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ORIGINAL COMMUNICATION

CSF Tau proteins reduce misdiagnosis of sporadic Creutzfeldt–Jakob disease suspected cases with inconclusive 14-3-3 result

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Abstract Cerebrospinal fluid (CSF) 14-3-3 protein supports sporadic Creutzfeldt–Jakob (sCJD) diagnosis, but often leads to weak-positive results and lacks standardization. In this study, we explored the added diagnostic value of Total Tau (t-Tau) and phosphorylated Tau (p-Tau) in sCJD diagnosis, particularly in the cases with inconclusive 14-3-3 result. 95 definite sCJD and 287 patients without prion disease (non-CJD) were included in this study. CSF samples were collected in routine clinical diagnosis and analysed for 14-3-3 detection by Western blot (WB). CSF t-Tau and p-Tau were quantified by commercial ELISA kits and *PRNP* and *APOE* genotyping assessed by PCR–RFLP. In a regression analysis of the whole cohort, 14-3-3 protein revealed an overall accuracy of 82 % (sensitivity = 96.7 %; specificity = 75.6 %) for sCJD. Regarding 14-3-3 clear positive results, we observed no added value either of t-Tau alone or p-Tau/t-Tau ratio in the model. On the other hand, considering 14-3-3 weak-positive cases, t-Tau protein increased the overall accuracy of 14-3-3 alone from 91 to 94 % and specificity from 74 to 93 % ($p < 0.05$), with no sensitivity improvement. However, inclusion of p-Tau/t-Tau ratio did not significantly improve the first model ($p = 0.0595$). Globally, t-Tau protein allowed a further discrimination of 65 % within 14-3-3 inconclusive results. Furthermore, *PRNP* MV genotype showed a trend to decrease 14-3-3 sensitivity ($p = 0.051$), but such effect was not seen on t-Tau protein. In light of these results, we suggest that t-Tau protein assay is of significant importance as a second marker in identifying 14-3-3 false-positive results among sCJD probable cases.

Keywords sCJD · CSF · 14-3-3 protein · Biomarkers · Tau protein · Phosphorylated Tau protein

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Abbreviations

sCJD	Sporadic Creutzfeldt–Jakob
Non-CJD	Non-prion disease
CSF	Cerebrospinal fluid
LP	Lumbar puncture
CNS	Central nervous system
t-Tau	Total Tau protein
p-Tau	Phosphorylated Tau protein
ELISA	Enzyme-linked immunosorbent assay
WB	Western blot
AD	Alzheimer's disease
APOE	Apolipoprotein E gene
PRNP	Prionic protein gene
PrPSc	Scrapie prion protein
EEG	Electroencephalogram
MRI	Magnetic resonance imaging
WHO	World health organization

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RPD   Rapidly progressive dementia
RT-QuIC   Real-time quaking-induced conversion

Introduction

Sporadic Creutzfeldt–Jakob disease (sCJD) is the most common human transmissible spongiform encephalopathy, causing a rapidly progressive neurodegeneration and ultimately leading to the patient's death within months to few years [1]. Like other human spongiform encephalopathies, it is characterized by the accumulation of pathological prion protein (PrPSc) in the central nervous tissue. Recent technical developments, allowing the reliable ultrasensitive detection of PrPSc in body fluids [2, 3], will hopefully provide a much more disease specific test during patient lifetime. However, to the moment, definitive diagnosis of sCJD remains highly dependent on neuropathological examination and immunochemical demonstration of PrPSc in brain tissue [4].

The probable diagnosis of sCJD is based, not only on the clinical features and course of the disease [5], (a rapidly progressive dementia with less than 2-year duration together with at least two of the following symptoms: myoclonus, ataxia, pyramidal or extrapyramidal signs, akinetic mutism, visual, and psychiatric disturbances), but also on electroencephalography (EEG), magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) findings [6]. The heterogeneity of symptoms, especially in early course of the disease, may resemble other irreversible rapidly progressive dementias (RPDs), however, a small proportion of patients may have a potentially treatable condition [7], and, therefore, an accurate and rapid diagnosis of these patients is of paramount importance.

