A new tetra-plex fluorimetric assay for the quantification of cerebrospinal fluid β-amyloid42, total-tau, phospho-tau and α-synuclein in the differential diagnosis of neurodegenerative dementia.

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Research

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Abstract

Background Differential diagnosis of neurodegenerative dementia is currently supported by biomarkers including cerebrospinal fluid (CSF) tests. Among them, CSF total-tau (t-tau), phosphorylated tau (p-tau) and β-amyloid42 (Aβ42) are considered core biomarkers of neurodegeneration. In the present work, we hypothesize that simultaneous assessment of these biomarkers together with CSF α-synuclein (α-syn) will significantly improve the differential diagnostic of Alzheimer's disease and other dementias. To that aim, we characterized the analytical and clinical performance of a new tetra-plex immunoassay that simultaneously quantifies CSF Aβ42, t-tau, p-tau and α-syn in the differential diagnosis of neurodegenerative dementia. Methods Biomarkers' concentrations were measured in neurological controls (n=38), Alzheimer's disease (n=35), Creutzfeldt-Jakob disease (n=37), vascular dementia (n=28), dementia with Lewy bodies/Parkinson's disease dementia (n=27) and frontotemporal dementia (n=34) using the new tetra-plex assay and established single-plex assays. Biomarker's performance was evaluated and diagnostic accuracy in the discrimination of diagnostic groups was determined using partial least squares discriminant analysis. Results The tetra-plex assay presented accuracies similar to individual single-plex assays with acceptable analytical performance. Significant correlations were observed between tetra-plex and single-plex assays. Using partial least squares discriminant analysis, Alzheimer's disease and Creutzfeldt-Jakob disease were well-differentiated, reaching high accuracies in the discrimination from the rest of diagnostic groups. Conclusions The new tetra-plex assay coupled with multivariate analytical approaches becomes a valuable asset for the differential diagnosis of neurodegenerative dementia and related applications.

Background

Neurodegenerative dementias are progressive and irreversible conditions characterized by the presence of cognitive and/or behavioral impairment that significantly affects daily life activities. Early diagnosis is of crucial importance to identify reversible causes of dementia, to intervene at the initial stages of the disease when available treatments are most effective, and to assist on the design of clinical trials. However, differential diagnosis still represents a main challenge due to the partial clinical overlap among diagnostic groups and the absence of disease-specific symptoms at onset (1). Differential diagnosis of neurodegenerative dementia is supported by biomarkers, which include neuroimaging and cerebrospinal fluid (CSF) tests. Currently, CSF biomarkers are part of the diagnostic criteria of Alzheimer's disease (AD) (2) and Creutzfeldt-Jakob disease (CJD) (3,4). Established core CSF markers for neurodegeneration are total-tau (t-tau), phospho-tau (p-tau) and β-amyloid42 (Aβ42). These markers are used for the diagnosis of AD and reflect the main neuropathological hallmarks of the disease. For CJD, gold standard biomarkers are 14-3-3 protein(5) and the real-time quacking induced conversion (RT-QuIC) assay (6), although alterations in the triplet of AD markers, especially t-tau are also reported (7). Some of the AD-related biomarkers are also altered in vascular dementia (VaD), Lewy body diseases (LBD) and fronto-temporal dementia (FTD), most likely due to the involvement of Aβ42 and tau in their pathogenesis. However, changes of biomarker profiles in these conditions are less obvious than in AD, rendering poor diagnostic value compared to their accuracy in discriminating AD(7,8).

A main focus in the biomarkers' field is the study of composite biomarkers that usually over-perform their individual constituents, displaying a higher diagnostic accuracy discriminating dementia types (9). In this regard, multiplexing technologies, which allow simultaneous quantification of several biomarkers, decrease analytic variability and present significant advantages in terms of time, costs and sample requirement. The multiple assessment of tau and amyloid peptide markers is useful in the discrimination of AD cases (10,11) and in the prediction of MCI-to-AD conversion (12), but this has not been scrutinized in the differential diagnosis of the spectrum of neurodegenerative dementia. Another focus of research is the discovery of new CSF biomarkers with potential clinical applicability. In this regard, α-synuclein (α-syn) is reported to be slightly to moderately reduced in DLB/PDD and other α-syn-related disorders (13,14) and highly increased in CJD (15–18). While contradictory data exist regarding the clinical relevance of α-syn in AD (15,19,20), high levels have been linked to early AD pathophysiology and to the onset of cognitive symptoms (21). To date, no data are available on

the potential benefit of α-syn quantification in the context of the differential diagnosis of neurodegenerative dementia either alone or in combination with core CSF neurodegenerative markers.

The aim of the present study is to investigate the pre-analytical and analytical performance of a new fluorimetric tetra-plex assay for the simultaneous quantification of Aβ42, t-tau, p-tau and α-syn in the differential diagnosis of the spectrum of neurodegenerative dementia, and to build up an analytical model able to discriminate the different types of dementias with the highest accuracy.

Materials And Methods

Study population:

The study included 199 patients classified as neurological controls (ND), Alzheimer's disease (AD), sporadic Creutzfeldt-Jakob disease (CJD), vascular dementia (VaD), Lewy body dementia including dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) cases, and frontotemporal dementia (FTD) collected at the Clinical Dementia Centers of Göttingen (Germany) and Coimbra (Portugal). Lumbar punctures were performed at the time of diagnosis.

The ND group was composed of cases diagnosed with non-primarily neurodegenerative neurological and psychiatric diseases and included the following diagnostic groups: depression, acute/chronic headache, peripheral polyneuropathy, psychosis, epilepsy, hypoxia, vasculitis, and benign intracranial hypertension. ND cases showed no subjective cognitive complaint and were diagnosed according to acknowledged standard neurologic findings based on the International Classification of Diseases and Related Health Problems, Tenth Edition definitions. AD was diagnosed according the National Institute on Aging–Alzheimer's Association workgroups criteria (2). Frontotemporal lobar degeneration was diagnosed according to the International Behavioral Variant FTD(bvFTD) Criteria Consortium for bvFTD (22). The diagnosis of DLB was based on the criteria of McKeith (23). PDD diagnosis was based on those cases that were initially diagnosed as Parkinson's disease (24) and later developed dementia following the task force of the Movement Disorder Society criteria (25). PDD was differentiated from other Parkinson-plus syndromes through established diagnostic criteria for corticobasal degeneration (26), DLB (23), progressive supranuclear palsy (27), and multiple system atrophy (28). VaD diagnosis was based on clinical and radiological criteria as described by Roman et al. (29). CJD was classified as probable or definite cases according to diagnostic consensus criteria (30).

