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RESEARCH ARTICLE

Cerebrospinal fluid p-tau181, 217, and 231 in definite Creutzfeldt–Jakob disease with and without concomitant pathologies

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Abstract

INTRODUCTION: The established cerebrospinal fluid (CSF) phosphorylated tau181 (p-tau181) may not reliably reflect concomitant Alzheimer's disease (AD) and primary age-related tauopathy (PART) found in Creutzfeldt–Jakob disease (CJD) at autopsy.

METHODS: We investigated CSF N-terminal p-tau181, p-tau217, and p-tau231 with in-house Simoa assays in definite CJD ($n = 29$), AD dementia ($n = 75$), mild cognitive impairment (MCI) due to AD ($n = 65$), and subjective cognitive decline (SCD, $n = 28$). Post-mortem examination performed in patients with CJD 1.3 (0.3–14.3) months after CSF collection revealed no co-pathology in 10, concomitant AD in 8, PART in 8, and other co-pathologies in 3 patients.

RESULTS: N-terminal p-tau was increased in CJD versus SCD ($p < 0.0001$) and correlated with total tau (t-tau) in the presence of AD and PART co-pathology (rho = 0.758–0.952, $p \leq 001$). Concentrations in CJD^{+AD} were indistinguishable from AD dementia, with the largest fold-change in p-tau217 (11.6), followed by p-tau231 and p-tau181 (3.2–4.5).

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DISCUSSION: Variable fold-changes and correlation with t-tau suggest that p-tau closely associates with neurodegeneration and concomitant AD in CJD.

KEYWORDS
Alzheimer's disease, cerebrospinal fluid, concomitant pathology, Creutzfeldt–Jakob disease, neuropathology, phosphorylated tau, p-tau181, p-tau217, p-tau231

Highlights

- N-terminal phosphorylated tau (p-tau) biomarkers are increased in Creutzfeldt–Jakob disease (CJD) with and without concomitant AD.
- P-tau217, p-tau231, and p-tau181 correlate with total tau (t-tau) and increase in the presence of amyloid beta (Aβ) co-pathology.
- N-terminal p-tau181 and p-tau231 in Aβ-negative CJD show variation among *PRNP* genotypes.
- Compared to mid-region–targeting p-tau181, cerebrospinal fluid (CSF) N-terminal p-tau has greater potential to reflect post-mortem neuropathology in the CJD brain.

1 | BACKGROUND

Creutzfeldt–Jakob disease (CJD) is a fatal, rapidly progressive neurodegenerative disease caused by misfolded proteins named prions (PrP^{Sc}).[1] The exact trigger of conformational changes and PrP^{Sc} aggregation in the most common, sporadic form (sCJD) is still unknown; however, methionine (M) and valine (V) homozygosity at *PRNP* codon 129 confer an increased risk of disease development.[2–4] Cerebrospinal fluid (CSF) biomarkers are valuable for accurate clinical diagnosis (possible or probable CJD). Real-time quacking-induced conversion (RT-QuIC) detects prions with almost 100% diagnostic specificity.[5,6] Similarly, neuronal damage biomarkers, 14-3-3 protein, and markedly increased CSF total tau (t-tau) are highly sensitive and specific in the context of a progressive neurological syndrome (dementia, cerebellar ataxia, and myoclonus).[5,7–10] Nevertheless, Alzheimer's disease (AD) was the main diagnosis associated with highly increased t-tau (>1000 ng/L) in a retrospective multicentric study.[11] In 10%–30% of cases, AD may also cause rapid cognitive decline and has thus been documented as one of the major CJD differential diagnoses by prion disease surveillance centers.[10,12,13] Reflecting AD neurofibrillary tangle (NFT) pathology,[14–16] conventional phosphorylated tau181 (p-tau181), containing mid-region epitopes is usually normal or only slightly increased in CJD,[17] which makes the CSF t-tau/p-tau181 ratio superior compared to t-tau alone in the differentiation of CJD and rapidly progressive AD.[8,18]

Definite CJD diagnosis requires immunohistochemical confirmation of prion deposition in the brain tissue.[6,7] Along with characteristic neuropathological changes, autopsy may also reveal concomitant pathologies, which are more challenging to assess during life due to fulminant and relatively short disease course. Independent studies have reported the co-existence of AD neuropathology in sCJD as well as high amounts of Aβ plaques without notable NFT pathology in familial CJD.[5,17,19–26] The distribution of Aβ plaques in the areas with spongiform degeneration and possible role of PrP in Aβ toxicity have lent credence to the assumption of shared pathogenic mechanisms in CJD and AD; however potential cross-seeding between both protein misfolding diseases remains controversial.[25–28]

Aside from AD, concomitant tau pathology compatible with primary age-related tauopathy (PART) is reported frequently in CJD.[17,25,29] PART is commonly observed in the brains of aged individuals without severe cognitive impairment and characterized by NFTs that are usually restricted to structures in the medial temporal lobe in the absence of Aβ pathology.[30–33] Neuropathological studies that thoroughly evaluated the spectrum of tau accumulation in CJD reported PART in up to 70% of cases.[17,25,34] Nevertheless, established CSF p-tau181 assays did not reliably reflect concomitant tauopathies found in CJD at autopsy.[17] The fact that CSF p-tau is not consistently increased in other tauopathies, despite the shared detrimental processes of aberrant tau phosphorylation and aggregation, is mostly explicable by distinct tau isoforms, anatomic and cellular distribution of tau deposits, as well as disease-associated phosphorylation, proteolytic cleavage, and higher p-tau secretion from AD-affected neurons.[35–38] However, paired helical filaments in PART show biochemical and structural features similar to those in AD.[39] Considering the parallels between PART and suspected non-AD pathophysiology (denoted by normal amyloid and abnormal tau and/or neurodegeneration biomarkers), it is not quite clear whether CSF p-tau can accurately discriminate between PART and AD or if PART truly is completely unrelated to AD.[30–32,40]

Recently, tremendous progress in the field of blood-based biomarkers of AD has been achieved by assays that quantify N-terminal-to-mid-region tau fragments, abundant both in CSF and blood. P-tau231, p-tau217, and p-tau181 based on N-terminal detection antibodies

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show increases early in the AD continuum, presumably because N-terminal tau fragments are released from neurons in response to incipient Aβ pathology.[41–43] Although these assays target some of the p-tau epitopes confirmed by immunohistochemistry in CJD brains with tauopathies,[17] CSF profiles of p-tau biomarkers in CJD have not been systematically evaluated. We performed a head-to-head characterization of N-terminal p-tau231, p-tau217, and p-tau181 in definite CJD versus AD to investigate their differential diagnostic performances during life as well as capacities to identify concomitant pathologies in CJD at autopsy.

R ESEARCH IN CONTEXT

1. **Systematic review**: We searched PubMed for publications on cerebrospinal fluid (CSF) phosphorylated tau (p-tau) biomarker profiles in definite Creutzfeldt–Jakob disease (CJD) with co-pathologies. Mid-region–targeting CSF p-tau181 that was investigated so far detected only advanced stages of concomitant Alzheimer's disease (AD). However, data on p-tau biomarkers, capable of identifying early AD neuropathology, is lacking.
2. **Interpretation**: Our results indicate that N-terminal-to-mid-region–targeting CSF p-tau181, p-tau217, and p-tau231 biomarkers become elevated in CJD, most evidently but not exclusively in those with concomitant AD at autopsy. Increased concentrations of p-tau biomarkers in CJD without detectable amyloid beta (Aβ) deposition could be reflective of primary age-related tauopathy (PART) or previously suggested prion-related tauopathy.
3. **Future directions**: Replication of the results in larger and multi-centric studies will be important to support clinical interpretation and application of the findings in this study.

