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## CSF biomarkers cutoffs: the importance of coincident neuropathological diseases

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### Abstract

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The effects of applying clinical versus neuropathological diagnosis and the inclusion of cases with coincident neuropathological diagnoses have not been assessed specifically when studying cerebrospinal fluid (CSF) biomarker classification cutoffs for patients with neurodegenerative diseases that cause dementia. Thus, 142 neuropathologically diagnosed neurodegenerative dementia patients [71 Alzheimer's disease (AD), 29 frontotemporal lobar degeneration (FTLD), 3 amyotrophic lateral sclerosis, 7 dementia with Lewy bodies, 32 of which cases also had coincident diagnoses] were studied. 96 % had enzyme-linked immunosorbant assay (ELISA) CSF data and 77 % had Luminex CSF data, with 43 and 46 controls for comparison, respectively. A $\beta$ <sub>42</sub>, total, and phosphorylated tau<sub>181</sub> were measured. Clinical and neuropathological diagnoses showed an 81.4 % overall agreement. Both assays showed high sensitivity and specificity to classify AD subjects against FTLD subjects and controls, and moderate sensitivity and specificity for classifying FTLD subjects against controls. However, among the cases with neuropathological diagnoses of AD plus another pathology (26.8 % of the sample), 69.4 % (ELISA) and 96.4 % (Luminex) were classified as AD according to their biomarker profiles. Use of clinical diagnosis instead of neuropathological diagnosis led to a 14–17 % underestimation of the biomarker accuracy. These results show that while CSF A $\beta$  and tau assays are useful for diagnosis of AD and neurodegenerative diseases even at MCI stages, CSF diagnostic analyte panels that establish a positive diagnosis of Lewy body disease and FTLD are also needed, and must be established based on neuropathological rather than clinical diagnoses.

## Keywords

Biomarker; Cerebrospinal fluid; Alzheimer's disease; Frontotemporal lobar degeneration; Amyloid beta; Tau

## Introduction

Alzheimer's disease (AD) is the most common dementia and the most frequent neurodegenerative disease [31]. The neuropathological hallmarks of AD are extracellular deposits of A $\beta$  in senile plaques and intracellular aggregates of tau protein in neurofibrillary tangles [3, 26, 32, 39]. The second most common cause of neurodegenerative disease dementia is frontotemporal lobar degeneration (FTLD), a heterogeneous group of neurodegenerative diseases characterized by deposits of TDP-43 (FTLD-TDP), tau (FTLD-TAU) or FUS (FTLD-FUS) [36]. Deposition of  $\alpha$ -synuclein in Lewy bodies defines the pathology of Parkinson's disease without (PD) or with dementia (PDD) [14, 17] and dementia with Lewy bodies (DLB) [37]. While the signature pathologies of these different forms of dementia are well defined, these pathologies frequently co-occur in patients with a neurodegenerative dementia. However, the presence of coincident neurodegenerative disease pathologies in a given patient classified clinically as having either AD, PD, PDD, DLB or one of the major forms of FTLD often is not anticipated prior to postmortem examination [30,31,53].

The identification of characteristic protein aggregates in the brains of patients with these diseases has led to the development of promising biochemical [5, 55] and neuro-imaging biomarkers [16, 45]. Cerebrospinal fluid (CSF) and positron emission tomography (PET) A $\beta$  measurements show a strong correlation with each other [60] and with AD-related A $\beta$  senile plaque pathology [27]. CSF phosphorylated tau (p-tau) and total tau (t-tau) are also established AD biomarkers and both CSF tau and A $\beta$  have recently been added to the clinical diagnostic criteria for mild cognitive impairment (MCI) and AD [2, 11, 38]. For FTLD patients there are few established neuroimaging or biochemical markers [24, 25], while biomarkers of PD, PDD and DLB are just emerging [41, 47, 57]. So far, the diagnostic accuracy of biomarkers has been determined primarily based on their correlation with

clinical diagnostic criteria [5, 48, 63]. Most studies on autopsy-confirmed cases have included small numbers of patients [4, 21, 56]. There are only three CSF biomarker studies with moderate [15] to large numbers (>100) of subjects with neuropathologically confirmed diagnoses [9,59]. However, none of these studies analyzed how overlapping neurodegenerative disease pathologies in patients with clinical evidence of dementia during life influence antemortem CSF biomarker levels and diagnostic accuracy established by postmortem neuropathology studies. Patients with coincident neuropathological diagnoses can represent an important and challenging part of the recruited subjects in clinical trials involving drugs directed specifically against deposits that are thought to be disease specific. Therefore, if a patient has AD and DLB, immunotherapy against amyloid or tau deposits could improve AD pathology, but would not affect  $\alpha$ -synuclein deposits and patients could still show cognitive decline even if there is a response of amyloid and tau pathologies to the treatment.

To enable more accurate diagnosis of co-occurring neurodegenerative diseases prior to death, we compared biomarker studies with post-mortem neuropathology studies in a cohort of patients with clinical dementia who had been followed with CSF sampling until death. First, we defined cases neuropathologically, differentiating those with a single neuropathological disease from those with coincident neuropathological diagnoses. In a second step, we built a classification algorithm using clinical controls and neuropathologically diagnosed cases with a single neuropathological disease, either FTLN or AD. This was done using a training-validation scheme that had three diagnostic categories: clinical controls, AD neuropathology, and FTLN neuropathology. In a third step, we tested our model in the complete sample that included the patients who had two coincident neuropathological diseases and obtained the different sensitivity and specificity values. Finally, we analyzed which would have been the estimated accuracy measures for the biomarkers if we used clinical diagnosis instead of neuropathological diagnosis and determine if there was a change in the classification algorithm. We included data for ELISA and Luminex platforms in the study so that the results would be useful for researchers that use either one of the platforms, but comparing these platforms was not the purpose of the study.

