test in differentiating NAD and AD patients relative to their CDR score.

	CDR [0.5 - 1]		*CDR* [2 - 3]	
	Tau	*Z*	Tau	*Z*
Cut-off	0.407 ng/ml	-0.148	0.407 ng/ml	-0.148
Sensitivity (%)	72.2	77.8	75	87.5
Specificity (%)	50	62.5	63.6	90.9
NPV	61.5	71.4	77.8	90.9
PPV	61.9	70	60	87.5
p-value (FDR)	(--): >0.1	(--): >0.1	(--): >0.1	(***): <0.002

This table shows the improvement of the differentiation NAD (Non-Alzheimer's Disease) *vs* AD (Alzheimer's Disease) in mild dementia (CDR: 0.5-1), and the higher significance of the test for moderate and severe dementia (CDR: 2-3) by using Z combination (***).

individual biomarkers in a combination with a specific one in order to delineate their discriminative impact of the global test. For instance, Roher et al. discarded PEDF and Hp from their final combination and selected the three most interesting ones (Aβ42, ApoA1 and haemopexin) (Roher et al., 2009). Our results can be explained by the fact that the variations of PEDF and Hp concentrations in our sample were too low to have by themselves a diagnostic value on a large cohort, but remained relevant in a combination test for individuals. Thus, it would be interesting to include also the measurement of the concentration of P-Tau, which improves the specificity for AD by decreasing false positive rate (Buerger et al., 2002; Buerger et al., 2005, Blennow and Hampel, 2003), and Aβ42 which is by far the most relevant biomarker in AD (Roher et al., 2009). Thus, we can hypothesize that adding PEDF and Hp to T-Tau, P-Tau and Aβ42 measurements would have led to a higher level of differentiation, especially in specificity improvement. Furthermore, by using a combination of Aβ42, P-Tau and T-Tau for discriminating AD from NAD, high sensitivities (85–94%) and specificities (83–100%) can be reached (Hansson et al., 2006), even at very early stages of AD (MCI, mild cognitive impairment) (Hansson et al., 2006). In our study, we have analysed the diagnostic value of Tau *vs* combination according to CDR status. We have shown that combination of the three markers allowed a discrimination between NAD and AD for the two ranges of CDR (Fig. 5), but especially in moderate and severe dementia, what Tau did not in separate groups. However, for very early stages (CDR = 0.5), the combination of the three markers did not seem to better discriminate than T-Tau alone. Thus, we assume that, in AD, changes in PEDF and Hp concentrations occur later, possibly after the increase in T-Tau level. Nevertheless, these proteins seem to reveal specific AD modifications that do not appear in FTD or VaD, what could be of importance for further treatments.

Conclusion

In conclusion, this pilot study shows that Hp and PEDF, two biomarkers discovered with a proteomic approach, may have a potential discriminatory value in AD and NAD diagnosis when combined with more pathology-specific markers like T-Tau. The potential diagnostic interest of

the combination of PEDF and Hp with T-Tau should now be confirmed in a larger clinical study.

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Declaration of interest

The authors report no declaration of interest.

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