2 | METHODS

2.1 | Description of p-tau biomarkers evaluated

Four p-tau biomarkers were studied, including (1) p-tau231, measured with an immunoassay based on antibodies targeting tau phosphorylated at threonine-231 for capture and N-terminal amino-acids 6-18 of tau for detection[42]; (2) p-tau217, measuring tau phosphorylated at threonine-217 and containing the N-terminal amino-acids 6-18 epitope[43–45]; (3) p-tau181, directed at N-terminal amino acids 6-18 and phosphorylated threonine-181[46]; and (4) Innotest p-tau181 (Fujirebio, Ghent, Belgium), currently used in clinical practice and targeting mid-region epitopes of threonine-181 p-tau[47] (Figure S1).

2.2 | Study participants

Participants were recruited from the Centre for Cognitive Impairments or hospital wards at the Department of Neurology, University Medical Centre, Ljubljana, Slovenia. The study cohort included 197 individuals diagnosed with subjective cognitive decline (SCD, $n = 28$), mild cognitive impairment (MCI) due to AD ($n = 65$), AD dementia ($n = 75$), and definite CJD ($n = 29$). Patients underwent comprehensive neurological examination, neuropsychological assessment, CSF analysis, and magnetic resonance imaging (MRI). Individuals with a self-perceived decline in cognition but no objective evidence of cognitive impairment through formal instrumental/neuropsychological testing, or any pathological MRI or CSF findings, were allocated to the SCD group. Clinical diagnosis of AD dementia and AD MCI was established according to dementia Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)[48] and Winblad & Peterson MCI diagnostic criteria,[49] considering the individual's level of independence in everyday functioning and performance against the norms for the patient's age as well as educational and language background. Furthermore, AD diagnosis was supported by typical CSF biomarker profiles; early profile with decreased Aβ42/Aβ40 ratio only was present in 58% of AD-MCI cases, whereas other AD MCI and all AD dementia patients had an advanced CSF profile (Aβ42/Aβ40 <0.063, p-tau181 >60 ng/L, tau >400 ng/L). None of the AD MCI and AD dementia patients had a rapidly progressive clinical course with markedly elevated CSF t-tau/p-tau181 ratio[6] or MRI findings considered characteristic of CJD (e.g., basal ganglia and cortical hyperintensities[7]). Electroencephalography (EEG) and CSF 14-3-3 protein analysis were done only in CJD-suspected cases.

2.3 | Neuropathological analysis

Definite CJD diagnosis was made upon neuropathological examination of formalin-fixed brains that confirmed the presence of protease-resistant PrP^{Sc} and excluded variant CJD. *PRNP* gene analysis was performed to identify mutations in genetic CJD and to determine the genotype at the polymorphic codon 129. Personal histories were evaluated to rule out potential iatrogenic cases. After brain section and macroscopic examination, extensive sampling was performed: hippocampi; amygdala; frontal, temporal, parietal, and occipital neocortex; basal ganglia; thalamus; cerebellum with dentate nucleus; midbrain; pons; and medulla were paraffin embedded and 5-micron thick sections were stained with haematoxylin and eosin. Spongiform degeneration of gray matter, neuronal drop out, and reactive astrogliosis requested immunohistochemical staining on prions using anti-PrP monoclonal anti-body 12F10 (Cayman Chemical) on selected samples from the hippocampus, cerebellum, basal ganglia, and visual cortex. Concomitant AD pathology was assessed based on Aβ and p-tau immunoreactivity on sections from the anterior and posterior part of the hippocampus and temporal and visual cortex, using monoclonal Aβ antibody, clone 6F/3D (Dako, Denmark), and monoclonal antibody against misfolded paired helical filament tau, clone AT8 (Thermo Scientific, USA). An automated Ventana Benchmark GX staining system was used for tau and Aβ immunohistochemistry. Staging of AD was achieved according to Braak.[50]

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2.4 | Measurement of CSF biomarkers

CSF was collected, processed, aliquoted, and stored at −80°C according to standard procedures.[51] Tubes were thawed at room temperature and briefly vortexed before use. Core AD biomarkers and 14-3-3 protein were assessed during routine diagnostic workup at the Department of Neurology, University Medical Centre, Ljubljana, Slovenia. After blinding and randomization, all other CSF biomarker analyses were performed at the Clinical Neurochemistry Laboratory, University of Gothenburg, Mölndal, Sweden.

2.4.1 | Core AD CSF biomarkers and 14-3-3 analysis

The Innotest PHOSPHO-TAU (181P) and Innotest hTAU Ag enzyme-linked immunosorbent assays (ELISAs) were used to quantify p-tau181 and t-tau, respectively. Samples with t-tau concentrations above the highest standard point were further diluted and re-assayed. Aβ42 and Aβ40 were measured using the Lumipulse G1200 platform from Fujirebio.[52] In CJD-suspected cases, gamma isoform of 14-3-3 protein was quantified using the *CircuLex* 14-3-3 gamma ELISA kit (MBL, Japan). Analyses were performed according to the manufacturer's instructions and passed both in-house and manufacturer-recommended quality control checks.

2.4.2 | In-house single molecule array (Simoa) assays

N-terminal p-tau231, p-tau217, and p-tau181 in CSF were measured using Simoa technology on the HD-X instrument platform from Quanterix (Billerica, MA, USA). The same detection mouse monoclonal antibody Tau12 (#806502, BioLegend), raised against the N-terminal epitope 6-18,[53] was used in all in-house assays The capture antibody was a mouse monoclonal antibody, ADx253, for p-tau231 and AT270 (#MN1050, Invitrogen) for p-tau181 assay. For the p-tau217 assay, rabbit polyclonal capture antibody (#44-744, Invitrogen)[54] was used in the original (190 samples analyzed), and p-tau217.3A7 capture antibody in the modified assay (7 CJD samples analyzed). A high correlation (Spearman rho = 0.964) between both p-tau217 assays was observed in a subset of samples analyzed with both versions of the assay, and because the data were linear, it was possible to apply a correction factor to p-tau217 measurements obtained with the modified assay only (seven samples). The assay calibrator in all p-tau assays was recombinant full-length tau-441 phosphorylated in vitro by glycogen synthase kinase 3β (#TO8-50FN, SignalChem). Calibrators and specimens were diluted with the assay diluent (Tau 2.0 buffer; #101556, Quanterix). Analytical and clinical validation of the assays have been described previously.[42,43,45,46] Signal variations within and between analytical runs were evaluated using minimally two internal quality control samples analyzed in duplicate at the beginning and the end of each run. The within- and between-run variations for p-tau231, p-tau181, and both p-tau217 assays were <13%, <10%, and <18.5%, respectively.

