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Research Report

Correlations between soluble α/β forms of amyloid precursor protein and A β 38, 40, and 42 in human cerebrospinal fluid

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ABSTRACT

Cerebrospinal fluid (CSF) biomarkers are now widely used for diagnosis of Alzheimer disease (AD) in atypical clinical forms, for differential and early diagnosis, or for stratification of patients in clinical trials. Among these biomarkers, different forms of amyloid peptides (A β) produced by the cleavage of a transmembrane precursor protein called APP (amyloid precursor protein) have a major role. A β peptides exist in different length the most common ones having 40 (A β 40), 42 (A β 42), or 38 (A β 38) amino acids in length. APP processing by gamma-secretase releases also an amino-terminal secreted fragment called sA β PP-beta while an alternative nonamyloidogenic cleavage of APP, through an alpha-secretase, liberates another fragment called sA β PP-alpha. To decipher the molecular and pathological mechanisms leading to the production and the detection of these entities is essential for the comprehension and the prevention of AD. In this report, we present the results of the

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multiplex measurement of CSF A β 38, A β 40, A β 42, sA β PP-alpha, and sA β PP-beta in 60 patients mostly with dementia eventually segregated between neurochemical dementia diagnostic (NDD) positive and negative groups. The NDD classification was based on our routine Tau, P-tau₁₈₁, and A β ₄₂ cutoff values. We confirmed previous findings regarding the correlation between sA β PP-alpha and sA β PP-beta, as well as the potential interest of these new biomarkers. We also studied the correlation between sA β PPs and A β peptides, as well as between A β peptides themselves. We observed a strong correlation between A β 38 and sA β PP-beta which suggested that the production of this peptide was in direct relation with β secretase activities. We also reported a strong correlation between A β 38 and A β 40, while A β 42 was correlated to these fragments only in nonpathological situations. These results enlighten the complex relationships between these molecular markers in both physiological and pathological situations. Our results are important for the further use of these analytes for AD diagnosis as well as for validating the cell biological hypotheses of APP processing and A β fragment production.

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1. Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized by significant cognitive deficits, language troubles, behavior changes and loss of functional autonomy. As the major cause of dementia in the elderly population, AD is approaching epidemic proportions in the Western industrialized countries due to aging of the population. AD is becoming a major public health issue with the increasing burden on caregivers and on the health care system. AD characteristic neuropathological lesions are represented by amyloid plaques and neurofibrillary tangles which coexist in the brain. The diagnosis of AD based on clinical, neuropsychological, morphological, as well as functional neuroimaging can remain dubious in a considerable number of cases delaying the diagnosis and the medical care. These data are already powerful but lead only to probability diagnosis. Recent new sets of diagnosis criteria including specific morphological (volumetric MRI), functional imagery (PET scan), and CSF biomarkers (total and phosphorylated Tau (P-tau) proteins and A β peptides) have been proposed to optimize the diagnosis (Dubois et al., 2007). When comparing AD patients to healthy controls, CSF concentrations of Tau and P-tau were reported to be increased, while A β 1–42 levels were decreased (Blennow and Hampel, 2003). Numerous studies have analysed the potential of CSF biomarkers in terms of positive diagnosis, differential diagnosis, and prognosis as predictive marker of the conversion (mild cognitive impairment (MCI) to AD) (Gabelle et al., 2009; Herukka et al., 2007; Mattsson et al., 2009). In combination and using several published ratios, these biomarkers reached a sensitivity and specificity for positive AD diagnosis beyond 80% (Ibach et al., 2006). We obtained similar results in our routine practice on more than 350 patients (unpublished results) using as cutoff values for P-tau and IATI (Innogenetics A β /Tau index), 60 and 0.8, respectively. The future development of therapeutic strategies aiming at slowing down or even blocking the neurodegenerative process could however benefit from earlier and more sensitive diagnosis AD biomarkers.