## Materials and methods

### Subjects

Subjects included 142 patients with clinical diagnoses of AD, DLB or frontotemporal dementia (FTD), and autopsy confirmation of their neuropathological diagnoses (Table 1). Neuropathological and clinical information [including mini-mental state exam (MMSE) and clinical dementia rating (CDR)], as well as CSF biomarker data, were gathered for these subjects, along with clinical and CSF biomarker data from 66 age-matched cognitively normal living controls. All data were obtained from the Penn Center for Neurodegenerative Disease Research (CNDR) Integrated Neurodegenerative Disease Database (INDD) [69]. CSF samples for the biomarker studies had been obtained at the Penn Memory Center (PMC), which is the clinical home of the AD Core Center (ADCC), and the Penn FTD Center (FTDC) [21]. CSF tau and A $\beta$  were assessed as described below. APOE genotype and general demographic information also were retrieved from the INDD.

The clinical diagnoses were established following clinical diagnostic criteria for AD [38], social/executive FTD (bv-FTD) [50], corticobasal syndrome (CBS) [22], primary progressive aphasia [20] and DLB [37] as previously reported [67, 68]. For the purposes of this study, patients diagnosed as CBS, bv-FTD, FTD-motor neuron disease, progressive non-fluent aphasia (PNFA) and semantic dementia (SD) were classified as FTD, while subjects with AD and logopenic progressive aphasia (LPA) were classified as AD. As per current

conventions, the term FTD was used for the clinical diagnosis and the term FTLT for the neuropathologically confirmed diagnoses. The age of onset of the disease was established after reviewing the clinical charts.

Written informed consent had been obtained for all patients using a protocol approved by the Institutional Review Board of the University of Pennsylvania.

### Biofluid collection/analysis

CSF samples were obtained as described previously and samples were immediately stored at  $-80^{\circ}\text{C}$  until analysis [21].

Samples had been previously analyzed using the ELISA assay (INNOTEST<sup>®</sup>, Innogenetics, Ghent, Belgium) or the Luminex xMAP platform (for research-use-only INNO-BIA AlzBio3<sup>™</sup> immunoassay reagents, Innogenetics, Ghent, Belgium) [21, 46, 56]. Monoclonal (MAb) capture and reporting antibodies used in the ELISA method for detection of t-tau and p-tau 181 in CSF were AT120/HT7 and BT2, HT7/AT270, respectively. The ELISA values for A $\beta_{1-42}$  were measured using an “in house” ELISA method [66] with MAb BAN-50 as the capture and MAb BC-05 as the reporting antibody [66]. The xMAP platform utilized the capture MAbs 4D7A3 (A $\beta_{1-42}$ ), AT120 (t-tau), and AT270 (p-tau 181) bound to color-specific beads. The biomarker analytes were detected using the reporting MAbs 3D6 (A $\beta_{1-42}$ ) and HT7 (t-tau, p-tau 181). Luminex measurements were obtained after dividing the samples into three different runs, two using the same kit lot number, and one with a different kit lot number; whereas ELISA measurements were obtained in 12 different runs between the years 1998 and 2009. 23 controls and 104 patients had measurements with both assays. In case of repeated ELISA measurements values from the largest runs were selected.

### Neuropathological analysis

Postmortem neuropathology analyses were performed on 12 central nervous system (CNS) regions: amygdala, hippocampus (CA1/subiculum), entorhinal cortex, midfrontal gyrus, angular gyrus, superior/middle temporal gyrus, cingulate gyrus, motor cortex, caudate nucleus, midbrain, medulla oblongata, and cervical spinal cord.

Sections were fixed and cut into 6–10  $\mu\text{m}$  sections, stained with hematoxylin and eosin to assess neuronal loss and gliosis and Thioflavin S to grade senile plaques semiquantitatively. Immunohistochemistry was performed with antibodies for tau,  $\alpha$ -synuclein, ubiquitin, and TDP-43 as previously described [18, 19, 42, 43, 62], as well as antibodies to FUS in FTLT cases as described [61]. Pathologies were then rated according to a semi-quantitative scale [19, 62]. Briefly, neurofibrillary tangles and senile plaques were graded according to NIA-Reagan criteria using Braak staging [6, 7] and the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) protocol, without considering subject age [39]. A neuropathological diagnosis of AD was assigned if the probability was intermediate or high [26]. The diagnoses of FTLT-TAU, FTLT-TDP and DLB were based on established criteria [36, 37]. FTLT-TAU cases included those with a diagnosis of argyrophilic grain disease (AGD), progressive supranuclear palsy (PSP), tangle predominant senile dementia (TPSD), corticobasal degeneration (CBD), and unclassifiable tau-positive FTLT (Unclas. T).

For cases with coincident neurodegenerative disease pathologies, i.e., those with more than one neuropathological diagnosis, the following terms were used: FTLT-TDP-Tau for FTLT with both TDP-43 and tau pathology, AD-FTLT for AD cases with a secondary diagnosis of FTLT-Tau or FTLT-TDP, FTLT-AD if the main diagnosis was FTLT with concomitant AD pathology, and AD-DLB for AD cases with a secondary diagnosis of DLB. Six of the seven cases with a clinical diagnosis of DLB also had a secondary neuropathological diagnosis of AD and were classified as DLB-AD.

## Statistical analysis

Normal distribution and homoscedasticity were tested when necessary. For normally distributed variables, a oneway ANOVA was used to test group differences and Tu-key's honest square difference was used for post hoc analyses. For comparison of two groups, Student's *T* test or Wilcoxon rank-sum test was used according to the presence or absence of a normal distribution. For categorical variables, Pearson  $\chi^2$  test was used. However, in cases with low number of expected counts Fisher's exact test was used. To study the relationship between CSF biomarkers and Braak and CERAD scores, an ordinal logistic regression was applied.