CSF tests:

For the simultaneous quantification of Aβ42, t-tau, p-tau and a-syn, we used a magnetic bead-based multiplex fluorimetric immunoassay custom panel containing the specific biomarkers previously analyzed with monoplex enzymatic assays. The custom 4-Plex Human Amyloid-Beta and Tau/Neuroscience Panel was developed by Merck-Millipore under the reference number SPR969 by combining two of their Milliplex MAP assays: Aβ42, t-tau, and p-tau biomarkers from the Human Amyloid Beta and Tau Magnetic Bead Panel (HNABTMAG-68K) assay and a-syn from the Human Neuroscience Magnetic Bead Panel 1 (HNS1MAG-95K) assay. Assay characteristics are indicated in Supplementary Methods (Additional File 1). For single-plex assays, colorimetric tests were used. T-tau was measured using the INNOTEST^{TM}hTAUAg Enzyme-Linked-ImmunoSorbent Assay (ELISA), p-tau (Thr181) using the INNOTEST^{TM}PHOSPHO-TAU(181P) ELISA and Aβ42 using the INNOTEST^{TM} AMYLOID(1-42) ELISA; all from Fujirebio. a-syn was measured using an ELISA kit from EUROIMMUN.

The stability of the four biomarkers under different pre-analytical conditions was analysed in four ND cases. CSF samples were stored in polypropylene tubes at RT and 4 °C for 1, 2, 3, 4, 5, and 14 days. In addition, these samples were subjected to 1, 2, and 3 repeated freezing and thawing cycles and tube transfers. Biomarker's concentrations at each time point or cycle were calculated as the percentage of control (time point zero), which was defined as 100%.

Statistical analysis:

Linear models were applied to compare the levels of each biomarker in the different neurodegenerative dementia groups. For normalization purposes, biomarker concentrations were log-transformed. Age and sex were included as covariates in the models. For multiple comparisons adjustment, Tukey method was applied. To evaluate inter-center variability as potential technical bias, two further LMs for each biomarker were applied: 1) including center as an additive effect into previous LMs, meaning systematic center difference in each dementia group, and 2) adding into 1) a dementia-center interaction term, denoting that center difference depend on dementia group. Likelihood-Ratio Tests (LRT) between nested models were applied to evaluate statistical significance. To assess the diagnostic accuracy of each biomarker, receiver operating characteristic curve analyses were carried out and areas under the curve (AUCs) with 95% confidence intervals (95% CI) were calculated. Spearman correlation coefficients were used to assess associations between continuous biomarker levels. To assess differences in the diagnostic accuracy of different analytical platforms across the pairs of diagnostic groups, ROC curves were compared with the pROC - R package (31). Passing-Bablock regression (32) was used to compare quantification methods. Diagnostic accuracy was also assessed under a multivariate perspective, through the simultaneous evaluation of all biomarkers and their composites (hereinafter referred as biomarkers as well). Partial least squares discriminant analysis (PLS-DA), a machine learning approach for classification purposes and appropriate for the analysis of high dimensionality data sets with multicollinearity (33) was applied as indicated in Supplementary Methods (Additional File 1). Evaluation of biomarkers' stability on different pre-analytical conditions was conducted with repeated measures ANOVA followed by Bonferroni post-hoc analysis. In all analyses, statistical significance was set at $p<0.05$.

Results

Characterization of a new tetra-plex assay for the quantification of CSF Aβ42, t-tau, p-tau and a-syn.

The general characteristics of the new tetra-plex assay are summarized in Supplementary Figure 1a (Additional File 2). Intra-coefficient of variation (CV) ranged from 7 to 11% and inter-CV ranged from 12 to 16%. The four biomarker assays showed a large dynamic range comprising more than three orders of magnitude, with fold changes ranging from ~175 to 1100 upper /lower limits of quantification. When tested up to 1:6 dilution from neat CSF, the assays displayed acceptable dilution linearity (101% to 114%), suggesting the absence of matrix-effects. Representative standard curves for each biomarker are shown in Supplementary Figure 1b (Additional File 2).

Influence of pre-analytic parameters on biomarkers measurements in the tetra-plex assay.

Treatment of CSF samples under different pre-analytical conditions revealed no significant differences on Aβ42, t-tau, p-tau and α-syn when CSF was stored at 4ºC up to 14 days (Figure 1a). In contrast, storage at RT led to a decrease on t-tau at 14 days ($p<0.01$) and on p-tau at 5 days ($p<0.05$) and 14 days ($p<0.001$) (Figure 1b). T-tau, p-tau and Aβ42 were stable up to three freeze/thawing (F/T) cycles, while α-syn was significantly lower after three F/T cycles ($p<0.05$) (Figure 1c). t-tau, p-tau and α-syn were stable up to three tube transfers, in contrast to Aβ42, whose concentrations were significantly lower after two ($p<0.05$) and three ($p<0.01$) transfers (Figure 1d).

Diagnostic performance of the tetra-plex assay in the differential diagnostic of neurodegenerative dementia.

In order to investigate the performance of the tetra-plex assay in the differential diagnostic of neurodegenerative dementia 199 CSF cases (ND: n=38, AD: n=35, CJD: n=37, VaD: n=28, DLB/PDD: n=27 and FTD: n=34) were quantified. After controlling for age and sex, significantly lower Ab42 concentrations were detected in AD ($p<0.01$) and DLB/PDD ($p<0.05$) compared to ND and in AD ($p<0.05$) compared to VaD (Figure 2a and 2b). T-tau concentrations were significantly higher in AD ($p<0.001$), CJD ($p<0.001$), FTD ($p<0.001$) and DLB/PDD ($p<0.01$) compared to ND, in CJD compared to AD ($p<0.001$), VaD ($p<0.001$), DLB/PDD ($p<0.001$) and FTD ($p<0.001$), and in AD compared to VaD ($p<0.01$) (Figure 2a and 2c). Compared to ND, p-tau concentrations were significantly higher in AD ($p<0.001$), CJD ($p<0.001$), VaD ($p<0.01$), DLB/PDD

(p<0.01) and FTD (p<0.01), in AD compared to CJD (p<0.01), VaD (p<0.001), DLB/PDD (p<0.001) and FTD (p<0.001), and in CJD compared to VaD (p<0.01) and FTD (p<0.01) (Figure 2a and 2d). Significantly higher a-syn concentrations were present in CJD compared to the rest of diagnostic groups (p<0.001) (Figure 2a and 2e). No other significant differences were detected for a-syn.