Since its introduction in the WHO diagnostic criteria for sCJD, CSF analysis has become increasingly important in the differential diagnosis of RPDs. Immunodetection of protein 14-3-3 in CSF was originally demonstrated to have a high sensitivity and specificity for sCJD [8, 9]. International collaborative studies [10], as well as single center studies [11–14], have shown that in the appropriate clinical circumstances, a positive 14-3-3 protein detection correlates with clinical diagnosis in 85–94 % of cases. However, this view has been challenged by findings of poor specificity [15, 16], and low sensitivity in autopsy-proven sCJD cases [17]. In addition, it has been shown that the sensitivity of 14-3-3 test is also influenced by disease duration [10, 12, 13] and by the prion molecular sub-type, with a decrease in sensitivity for PrP-type 2 and *PRNP* codon 129 heterozygotes [10, 13, 14, 18].

Another major limitation of the 14-3-3 analysis is the lack of standardization of the assay between laboratories. The analysis of 14-3-3 protein is usually done using Western blotting (WB), which is usually analysed in a qualitative manner, prone to interrater variability. Interpretation of borderline results (weak-positive) is also a subjective issue, with less than half of the patients with weak-positive CSF 14-3-3 results corresponding to sCJD cases, suggesting that the diagnostic utility of weak-positive 14-3-3 results is limited [19]. Moreover, during the last years, a marked increase in 14-3-3 test referrals for sCJD diagnosis in various reference laboratories has been observed [20, 21].

To overcome the limitations of the 14-3-3, other brain-derived proteins have been studied in the CSF of sCJD patients, and total Tau protein (t-Tau) has shown promising results. CSF t-Tau reaches extremely high levels in sCJD, probably reflecting the extent of the neurodegenerative process, and its clinical utility has been previously reported by us [14] and others [11, 12, 22, 23], with sensitivity ranging from 87 to 94 % and specificities of 90–100 %. Interestingly, recently published studies have suggested t-Tau as the single best marker for sCJD [24, 25], particularly in early stage sCJD [26], with increased specificity compared to 14-3-3 [19, 27]. As specificity is highly dependent on the population investigated (group of patients with a defined neurological condition vs. control group of individuals without dementia vs. cohort of mixed pathologies with a clinical suspicion of sCJD), it is very difficult to compare individual studies on t-Tau specificity. In fact, several cut-off levels for CSF t-Tau in sCJD have been proposed, but vary between centers. Therefore, the comparative value between t-Tau and 14-3-3 detection is still an open question, and only limited information on t-Tau in the differential diagnosis of RPD is available [28].

Unlike what happens in Alzheimer's disease (AD), hyperphosphorylation of Tau and formation of neurofibrillary tangles do not occur in sCJD, and so, phosphorylated Tau (p-Tau) alone does not seem to be a useful marker for sCJD. However, the ratio between p-Tau and t-Tau levels (p-Tau/t-Tau) has shown to improve discrimination between AD or other RPDs and sCJD, with lower levels in favour of the latter [14, 24, 29, 30]. Only limited information is available on p-Tau/t-Tau ratio optimal cut-off levels and its comparative value in relation to 14-3-3 or t-Tau alone [31]. Along with the introduction of these alternative CSF markers in laboratories worldwide, questions about the sensitivity, specificity, and added value of these assays, although recognised as extremely useful, have been raising.

In this study, conducted in the framework of the Portuguese Epidemiological Surveillance Program for Human Prion Diseases and of a Joint Programming for Neurodegenerative Diseases (JPND) European project (DEMTEST—biomarker-based diagnosis of rapid progressive dementias—optimisation of diagnostic protocols),

we aim to evaluate the added value of Tau proteins (t-Tau and the p-Tau/t-Tau ratio), next to the conventional sCJD biomarker 14-3-3, in a population of patients with a clinical suspicion of sCJD. In parallel, we also focus on the discriminatory potential between sCJD and non-CJD patients, particularly in cases with a weak-positive 14-3-3 result. The influence of *APOE* and *PRNP* genetical profile on the diagnostic accuracy of the CSF markers is also explored.

Materials and methods

Patient characterization

Patients with a clinical suspicion of CJD, recruited from different hospitals across the country, were included in this study. Their CSF and blood samples were collected as part of their routine clinical diagnosis investigation and sent to our laboratory for the detection of 14-3-3 protein in CSF. Of the 872 samples received between April 2000 and December 2012, 675 were also submitted to t-Tau and p-Tau quantification. Within these, follow-up diagnosis from 382 cases was reported back to the laboratory by the responsible physician. Herein, we only report the results from the patients for whom a confirmatory diagnosis was available: 95 neuropathologically confirmed sporadic CJD (sCJD) and 287 patients proved to have an alternative diagnosis (non-CJD), as this was our inclusion criteria. Therefore, all probable and possible cases as well as the two cases of variant CJD [32] and three of Fatal Familiar Insomnia (Santana et al., personal communication) that have been identified in our population, were excluded from this study.