For logistic regression analysis, the sample was divided into training-validation (70 % of the sample) and test sets (30 % of the sample). A stepwise logistic regression model using forward and backward steps with a leave-group-out cross-validation (data were randomly split ten times into a 70 % training and 30 % validation set) was used to select the ideal combination of biomarkers for the different models in the training-validation set, using accuracy and kappa index as performance metrics [33, 65]. The logistic regression model rendered a probability value for AD for each subject ranging from 0 to 1. Age,  $A\beta_{42}$ , p-tau, and t-tau were introduced as potential predictors in the model and the best model was compared against the corresponding ratio (t-tau/ $A\beta_{42}$  or p-tau/ $A\beta_{42}$ ). In no case did the ratio offer equal or better performance than the use of the combination of the single analytes. The obtained logistic regression model was then applied to the test set to obtain sensitivity, specificity, accuracy, kappa index, and the area under the curve (AUC) in the receiver operating characteristic (ROC) curve. ROC curves and density plots were used to illustrate the results [51]. The initial analysis classified neuropathologically confirmed AD cases against neuropathologically confirmed FTLN and clinically diagnosed control cases. A second model was then derived to classify control cases against FTLN cases.

The obtained regression models were then applied in the whole sample, including the cases with coincident neuropathological diagnoses. Thereafter, sensitivity and specificity measures for different settings were obtained (i.e., for the MCI and dementia settings). Taking the original sample of the first analysis again, we then used the clinical diagnoses in a new logistic regression model. Metrics obtained with this model and the probabilities obtained in both models (the neuropathological and clinical) were compared. All statistical tests were two-sided. Statistical significance was set at the 0.05 level. Statistical analyses were performed using IBM SPSS statistics v. 19 and R 2.13.1 [23,33,49,51].

## Results

A total of 142 clinically diagnosed neurodegenerative dementia patients with an autopsy-confirmed diagnosis of one or more neurodegenerative diseases and antemortem CSF tau and  $A\beta$  measurements were included in this study (Table 1). Of the neuropathologically confirmed cases, 71.1% showed a single type of neurodegenerative disease pathology indicative of a single diagnosis (i.e. AD, FTLN-TDP, FTLN-TAU, or DLB), while 28.9 % of cases presented with an overlap of different neurodegenerative disease pathologies. The group with overlapping neuropathological diagnoses included: 24 AD-DLB, 2 AD-FTLN, 6 FTLN-AD, 3 with both subtypes of FTLN, and 6 DLB-AD. Only 1 out of the 7 cases with a main neuropathological diagnosis of DLB did not have a secondary diagnosis of AD. For further analysis this case was grouped with the DLB-AD cases. In the FTLN-TAU and in the FTLN-TDP groups, there was one subject per group that had a CERAD C classification; however, in both cases the tau pathology corresponding to AD was classified as Braak stage I-II. Additionally, 43 cognitively normal living controls were available that had ELISA  $A\beta$  and tau data (median follow-up 48 months) and 46 with Luminex data for these CSF biomarkers (median follow-up 35 months). None of the controls progressed to MCI or AD.



Of these, 23 controls were included in both assays. The mean time between onset of cognitive symptoms and time of CSF sampling was 2.8 years (SD 2.1). The median time lapse between the MMSE scoring and CSF sampling was 1.3 months (0.8–3.3 months). The different groups of patients with neurodegenerative diseases did not show differences in MMSE scores at the time of CSF sampling ( $\chi^2 = 11.3$ ,  $p = 0.13$ ), and all these groups had lower MMSE scores than the control group. The overall median MMSE score for patients with a neurodegenerative disease at CSF sampling was 22 (15.0–26.0), and 41.4 % of them had a MMSE higher than 23. At CSF sampling, 36 patients did not meet dementia criteria: Five cases [including the three amyotrophic lateral sclerosis (ALS) cases] were cognitively normal, 27 had an MCI diagnosis, three had a primary progressive aphasia (PPA) diagnoses, and one a behavioral FTD diagnosis at the time of CSF sampling. The other 106 neuropathological cases had a dementia diagnosis. CDR was available for 55 of these patients (40 CDR 1.0, 8 CDR 2.0, and 7 CDR 3.0). Overall, the groups differed in the percentage of APOE4 positive subjects (one or more APOE  $\epsilon$ 4 allele) [ $\chi^2(9, n = 204) = 47.4$ ,  $p < 0.0001$ ]. Subjects with FTLT-DTP had a lower frequency of APOE4 than those with AD and AD-DLB, Controls and FTLT-TAU subjects had a lower APOE4 frequency than AD, AD-FTLT, FTLT-AD, DLB-AD, and AD-DLB.

There was no statistically significant difference regarding the time interval from CSF sampling to death ( $H(8,134) = 1.8$ ,  $p = 0.08$ ) or regarding age ( $H(9,198) = 1.44$ ,  $p = 0.17$ ) between the groups. The overall agreement between clinical and neuropathological diagnosis was 81.4 % (Supplementary Table 1). The CSF biomarker values for the Luminex and ELISA assays stratified by neuropathological diagnosis are represented in Fig. 1 and supplementary figure 1, respectively. Supplementary figure 2 represents the CSF biomarker values according to Braak and CERAD scoring. Supplementary table 2 summarizes the ordinal logistic regression analyses used to assess the association between the biomarkers and neuropathological scores, adjusting for time.

### **Classification cutoffs based on subjects with a single neuropathological diagnosis and clinically diagnosed controls**

The three measured biomarkers in both assays differed in subjects with “pure” AD, FTLT-DTP, and/or FTLT-TAU and controls ( $p < 0.0001$ ). The post hoc analysis of FTLT subjects and controls differed in all measurements from AD subjects. However, controls and FTLT subjects only differed in A $\beta$ <sub>42</sub> measurements in the ELISA and Luminex assays (Supplementary table 3).

The logistic regression model that best distinguished AD from the group combining controls and FTLT cases showed an AUC of 0.96 for ELISA and for Luminex in the test set (Table 2). For distinguishing FTLT from AD patients, the AUC for these two groups were 0.96 and 0.98 for ELISA and Luminex, respectively. The Luminex and ELISA assays showed moderate sensitivity and specificity for classifying FTLT patients compared to controls. AUC, accuracy, kappa, sensitivity, and specificity scores are summarized in Table 2. The AUC and the density plots obtained in the test set are depicted in Fig. 2. The AUC is represented with the 95 % confidence interval (CI) shaded in blue and the selected cutoff is depicted in the curve, including the 95 % CI for specificity and sensitivity of the cutoffs. The density plots show the distribution for the predicted probabilities for the different groups by the logistic regression model. In addition, the accuracy and AUC results from the models obtained using single analytes and the selected combination in the training-validation sample are summarized in supplementary table 4. The coefficients of the selected logistic regression models are shown in supplementary table 5.