2.5 | Statistical analysis

Data analysis was done using Prism version 10.0.3 (GraphPad Software, San Diego, CA, USA), R Studio version 2023.3.0.386 (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA, URL: http://www.rstudio.com/), and MedCalc version 22.014. Non-parametric tests were used for biomarker data that did not follow a normal distribution (Shapiro–Wilk test for normality). Group differences were examined using a two-tailed Mann–Whitney test (two categories) or the Kruskal–Wallis test (three or more groups) followed by the Dwass-Steel-Critchlow-Fligner test for multiple comparisons. Associations between continuous variables were assessed with Spearman correlation. Receiver-operating characteristic (ROC) curve and area under the curve (AUC) analyses were used to examine the ability of biomarkers to differentiate between diagnostic groups and DeLong's test (MedCalc version 22.014) to compare AUC for different biomarkers. To investigate associations between categorical variables, chi-square or Fisher's exact tests were used depending on the groups compared (all participants or CJD patients only). Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Demographic and clinical characteristics

The mean age in the study cohort ($n = 197$) was 71.6 years (SD = 8.2 years), with 56% women. There were no statistically significant differences in sex distribution among the groups and no differences in age between the AD, AD-MCI, and the entire CJD patient group. However, AD and AD-MCI patients were older than individuals with SCD ($p = 0.014$ and $p = 0.008$, respectively) and older than CJD cases with no co-pathology at autopsy ($p = 0.0001$; Table 1). Furthermore, CJD patients with AD or PART co-pathology at autopsy were older than CJD patients without concomitant pathologies ($p = 0.005$ and $p = 0.030$, respectively), (Table 1). Global cognition (as estimated by Mini-Mental State Examination [MMSE]) was significantly impaired in AD dementia (MMSE median 20 [4–26]) compared to AD-MCI (26 [20–30]; $p < 0.0001$) and SCD (29 [24–30]; $p < 0.0001$), as well as in AD-MCI compared to SCD ($p = 0.01$). Nearly all CJD patients presented with rapidly progressive dementia (97%) and myoclonus (80%), whereas clinical manifestation with cerebellar ataxia was seen in 59%, mutism in 52%, and visual symptoms in 34% of cases.

3.2 | Pathological data of the CJD cohort

In CJD, neuropathological examination after death was done at a mean age of 67.6 ± 11.2 years and an estimated median disease duration of 3.0 months (range 0.9–26.0 months). Except for the two *PRNP* gene mutation carriers, all CJD patients were sporadic cases. As anticipated, the most common genotype at the polymorphic *PRNP*

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TABLE 1 Demographic and CSF biomarker characteristics of the study cohort.

	SCD ($n = 28$)	AD-MCI ($n = 65$)	AD dementia ($n = 75$)	CJD ($n = 29$)	CJD^{only} ($n = 10$)	CJD^{+PART} ($n = 8$)	CJD^{+AD} ($n = 8$)
Age, years, *mean (SD)*	68.3 (5.7)[a,b]	72.9 (6.7)[c]	73.2 (7.9)[c]	67.4 (11.4)	57.3 (7.4)[a,b,c,e,f]	72.8 (8.2)[g]	77.8 (4.9)[c,g]
Females, *n (%)*	16 (57.1%)	42 (64.6%)	40 (53.3%)	13 (44.8%)	4 (40.0%)	3 (37.5%)	5 (62.5%)
Aβ42/40, *median (IQR)*	0.093[a,b] (0.088–0.097)	0.041[c,d] (0.038–0.048)	0.038[c,d] (0.032–0.043)	0.089[a,b] (0.076–0.103)	0.093[a,b,e] (0.087–0.123)	0.103[a,b] (0.084–0.178)	0.053[b,c,g] (0.044–0.085)
T-tau, pg/mL *median (IQR)*	227[a,b,d,e,f,g] (182–272)	382[b,c,d,e,f,g] (314–652)	798[a,c,d,e,f,g] (595–972)	6482[a,b,c] (2522–11498)	9045[a,b,c] (1041–11662)	5495[a,b,c] (2136–10415)	5315[a,b,c] (3742–19785)
T-tau/mid-region p-tau181 *median (IQR)*	6.3[a,b,d,e,f,g] (5.5–9.5)	7.3[b,c,d,e,f,g] (6.7–10.6)	8.3[a,c,d,e,f,g] (7.6–10.7)	162.1[a,b,c] (112–481)	196.6[a,b,c] (154.3–278.0)	146.1[a,b,c] (108.9–208.2)	262.9[a,b,c] (96.6–481)
Mid-region p-tau181, pg/mL *median (IQR)*	41[a,b,d,e] (31–47)	56[b,c] (50–98)	103[a,c,d,e,f,g] (86–130)	53[b,c] (40–72)	50[b] (36–57)	41[b] (27–90)	65[b,c] (51–78)
N-terminal p-tau181, pg/mL *median (IQR)*	261[a,b,d,e,g] (197–303)	573[b,c] (380–1062)	1316[a,c,f,g] (1064–1507)	726[b,c] (374–988)	601[b,c] (352–818)	380[b] (205–1045)	821[c] (742–1304)
N-terminal p-tau217, pg/mL, *median (IQR)*	2.7[a,b,d,e,f,g] (1.5–5.3)	18.2[b,c,e] (9.7–31.2)	37.6[a,c] (26.3–48.5)	26.8[a,c] (17.9–42.2)	22.9[c] (16.2–40.2)	22.5[c] (13.3–34.6)	38.9[a,c] (25.4–64.9)
N-terminal p-tau231, pg/mL *median (IQR)*	232[a,b,d,e,f,g] (135–298)	829[b,c] (570–964)	1026[a,c] (779–1272)	869[c] (491–1021)	608[c] (437–1021)	639[c] (449–953)	1011[c] (709–1421)

Note: Data are summarized as mean and standard deviation (SD) (age) or median and interquartile range (IQR) (CSF biomarkers). Differences between groups were tested using analysis of variance followed by the Dwass-Steel-Critchlow-Fligner test for multiple comparisons. Significant differences compared to:
Abbreviations: AD, Alzheimer's disease; CJD, Creutzfeldt–Jakob disease; IQR, interquartile range; MCI, mild cognitive impairment; PART, primary age-related tauopathy; p-tau181(231, 217), tau phosphorylated at threonine-181, threonine-231, or threonine-217; SCD, subjective cognitive decline; SD, standard deviation.
[a]AD-MCI.
[b]AD dementia.
[c]SCD.
[d]entire CJD group.
[e]CJD with AD co-pathology.
[f]CJD with PART co-pathology.
[g]CJD without co-pathology.

codon was 129 MM (76% of cases), followed by VV (14%) and MV (10%) subtypes. Disease progression was somewhat slower, and time from CSF collection to death less brief in MV + VV genotypes compared to MM subtypes (7 months vs 3 months, $p = 0.048$; and 3 months vs 1 month, $p = 0.016$, respectively). Concomitant AD pathology was reported in eight (28%) CJD patients (CJD^{+AD}); a further eight patients (28%) had NFT pathology in the absence of Aβ (CJD^{+PART}). Lewy body pathology was found in two and concurrent hypoxic brain injury in one CJD patient. In 10 patients (34%) no other pathologies were identified (CJD^{only}), (Figure S2). All CJD^{+AD} patients were of the MM subtype; however, concomitant AD or PART pathology was not associated with genotype at the *PRNP* codon 129 (Fisher's exact test, $p = 0.340$). AD and PART co-pathology was observed more frequently at advanced age but did not associate with disease duration. Concerning the clinical manifestation of CJD, concomitant pathologies were found in 50% of patients with pronounced cerebellar signs and 91% of cases without cerebellar ataxia (Fisher's exact test, $p = 0.044$).