The diagnosis of definite sCJD was made according to the standard international agreed criteria [6], including post-mortem neuropathological confirmation or brain biopsy. In the non-CJD group, the appropriate diagnosis criteria were used. This group included patients with other neurodegenerative diseases ($n = 181$, 63 %), metabolic encephalopathies ($n = 33$, 11.5 %), CNS inflammatory/infectious diseases ($n = 16$, 5.6 %), psychiatric conditions ($n = 18$, 6.3 %), neoplastic syndromes ($n = 8$, 2.8 %), autoimmune diseases ($n = 8$, 2.8 %), vascular diseases ($n = 6$, 2.1 %), epilepsy ($n = 4$, 1.4 %), and other diseases ($n = 13$, 4.5 %). These clinical probable diagnoses were established by a senior neurologist, considering clinical data review, ancillary investigation, and evolution.

CSF proteins evaluation: 14-3-3, t-Tau, and p-Tau

CSF samples, collected in sterile polypropylene tubes, were centrifuged at 2500 rpm for 5 min, aliquoted and stored at -80 °C until analysis. Immunodetection of protein 14-3-3 in CSF was done as described previously [14]. Briefly, CSF proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and detection was carried out by incubation with mouse anti-14-3-3 beta monoclonal antibody (sc-1657, Santa Cruz Biotechnology, USA) followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin (DakoCytomation, Denmark). Membranes were developed by enhanced chemiluminescence (SuperSignal, West Pico, PIERCE, USA) and imaged on film (Eastman Kodak Company, Rochester, NY, USA). A positive and a weak-positive control (CSF from patients with histopathologically confirmed sporadic CJD with a strong- or a weak-protein 14-3-3 signal, respectively) and a negative control (CSF from a patient without histological evidence of CJD and showing no protein 14-3-3 signal) were included in the run. All samples were tested twice and the result evaluated by three independent observers. In cases of contradictory results or when an agreement between the observers could not be reached, a third test was made to establish a final result. As an external quality control measure, we participated in ring trials for WB 14-3-3 detection, where intervariability across centers was addressed.

CSF t-Tau and p-Tau-181 were measured by commercially available sandwich ELISA kits (Innotest, Innogenetics, Ghent, Belgium), according to the manufacturer instructions, as previously reported [14]. Whenever the readings for t-Tau were above the highest standard used in the calibration curve (1200 pg/mL), CSF sample was appropriately diluted to give readings within the linear part of the calibration curve. All samples were analysed in duplicate, and the p-Tau to t-Tau ratio was calculated. External quality control of the assays was performed under the scope of the Alzheimer's Association Quality Control Program for CSF Biomarkers [33], using trial samples three times a year, including a long-term storage CSF control which has been the same since 2011 and tested several times with reproducible values.

All assays were performed sequentially in a clinical routine setting.

APOE and *PRNP* genotyping

A sub-set of 243 patients was genotyped for *APOE* (59 sCJD and 184 non-CJD) and 196 patients for *PRNP* (62 sCJD and 134 non-CJD). Blood samples were collected into EDTA tubes and DNA was isolated from whole blood using a commercial kit (Roche Diagnostics GmbH, Manheim, Germany), as described by the manufacturer.

The analysis of the two polymorphisms at codons 112 and 158 of the *APOE* gene (rs429358 and rs7412) was determined by polymerase chain reaction–restriction

fragment length polymorphisms (PCR–RFLP) assay, as previously described [34].

The analysis of the PRNP M129Vpolymorphism (rs1799990) was performed by PCR using specific primers (F5′-GAC AGC CTC ATG GTG GTG GC and R5′-CACA TCT GCT CAA CCA CGC GC) followed by restriction NspI digestion. The resulting fragments were separated by electrophoresis and gel interpretation was performed independently by two observers.