Recently, Lewczuk et al. (2010) brought to light the interest of CSF soluble amyloid precursor proteins (sAPP) as novel potential biomarkers in the positive diagnosis of AD. sAPPs are

generated from APP following α and β cleavages (Kang et al., 1987). The nonamyloidogenic pathway that cleaves off APP in position α (within the sequence corresponding to A β) leads to the α -secretase-cleaved soluble APP (sAPP α) fragment. The β -secretase action on APP leads to the β -secretase-cleaved soluble APP (sAPP β). This β -secretase activity (BACE), combined with γ -secretase cleavages releases the three major peptide fragments A β 40, A β 42, and A β 38 (Wiltfang et al., 2002) in a kinetics that is not yet fully elucidated. It is well known that most of the secreted A β fragments correspond to A β 40, however, in AD, a prominent pathogenic role is given to A β 42 that aggregates much more rapidly due to the presence of two additional hydrophobic amino acids, and represents the main constituent of senile plaques (Iwatsubo et al., 1994). While investigating presenilin mutations and γ -secretase inhibitors, Page et al. (2008) observed that while A β 42 and A β 40 productions were strongly related, A β 38 generation by γ -secretase was not dependent on the previous entities.

The objective of the current study was to investigate the relationship between sAPPs and A β fragments in different pathophysiological contexts. Our results confirmed the potential interest of sAPPs as AD biomarkers and provide important information on the complex correlation between the production of A β fragments and sAPPs.

2. Results

2.1. sAPP α / β correlation and values in NDD groups

A significant correlation (Fig. 1A; $r=0.87$, $P<0.0001$) was observed between the values of sAPP α and sAPP β in our population, independently of the NDD biological status. When concentration of the biomarkers were compared between NDD groups, higher mean values were obtained in NDD+ (Figs. 1B and C) with a difference with NDD– significant for sAPP β . These results were reminding of those of Lewczuk et al. (2010) who analyzed a larger population and therefore obtained differences with lower P values.

The potential interest of sAPPs for dementia diagnosis could not be fully investigated in the present study that

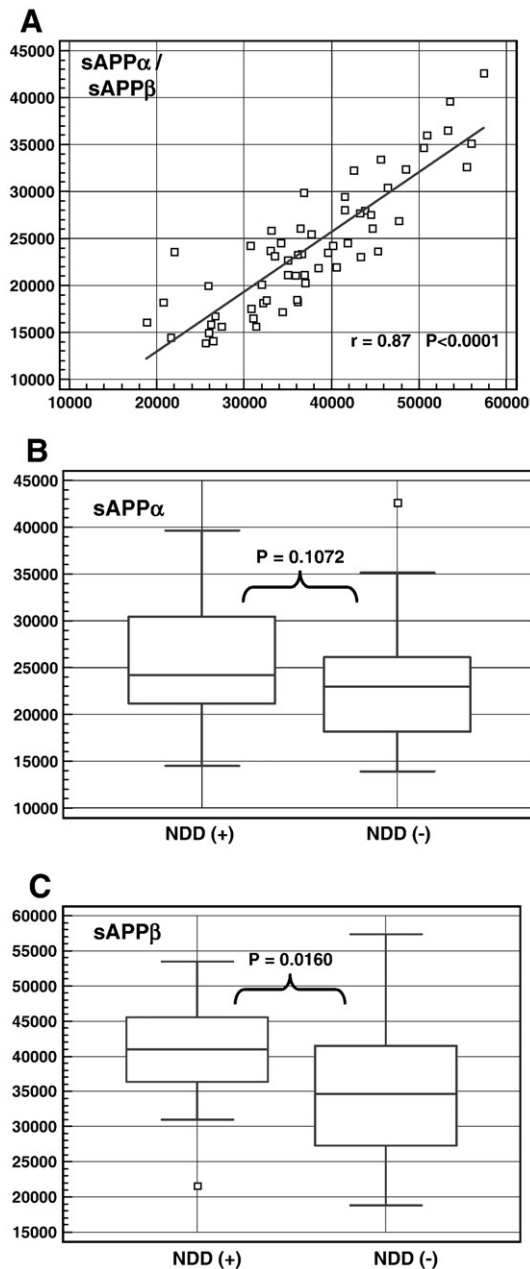


Fig. 1 – (A) Correlation between soluble amyloid precursor protein α (sAPP α) and soluble amyloid precursor protein β (sAPP β) in cerebrospinal fluid (CSF) samples in whole population (patients with neurochemical dementia diagnostic (NDD) positive and negative results). A statistically significant correlation was observed with $r=0.87$ and $P<0.001$. (B and C) Comparison of the CSF sAPP α/β concentrations between the NDD+ and the NDD- groups. A difference in mean concentration was observed and was significant for sAPP β ($P=0.0160$).