Correlation analyses between the four biomarkers were studied in the total population. Modest, but significant correlations were detected between Aβ42 and a-syn (rho=0.17, p=0.036), t-tau and p-tau (rho=0.18, p=0.017) and p-tau and a-syn (rho=0.19, p=0.011), while a strong significant correlation was detected between t-tau and a-syn (rho=0.80, p<0.001) as shown in Supplementary Figure 2 (Additional File 2).

Comparison of the diagnostic accuracies obtained with the tetra-plex assay and the single-plex assays.

In order to determine the performance of the fluorimetric tetra-plex assay and compared it to established commercial single-plex assays, AUCs and associated p values were calculated. For Aβ42, significant differences between assays were detected neither in the discrimination of ND from neurodegenerative dementias (Figure 3a), nor in the discrimination of neurodegenerative dementia groups among them (Supplementary Figure 3- Additional File 2). T-tau in the tetra-plex assay was better discriminating ND from VaD (p=0.029), DLB/PDD (p<0.001) and FTD (p=0.002) than single-plex t-tau (Figure 3a), and tetra-plex p-tau was better discriminating ND from all neurodegenerative dementias than single-plex p-tau (ND vs. AD: p=0.003, ND vs. CJD: p=0.009, ND vs. VaD: p=0.049, ND vs. DLB/PDD: p=0.012 and, ND vs. FTD: p<0.001) (Figure 3a). In contrast, neither tetra-plex t-tau nor tetra-plex p-tau displayed better accuracies discriminating neurodegenerative dementias than the corresponding single-plex assays, with the exception of AD vs. CJD (p=0.001) and AD vs. FTD (p=0.018) comparisons for p-tau (Supplementary Figure 3- Additional File 2). Regarding a-syn, the tetra-plex assay displayed significantly lower accuracy in discriminating ND from AD cases compared with the single-plex assay (p=0.038), although both methods showed poor AUC values (0.53 and 0.69, respectively) discriminating these diagnostic groups.

A moderate but significant correlation was observed between tetra-plex and single-plex methods for Ab42 (r=0.61 (95%CI=0.51-0.69), p<0.001) (Figure 3b), while a strong agreement was detected for t-tau (r=0.83 (95% CI=0.78-0.87), p<0.001) (Figure 3c), p-tau (r=0.80 (95% CI=0.74-0.85), p<0.001) (Figure 3d) and a-syn r = 0.72 (95% CI=0.64-0.78), p<0.001 (Figure 3e). Interestingly, for Aβ42, the most discrepant values according to Cook's distance (Supplementary Figure 4- Additional File 2) were two ND cases with normal Aβ42 concentrations according to tetra-plex assay (654 pg/mL and 745 pg/mL) and low Aβ42 concentrations according to the single-plex assay (125 pg/mL and 235 pg/mL). Thus, in cases with discrepant Aβ42 concentrations, these were associated to its correct diagnosis when using the tetra-plex assay. In spite of significant correlation between concentrations in both types of assay, Passing-Bablok regressions indicated that in all cases there are proportional and systematic differences between tetra-plex and single-plex assays (Figure 3b-e). Therefore, both methods are not interchangeable for the quantification of the four biomarkers.

Next, we aimed to ascertain whether a-syn inclusion added value to the group of core neurodegenerative markers (t-tau, p-tau and Ab42) in the discrimination of diagnostic groups. To address this issue, principal component analysis (PCA) plots were performed in the presence or absence of a-syn (Supplementary Figure 5- Additional File 2). Since biomarkers are best defined to differentiate AD, CJD and ND, and not so much for the rest of the diagnoses, analyses are focused in these three diagnostic groups to simplify the visualization of discriminatory performance in the presence or absence of a-syn. When a-syn was included as a biomarker, a better separation between diagnoses was achieved (Supplementary Figure 5- Additional File 2).

Diagnostic accuracy of the tetra-plex assay using PLS-DA.

We built PLS-DA models to discriminate the 15 pairs of diagnostic groups (Table 1). As expected, ND, AD and CJD were best differentiated, reaching accuracies, sensitivities and specificities over 90%. These groups showed as well good

discrimination performance against DLB/PDD, FTD and VaD diagnoses, with diagnostic measures significantly higher than those obtained by random chance (last column in Table 1). These latter groups failed to discriminate one from each other showing accuracies similar to random non-informative data-based accuracies, although moderate accuracies were achieved when compared to the ND group (ND vs. DLB/PDD: 81%, ND vs. FTD 75.5% and ND vs. VaD: 70.5%).

PLS-DA separation performance of each diagnostic pair through the first two components is displayed in Figure 4. All pairs showed a clear separation except for DLB/PDD, FTD and VaD combinations. The combinations of neurodegenerative markers contributing the most (VIP > 1) to the diagnostic accuracy are shown in Table 1. Importantly, all biomarkers contributed to the discrimination performance of the PLS-DA models, and composite markers contributed better than single markers.

Discussion

In this study, we describe the analytical and clinical performance of a new tetra-plex immunoassay that simultaneously quantifies human CSF Aβ42, t-tau, p-tau and a-syn on a fluorimetric platform. Coefficient of variability between sample replicates and dilution linearity was among acceptable ranges (34), which indicates that the method performance fulfills the requirements for its intended use. Additionally, compared to single-plex methods, the tetra-plex assay decreases analytical variability, is faster (6 hours), reduces workflow and the amount of sample required (25mL in a 1:3 dilution for the four biomarkers), is less expensive, and provides larger dynamic ranges. Furthermore, influence of pre-analytical factors on biomarker measurements revealed the stability of the four biomarkers in front to different storage temperatures, freeze-thawing cycles and tube transfers, with the exception of the well-known impact of tube transfer on Aβ42 concentrations (35) and freeze-thaw cycles on a-syn (36), observations that were validated in our study.