Statistical analysis

Statistical analysis was performed using the program Statistical Package for the Social Sciences (SPSS, version 21.0) (IBM SPSS, Chicago, IL) and graphs built using GraphPad Prism 6.0. Normality of continuous variables was assessed by the Kolmogorov–Smirnov test. Differences in demographical, clinical, and biochemical continuous variables between diagnostic groups were examined using either Student's t test (for variables with normal distribution) or the Mann–Whitney U (for variables that did not show normal distribution). Kruskal–Wallis followed by Mann–Whitney U was used for comparing protein levels between PRNP genotypes. χ^2 test was used to assess differences between categorical variables. Logistic regression analysis was used to identify the best sub-set of biomarkers for pairwise discrimination. The chosen method was "enter", diagnostic group (sCJD or non-CJD) was set as the dependent variable, and age, gender, and disease duration were set as independent variables. Tau protein and p-Tau to t-Tau ratio were added one at a time to the first model (just 14-3-3). The first combination of variables resulted in Model 2 and the other in Model 3. Sensitivity, specificity, positive and negative likelihood ratios (LR+/LR−), and overall accuracy were derived from the models. Receiver-operating characteristics (ROC) curve analysis was used to evaluate not only the diagnostic accuracy of the different isolated markers, but also the predicted probabilities derived from the models. Those ROC curves were further compared according to the AUC comparison method of Hanley and McNeil, 1983, using MedCalc (version 13.1) (MedCalc Software, Mariakerke). Optimal cut-off values for each parameter that yielded the highest Youden index [sensitivity + (specificity − 1)] were selected. A decision tree was made using the Chi-squared automatic interaction detection method (CHAID). The maximum tree depth was set to three levels, the significance for splitting nodes and merging categories was set to 0.05, the maximum number of iterations was 100, the minimum number of cases in parent nodes was 10 and for a child node 5, and the minimum change in expected cell frequencies was 0.001. CJD population was targeted as our classification variable (with positive state). We have inserted 14-3-3, t-Tau and p-Tau as classifying variables, and the model decided which biomarker was best to reach a final diagnosis and displayed the number of cases corresponding to CJD diagnosis or other (non-CJD).

Results

Clinical and molecular characterization of the patients

The principal features of all patients for whom a final diagnosis was available are summarized in Table 1. There were small, but significant differences regarding gender distribution and age between the sCJD and the non-CJD group, with 83 % of sCJD patients being older than 60 years at the time of lumbar puncture vs. 65 % of the non-CJD group ($p = 0.03$). Disease duration at the time of lumbar puncture was also significantly shorter in the sCJD group, with 86 % of sCJD patients having a disease duration inferior to 12 months (70 % in the non-CJD group; $p = 0.001$). Most patients had a negative family history of dementia (98 % of sCJD and 92 % of non-CJD patients; $p > 0.05$). We also assessed the prevalence of symptoms in sCJD and non-CJD patients at the time of lumbar puncture (not shown). Dementia was present in all patients, and at least one other symptom was present in all sCJD patients and over 90 % of non-CJD patients. Myoclonus, ataxia, and akinetic mutism were significantly more common in the sCJD group (74, 74 and 41 %, respectively, vs. 35, 26 and 18 % in non-CJD patients, $p < 0.0001$ for all comparisons). Extrapyramidal or pyramidal signs and psychiatric problems were equally found in both groups (55 % in sCJD vs. 49 % in non-CJD and 59 % in sCJD vs. 67 % in non-CJD). Visual problems were the least common of symptoms, showing also no difference between sCJD and non-CJD patients (31 % in sCJD vs. 21 % in non-CJD).

Concerning molecular characteristics, PRNP codon 129 genotyping was available in 196 patients (62 sCJD and 134 non-CJD) (see Table 1). MM genotype was the most prevalent, accounting for half the total number of patients. Although MM genotype frequency was higher in sCJD patients (61 vs. 46 % in non-CJD), there were no significant differences between groups, even when considering homozygous patients altogether (MM + VV) vs. heterozygous (MV). Within the sCJD group, there was a trend for individuals with MM genotype to be older at time of diagnosis and the heterozygous to present longer disease duration ($p = 0.051$; not shown). Information regarding the APOE polymorphism was available in 243 patients (59 sCJD and 184 non-CJD). The distribution of the different APOE genotypes was neither statistically different between the diagnostic groups, nor there were differences regarding