3.3 | CSF biomarkers of Aβ deposition and neurodegeneration differentiate between AD dementia and CJD

The CSF Aβ42/Aβ40 ratio was significantly decreased in AD-MCI and AD dementia patients compared to SCD and CJD ($p < 0.0001$ for all comparisons) (Figure 1A), and therefore differentiated accurately between AD dementia and CJD (AUC = 96.6% [95% confidence interval [CI] 92.7%–100%]) (Figure 2A). Neuropathological classification of CJD cases based on the concomitant pathology revealed a decreased CSF Aβ42/Aβ40 ratio in five patients with AD co-pathology at autopsy (63% of CJD^{+AD} cases). Although the CSF Aβ42/Aβ40 ratio was not as low in CJD^{+AD} as in AD dementia ($p = 0.006$) it was still significantly decreased compared to SCD and CJD^{only} ($p = 0.005$ and $p = 0.023$, respectively) but not (likely due to the small sample number) versus CJD^{+PART} (Figure 1A).

Predictably, CSF t-tau was markedly increased in CJD, with observed 28.3-fold changes against the SCD mean, in contrast to

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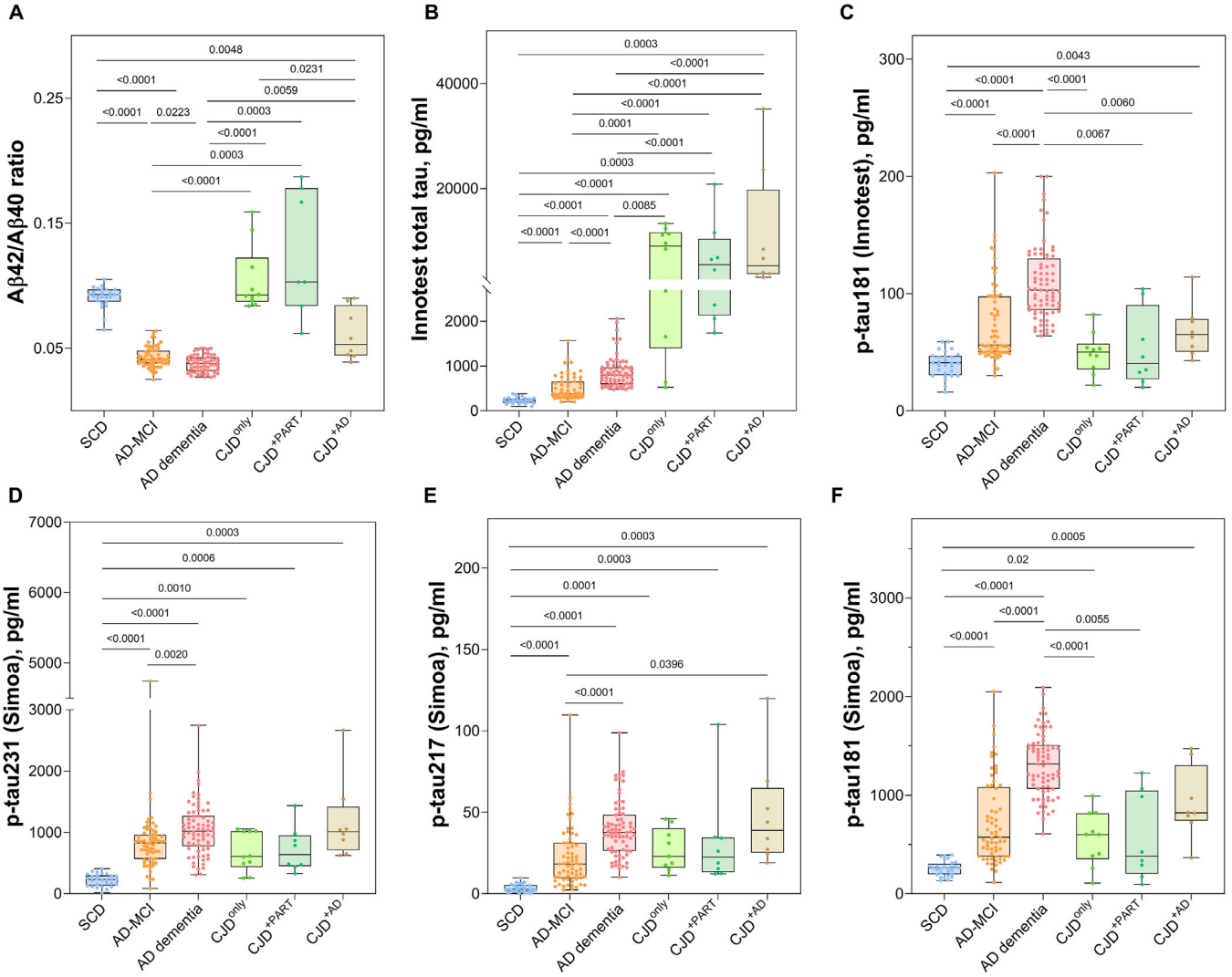

FIGURE 1 Cerebrospinal fluid biomarkers in patient groups. (A–C) show established biomarkers, amyloid ratio, and total and mid-region p-tau181, respectively. (D–F) show concentrations of distinctly phosphorylated (threonine-231, threonine-217, and threonine-181) N-terminal p-tau biomarkers. Group differences were compared using analysis of variance followed by the Dwass-Steel-Critchlow-Fligner test for multiple comparisons. AD, Alzheimer's disease; CJD, Creutzfeldt–Jakob disease; MCI, mild cognitive impairment; PART, primary age-related tauopathy; SCD, subjective cognitive decline.

3.5- and 1.7-fold increased t-tau in AD dementia and AD-MCI, respectively (Table S1, Figure 2B). Accordingly, t-tau discriminated between CJD and AD dementia with high accuracy (AUC = 92.8% [95% CI 84.9%–100%]), which was further improved by the t-tau/p-tau181 (Innotest) ratio (AUC = 100%; $p = 0.08$; Figure 2A). The CSF t-tau and t-tau/p-tau181 (Innotest) ratio were similar across CJD subgroups, irrespective of the co-pathology (Figure 1B, Figure 2B) or genotype at the polymorphic codon 129. In CJD, t-tau correlated strongly with CSF levels of 14-3-3 protein (Spearman rho = 0.95; $p < 0.0001$). Furthermore, both neurodegeneration biomarkers showed an inverse correlation with survival after the lumbar puncture (Spearman rho = –0.374; $p = 0.046$ and Spearman rho = –0.389; $p = 0.037$, for t-tau and 14-3-3 protein, respectively) but not with estimated disease duration.