When the tetra-plex assay was tested for its ability to discriminate neurodegenerative dementias from ND and from each diagnostic group, in general we obtained accuracies similar to those previously reported using well-established single-plex assays (7,8). Indeed, the measurements were highly correlated between the two assay platforms despite the fact that absolute values were different, in agreement with a previous study comparing colorimetric single-plex assays with a fluorimetric triplex assay for t-tau, p-tau and Ab42 (11). Nevertheless, a striking difference was the significantly better performance of t-tau and p-tau in the tetra-plex assay in discriminating ND from different type of dementias. This was particularly relevant in the discrimination from VaD, DLB/PDD and FTD. These three diseases are orphan of clinically relevant CSF diagnostic biomarkers and, whereas single-plex quantification only resulted in very poor AUC values (0.67 at the best case with p-tau for the pair ND vs. VaD), we managed to increase these values to moderate levels (AUCs ranged between 0.72 and 0.82) with t-tau and p-tau. This characteristic becomes very useful in specific contexts of use where biomarkers' differences between pathological and basal levels are required, such as inclusion criteria or evaluation of efficacy in clinical trials.

A challenge that multiplexing platforms pose in the differential diagnostic context (multiple biomarkers and multiple diagnostic groups) is data analysis, which should be easily interpretable on clinical grounds. The use of PLS-DA versus classical approaches based on biomarker analysis at univariate level simplifies the optimum search for biomarkers or their combinations in the process of diagnostic discrimination. Using this approach, we built up a versatile algorithm that resulted in the discrimination of CJD and AD from the rest of diagnostic groups with high accuracy. In contrast, the combination of the four biomarkers in our multivariate analysis was not able to discriminate FTD, DLB/PDD and VaD groups among them, despite the fact that moderate accuracies were achieved for their discrimination from ND cases.

One of the potentials of the present methodology lays in future trials where many more biomarkers together may be considered for the possible discrimination of diseases, facing therefore the challenging problem of "Big Data" analysis. The method will also gain power when more data are available, as statistical learning will be more accurate. By contrast, a limitation of this approach is the inherent complexity in the interpretation of the model, which is how the biomarkers

interact with each other for the discrimination of the diseases. This is the well-known trade-off between prediction accuracy and model interpretability in statistical learning methods. When the aim is to understand the relationship between biomarkers and diagnostic groups, restrictive models are more interpretable. However, when prediction is the goal and interpretability is less relevant, the use of flexible models render better accuracies.

An important aspect to take into account when using the present approach is the need to evaluate and validate the added value of each biomarker as a contributing discriminatory variable of the model. Among the measured proteins, t-tau, p-tau and Ab42 are widely described core neurodegenerative biomarkers included in the diagnostic criteria of AD (37) and the utility of their simultaneous quantification using multi-plexing technologies was previously reported, with accuracies in range of those achieved by single-plex assays (10,38). Additionally, t-tau and p-tau/tau ratio are supportive biomarkers for the differential diagnosis of CJD (7,39).

An innovation of this work is the addition of a-syn in the existing tri-plex quantification platform. This protein is known to be an excellent biomarker for CJD (15) that has been recently validated for its clinical use (40). a-syn is also slightly to moderately decreased in DLB/PDD compared to controls and AD, but with poor discriminatory value (41,42), although we didn't detect significant differences between DLB/PDD cases and the rest of the diagnostic groups. Importantly, it was unknown whether a-syn could contribute to improve the accuracy of t-tau, p-tau and Ab42 in the differential diagnostic context of neurodegenerative dementia. Here, we observed that a-syn: i) significantly contributes to the discrimination of most of diagnostic pairs in the PLS-DA analysis (similar to Ab42, t-tau and p-tau), with VIPs > 1, ii) improves the classification of ND, AD and CJD groups as shown on PCA plots and iii) displays high accuracy discriminating CJD from the rest of the diagnostic groups. While discrepant observations have been reported regarding CSF a-syn concentrations in AD (that could be attributed to technical and clinical differences between studies) (43,44), using our fluorimetric assay, CSF a-syn concentrations were not changed in AD compared to controls. This observation is especially relevant for the discrimination of AD from CJD cases. Indeed, a-syn over performs t-tau in terms of specificity (a-syn is exclusively increased in CJD), in contrast to t-tau, which presents a partial overlap on its concentrations between both diseases, as reported before (7,45,46). Overall, these findings indicate that the inclusion of a-syn provides added value to the present biomarker platform. Interestingly, a-syn displayed a high correlation with t-tau values (r=0.80), suggesting that both markers reflect the same physio-pathological alterations in the brain tissue.

A limitation of our study is the modest amount of cases, which impedes an optimal validation of the model. However, the moderate accuracy of the studied biomarkers in the discrimination of DLB/PDD, VaD and FTD cases suggests that the inclusion of additional biomarkers for these conditions, rather than increasing the number of cases, will be necessary to improve diagnostic accuracies. We postulate that the new tetra-plex assay could be an optimal platform to combine with future biomarkers for these conditions. These new biomarkers should be sensitive enough to detect alterations on these groups, whereas, lack of specificity can be overcome through the combination of all biomarkers included in the PLS-DA model. In this regard, we propose the inclusion of biomarkers such as neurofilament light chain, recently reported to be increased in VaD and FTD(47,48), as a way to improve diagnostic accuracies of both conditions using a similar analytical multivariate approach.

Conclusions

In total, we demonstrate that the implementation of a new tetra-plex assay coupled to the application of multivariate analytical approaches is a valuable asset for the differential diagnosis of neurodegenerative dementias and related applications such as patient stratification and data interpretation in clinical trials.