Table 1 Clinical features and principal investigation findings of all patients

	SCJD ($n = 95$)	Non-CJD ($n = 287$)
Gender (M/F)	45/50	163/124
Age, years[a] (min–max)	67.6 ± 9.7* (34–87)	63.8 ± 13.6 (19–91)
Duration disease, months[a] (min–max)	4.7 ± 4.8*** (1–21)	12.2 ± 18.5 (1–120)
ApoE genotype (59 CJD; 184 non-CJD)	ε2ε3—5 (8.5 %)	ε2ε3–18 (10 %)
	ε2ε4—0	ε2ε4–1 (0.5 %)
	ε3ε3—43 (73 %)	ε3ε3—117 (63.5 %)
	ε3ε4—11 (18.5 %)	ε3ε4—42 (23 %)
	ε4ε4—0	ε4ε4—6 (3 %)
PRNP codon 129 genotype (62 CJD; 134 non-CJD)	MM—38 (61 %)	MM—61 (46 %)
	MV—16 (26 %)	MV–51 (38 %)
	VV—8 (13 %)	VV—22 (16 %)
EEG—typical/total (%)	38/67 (57)***	14/170 (8)
MRI—characteristic/total (%)	21/43 (49)***	9/134 (7)
14-3-3 protein (pos/Wp/neg)	68/25/2***	3/83/201
t-Tau (pg/mL)[b]	7417 ± 9801***	276.5 ± 1502
p-Tau (pg/mL)[b]	35.5 ± 24.0**	25.5 ± 25.5
p-Tau/t-Tau (×100)[b]	0.53 ± 2.3***	12 ± 8.4

Pos positive, *Wp* weak-positive, *neg* negative

* $p < 0.05$ vs. non-CJD; ** $p < 0.01$ vs. non-CJD; *** $p < 0.0001$ vs. non-CJD

[a] Data are expressed as mean ± SD

[b] Data are expressed as median ± SD

the frequency of the ε4 allele. The most prevalent form was ε3ε3 (65.8 % of all patients) followed by ε3ε4 (21.8 %). Only 18.5 % of sCJD patients carried one ε4 allele, and there were no ε4 homozygous patients, whereas in the non-CJD group, 26.5 % had at least one ε4 allele, with six homozygous patients.

Results regarding diagnostic tests, other than the 14-3-3 assay, performed in sCJD suspected cases, are also shown in Table 1. An EEG result was available for 58 sCJD and 158 non-CJD patients. The number of positive EEGs in the sCJD group was statistically higher than the number of typical sCJD-EEG results in the non-CJD group, resulting in a sensitivity of 55 % and a specificity of 92 %. Cerebral MRI had been performed in only 35 sCJD and 135 non-CJD patients, showing a rather low sensitivity (40 %), but a very high specificity (93 %).

CSF biomarkers: 14-3-3, t-Tau, and p-Tau

CSF 14-3-3 immunoblot showed a very high sensitivity (98 %), with 93 out of 95 sCJD patients presenting with a signal in the WB. Within 14-3-3 positive sCJD samples, 68 gave a clear positive result (72 %), while 25 were weak-positive signals (26 %). Only 2 sCJD patients had a negative 14-3-3 result: one was a young woman (age 47), with an isolated dementia evolving over a two-year period, with no

investigational features, except for the MRI, suggestive of sCJD and *PRNP* genotype MV, whose final diagnosis was made following brain biopsy [18]; the other was a 73-year-old male, presenting with dementia, extrapyramidal signs, psychiatric problems and a typical sCJD-EEG, 1-year disease duration at time of LP, and also with a *PRNP* MV genotype. In the non-CJD groups, only 3 patients (1 %) showed a clear positive 14-3-3 band, but 83 (29 %) presented with weak-positive signals, resulting in a rather low specificity of 70 %. 14-3-3 positive and weak-positive non-CJD cases, divided by the different diagnostic sub-groups are shown in Table 2. Overall, around 25–40 % of patients with other neurodegenerative, inflammatory/infectious, metabolic, epileptic, or vascular diseases gave a weak/positive signal, while in psychiatric and autoimmune conditions, weak/positive signals were seen in approximately 15 % of patients. In patients with the neoplastic conditions, more than half had a weak-positive 14-3-3 result. The three non-CJD patients that gave a clear 14-3-3 positive signal were all cases of encephalopathies: one patient in post-anoxic state in context of prolonged ill partial complex; one autoimmune, associated with presence of antibodies anti-potassium voltage channels, and the one case of Wernicke encephalopathy. Amongst the group of neurodegenerative disorders, AD was the most prevalent ($n = 58$; 32 %), followed by frontotemporal lobar degeneration (FTLD,

Table 2 Findings for tested protein markers according to the different sub-groups within non-CJD patients