Although DLB-AD subjects showed lower t-tau levels in the ELISA assay, no variable was entered in the ELISA regression stepwise model, indicating that none of them was useful for classification of patients.

### Cutoffs applied to all cases, including cases with coincident neurodegenerative disease pathologies

We applied the obtained regression models that included control subjects in the whole sample. Based on the previous ELISA cutoffs, 69.4 and 96.4 % of the cases with coincident neuropathological diagnoses (that included AD) were classified as AD by the ELISA and Luminex CSF measurements, respectively (Fig. 3). In both assays, the only DLB case that did not have AD pathology was classified as belonging to the FTLN-control group.

We determined the diagnostic accuracy for distinguishing neuropathologically confirmed cases with concomitant AD pathology versus those cases without concomitant AD pathology. ELISA cutoffs showed a sensitivity of 90.1 % and a specificity of 80.8 % to differentiate subjects with AD pathology (main or secondary) against FTLN cases without an AD diagnosis, while Luminex results were 96.7 and 88.2 %.

We also compared the controls against all the neuropathologically diagnosed cases, finding a sensitivity of 85.6 % and a specificity of 76.7 % to identify subjects with a neurodegenerative disease with ELISA assay; while with the Luminex assay these values were 93.3 and 84.8 %. When classifying AD subjects against FTLN subjects and controls, the sensitivity and specificity were 74.3 and 89.9 % for ELISA assay and 95.6 and 87.3 % for Luminex assay (Table 3).

To test if biomarkers showed the same accuracy in subjects with less cognitive impairment, we divided the groups of patients with a main diagnosis of AD or FTLN and no secondary neuropathological diagnosis according to their MMSE scores. Therefore, those subjects with an MMSE below or equal to 24 were compared to those with an MMSE higher than 24. We found that the proportion of patients correctly classified by main diagnosis did not differ in MMSE score, neither in the ELISA [AD:  $\chi^2(1, n = 62) = 1.1, p = 0.29$ ; FTLN ( $n = 25$ ):  $p = 1.0$ ], nor in the Luminex model [AD:  $\chi^2(1, n = 60) = 1.4, p = 0.24$ ; FTLN:  $p = 1.0$ ].

### Classification cutoffs based on clinical diagnosis

As seen in Table 2 the obtained sensitivity and specificity for the biomarker classification formulas based on clinical diagnosis were worse than the ones based on neuropathological diagnosis. When the predicted probability of each subject obtained by the clinical classification model was compared with the predicted probability based on the neuropathological model, there was a median change in the predicted probability of 3.0 % in the ELISA assay (1st and 3rd quartiles: 0.3–22.2 %) and 12.8 % in the Luminex assay (1st and 3rd quartiles: 1.0–35.2 %).

In addition we compared the diagnosis based on clinical, neuropathological, and biomarker criteria using the Luminex assay for the biomarker values. As shown in supplementary table 6 Luminex classification algorithm predicts the neuropathological diagnosis more accurately than the clinical diagnosis in 13 patients, while it incorrectly assigned a diagnosis compared to the clinical and neuropathological diagnosis in two cases.

## Discussion

This is the first study to use CSF biomarkers to assess the relevance and classification of coincident neurodegenerative disease pathologies, as determined by autopsy-confirmed diagnoses in a large neuropathological cohort. We show that patients with overlapping

diagnoses represent an important subgroup in clinical cohorts, but are often not identified correctly with the current biomarkers. It is also the first study to demonstrate that using samples classified by clinical diagnosis leads to an underestimation of biomarker sensitivity and specificity values and shifts the cutoffs. Our results confirm that A $\beta$ <sub>42</sub>, p-tau and t-tau as measured by ELISA and 'Luminex assays enable the differentiation of AD from FTLD in a sample of demented patients and that AD can be distinguished from FTLD and controls in a cohort sample of MCI and early dementia patients, i.e., at early disease stages. In summary, these biomarkers are helpful to distinguish subjects with an underlying neurodegenerative disease (FTLD, AD or both) from cognitively normal controls.

The cases studied here included patients with typical AD pathology overlapping with FTLD-TDP or  $\alpha$ -synuclein proteinopathies. In our sample we found that 28.9 % of the cases presented with multiple neurodegenerative disease pathologies, in agreement with previously described samples [13, 31, 44, 52].

Our study demonstrates that the patients studied here were mainly classified as AD by a diagnostic biomarker panel, which includes p-tau, t-tau and A $\beta$ , regardless of the presence of other co-morbid neurodegenerative pathologies. While this may not be surprising since the biomarkers studied here are among the most informative AD biomarkers, it is an important observation that is highly relevant for neurodegenerative disease clinical trials because disease modifying therapies specifically directed against amyloid or tau might reduce the burden of tangles or plaques, but may not affect deposits of other disease proteins. In the absence of cognitive improvement, this could be mistakenly interpreted as a failure of the treatment to ameliorate plaque and tangle pathology rather than a failure to target the appropriate protein aggregate. Therefore, it is critically important to discover and validate biomarkers for non-AD pathologies.