List Of Abbreviations

CSF cerebrospinal fluid

AD Alzheimer's disease

CJD Creutzfeldt-Jakob disease

t-tau total-tau

p-tau phosphorylated tau

Aβ42 β-amyloid42

RT-QuIC real-time quacking induced conversion

VaD vascular dementia

LBD Lewy body diseases

FTD fronto-temporal dementia

α-syn α-synuclein

ND neurological controls

DLB dementia with Lewy bodies

PDD Parkinson's disease dementia

bvFTD Behavioural Variant FTD

LM Linear models

LRT Likelihood Ratio Tests

AUCs areas under the curve

95%CI 95% confidence intervals

ROC Receiver operating characteristic

PLS-DA Partial least squares discriminant analysis

CV Intra-coefficient of variation

RT room-temperature

F/T freeze/thawing

PCA principal component analysis

VIP Variable importance for the projection

SD Standard Deviation

f female

m male

Declarations

Ethics approval and consent to participate:

Written informed consent was obtained from all study participants or their legal guardians. The study was conducted according to the revised Declaration of Helsinki and Good Clinical Practice guidelines, and was approved by all local Ethics committees (Reference numbers 11/11/93, 5/09/08, 9/06/08, 19/11/09, Universitätsmedizin Göttingen, Germany and HUC-43-09, University of Coimbra, Portugal).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions:

FL designed the study. DD-L, GE, AV-P, JADR, EM, IF and FL participated in the acquisition and analysis of data. PH, MS, IS, IB, IZ and FL collected and characterized biological samples and contributed to data interpretation. GE and FL drafted the manuscript and the figures. All authors critically revised the manuscript.

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Tables

Table 1: Diagnostic accuracy of the tetra-plex assay using PLS-DA

	Biomarker (VIP>1)	Median accuracy based on 1000 permutations (2.5-97.5 percentiles)	Median accuracy based on 1000 permutations with random non informative data (2.5-97.5 percentiles)	Median Sensitivity based on 1000 permutations (2.5-97.5 percentiles)	Median Specificity based on 1000 permutations (2.5-97.5 percentiles)
ND vs. AD	p-tau/α-syn Aβ42/p-tau t-tau/α-syn Aβ42/t-tau (Aβ42 · α-syn)/p-tau (Aβ42 · α-syn)/t-tau p-tau (t-tau · p-tau)/ α-syn t-tau	95·5 (90·0-100)	43·2 (24·1-61·8)	100 (80·0-100)	100 (90·0-100)
ND vs. CJD	Aβ42/t-tau t-tau/α-syn (t-tau · p-tau)/ α-syn t-tau p-tau Aβ42/p-tau (Aβ42 · α-syn)/t-tau Aβ42/α-syn (Aβ42 · p-tau)/t-tau t-tau/p-tau	95·5 (86·4-100)	50·0 (31·8-68·2)	100 (72·7-100)	100 (90·9-100)
ND vs. VaD	t-tau/α-syn p-tau/α-syn p-tau (t-tau · p-tau)/ α-syn	70·5 (53·4-89·2)	46·0 (27·3-62·5)	90·9 (72·7-100)	50·0 (12·5-90·9)
ND vs. DLB/PDD	Aβ42/t-tau (Aβ42 · α-syn)/t-tau Aβ42/p-tau (Aβ42 · α-syn)/p-tau t-tau/α-syn p-tau (t-tau · p-tau)/ α-syn (t-tau · p-tau)/ Aβ42	81·3 (63·1-95·5)	52·3 (33·5-70·5)	75·0 (37·5-100)	90·9 (63·6-100)
ND vs. FTD	Aβ42/p-tau Aβ42/t-tau (t-tau · α-syn)/ Aβ42	75·5 (57·3-90·5)	55·9 (36·8-71·4)	70·0 (40·0-100)	81·8 (54·5-100)
AD vs. CJD	p-tau/α-syn t-tau Aβ42/t-tau t-tau/p-tau (Aβ42 · t-tau)/p-tau α-syn	90·5 (76·4-100)	57·3 (38·2-76·2)	90·0 (70·0-100)	90·9 (72·7-100)
AD vs. VaD	Aβ42/p-tau Aβ42/α-syn p-tau	82·5 (65·0-95·0)	56·3 (32·5-77·5)	90·0 (70·0-100)	7 75·0 (37·5-100)

	(t-tau · p-tau)/ α-syn				
AD vs. DLB/PDD	p-tau (p-tau · α-syn)/t-tau Aβ42/p-tau p-tau/α-syn (Aβ42 · α-syn)/p-tau t-tau	70·0 (49·8-88·8)	37·5 (20·0-55·0)	80·0 (50·0-100)	62·5 (25·0-87·5)
AD vs. FTD	p-tau/α-syn Aβ42/p-tau (Aβ42 · α-syn)/p-tau (t-tau · p-tau)/ α-syn p-tau	85·0 (70·0-95·0)	55·0 (35·0-75·0)	90·0 (60·0-100)	90·0 (60·0-100)
CJD vs. VaD	t-tau t-tau/α-syn Aβ42/α-syn t-tau/p-tau α-syn	93·8 (78·4-100)	46·0 (27·3-65·9)	100 (81·8-100)	87·5 (62·5-100)
CJD vs. DLB/PDD	t-tau p-tau/α-syn Aβ42/t-tau t-tau/p-tau	93·8 (76·7-100)	46·0 (27·8-65·9)	100 (81·8-100)	87·5 (62·5-100)
CJD vs. FTD	t-tau Aβ42/t-tau t-tau/α-syn (t-tau · p-tau)/ α-syn Aβ42/α-syn t-tau/p-tau	95·0 (80·5-100)	61·4 (41·3-76·4)	90·9 (72·7-100)	100 (70·0-100)
VaD vs. DLB/PDD	none	56·3 (37·5-75·0)	62·5 (37·5-81·3)	62·5 (25·0-87·5)	62·5 (25·0-87·5)
VaD vs. FTD	none	50·0 (32·5-68·8)	53·8 (31·3-73·8)	70·0 (40·0-100)	37·5 (40·0-75·0)
DLB/PDD vs. FTD	none	60·0 (41·2-81·3)	50·0 (31·3-66·3)	75·0 (40·0-100)	50·0 (12·5-87·5)

Table 1. Diagnostic accuracy of the tetra-plex assay using PLS-DA. PLS-DAs were constructed based on training datasets. Variable importance for the projection (VIP) criterion was used to identify which biomarkers contribute most on the classification performance. Accuracy, sensitivity and specificity diagnostic measures are indicated. The training and test sets random partitions were generated 100 times and statistical summaries (median, 2.5^{th} and 97.5^{th} quintiles, termed here 95% Confidence Interval) were computed for each diagnostic measure. Accuracies with random non informative data were obtained based on a permutation test involving 1000 data sets constructed by randomly reassigning class labels at each individual, then performing a PLS-DA on the new randomized training data sets and computing diagnostic measures in their respective 1000 randomized test sets. ND, neurological controls; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; VaD, vascular dementia; DLB/PDD, dementia with Lewy bodies and Parkinson's disease dementia; FTD, frontotemporal dementia. Ab42, β-amyloid42; t-tau, total-tau, p-tau, phospho-tau and a-syn, a-synuclein.