Clinical group, N (% non-CJD)	Age (years)	Dis. duration, (months)	14-3-3 pos/Wp, N (%)	t-Tau pos, N (%)	p-Tau/t-Tau pos, N (%)
Neurodegenerative 181 (63.0 %)	66.6 ± 11.7	15.4 ± 20.2	0/51 (28.2 %)	12 (6.6 %)	12 (6.6 %)
Metabolic 33 (11.5 %)	58.1 ± 12.4	9.6 ± 22.7	1/13 (43.8 %)	3 (9.4 %)	5 (15.6 %)
Inflammatory/infectious 16 (5.6 %)	60.3 ± 15.9	7.2 ± 3.9	0/5 (31.2 %)	0 (0 %)	2 (12.5 %)
Psychiatric 18 (6.3 %)	53.6 ± 17.3	7.2 ± 8.6	0/3 (16.7 %)	0 (0 %)	1 (5.6 %)
Neoplastic 8 (2.8 %)	65.4 ± 11.7	1.9 ± 0.8	0/5 (62.5 %)	0 (0 %)	1 (12.5 %)
Autoimmune 8 (2.8 %)	52.1 ± 23.5	5.3 ± 3.8	1/0 (12.5 %)	1 (12.5 %)	1 (12.5 %)
Vascular 6 (2.1 %)	69.8 ± 9.3	3.0 ± 0.7	0/3 (50 %)	0 (0 %)	1 (25 %)
Epilepsy 4 (1.4 %)	72.5 ± 4.6	4.0 ± 2.2	1/0 (25 %)	1 (25 %)	1 (25 %)
Other diseases 13 (4.5 %)	61.5 ± 14.6	2.7 ± 1.9	0/3 (23.1 %)	2 (15.4 %)	2 (15.4 %)

Age and disease duration were assessed at the time of lumbar puncture and are expressed as mean ± SD

14-3-3 pos/Wp: number of positive and weak-positive cases out of totals

t-Tau pos: >1039 pg/mL

p-Tau/t-Tau pos: <4.36

$n = 33$; 18 %); vascular dementia ($n = 23$; 13 %); Lewy body dementia ($n = 20$; 11 %); Parkinson's disease ($n = 9$; 5 %), mixed dementia ($n = 7$; 4 %); corticobasal degeneration ($n = 7$; 4 %); multiple system atrophy ($n = 6$; 3 %); progressive supranuclear palsy ($n = 4$; 2 %), and other neurodegenerative dementias ($n = 14$; 8 %). In all these neurodegenerative disorders sub-groups, 20–40 % of patients had a weak-positive 14-3-3 result (not shown), except for the 4 patients with progressive supranuclear palsy, that were all negative for 14-3-3.

CSF concentrations of t-Tau and p-Tau, as well as the p-Tau/t-Tau ratio for both sCJD and non-CJD patients are shown in Table 1 and in Fig. 1. CSF t-Tau was, as we had previously reported [14], markedly increased in sCJD patients ($p < 0.0001$), whereas CSF p-Tau levels were only slightly increased ($p = 0.012$). These differences in CSF t-Tau and p-Tau resulted in a marked decrease in the p-Tau/t-Tau ratio in the sCJD group ($p < 0.0001$). Single marker ROC curve analysis depicted that t-Tau alone (cut-off = 1039 pg/mL) could distinguish sCJD from non-CJD patients with a sensitivity of 95 % and a specificity of 94 %, with an AUC of 0.983 (95 % CI 0.972–0.994; $p < 0.001$). p-Tau did not reach enough discrimination power, as the AUC from its ROC curve was below 0.8 and for that reason was next discarded for the regression analysis. For the p-Tau/t-Tau ratio (cutoff = 0.0436),

sensitivity was 96 % and specificity 91 %, with and AUC of 0.972 (95 % CI 0.950–0.994; $p < 0.001$), not statistically different from the ROC curve obtained with t-Tau ($p = 0.203$). Although sensitivity was slightly lower as compared with the 14-3-3 immunoblot, an increase in specificity was found for Tau proteins. This increase in specificity was seen across almost all the different diagnostic sub-groups within non-CJD patients (Table 2), with less than 10 % of patients with other neurodegenerative and metabolic diseases testing positive for t-Tau, and none in the inflammatory/infectious, psychiatric, neoplastic, and vascular conditions groups. No increase in specificity was seen in the small group of patients with autoimmune or epileptic encephalopathies, with the two patients that were clear positive for 14-3-3 also testing positive for t-Tau and p-Tau/t-Tau ratio.