3.4 | CSF N-terminal p-tau biomarkers are increased in CJD

All p-tau biomarkers were increased in AD compared with SCD ($p < 0.0001$) and AD-MCI ($p \leq 0.002$) (Figure 1C–F); however, they were also elevated in CJD versus SCD ($p < 0.0001$ for N-terminal p-tau biomarkers; $p = 0.03$ for mid-region p-tau181) with the largest magnitude of change versus the SCD mean observed for p-tau217 (8.0-fold; Figure 2B, Table S1). N-terminal p-tau181 and p-tau231 levels in CJD were not significantly different from those found in AD-MCI, whereas p-tau217 was higher compared to AD-MCI ($p = 0.02$). Moreover, p-tau231 and p-tau217 did not differ significantly from concentrations observed in AD dementia (Table 1), which resulted in their modest and significantly lower discriminatory accuracy (AUC = 67.5% and 65.5%,

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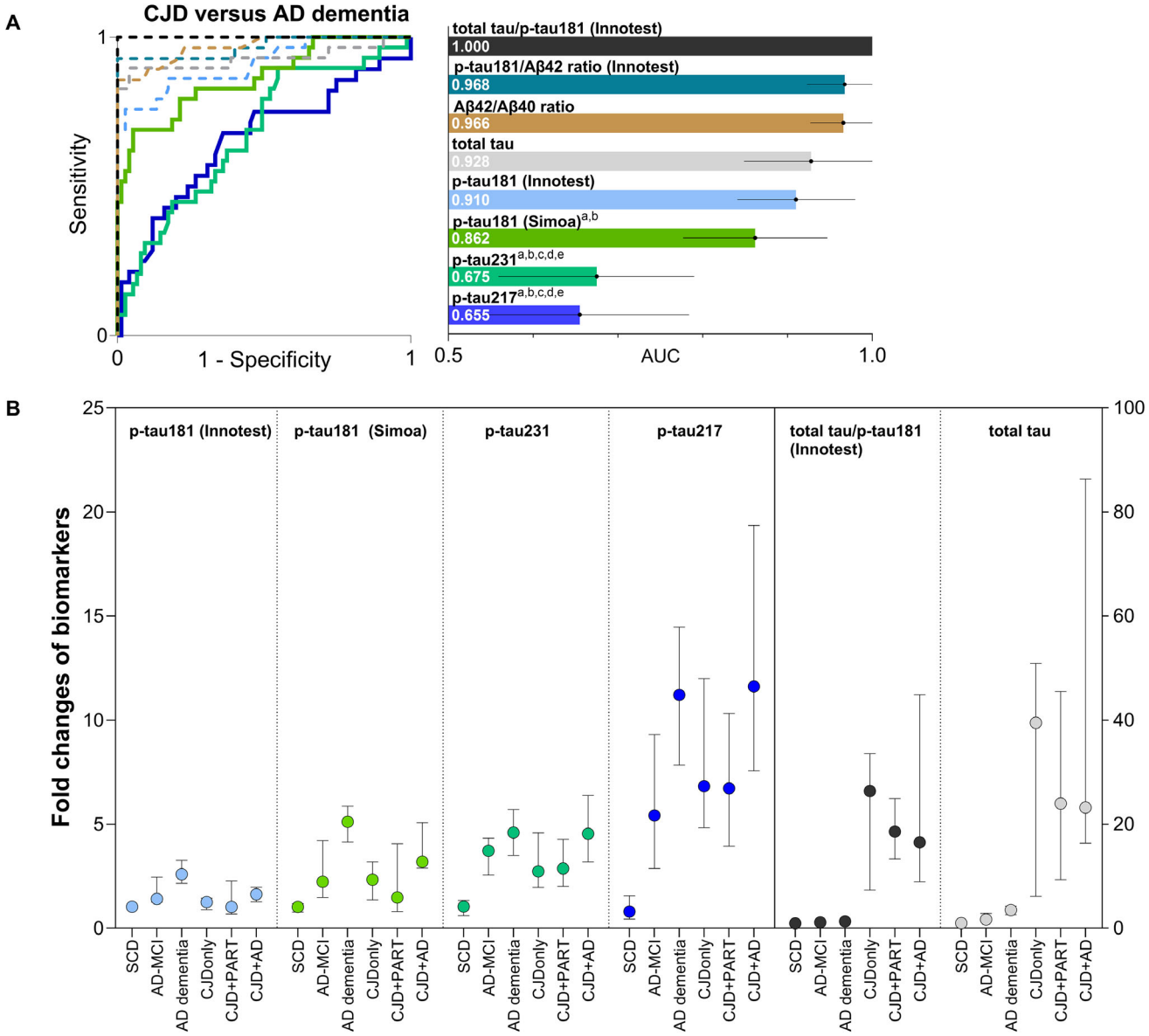

FIGURE 2 (A) Accuracies (ROC analyses) of CSF biomarkers to discriminate between CJD and AD dementia patients. The AUC and 95% confidence intervals for each biomarker are depicted. Discriminatory accuracies were compared with the DeLong test. Significant differences compared to: [a]total tau/p-tau181 (Innotest) ratio, [b]Aβ42/Aβ40 ratio and mid-region p-tau181/Aβ42 ratio, [c]total tau, [d]p-tau181 (Innotest), and [e]p-tau181 (Simoa). Fold-changes of CSF biomarkers in patient groups. (B) Fold-changes of CSF biomarkers against the SCD mean. T-tau and t-tau/p-tau181 ratio are plotted against the right y-axis. AD, Alzheimer's disease; AUC, area under the curve; CSF, cerebrospinal fluid; CJD, Creutzfeldt–Jakob disease; MCI, mild cognitive impairment; PART, primary age-related tauopathy; p-tau181(231, 217), tau phosphorylated at threonine-181, threonine-231 or threonine-217; ROC, receiver-operating characteristic; SCD, subjective cognitive decline.

respectively) compared with the established biomarkers as well as versus N-terminal p-tau181 (AUC = 86.2%) (Figure 2A). Accordingly, using N-terminal p-tau concentrations to generate the p-tau/Aβ42 ratio (U.S. Food and Drug Administration (FDA)–approved test in the assessment of AD pathology on Elecsys platform) led to less-accurate biomarkers (Figure S3) compared to the mid-region p-tau181/Aβ42 ratio (Figure 2A).

When the presence of different co-pathologies in CJD were accounted for, concentrations of N-terminal p-tau biomarkers were the highest in patients with concomitant AD (CJD^{+AD}); p-tau181 and p-tau231 in CJD^{+AD} did not differ significantly from AD dementia or AD-MCI, whereas p-tau217 concentrations were higher compared to AD-MCI ($p = 0.040$) (Figure 1D–F). Median concentrations of p-tau231 and p-tau217 in CJD^{+AD} were more than 60% and of N-terminal p-tau181 nearly 40% higher than in CJD without co-pathologies (CJD^{only}) (Table 1). Nevertheless, differences between CJD^{+AD} and CJD^{only} were not statistically significant. Despite generally lower p-tau concentrations in CJD without Aβ co-pathology, p-tau217 and p-tau231 in CJD^{only} and CJD^{+PART} were comparable to AD-MCI and did not differ significantly from AD dementia (Figure 1D–E). N-terminal

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TABLE 2 Spearman's correlations of N-terminal p-tau biomarkers in patient groups.