As shown here, the bias introduced by the use of clinical categories when establishing biomarker cutoffs can have an important effect on the calculated sensitivity and specificity of CSF tau and A $\beta$  biomarker-based disease classification. Indeed, this may help explain the variability of published results validating these biomarkers based on clinical diagnosis and the variation in these biomarker cutoffs and estimated group probabilities. Recently, a large clinical sample with a small subset of neuropathological cases found that 34 % of the patients diagnosed as dementia of the non-AD type were found to have an AD CSF biomarker profile, and were thought to be incorrectly classified by these biomarkers and, therefore, the specificity of these biomarker was not deemed to be good [54]. The non-AD dementia clinical diagnoses in this study that showed a higher percentage of AD CSF profile were FTD and CBS patients that in our cohort have a 65.2 and 33 % clinical-pathological agreement, similar to other studies [34]. Thus, these clinical diagnoses may not be accurate for establishing CSF biomarker cutoffs or predicting the nature of the underlying neuropathology. Here, we demonstrate that the use of clinical diagnoses instead of neuropathological diagnoses underestimates at about 10–20 % sensitivity and specificity values for CSF tau and A $\beta$  biomarkers and that cohorts with larger numbers of subjects with neuropathological diagnoses are needed to establish the real accuracy of biomarkers as well as to establish cutoffs for classification.

These analyses point to the urgent need for reliable and informative CSF biomarkers that can be used to identify more homogeneous samples of AD or FTD patients with respect to underlying neuropathology for clinical trials, as well as for imaging, genetic and other studies of neurodegenerative dementias. Indeed, this study emphasizes the importance of recognizing that a large subset of patients who are classified as AD by currently available CSF biomarker measurements also may have other concomitant neurodegenerative disorders that may contribute to their cognitive impairments.



We have developed two classification algorithms. The first was designed for a dementia stage that would consist of patients with an underlying AD or FTLN neuropathological diagnosis (and a small subset of DLB cases without AD). The second was designed for earlier stages and offers three diagnostic categories: AD neuropathology, FTLN neuropathology, and a group that potentially has no neurodegenerative disease. This would allow classification of patients in an MCI stage as having or not having an underlying AD neuropathology, which would be useful for clinical trials, or as having or not having any neurodegenerative disease. In our cohort, we observed that 25.4 % of the subjects had an MCI diagnosis and another 28.2 % had a dementia diagnosis with a CDR of 1.0. The diagnostic accuracy did not differ in the group of more impaired patients when compared with the less impaired patients. This is in agreement with other studies that show that CSF A $\beta$  and tau levels are stable in MCI and AD patients [29, 35] and indicates that established CSF tau and A $\beta$  cutoffs are useful not only at the dementia stage but also in the MCI stage of disease. However, recently a study that included MCI patients with a median follow-up of 9.2 years, found that early converters (less than 5 years) had higher t-tau and p-tau levels than late converters (more than 5 years), while A $\beta$  showed no changes [8]. Most of our MCI patients converted to dementia in less than 5 years (data not shown); therefore, our model cannot be inferred to apply to the early stages of MCI without further studies.

This study has several strengths. First it is the largest cohort of subjects with a neuropathologically confirmed diagnosis from whom ante-mortem CSF tau and A $\beta$  measures were obtained. Only two other studies have reported similar data from >100 subjects [9,59]. Second, we present results for the two most commonly used biomarker platforms, i.e. Luminex and ELISA and give the classification algorithm for both assays. Third, the control subjects were closely age matched to the other groups and since the patients had postmortem neuropathological confirmation of their diagnoses, we were able to study a patient cohort that had well established coincident neuropathological diagnoses. Finally, the large sample size here allowed us to divide our cohort into a training-validation and a test set.

All the subjects with a main diagnosis of DLB in our sample, except one, had a secondary diagnosis of AD and were classified as cases with coincident neuropathological diseases. Other studies report DLB cases without an AD diagnosis; however, in our series of 1,240 neuropathological cases in the CNDR INDD, there are 81 cases with a coincident AD diagnosis but only 11 cases (12 % of all DLB cases) with a main diagnosis of pure DLB with no coincident AD diagnosis. The fact that only 3 % of DLB cases did not have a coincident AD neuropathological diagnosis could be due to a selection bias of cases based on a dementia clinic recruitment of the sample. This skewed representation might be due to a referral bias of our patients to a dementia clinic, which may result in a significant overlap between DLB and AD pathology in many patients drawn from such clinics. Finally, based on the small numbers of DLB cases studied here, we cannot make any claims on the overall CSF signature of DLB patients. We confirm previous results of lower CSF t-tau levels in cases with DLB (data not shown) [63], but the overlap of CSF biomarker measurements together with a lower prevalence of DLB present a significant challenge for improving the recognition of DLB based on CSF biomarkers [58]. However, it is possible that measuring other species of A $\beta$ , including A $\beta$ <sub>1-40ox</sub>, could improve the classification of DLB using CSF biomarkers [40].

One of the weaknesses of this study and previous studies is the lack of neuropathologically diagnosed controls. Previous studies using unsupervised analyses have shown that up to 36 % of cognitively normal subjects have a pathological AD CSF signature [10] and this is in agreement with a long preclinical or prodromal phase of AD during which time AD neuropathology is accumulating [1, 28] and the observation that CSF A $\beta$  measures are most

dynamic in the MCI stage of AD but are stable when AD dementia becomes clinically manifest [27, 35, 64]. Thus, it is possible that some of the cognitively normal subjects, classified as AD by the CSF measurements, are asymptomatic subjects at risk for AD [12] and these subjects may have shifted our cutoffs. While we also indicated that biomarkers could improve clinical diagnoses, we acknowledge that for the comparison shown in supplementary table 5 we used some subjects who had been used to train the logistic regression models. Finally, cases with vascular dementia were not included in our study and we therefore have mainly focused on neurodegenerative diseases in this study.

In summary, our study demonstrates the importance of using neuropathological diagnoses for establishing biomarker cutoffs and provides important new insights into the interpretation of CSF biomarkers for the classification of neurodegenerative dementias. We also emphasize the important clinical need to develop specific biomarkers for PD, PDD and DLB as well as other forms of FTLN in order to be able to accurately classify subjects with other coincident neurodegenerative disease pathologies as the basis for their neurodegenerative dementia. In addition, studies analyzing biomarkers in early stages of the disease are needed because of the dynamic characteristics of some biomarkers. Finally, this study underlines the critical importance of CSF biomarker assay standardization to increase accuracy for the early diagnosis of neurodegenerative diseases, especially since many patients with a neurodegenerative dementia will show evidence of coincident neurodegenerative disease pathologies and more than one neuropathologically confirmed neurodegenerative disorder as the underlying basis for their cognitive impairment.