focused on the relationship between CSF biological values. However, receiver operating characteristic (ROC) curve of sAPPs for NDD+ resulted in cutoff values with sensitivity of 68.2% and specificity of 57.9% for sAPP α and 81.8% and 63.2% for sAPP β . As illustrated Table 1, MMS status was different

between NDD+ and NDD- while no significant difference was observed for the age or the CSF total protein content.

2.2. Correlation between sAPP α/β and A β 38, 40, and 42

Correlations between sAPP α/β and A β 38, 40, and 42 were plotted, and Spearman factors were calculated for each pair of biomarkers (Fig. 2). Interestingly, we observed for the first time to our knowledge a positive correlation between sAPPs and A β 38 or A β 40 independently of the NDD status. This was particularly significant for sAPP β and A β 38 ($r=0.72$, $P<0.0001$) as well as for sAPP α and A β 38 which was unexpected as this sAPP fragment is not generated in the same processing pathway as A β peptides. The significance of sAPP correlation was however lower with A β 40 than with A β 38 and was not significant at all with A β 42 (compare Figs. 2 AD, BE, and CF). As A β 42 and in a lesser extend A β 40 are involved in AD pathogenesis and CSF levels of these biomarkers are known to be modified in the disease (Blennow and Hampel, 2003), the analysis was restricted to the NDD- group (Fig. 3). This resulted in an increase in correlation factors and a statistically significant correlation also between sAPPs and A β 42.

2.3. Correlation between A β 38, 40, and 42

Correlation between the levels of A β 38, 40, and 42 were plotted, and Spearman factors were calculated for each pair of biomarkers (Figs. 4A, B, and C). Interestingly, we observed that A β 38 and A β 40 were strongly correlated ($r=0.90$, $P<0.0001$). There was no correlation with A β 42 in the total population; however, when correlation was restricted to the NDD- group (Fig. 4D), the correlation factor increased and was statistically significant for A β 38 and A β 42.

3. Discussion

In this report, we present in a series of neurochemical dementia diagnostic (NDD) positive and negative CSF samples the results of multiplex assays for soluble forms of amyloid precursor proteins (sAPPs) and A β peptides. Data were collected in two independent laboratories using similar and controlled preanalytic and analytic conditions (Lehmann et al., 2009).

Importantly, a recent investigation on sAPP α and A β CSF levels already confirmed the specificity of the detection assays used in the present study (Lewczuk et al., 2010). This previous work revealed a surprising correlation between these two entities. We independently confirmed this correlation (Fig. 1) which was intriguing because it did not correspond to what would be intuitively expected. One could believe in fact that the production of sAPP β would be inversely proportional to that of sAPP α since their metabolic pathways are exclusive. Moreover, these fragments have opposite impact (toxic vs. neuroprotective). To our knowledge, no precise metabolic regulation has been demonstrated between α and β APP cleavages in the literature, and further studies would be needed to explain this positive correlation.

We also observed a slight increase in sAPP levels in NDD+ samples compared to NDD- with a statistically significant

Table 1 – Demographic and biologic data of the NDD groups.