	N-terminal p-tau181	N-terminal p-tau217	N-terminal p-tau231
(a) With mid-region p-tau181			
Entire cohort (n = 197)	0.903 ($p < 0.0001$)	0.813 ($p < 0.0001$)	0.656 ($p < 0.0001$)
SCD ($n = 28$)	0.801 ($p < 0.0001$)	0.238	0.682 ($p = 0.0001$)
AD-MCI ($n = 65$)	0.825 ($p < 0.0001$)	0.774 ($p < 0.0001$)	0.261 ($p = 0.0355$)
AD dementia ($n = 75$)	0.741 ($p < 0.0001$)	0.677 ($p < 0.0001$)	0.557 ($p < 0.0001$)
CJD ($n = 29$)	0.816 ($p < 0.0001$)	0.742 ($p < 0.0001$)	0.743 ($p < 0.0001$)
(b) With total tau			
Entire cohort (n = 197)	0.645 ($p < 0.0001$)	0.752 ($p < 0.0001$)	0.532 ($p < 0.0001$)
SCD ($n = 28$)	0.704 ($p < 0.0001$)	0.329	0.626 ($p = 0.0004$)
AD-MCI ($n = 65$)	0.787 ($p < 0.0001$)	0.744 ($p < 0.0001$)	0.216
AD dementia ($n = 75$)	0.760 ($p < 0.0001$)	0.652 ($p < 0.0001$)	0.569 ($p < 0.0001$)
CJD ($n = 29$)	0.504 ($p = 0.0053$)	0.655 ($p = 0.0002$)	0.467 ($p = 0.0107$)
(c) With amyloid ratio			
Entire cohort (n = 197)	−0.653 ($p < 0.0001$)	−0.532 ($p < 0.0001$)	−0.544 ($p < 0.0001$)
SCD ($n = 28$)	−0.083	0.091	−0.240
AD-MCI ($n = 65$)	−0.516 ($p < 0.0001$)	−0.460 ($p = 0.0002$)	−0.366 ($p = 0.0038$)
AD dementia ($n = 75$)	−0.261	−0.334 ($p = 0.0111$)	−0.154
CJD ($n = 29$)	−0.533 ($p = 0.0035$)	−0.200	−0.364

Abbreviations: AD, Alzheimer's disease; CJD, Creutzfeldt–Jakob disease; MCI, mild cognitive impairment; p-tau181(231, 217), tau phosphorylated at threonine-181, threonine-231, or threonine-217; SCD, subjective cognitive decline.

p-tau181 in CJD^{only} and CJD^{+PART} showed less remarkable increases than in AD dementia ($p < 0.0001$ and $p = 0.0055$, respectively); even so, concentrations were not significantly different from AD-MCI (Figure 1F). N-terminal p-tau biomarkers in CJD were not associated with age, time from lumbar puncture to death (as seen with CSF t-tau), or estimated disease duration. They also did not differ between clinical manifestations of CJD (presence or absence of cerebellar signs, visual symptoms, or mutism).

3.5 | Association of N-terminal p-tau biomarkers with the established CSF biomarkers

Strong correlations between mid-region p-tau181 and N-terminal p-tau biomarkers were observed in the entire study cohort (Spearman's rho = 0.903, 0.813, and 0.656 for p-tau181, p-tau217, and p-tau231, respectively; $p < 0.0001$) and were present in each of the patient groups except for p-tau231 in AD-MCI and p-tau-217 in SCD, which showed only weak or no correlation with mid-region p-tau181, respectively (Table 2). Similarly, N-terminal p-tau biomarkers correlated significantly with t-tau in the entire cohort (Spearman's rho = 0.645, 0.752, and 0.532 for p-tau181, p-tau217 and p-tau231, respectively; $p < 0.0001$) and in patient groups, again except for p-tau-217 in SCD and p-tau231 in AD-MCI (likely due to still normal concentrations of t-tau in nearly half of the patients [see above] and already increased p-tau231). On a cohort level, there was an inverse correlation between all p-tau biomarkers and Aβ42/Aβ40 ratio (Spearman's rho = −0.532 to −0.653; $p < 0.0001$), which was also present in AD-MCI, whereas only individual p-tau forms associated significantly with Aβ ratio in AD dementia (p-tau217; Spearman's rho = −0.334, $p = 0.011$) and in CJD (p-tau181; Spearman's rho = −0.533; $p = 0.004$ and −0.409; $p = 0.031$ for N-terminal and mid-region form respectively) (Table 2), suggesting different time points or stages of tau phosphorylation in response to Aβ plaques in these patient groups.

To better explain increased N-terminal p-tau biomarkers in CJD patients with or without concomitant pathology, we also investigated the associations of N-terminal p-tau biomarkers with the established CSF biomarkers in the CJD subgroups (Table S2). Of interest, the strongest correlations between N-terminal p-tau biomarkers and t-tau were observed in CJD^{+PART} (Spearman's rho = 0.758; $p = 0.015$, 0.867; $p = 0.002$ and 0.952; $p < 0.001$ for p-tau181, p-tau217 and p-tau231, respectively). In CJD^{+AD}, moderate to strong correlations with t-tau were observed (Spearman's rho = 0.619; p = ns, 0.786; $p = 0.028$ and 0.476; p = ns for p-tau181, p-tau217 and p-tau231, respectively), whereas only weak correlations of N-terminal p-tau biomarkers with t-tau existed in CJD^{only} (Spearman's rho = 0.200–0.430; $p > 0.05$), suggesting that they closely associate with neurodegeneration only in the presence of NFT pathology (CJD^{+PART} and CJD^{+AD}) (Table S2).

3.6 | Effect of CJD neuropathology on p-tau biomarkers

Because some *PRNP* mutations were previously reported to affect Aβ and tau accumulation in CJD,[26,55] we next compared CSF p-tau

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TABLE 3 CSF p-tau biomarkers in sporadic CJD without concomitant Alzheimer's disease pathology, and across *PRNP* codon 129 genotype.

PRNP codon 129 genotype	MM ($n = 13$[a])	MV + VV ($n = 6$)	*p*-value[b]
mid-region p-tau181 (pg/mL), *median (IQR)*	46 (28–54)	60 (45–100)	0.055
N-terminal p-tau181 (pg/mL), *median (IQR)*	407 (276–615)	1028 (889–1339)	0.002
N-terminal p-tau217 (pg/mL), *median (IQR)*	18.9 (13.1–31.8)	28.7 (17.3–34.8)	0.336
N-terminal p-tau231 (pg/mL), *median (IQR)*	488 (341–736)	932 (746–991)	0.022

Abbreviations: CSF, cerebrospinal fluid; CJD, Creutzfeldt–Jakob disease; MM, MV, and VV, methionine/methionine, methionine/valine, or valine/valine polymorphic combination at codon 129 of the *PRNP* gene; *PRNP*, prion protein gene; p-tau181(231, 217), tau phosphorylated at threonine-181, threonine-231, or threonine-217.

[a]To investigate only the effect of the *PRNP* codon 129 genotype on CSF p-tau concentrations, CJD cases with concomitant AD (all homozygous for methionine at codon 129) were excluded.

[b]Differences between methionine homozygous (MM) versus valine hetero- and homozygous (MV + VV) genotypes were tested using the Mann–Whitney *U* test.

concentrations only in sporadic cases ($n = 27$), across *PRNP* codon 129 genotypes and with respect to concomitant Aβ and NFT pathology.