Added value of combining CSF markers

To assess the value of adding CSF Tau proteins to 14-3-3 in the diagnostic workout of patients with a clinical suspicion of sCJD, we performed the multiple logistic regression analysis with diagnosis (sCJD vs. non-CJD) as the dependent variable and age, gender, duration of disease, and either CSF 14-3-3 alone or with t-Tau and p-Tau/t-Tau ratio as independent categorical variables (Table 3). The model
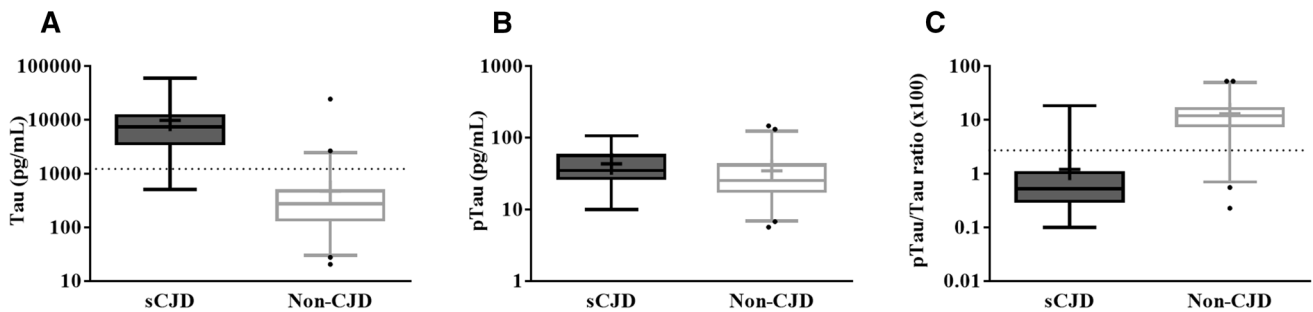
Fig. 1 *Box plots* of CSF: **a** t-Tau, **b** p-Tau, and **c** p-Tau 181/t-Tau (×100) ratio in sCJD and non-CJD patients. Plots show 10th, 25th, 50th, 75th, and 90th percentiles and outliers. The *dotted line* represents the optimal cut-off levels between sCJD with non-CJD patients, as determined by the ROC curve analysis for: **a** Tau >1039 pg/mL and **c** p-Tau/t-Tau ratio ×100 < 4.36. ***p < 0.0001 vs. non-CJD. Y axis displayed in log 10 scale

Table 3 Logistic regression models for distinction between sCJD and non-CJD patients

Variables included	Sens (%)	Spec (%)	AUC (95 % CI)	OA (%)	LR(+)	LR(−)
All cases (n = 354)						
Model 1	96.7	75.6	0.882 (0.845–0.918)	81.9	3.96	0.04
Model 2	94.5	94.6	0.966 (0.946–0.985)***	94.3	17.5	0.06
Model 3	96.7	93.0	0.961 (0.935–0.987)***	94.0	13.8	0.04
14-3-3 pos and neg (n = 255)						
Model 1	97.0	98.4	0.973 (0.943–1.000)	98.0	60.6	0.03
Model 2	98.5	96.2	0.990 (0.977–1.000)	98.0	25.9	0.02
Model 3	97.0	98.4	0.979 (0.950–1.000)	98.0	60.6	0.03
14-3-3 Wp and neg (n = 286)						
Model 1	92.6	74.1	0.832 (0.758–0.906)	90.6	3.58	0.1
Model 2	92.6	93.3	0.948 (0.908–0.988)*	94.3	13.8	0.08
Model 3	92.6	94.1	0.936 (0.868–1.000)	93.6	15.7	0.08

Model 1: 14-3-3 only; Model 2: 14-3-3 + Tau; and Model 3: 14-3-3 + p-Tau/t-Tau ratio. LR(+) = positive likelihood ratio (sensitivity − (1 − specificity)); LR(−) = negative likelihood ratio ((1 − sensitivity)/specificity). AUC = area under the curve, determined by the ROC analysis of the predicted probability determined by the models. All regression models included age, duration of disease, and gender as covariates

* p < 0.05 vs. model, including just the 14-3-3 assay; *** p < 0.0001 vs. model, including just the 14-3-3 assay

that included 14-3-3 (both positive and weak-positive results considered as positive) as the only CSF marker, presented a sensitivity of 97 % and specificity of 76 % with an overall accuracy of 82 %, a LR+ of 3.96 and a LR− of 0.04. Adding CSF t-Tau to the model (positive if >1039 pg/mL), slightly decreased sensitivity to 95 %, but significantly increased specificity also to 94.6 %, LR+ to 17.5, LR− to 0.06 and overall accuracy to 94 % (p < 0.0001). The logistic regression model obtained by addition of p-Tau/t-Tau ratio (positive if <0.0436) was also statistically different from the one containing only 14-3-3 maintaining sensitivity in 97 % and improving overall accuracy to 94 % and specificity to 93 % (p < 0.0001). However, it was not better than t-Tau as a second biomarker.