Figures

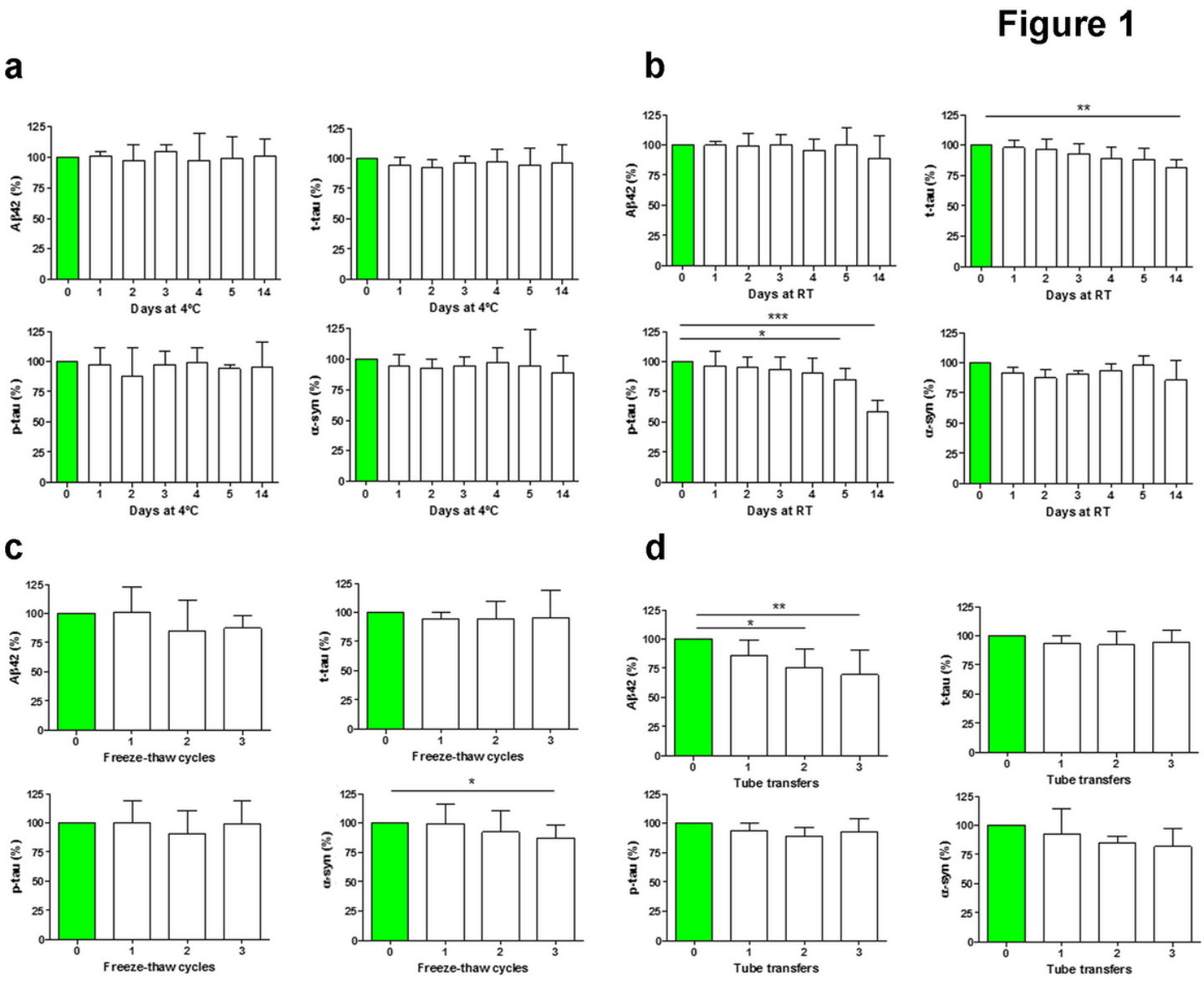

Figure 1

Effect of different storage conditions on CSF t-tau, p-tau, α-syn, and Aβ42 concentrations. The stability of t-tau, p-tau, α-syn, and Aβ42 under pre-analytical conditions: (a) storage at 4ºC, (b) storage at room temperature (RT), (c) freeze/thaw cycles and, (d) tube transfers in four ND cases stored in polypropylene tubes. Biomarkers concentrations were determined in the tetra-plex assay as indicated in Material and Methods and are shown relative to the reference sample (time point 0), which was set as 100%. Error bars represent standard deviations (SD). Repeated measures ANOVA followed by Bonferroni post-hoc analysis was applied. * p<0.05, **p<0.01 and ***p<0.001. A□42, β-amyloid42; t-tau, total-tau, p-tau, phospho-tau and □-syn, □-synuclein.

Figure 2

a

	n	Age	Sex (f/m)	A β42 (pg/mL)	t-tau (pg/mL)	p-tau (pg/mL)	α-syn (pg/mL)
ND	38	64 ± 11	19/19	432 ± 243	622 ± 532	17 ± 8	4099 ± 1764
AD	35	68 ± 7	16/19	230 ± 191	1803 ± 1127	98 ± 68	4373 ± 2412
CJD	37	68 ± 8	16/21	323 ± 235	12378 ± 8163	56 ± 33	22509 ± 22060
VaD	28	71 ± 7	11/17	392 ± 245	1010 ± 699	38 ± 33	3959 ± 2248
DLB/PDD	27	70 ± 9	13/14	256 ± 227	1169 ± 693	43 ± 35	3890 ± 2677
FTD	34	67 ± 8	16/18	353 ± 201	1182 ± 527	35 ± 28	5413 ± 3418