N-terminal p-tau181 varied significantly among CJD subtypes, with higher median concentrations observed in the less-common MV and VV genotypes ($p = 0.015$; MV + VV vs MM genotype), which were not associated with concomitant AD at autopsy but had a somewhat longer disease duration. To eliminate the influence of underlying AD and further clarify how CSF p-tau is affected by polymorphisms at *PRNP* codon 129, concentrations were again compared in a subset of patients without eight CJD^{+AD} cases (all methionine homozygotes). Appreciable differences in N-terminal p-tau181 and p-tau231 in MM versus MV + VV genotypes were found ($p = 0.002$ and $p = 0.022$, respectively), whereas mid-region p-tau181 and p-tau217 did not differ significantly (Table 3).

Because of the observed variation in p-tau biomarkers among CJD subtypes, the effect of concomitant Aβ and NFT pathology was investigated in the largest homogeneous subgroup—cases with 129 MM genotype ($n = 21$). All p-tau biomarkers were significantly higher in Aβ-positive (all CJD^{+AD} cases, $n = 8$) than in Aβ-negative patients (6 CJD^{+PART}, 6 CJD^{only}, 1 CJD with Lewy body pathology; Figure S4), but not in the presence of tau accumulation in general (CJD^{+AD} + CJD^{+PART} vs NFT-negative), indicating further increased concentrations along with Aβ deposition, which were already noticeable from comparisons between CJD subgroups that included different disease subtypes (Figure 1C–F, Table 1).

Comparing all sporadic cases irrespective of the codon 129 genotype yielded similar results for p-tau217 and p-tau231 (Aβ-negative vs Aβ-positive $p = 0.022$ and $p = 0.009$, respectively), but a greater overlap in N-terminal p-tau181 between Aβ-positive and Aβ-negative patients ($p = 0.147$), which could be attributed to higher concentrations in MV + VV genotypes (described above) even in the absence of Aβ pathology. Consequently, the two p-tau forms with only modest discrimination between CJD and AD dementia—p-tau217 and p-tau231—were more accurate at identifying concomitant Aβ pathology in sporadic CJD (AUC = 78.5 and 81.6% for p-tau217 and p-tau231, respectively) than p-tau181 (AUC = 68.4% and 74.0% for N-terminal and mid-region form, respectively) and performed almost as well as Aβ ratio (AUC = 90.8%; statistical significance of differences between AUCs was not tested due to sample size limitation—only sporadic CJD cases).

4 | DISCUSSION

The envisioned widespread application of N-terminal p-tau biomarkers in clinical practice requires real-world data on their performance, especially in patients with mixed pathologies and overlapping clinical features. We had an opportunity to study N-terminal p-tau181, p-tau217, and p-tau231 in the CSF of autopsy-verified CJD with AD, PART, or no detectable co-pathology in comparison with AD, generally considered in CJD differential diagnosis.

Neuropathological characteristics of our CJD patients were consistent with previous findings of more common tau rather than Aβ co-pathology in sCJD.[17,25,34] Additional NFT pathology was found in 16 of 29 (55%) of the CJD patients; in half of these (28%), the diagnosis of concomitant AD was established, which resembles formerly reported frequencies of concurrent non-AD tau pathology/PART (20%–70%) and Aβ deposition (30%–50%) in CJD brains.[17,25] In the present study, only 63% of the CJD patients with parenchymal Aβ pathology had decreased CSF Aβ42/Aβ40 ratio; yet this concordance was better compared to a study using Aβ42 alone as a surrogate for brain amyloidosis.[5] Although all our CJD^{+AD} cases were homozygous for methionine at *PRNP* codon 129, the prevalence of NFT and Aβ co-pathology did not differ significantly across CJD subtypes, nor was it associated with disease duration. The only notable distinction was the older age of CJD patients with concomitant AD/PART compared with CJD^{only}, which concurs well with earlier observations[17,25] and further supports the notion of a largely independent pathogenic mechanisms in AD and sCJD.

Our results confirm the value of conventional CSF biomarkers to discriminate between CJD and AD.[5,8,11,14] T-tau and p-tau181, targeting mid-region tau fragments, accurately separated CJD from AD dementia; likewise, nearly perfect discrimination was achieved by Aβ42/Aβ40 and t-tau/p-tau181 ratio. However, N-terminal p-tau forms had lower differential diagnostic performance in our study; particularly p-tau217 and p-tau231 were outperformed by all other biomarkers as they were similarly increased in AD dementia and CJD. This seems to contradict the previously reported exceptional diagnostic accuracy of novel p-tau biomarkers for AD against non-AD dementia.[42,43,46,56–58] Nevertheless, the rarity of CJD and its rapid progression impedes systematic comparisons in clinical settings, which is probably why CJD patients were not included in the aforementioned research cohorts. Available data on CSF p-tau in CJD thus far were limited to mid-region p-tau181, which was rarely increased above the threshold[8,17,59] and

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was questioned for its reliability to capture the tau pathology present in CJD brain.[17] It was, however, found to correlate with the level of concomitant AD-related NFT pathology in a large neuropathological study, showing that a majority of CJD cases with Braak stages III-IV had elevated mid-region p-tau181, whereas most of the CJD brains with Braak stages I-II were associated with normal p-tau181.[5] In agreement with this, Innotest p-tau181 in our study was increased in CJD^{+AD} compared with SCD, but less prominently than in AD dementia, surpassing the threshold in only 5 of 8 CJD^{+AD} cases.

As hypothesized, N-terminal p-tau biomarkers increased with the presence of AD co-pathology in CJD and were significantly higher in patients with Aβ immunoreactivity at autopsy than in Aβ-negative cases. In CJD^{+AD}, p-tau231, p-tau217, and p-tau181 were indistinguishable from concentrations in AD dementia, indicating that concomitant proteinopathy did not affect their sensitivity to AD neuropathology. The greater magnitude of change in p-tau231 and p-tau217 compared with p-tau181 in CJD with NFT pathology (CJD^{+AD} and CJD^{+PART}) aligns well with a neuropathological report showing immunohistochemistry with AT180 (p-tau231) or AT100 (p-tau212 and p-tau214) rather than AT270 (p-tau181) antibody better reveals widespread tauopathy in CJD.[17] Furthermore, incipient Aβ pathology and shared neuropathological characteristics of PART and presymptomatic AD[31,39] could explain some of the overlaps between our CJD patients. Because NFT pathology in the medial temporal lobe is typical of PART but also belongs to the AD continuum, it was suggested that certain cases with PART may eventually develop Aβ pathology, as prediction on the future progress to AD is still unreliable in the early stages of NFT degeneration.[17,39] Notably, N-terminal p-tau forms with the lowest capacity to distinguish between CJD and AD dementia herein also seemed more accurate than p-tau181 at identifying concomitant Aβ pathology in our CJD cases; p-tau217 and p-tau231 have been found to increase already at the beginning of the AD continuum,[41–43] in parallel with brain tau (plasma p-tau231[42]) and Aβ deposition in the initial accumulating regions (plasma p-tau231 and p-tau217[41,42]). Because these biomarkers reflect subtle changes in Aβ deposition, their CSF concentrations could presumably increase even before the overt Aβ plaque formation allows for accurate classification of Aβ positivity.