	MMS	Age	CSF Total Protein
NND+	16.4 ± 6.4	66.5 ± 7.9	0.40 ± 0.19
NND–	21.5 ± 6.3	67.3 ± 11.3	0.41 ± 0.14
P value	<0.01	0.730	0.917

difference only for sAPP β (Fig. 1; $P < 0.02$). A higher level of sAPP β in patients has been previously described (Lewczuk et al., 2010) but is still controversial (Hock et al., 1998; Lannfelt et al., 1995; Olsson et al., 2003; Palmert et al., 1990; Zetterberg et al., 2008). In previous studies, for example, no significant increase of sAPP using Western Blotting or sandwich enzyme-

linked immunosorbent assay has been observed in AD patients (Olsson et al., 2003; Sennvik et al., 2000). In familial AD, symptomatic patients with APP mutations had lower sAPP α (Lannfelt et al., 1995) and low levels of this fragment were correlated to poor performance on neuropsychological tests (Almkvist et al., 1997). This discrepancy with recent data,

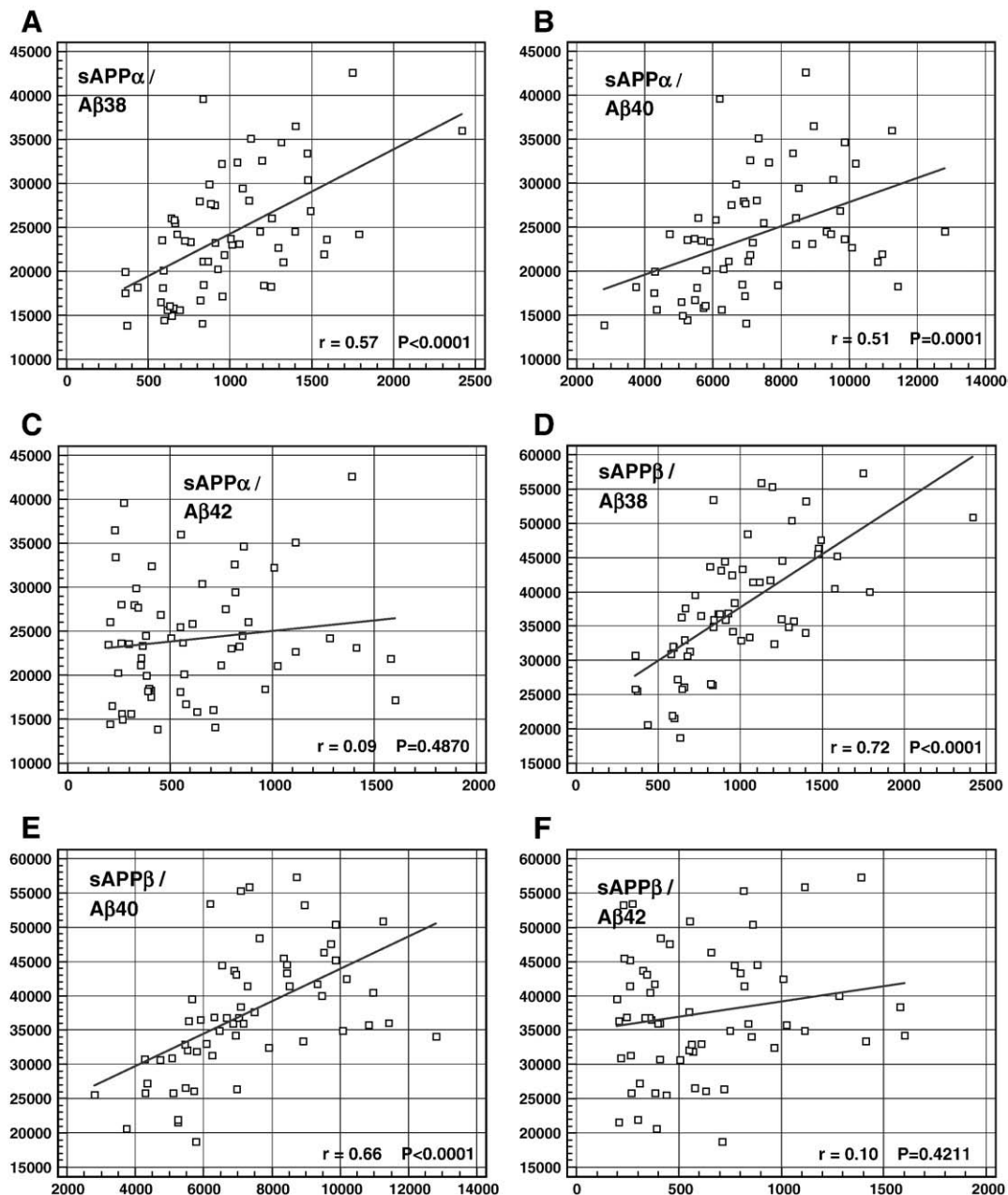


Fig. 2 – Correlation plots between CSF soluble amyloid precursor protein α/β (sAPP α/β) and A β 38 (A, D), A β 40 (B, E), and A β 42 (C, F) peptides in the whole population. A statistically significant correlation was present only for A β 38 and A β 40 (A, B, D, and E).

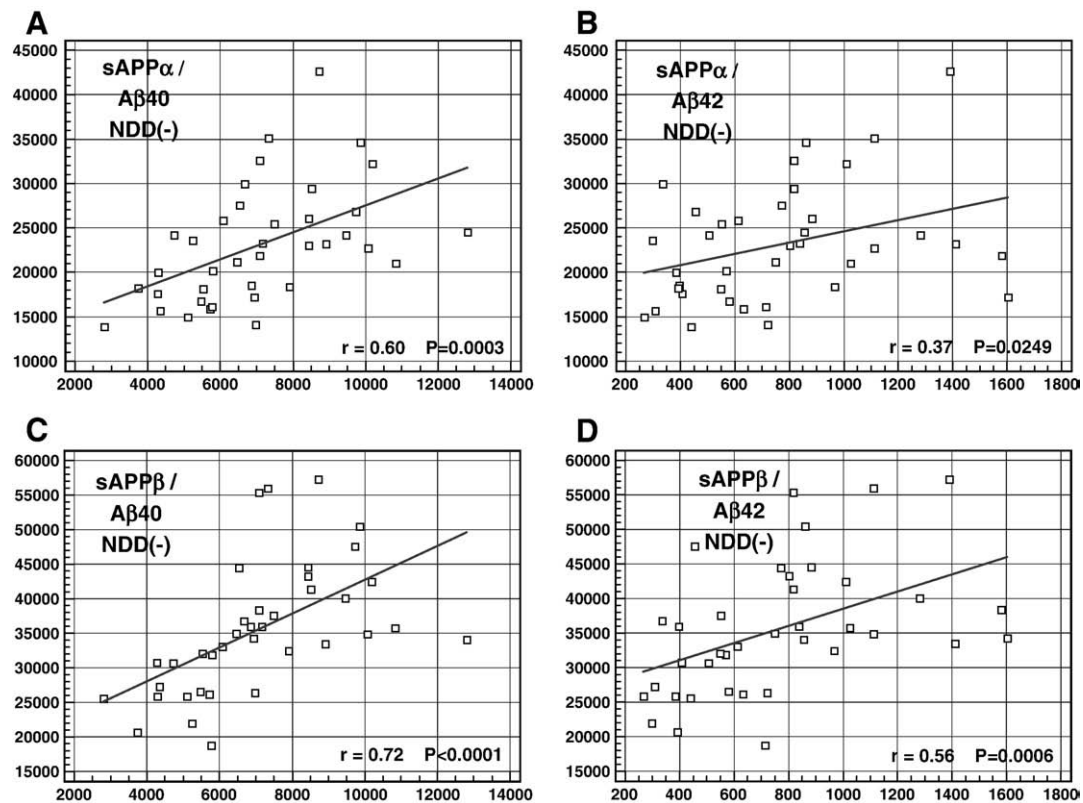


Fig. 3 – Correlation plots between CSF soluble amyloid precursor protein α/β (sAPP α/β) and A β 40 (A, C) and A β 42 (B, D) peptides in the NDD– population. A statistically significant correlation was present in particular for sAPP β and A β 40 (C).