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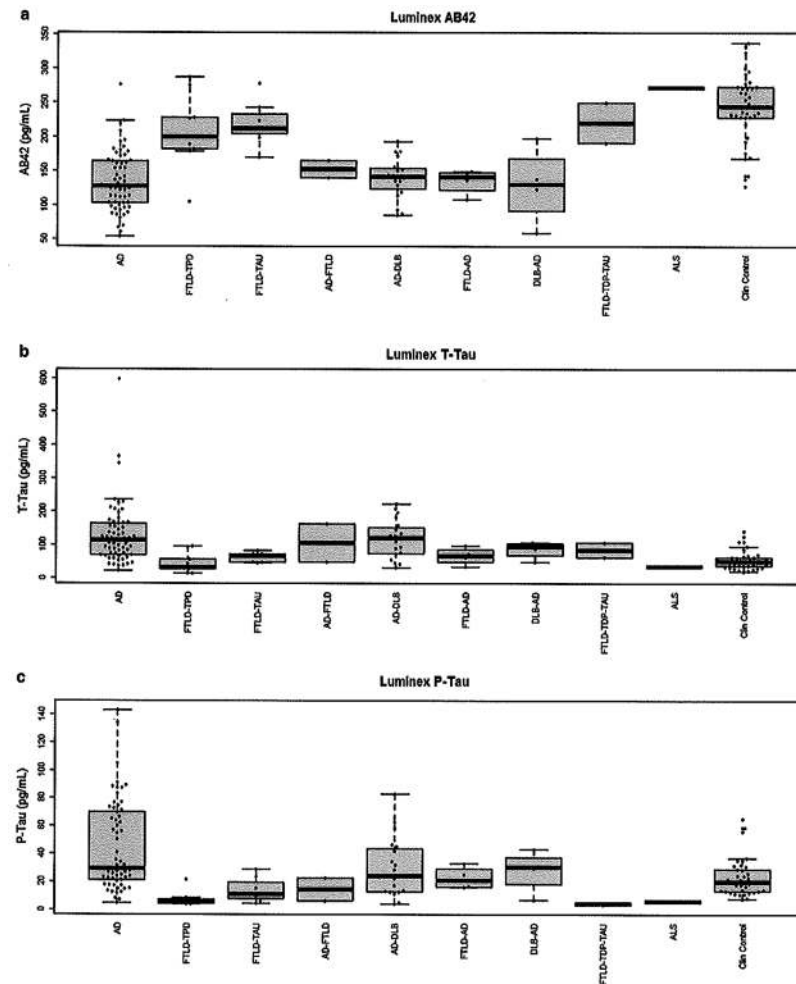
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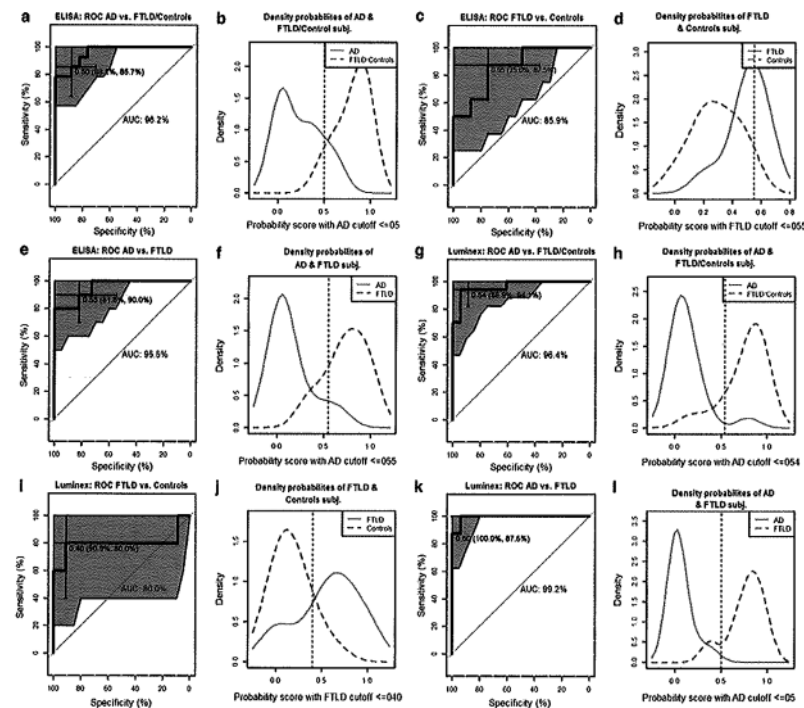
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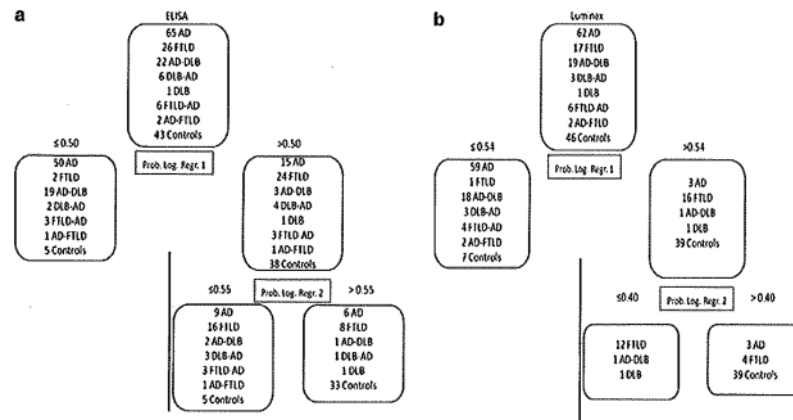