Our results further underscore the heterogeneity of CJD and support the assumption of prion-related tau phosphorylation, which could explain higher CSF p-tau in the presence of V129 allele in this study. The findings of elevated N-terminal p-tau181 and p-tau231 in MV + VV compared to MM genotypes, despite that the former were not associated with AD co-pathology, are similar to the previously described increase in mid-region p-tau181 in MV2K and VV2 subtypes.[5] Of interest, V2 strain-related subtypes were also shown to be particularly affected by prion-specific tauopathy and often exhibit higher PrP^{Sc} burden compared to MM(V)1 subtypes.[5] CSF p-tau concentrations were thus proposed to reflect tiny, rod- or dot-shaped tau deposits that correlate with PrP^{Sc} burden and can be found in examined brain tissues (including the areas with spongiform degeneration[5]), irrespective of Aβ plaques.[5,60] Although PrP^{Sc} typing was not performed in our patients and we did not quantify abnormal tau deposition in association with prion protein load, it was still intriguing to find increased N-terminal p-tau181 and p-tau231 in genotypes with somewhat slower disease progression, suggesting that disease duration itself could contribute to a higher extent of site-specific tau phosphorylation.

In line with formerly published data,[8,61] neurodegeneration biomarkers (t-tau and 14-3-3 protein) in our CJD patients were inversely associated with survival after lumbar puncture. N-terminal p-tau biomarkers correlated strongly with mid-region p-tau181 and t-tau in CJD; however, they did not inform survival in these patients. Furthermore, significant correlations between N-terminal p-tau and t-tau in our CJD^{+PART} and CJD^{+AD} but not in CJD^{only}, suggested they closely associate with neurodegeneration principally in the presence of NFT pathology. Despite concomitant AD being more frequently observed in older CJD and understandably less common in cases with pronounced subcortical clinical manifestation (i.e., more cerebellar signs), we found no correlation of N-terminal p-tau biomarkers with age or any specific clinical feature. Consistent with a study measuring mid-region p-tau181 in sCJD,[5] our p-tau biomarkers did not associate with estimated disease duration. Nevertheless, a subgroup of sCJD patients with high p-tau181 levels who presented with early akinetic mutism and shorter disease duration was reported by another group.[62] Given their capacity to reveal concomitant pathologies, the prognostic potential of N-terminal p-tau biomarkers in sCJD should be investigated further alongside known disease modifiers (e.g., codon 129 genotype).

Our results demonstrate that increases in N-terminal p-tau in CJD exceed fold-changes usually observed with the conventional p-tau181 assays and lie within the concentration range of the AD continuum. Whether this has to do with high analytical sensitivity or measured tau fragment, elevated N-terminal p-tau in CJD should be viewed in parallel with a considerably higher magnitude of change in t-tau. Because CSF and plasma p-tau were shown to correlate[46,63,64] and likely follow similar dynamics in both compartments,[63] complementary use of blood-based p-tau and neurodegeneration biomarkers (e.g., neurofilaments or recently proposed brain-derived tau[65,66]) would be advantageous to differentiate between AD and AD with concomitant proteinopathies. Furthermore, our results highlight that applying only simplified ratios, such as the FDA-approved Aβ42/Aβ40 or mid-region p-tau181/Aβ42 ratio without considering CSF biomarkers of neurodegeneration or additional diagnostic evaluations, may delay the detection of other conditions accompanying AD.

CJD classification was limited to the *PRNP* genotype in our study, and the presence of NFT and Aβ pathology was only descriptively recorded, although PrP typing and semi-quantitative assessment of tau, Aβ, and prion protein burden would have provided better neuropathological correlates of CSF findings. Admittedly, CJD subgroups were small, preventing us from detecting significant differences despite visible variation among subgroups. Nevertheless, our results are consistent with and expand on some of the previous studies. Furthermore, sCJD is extremely rare, with an estimated annual incidence of 1–2 cases per million in our country[67] and worldwide.[1,13] As the main referral center, we were able to include almost all neuropathologically confirmed cases that were reported to the national surveillance unit and still had available CSF sample. Study strengths also include its

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focus on clinical setting and measuring N-terminal p-tau biomarkers on identical analytical platform, which enabled head-to-head comparison of different phosphorylation sites. Finally, our immunoassays targeted some of the p-tau epitopes previously detected in CJD brain by immunohistochemistry.

In summary, our study provides confirmatory and novel evidence of increased CSF p-tau in definite CJD with and without concomitant pathologies. Although conventional mid-region p-tau181 and t-tau/p-tau181 ratio remain the most important biomarkers to discriminate CJD from AD, N-terminal p-tau biomarkers are closely associated with neurodegeneration and concomitant AD and possibly respond to prion-related tauopathy in CJD. Replication of our findings in independent cohorts could augment the value of N-terminal p-tau biomarkers in the diagnosis and management of CJD with concomitant tauopathies.

AUTHOR CONTRIBUTIONS

Thomas K. Karikari, Saša Čučnik, and Kaj Blennow are co-senior authors. Andreja Emeršič, Thomas K. Karikari, Nicholas J. Ashton, Uroš Rot, Saša Čučnik, Milica Gregorič Kramberger, Henrik Zetterberg, and Kaj Blennow conceptualized and coordinated the study. Andreja Emeršič, Thomas K. Karikari, Nicholas J. Ashton, Agathe Vrillon, Juan Lantero-Rodriguez, Fernando Gonzalez-Ortiz, and Przemysław R. Kac performed CSF biomarker analyses. Andreja Emeršič, Thomas K. Karikari, Maciej Dulewicz, Jörg Hanrieder, Uroš Rot, Eugeen Vanmechelen, Henrik Zetterberg, Saša Čučnik, and Kaj Blennow are responsible for the analysis or interpretation of data. Eugeen Vanmechelen provided reagents for the p-tau231 assay. Milica Gregorič Kramberger and Uroš Rot contributed to patient recruitment and diagnosis. Jernej Mlakar provided and interpreted neuropathological diagnostic assessments. Andreja Emeršič and Thomas K. Karikari wrote the first manuscript draft. All authors critically revised the manuscript for important intellectual input and approved the final version for submission.

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CONFLICT OF INTEREST STATEMENT

H.Z. has served on scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Amylyx, Annexon, Apellis, Artery Therapeutics, AZTherapies, Cognito Therapeutics, CogRx, Denali, Eisai, Merry Life, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, and has given lectures in symposia sponsored by Alzecure, Biogen, Cellectricon, Fujirebio, Lilly, Novo Nordisk, and Roche. K.B. has served as a consultant and on advisory boards for AC Immune, Acumen, ALZPath, AriBio, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served on data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials, and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai, and Roche Diagnostics. H.Z. and K.B. are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. E.V. is a co-founder of ADx NeuroSciences. The other authors declare no competing interests. Author disclosures are available in the Supporting Information.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

CONSENT STATEMENT

This study was performed following the principles of the Declaration of Helsinki. Informed consent for biobanking and research use of left-over diagnostic samples was obtained from study participants or legal guardians. Approval of the study protocol was granted by the Medical Ethics Committee of the Republic of Slovenia, Ministry of Health (0120-308/2021/3 and 0120-342/2021/6).

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