including our, might be related to differences in population number, preanalytics or analytical methods. In addition, we lacked here a cognitively healthy control group which would be needed to really evaluate the clinical interest and relevance of sAPP measurement. In any case, a higher concentration of sAPP in neurochemical pathological group could be explained by several hypotheses. Firstly, the intrinsic amount of APP available for metabolic processing could be increased in patients with symptomatic or asymptomatic AD amyloid lesions. This hypothesis is supported by the observation from Lewczuk et al. (2010) that sAPPs were higher in MCI/NDD+ than in MCI/NDD– and that no significant difference has been detected between MCI/NDD+ and AD/NDD+. This infers that pathological processes and neurochemical alterations precede clinical symptoms by several years, giving rationale for the use of CSF sAPP biomarkers. It was not the purpose of our study, which focussed on biological data, to evaluate the interest of sAPP β for AD diagnosis. However, with an optimal cutoff, a sensitivity of 78.6% and a specificity of 62.5% were observed for the positive diagnosis of AD, the area under the ROC curve being at 0.68.

One explanation for our data could be related to a feed back regulation of APP level by A β concentrations. As soluble A β 42 levels decrease in AD this could result in a positive production signal for APP and/or for secretase activities as observed for BACE1 in patients with AD and MCI/AD (Blennow, 2005; Zhong et al., 2007). Finally, a modification of the degradation/clearance of these sAPP fragments in AD could also account for our results.

The second major outcome of our study relates to the intricate relationship between sAPP fragments and A β peptide levels in human CSF. A strong correlation was indeed observed between A β 38 and sAPP β and, in a lesser extent, with sAPP α or with A β 40 (Fig. 2). This suggested that A β 38 production was in direct relation with β secretase activity even in pathological situations. This result was of high interest with in mind the follow-up of the activity of the β -secretase in therapeutic strategies targeting this metabolic pathway. sAPP α and β being correlated, it made sense to also find a limited correlation between A β 38 and sAPP α . Noteworthy, the correlation of sAPP β with A β 40 and A β 42 was really significant only in NND– samples (Fig. 3). One interpretation of these data is that the three peptides were physiologically generated in parallel through the normal processing of APP. In the presence of an amyloid process like AD, the peptides would have different fates. A β 42 that is particularly prone to aggregation would therefore have its concentration modified in the disease (Blennow et al., 2006). The differential behaviour of the different A β species was also illustrated by the strong correlation existing in all situations between A β 38 and A β 40 (Fig. 4), while A β 42 was correlated to these fragments only in non pathological situations (NDD–). These results are important for hypothesis on APP processing and pathologic involvement. They suggest a strong physiological coregulation between the generations of the different A β entities which are all produced by the same secretase (Vassar et al., 1999). This argued also against a nonspecific degradation/conversion of the longer A β peptides into the smaller ones. The decrease