**Fig. 1.**  
Levels of Aβ<sub>42</sub>, t- and p-tau as measured by Luminex assay



**Fig. 2.**

ROC curve and density probabilities of the classification groups based on the probabilities obtained in the regression models. The values inside the parenthesis in the AUC curves indicate the specificity and sensitivity of the selected cutoff. The ROC curves and density plots represent values and distributions for AD versus FTLD and controls (**a, b**), FTLD versus controls (**c, d**) and AD versus FTLD versus AD (**e, f**) using ELISA. For the Luminex assay the ROC curves and density plots represent values and distributions for AD versus FTLD and controls (**g, h**), FTLD versus controls (**i, j**) and AD versus FTLD versus AD (**k, l**)

**Fig. 3.**

Classification of the cases based on ELISA (**a**) and Luminex assays (**b**) using the logistic regression models based on neuropathological diagnoses. The *upper box* represents all the cases available for the corresponding platform and the *boxes* below show how cases were classified. *Prob. Log. Regr.* probability obtained in the logistic regression model

Characteristics of the neuropathological and clinical diagnosed subjects included in the study

	Neuropathological						Controls				
	"Pure" AD (n = 71)	AD-DLB (n = 24)	AD-FTLD (n = 2)	FTLD-TDP (n = 13)	ALS (n = 3)	FTLD-TAU (n = 13)	FTLD-TDP-TAU (n = 3)	FTLD-AD (n = 6)	DLB-AD (n = 6) DLB (n = 1)	ELISA (n = 43)	Luminex (n = 46)
ELISA	66 (93.0 %)	24 (100 %)	2 (100 %)	13 (100 %)	2 (66.7 %)	13 (100 %)	3 (100 %)	6 (100 %)	7 (100 %)	43	–
Luminex	62 (87.3 %)	19 (79.2 %)	2 (100 %)	9 (69.2 %)	1 (33.3 %)	7 (53.8 %)	2 (66.7 %)	4 (66.7 %)	4 (57 %)	–	46
Neuropathological diagnosis	–	–	2 AD-PSP		–	2AGD 5 CBD 1 PGRN 2 PSP 2 TPSPD 1 Unclas. T	3 FTLD-TDP and AGD	1 AGD-AD 4 FTLD-TDP-AD 1 PSP-AD	1 DLB 6 DLB-AD		
CERAD staging	4 % B 96 % C	8 % B 92 % C	50 % B 50 % C	92 % No pl. 8 % A	100 % No pl.	69 % No pl. 8 % A 8 % B 15 % C	67 % No pl. 33 % C	33 % B 67 % C	14 % A 29 % B 57 % C	–	–
Braak staging	III–IV 7 % V–VI 93 %	100 % V–VI	50 % III–IV 50 % V–VI	61 % 0 31 % I–II 8 % III–IV	67 % 0 33 % I–II	–	67 % I–II 33 % III–IV	50 % I–II 17 % III–IV 33.3 % V–VI	14 % I–II 72 % III–IV 14 % V–VI	–	–
Age at onset of disease	65.1 (10.6)	64.3 (11.5)	77.0 (1.4)	60.8 (8.3)	–	64.2 (9.9)	63.7 (16.0)	66.3 (9.9)	66.7 (6.4)	–	–
Age at CSF (years)	68.9 (9.5)	67.9 (10.7)	81.0 (1.4)	61.7 (8.6)	55.3 (9.5)	66.6 (10.6)	64.7 (14.5)	68.1 (6.4)	71.6 (9.0)	69.7 (10.1)	69.4 (10.8)
Time disease onset-CSF sampling (years)	3.1 (2.3)	3.3 (2.0)	4.0 (2.8)	1.9 (1.7)	–	3.5 (2.0)	1.0 (0)	2.8 (1.2)	1.4 (0.8)	–	–
Follow up time after CSF sampling (months) <sup>a</sup>	66.0 (33.6)	73.9 (45.3)	45.5 (46.0)	42.31 (30.1)	13.3 (9.0)	60.9 (40.9)	31.3 (13.6)	62.6 (28.5)	53.3 (31.0)	48.0 (44.0–57.3)	35 (23.8–46.3)
Closest MMSE to CSF sampling	23.0 (15.0–26.0)	19.0(15.0–23.0)	13.5 (2.0–25.0)	26.0 (19.5–28.0)	–	23.0 (13.0–26.0)	22.0(18.0–23.5)	19.5 (16.0–25.0)	26.0 (24.5–28.0)	30.0 (29.0–30.0)	30.0 (29.0–30.0)
Closest CDR to CSF sampling <sup>b</sup>	0.5: 31.7 % 1.0: 56.1 % 2.0: 2.4 % 3.0: 9.8 %	0: 4.8 % 0.5: 23.8 % 1.0: 38.1 % 2.0: 23.8 % 3.0: 9.5 %	1.0: 100 %	0: 14.3 % 0.5: 57.1 % 1.0: 14.3 % 2.0: 14.3 %	0: 100 %	1.0: 50 % 2.0: 25 % 3.0: 25 %	–	1.0: 100.0%	0.5: 100 %	–	–
APOE4 pos.	51.4 %	77.3 %	100 %	33 %	33.3 %	0 %	0 %	66.7 %	71.4 %	25.6 %	17.4 %
Aβ <sub>42</sub> EL	287.1 (207.6–356.4)	254.8 (165.7–10.9)	335.3 (297.2–373.4)	338.9 (281.8–399.9)	421.1 (415.7–426.6)	438.6 (389.6–580.6)	425.2 (413.9–428.6)	268.1 (237.0–297.7)	304.4 (223.9–501.0)	554.7 (449.1–668.5)	–
t-tau <sub>EL</sub>	547.1 (387.4–876.3)	494.2 (326.1–788.7)	540.5 (206.1–875.0)	157.5 (106.9–313.9)	136.4 (133.9–138.9)	216.8 (106.9–313.9)	260.2 (257.1–347.1)	297.9 (250.8–421.5)	229.7 (150.0–437.8)	226.6 (183.0–350.1)	–
p-tau <sub>EL</sub>	88.4 (57.3–123.4)	72.4 (58.9–87.0)	62.6 (32.5–92.8)	34.7 (31.8–37.7)	36.8 (32.0–41.5)	46.0 (36.6–55.1)	36.8 (34.2–95.0)	52.83 (43.7–53.2)	73.1 (64.1–78.6)	51.5 (41.6–62.1)	–
Aβ <sub>42</sub> Lum	128.1 (103.0–164.0)	142.0(123.9–154.1)	152.5 (140.0–165.0)	199.8 (182.0–228.0)	272	212 (204.5–233.1)	220.4(191.3–249.4)	131.0(122.0–147.5)	130.7 (91.3–167.8)	–	2465 (228.5–278.5)
t-tau <sub>Lum</sub>	113.5(70.0–164.0)	120.0(73.9–151.5)	106.0 (49.0–163.0)	33.0(27.2–56.3)	36	67.0 (47.4–72.2)	84.5 (62.4–106.5)	67.0 (48.0–84.5)	95.0 (68.7–103.7)	–	50.4 (38.1–65.2)
p-tau <sub>Lum</sub>	29.5 (21.2–70.0)	24.2 (12.9–43.6)	14.3 (6.2–22.5)	5.5 (4.5–7.3)	6	10.9 (7.8–19.4)	4.3 (3.6–5.1)	20.9 (16.4–29.1)	30.3 (18.2–37.2)	–	20.3 (13.6–31.7)