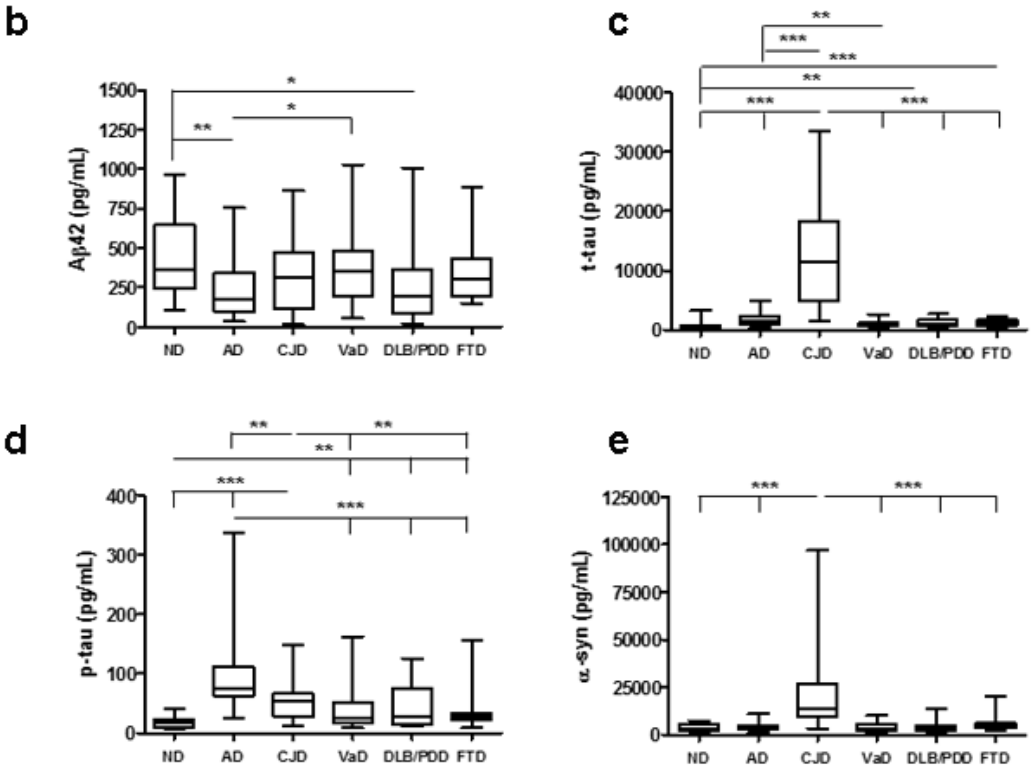

Figure 2

CSF A□42, t-tau, p-tau and □-syn quantification using a fluorimetric tetra-plex assay in neurodegenerative dementias. (a) Demographic and CSF biomarker characteristics of the cases used in the present study. Biomarkers were quantified with the new fluorimetric tetra-plex assay described in the study. Number of cases (n), age (mean value ± standard deviation (SD)), sex (f: female, m: male) and concentrations of CSF A□42, t-tau, p-tau and □-syn (mean value ± SD) are indicated. Data from biomarker's quantifications in the different diagnostic groups, (b) A□42, (c) t-tau, (d) p-tau and (e) □-syn, displayed as box and whiskers plots. *p<0.05, **p<0.01, ***p<0.001. ND, neurological controls; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; VaD, vascular dementia; DLB/PDD, dementia with Lewy bodies and Parkinson's disease dementia; FTD, frontotemporal dementia. A□42, β-amyloid42; t-tau, total-tau, p-tau, phospho-tau and □-syn, □-synuclein.

Figure 3

a

	Aβ42			t-tau		
	Tetra-plex (fluorimetric)	Single-plex (colorimetric)	pvalue	Tetra-plex (fluorimetric)	Single-plex (colorimetric)	pvalue
ND vs. AD	0·78 (0·68-0·89)	0·71 (0·59-0·83)	0·222	0·89 (0·81-0·97)	0·87 (0·78-0·96)	0·708
ND vs. CJD	0·61 (0·48-0·73)	0·51 (0·38-0·64)	0·400	0·99 (0·99-1)	0·99 (0·99-1)	1
ND vs. VaD	0·54 (0·40-0·69)	0·51 (0·36-0·65)	0·616	0·72 (0·59-0·85)	0·55 (0·41-0·69)	**0·029**
ND vs. DLB/PDD	0·73 (0·60-0·85)	0·65 (0·51-0·79)	0·179	0·77 (0·65-0·89)	0·59 (0·44-0·74)	**<0·001**
ND vs. FTD	0·60 (0·47-0·73)	0·63 (0·50-0·77)	0·778	0·82 (0·72-0·91)	0·65 (0·52-0·78)	**0·002**

	p-tau			α-syn		
	Tetra-plex (fluorimetric)	Single-plex (colorimetric)	pvalue	Tetra-plex (fluorimetric)	Single-plex (colorimetric)	pvalue
ND vs. AD	0·99 (0·98-1)	0·88 (0·80-0·96)	**0·003**	0·53 (0·39-0·67)	0·69 (0·57-0·81)	**0·038**
ND vs. CJD	0·93 (0·88-0·99)	0·83 (0·74-0·93)	**0·009**	0·94 (0·88-0·99)	0·96 (0·92-1)	0·444
ND vs. VaD	0·77 (0·65-0·88)	0·67 (0·54-0·80)	**0·049**	0·53 (0·39-0·68)	0·65 (0·53-0·79)	0·297
ND vs. DLB/PDD	0·74 (0·61-0·87)	0·62 (0·47-0·76)	**0·012**	0·60 (0·46-0·73)	0·52 (0·35-0·65)	0·507
ND vs. FTD	0·80 (0·69-0·90)	0·63 (0·50-0·76)	**<0·001**	0·60 (0·47-0·74)	0·63 (0·50-0·77)	0·720