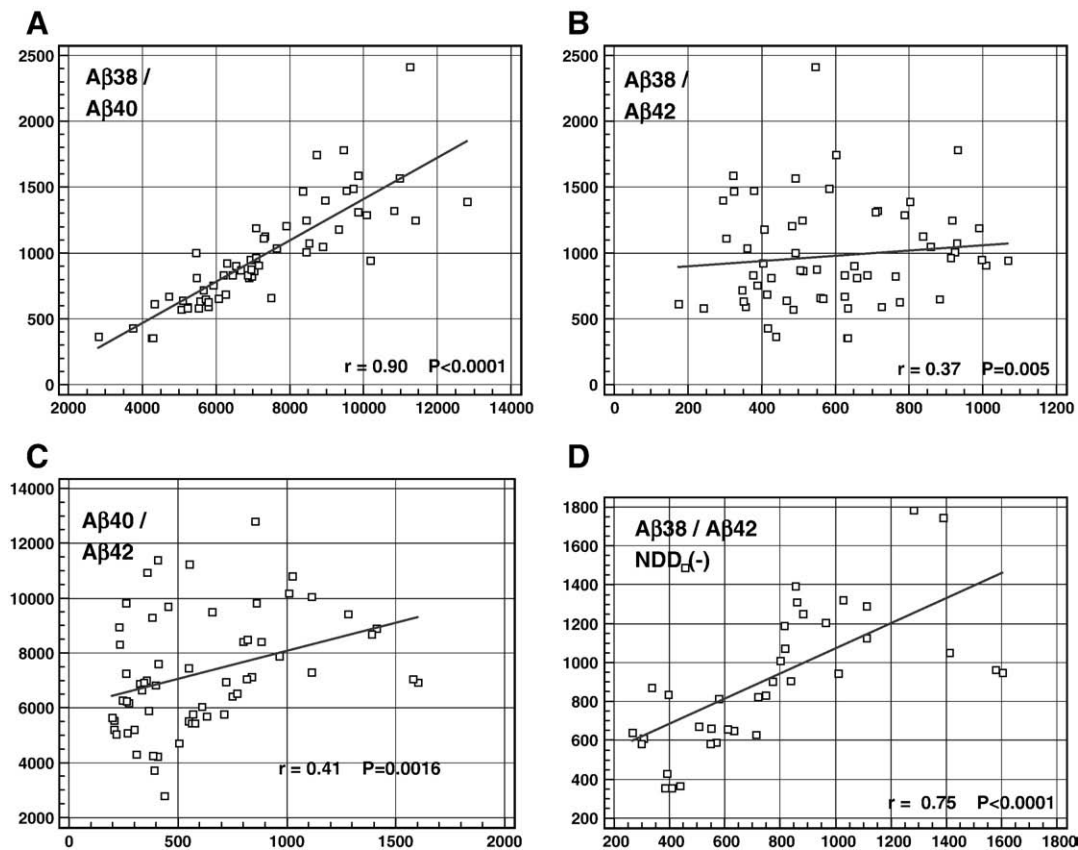


Fig. 4 – (A–D) Correlation plots between CSF A β 38, A β 40, and A β 42 peptides. A strong correlation was observed in the whole population between A β 38 and A β 40 (A), while the correlation between A β 38 and A β 42 (D) was significant only in NDD– patients.

of A β 42 observed in NDD+ patients (that have also increased P-tau levels) illustrated the strong involvement of this peptide in the pathology and the loss of correlation with the other peptides in presence of neuropathological changes. Our observations contribute to the biochemical rationale for using ratio between A β 42 and A β 40 (or A β 38) to improve AD diagnosis (Welge et al., 2009; Wiltfang et al., 2007). In fact, as the baseline level of A β production seems variable between individuals leading to possible misdiagnosis, the fact that A β fragments have different fates allows using ratios independently of baseline production.

In conclusion, our correlations between sAPPs and A β peptides, as well as between A β peptides themselves enlightened the complex relationships between these molecular markers in both physiological and pathological situations. Our results are important for the further use of these analytes for AD diagnosis, as well as to validated cell biological hypotheses of APP processing and A β fragment production.

4. Experimental procedures

4.1. Subjects and study design

Patients included in this study were selected in two independent cohorts of subjects referring to the Neurological Units of

Montpellier/Nîmes and Lyon for cognitive or behavioural troubles and who gave their consent to include their biological samples into a research biobanks in the field of dementia. All patients underwent a standardized clinical and paraclinical investigations including anamnestic, physical and neurological examination, screening laboratory tests, neuropsychological assessment, brain morphological imaging (CT or MRI) or functional imaging (SPECT) evaluations, and also CSF Tau, P-tau₁₈₁, and A β 42 analyses using Innogenetics™ ELISA kits.

All the clinical diagnoses (38 NDD–=26 DFT, 3 DCL, 7 DTA, 1 psy, and 1 other and 22 NDD+=1 DFT and 21 DTA) were validated an expert in the field of dementia and during consensus multidisciplinary evaluation. For AD, diagnosis criteria of the National Institute of Neurological Disease and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) (McKhann et al., 1984) were used. For frontotemporal lobar dementia (FTD), the diagnosis criteria were based on the Lund and Manchester criteria established in 1994 (Groups, 1994) and revised by McKhann et al. (2001) and Neary et al. (2005). The patients with Lewy body dementia were validated using the international clinical diagnosis consensus criteria (McKeith et al., 2005). The diagnosis status of the patients was discussed and confirmed during a multidisciplinary evaluation integrating imagery and clinical/neuropsychological data. Sixty CSF samples with possible and probable AD (n=28), FTD (n=27),

LBD ($n=3$), and other neurological disease ($n=2$) were analyzed. As the purpose of the investigation was primarily the biological relationships between the studied analytes, rather than their diagnosis interest, the samples were divided as in Lewczuk et al. (2010) between neurochemical dementia diagnosis-positive (NDD+) and negative (NDD-) using our routine P-tau and IATI (Innogenetics $A\beta$ /Tau index) cutoff values of 60 and 0.8, respectively. A total of 22 samples complied with NDD+ conditions, the 38 remaining being in the NDD- group.