Continuous variables are expressed as mean (SD), except biomarker measurements (Aβ and tau). MMSE scores, and clinical controls follow up time that are shown as median (1<sup>st</sup> quartile–3<sup>rd</sup> quartile). Categorical variables are expressed as frequency and percentage. In the groups with two neuropathological diagnoses the first represents the main diagnosis and the second the secondary diagnosis

PGRN/Progranulin mutation

<sup>a</sup>For neuropathological cases represents time until death and for the clinical controls time until last follow up (available for 79 % and 83 % of the ELISA and Luminex clinical controls, respectively)

<sup>b</sup>CDR available for: 58 % AD, 54 % FTLD-TDP, 31 % FTLD-TAU, 50 % AD-FTLD, 88 % AD-DLB, 67 % FTLD-AD, 43 % DLB-AD, 0 % FTLD-TDP-TAU, 100 % ALS



**Table 2**  
Best biomarker combination results for classification of patients in the different groups

Assay	AD vs. FTLD & Controls		FTLD vs. controls		AD vs. FTLD		Clinical AD vs. clinical FTLD	
	ELISA	Luminex	ELISA	Luminex	ELISA	Luminex	ELISA	Luminex
Selected biomarkers	t-tau and A $\beta$ <sub>42</sub>	p-tau and A $\beta$ <sub>42</sub>	A $\beta$ <sub>42</sub>	t-tau, p-tau and A $\beta$ <sub>42</sub>	t-tau and A $\beta$ <sub>42</sub>	p-tau and A $\beta$ <sub>42</sub>	t-tau and p-tau	t-tau and p-tau
AUC	0.96 (0.91–1.0)	0.96 (0.91–1.0)	0.86 (0.68–1.0)	0.80 (0.45–1.0)	0.96 (0.88–1.0)	0.98 (0.96–1.0)	0.80 (0.61–0.99)	0.87 (0.72–1.0)
Accuracy	87.1 % (70.2–96.4 %)	91.3 % (76.9–98.2 %)	81.3 % (54.4–95.9 %)	87.5 % (61.7–98.4 %)	85.7 % (63.7–97.0 %)	95.8 % (78.9–99.9 %)	71.4 % (47.8–88.7 %)	78.3 % (56.3–92.6 %)
Kappa	0.74 (0.49–0.98)	0.83 (0.64–1.0)	0.63 (0.24–1.0)	0.71 (0.33–1.0)	0.71 (0.42–1.0)	0.90 (0.72–1.0)	0.38 (0.0–0.80)	0.55 (0.21–0.90)
Sensitivity	85.7 % (57.2–98.2 %)	94.1 % (71.3–99.9 %)	87.5 % (47.3–99.7 %)	80.0 % (28.4–99.5 %)	90.0 % (55.5–99.7 %)	100.0 % (79.4–100.0 %)	68.8 % (41.3–89.0 %)	78.6 % (49.2–95.3 %)
Specificity	88.2 % (63.6–98.5 %)	88.9 % (65.3–98.6 %)	75.0 % (34.9–96.8 %)	90.9 % (58.7–99.8 %)	81.8 % (48.2–97.7 %)	87.5 % (47.3–99.7 %)	80.0 % (28.4–99.5 %)	77.8 % (40.0–97.2 %)
Probability cutoff	≤0.50	≤0.54	≤0.55	≤0.40	≤0.55	≤0.50	≤0.50	≤0.50

The values in parentheses represent the 95 % confidence interval

**Table 3**

Sensitivity and specificity of the cutoffs for the different diagnoses (Subjects with a main or secondary AD diagnosis, FTLD without AD, and controls) against other dementias and controls

	<b>ELISA</b>		<b>Luminex</b>	
	<b>Sensitivity</b>	<b>Specificity</b>	<b>Sensitivity</b>	<b>Specificity</b>
Neurodegenerative disease vs. Controls <sup>a</sup>	85.6 % (77.9–91.4 %)	76.7 % (61.4–88.2 %)	93.3 % (87.1–97.4 %)	84.8 % (71.1–93.7 %)
AD and AD mixed vs. pure FTLD <sup>b</sup>	90.1 % (82.5–95.1 %)	80.8 % (60.6–93.4 %)	96.7 % (90.7–99.3 %)	88.2 % (63.6–98.5 %)
AD and AD mixed vs. pure FTLD and Controls <sup>a</sup>	74.3 % (64.6–82.4 %)	89.9 % (80.2–95.8 %)	95.6 % (89.0–98.8 %)	87.3 % (76.5–94.4 %)

The values in parentheses represent the 95 % confidence interval

<sup>a</sup>Formula derived from comparing AD, FTLD and controls

<sup>b</sup>Formula derived from comparing AD and FTLD