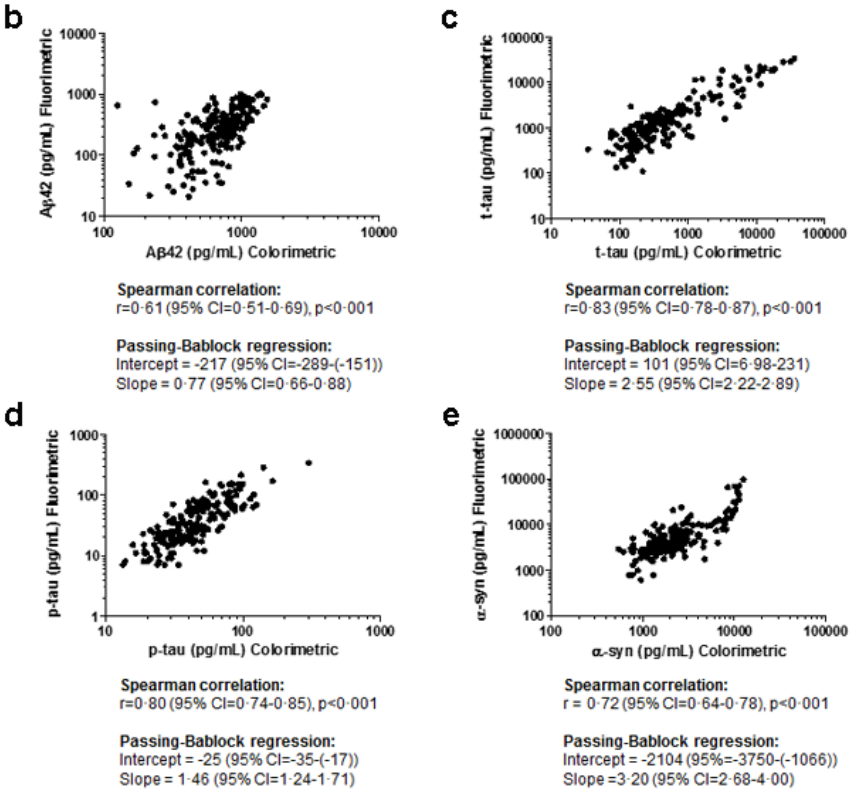

Figure 3

Comparison of diagnostic performance between tetra-plex (fluorimetric) and single-plex (colorimetric) assays. (a) Area Under the Curve (AUC) derived from receiver operating characteristic curves with 95% confidence interval (95% CI) for the comparison between the tetra-plex and single-plex assays. Accuracy was calculated for the four biomarkers (A□42, t-tau, p-tau and □-syn) in the comparison between ND, AD, CJD, VaD, DLB/PDD and FTD. Differences between AUCs derived from tetra-plex and single-plex assays are reported. Statistically significant values (considered as p<0.05) are indicated in bold. (b-e) Spearman correlation coefficient was used to determine biomarker's correlations between methods and Passing-Bablok regression to prove methods equality. ND, neurological controls; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; VaD, vascular dementia; DLB/PDD, dementia with Lewy bodies and Parkinson's disease dementia; FTD, frontotemporal dementia. A□42, β-amyloid42; t-tau, total-tau, p-tau, phospho-tau and □-syn, □-synuclein.

Figure 4

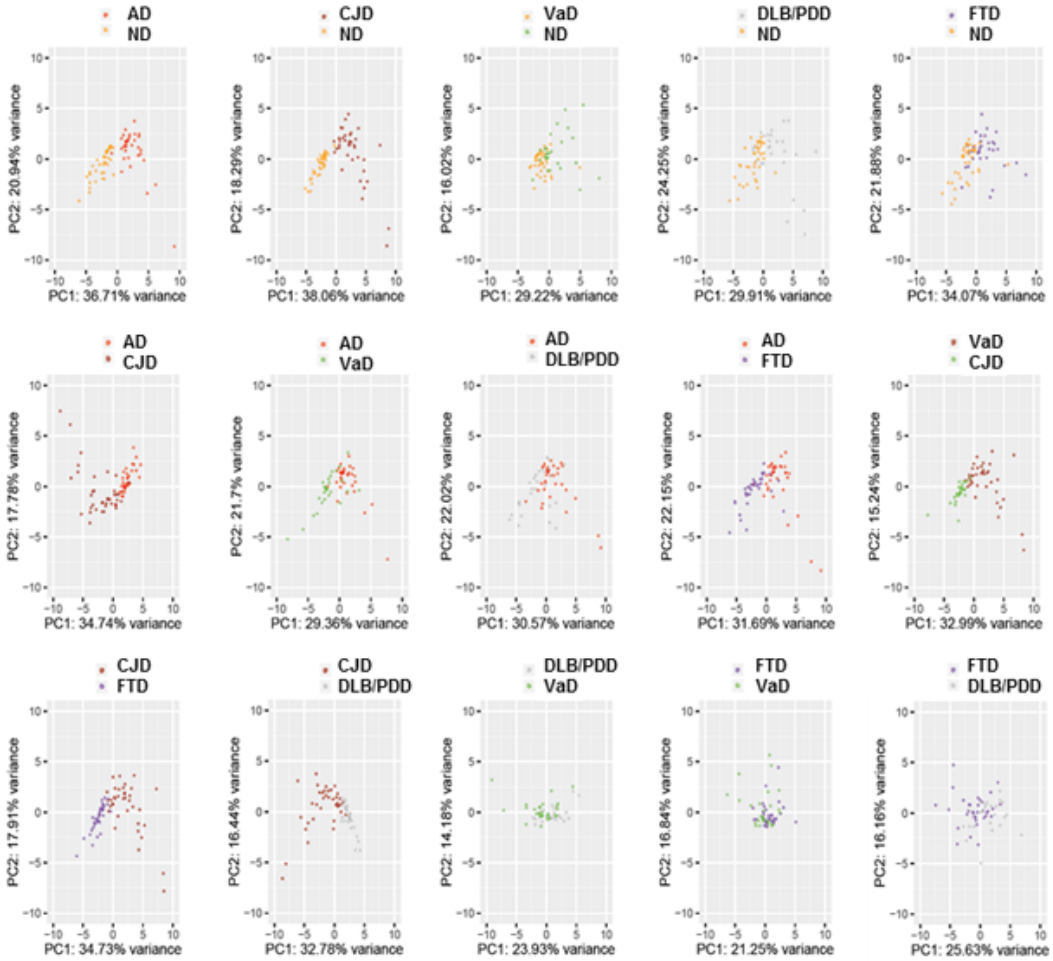

Figure 4

Diagnostic pair wise discriminant analysis. First two PLS-DA component scores showing the degree of separation between diagnostic groups. Each component score is constructed as a linear combination of all biomarkers considered in the analysis: A□42, t-tau, p-tau and □-syn, as well as their composites. The first two component scores together explain between 50% and 60% of the whole set of biomarkers variability across the different pairwise discriminant analysis. ND, neurological controls; AD, Alzheimer's disease; CJD, Creutzfeldt-Jakob disease; VaD, vascular dementia; DLB/PDD, dementia with Lewy bodies and Parkinson's disease dementia; FTD, frontotemporal dementia.

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