4.2. CSF samples and routine assays

Lumbar puncture (LP) was performed in standardized conditions. LP was carried out at the L3/L4 or L4/L5 interspace after exclusion of the potential contraindications. CSF samples were taken directly in similar polypropylene tubes to avoid variation in adsorption of biomarkers to the container surface. All samples were transferred to the biochemical laboratories (CHU Montpellier/Nîmes, Hospices Civils de Lyon) in less than 4 hours. The CSF was centrifuged ($1000\times g$, 10 min, at 4–8 °C, without break), and the supernatant was aliquoted by 0.5 mL in polypropylene tubes before storage at –80 °C. The three routine CSF biomarkers Tau, P-tau₁₈₁, and $A\beta$ 42 were determined with a standardized commercially available ELISA Kit (Innotest beta-amyloid 1–42 (Vanderstichele et al., 2000), Innotest hTau-Ag (Vandermeeren et al., 1993), and Innotest Phosphotau(181P) (Vanmechelen et al., 2000), Innogenetics, Ghent, Belgium) in both laboratories. Quality control program organised by Hospices Civils de Lyon was used to study inter laboratories variability, using two CSF samples. The IATI score was calculated using the formula $IATI = A\beta 42 / (240 + 1.18 \times \text{tau})$ as described (Hulstaert et al., 1999). Routine CSF analyses included glucose, total protein and cytology. Only samples that were assessed as red cells count $<2000/\text{mm}^3$ were used for analyses.

4.3. Multiplex assays of CSF sAPP α/β and A β 38, A β 40, A β 42

Detection of these analytes was performed independently in the two laboratories using multiplex kits purchased from Meso Scale Discovery (ref: K11120E, K11148E). All reagents were provided with the kits. They contained a 96-well plate with two carbon electrodes precoated with either anti APP antibody 22C11 (sAPP α/β) or antibodies specific for A β 38, A β 40, A β 42. The wells were incubated for 1 hour with the block solution (bovine serum albumin) and washed four times with the Tris Wash Buffer (TWB). For sAPPs duplex assay, 25 μl of standards (dilution of recombinant sAPPs) and samples were then added to all wells, and the plate was sealed and incubated for 1 hour at room temperature on an orbital shaker (300 rpm). At the end of the incubation, the wells were washed four times using the TWB. Detection antibody (sAPP α/β) was added at 25 μl per well, and the plate was sealed and incubated for 1 hour at room temperature on an orbital shaker (300 rpm). For A β triplex assay, 25 μl of standards (dilution of recombinant A β) and samples were added at the same time of addition of 25 μl of detection antibody (6E10) to all wells, and the plates were incubated during 2 hours. At the end of the incubation,

the plate was washed four times as before. About 150 μl of the MSD Read Buffer T was added to each well, and the MSD plates were measured on the MSD Sector Imager 6000 plate reader. The raw data were measured as electrochemiluminescence signal (light) detected by photodetectors and analysed using the Discovery Workbench 3.0 software (MSD). A 4-parameter logistic fit curve was generated for each analyte using the standards and the concentration of each sample calculated. The intra-assay coefficient of variation ranged from 0.2% to 15.9%. The interlaboratory coefficient of variation ranged from 0.3% to 17%.

4.4. Statistical analysis

If not stated otherwise, the results were presented as medians and interquartile ranges. The Mann–Whitney–Wilcoxon test was used to test the significance of the difference between two sample groups. Correlations between measured values were analyzed with Spearman's correlation factor. Differences were considered significant if $P < 0.05$. Analyses were performed with MedCalc (3.0).

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