To further explore the added value of Tau in relation to the 14-3-3 protein assay, we repeated the above analysis, but using either only 14-3-3 clear positive vs. negative cases or 14-3-3 weak-positive vs. negative cases. When only 14-3-3 clear positive and negative cases were selected, the first model (including only age, gender duration of disease, and 14-3-3), reached an overall accuracy of 98 %, with optimum sensitivity and specificity levels of 97 % and 98 %, respectively, an LR+ of 61.0 and an LR− of 0.03. Adding just t-Tau or p-Tau/t-Tau ratio to this model did not change significantly any of the diagnostic accuracy parameters (see Table 3; p > 0.05 for all comparisons between the models). However, when we considered only 14-3-3 weak-positive and negative cases in the logistic regression analysis, we observed that the first model could be significantly improved by the addition of only t-Tau, that increased specificity from 74 to 93 % (p < 0.05). Addition of p-Tau/t-Tau ratio improved specificity from 74

to 94 % and overall accuracy from 91 to 94 %, although not significantly ($p = 0.0595$) and had no impact on sensitivity.

Subsequently, the three CSF assays (14-3-3, t-Tau, and p-Tau/t-Tau ratio) were entered in a decision tree model to discriminate between the two diagnostic groups (sCJD vs. non-CJD). As the 14-3-3 assay is the only CSF marker currently included by the WHO in the diagnostic criteria for sCJD, we initially decided to force 14-3-3 protein as the first variable of the model. As shown in Fig. 2a, the final decision model retained only CSF 14-3-3 and t-Tau. In a first step, the population was divided into three groups according to the 14-3-3 assay, with the group with a negative 14-3-3 result retaining 70 % of non-CJD patients (201/287) and just 2 sCJD patients (2 %). On the other hand, the group with a positive 14-3-3 result retained 72 % of sCJD patients (68/95) and only 3 non-CJD patients (1 %). The group with a weak-positive 14-3-3 result (including 108 subjects; 28 % of the study population) was then further divided into three sub-groups, according to the t-Tau levels, with the sub-group with the lowest t-Tau levels accounting for 52 of the remaining 83 non-CJD patients, and the group with the highest t-Tau levels retaining 18 of the remaining 25 sCJD patients. The sub-group with intermediate t-Tau levels (38 subjects) remained 31 non-CJD patients and 7 sCJD subjects, without a conclusive result. Overall, this decision tree model correctly classified 89 % of the study population, including 253 non-CJD (88 %) and 86 sCJD patients (91 %).

When 14-3-3 protein was not forced as the first variable in the decision tree model (Fig. 2b), this resulted in a model with a higher overall diagnostic accuracy (96 %) that classified correctly 285 non-CJD (99 %) and 83 sCJD patients (87 %). However, as a first variable, the model chose t-Tau, dividing the population into three groups: low t-Tau (<901 pg/mL), that included 92 % of the non-CJD patients (265/287) and only 5 sCJD patients (5 %); intermediate t-Tau ($901 \leq$ t-Tau ≤ 2983), that retained 38 subjects and was further divided according to the 14-3-3 assay as positive (10 sCJD and 1 non-CJD) or weak-positive + negative (20 non-CJD and 7 sCJD); and high t-Tau (>2983 pg/mL), that retained the remaining 73 sCJD patients (77 %) and only 1 non-CJD individuals (0.3 %).

Influence of age and disease duration on protein markers

Age at the time of diagnosis (<60 vs. $\geq$60 years) did not influence the sensitivity of either of the CSF markers tested for sCJD patients (Fig. 3a), nor it had any effect on the mean values of t-Tau, p-Tau, or the p-Tau/t-Tau ratio (not shown; $p > 0.05$ for all comparisons). When we divided sCJD patients according to disease duration at the time of lumbar

Fig. 2 Decision tree models using CSF 14-3-3 and t-Tau to ▶ discriminate between sCJD and non-CJD patients, forcing or not 14-3-3 as the first variable in the model. sCJD is targeted as state variable. Correctly classified patients are depicted in *bold*. **a** 14-3-3 is forced as first variable and Tau is added by the model only to weak-positive cases. **b** Without forcing any variable, the decision model sets Tau as classifying variable and further adds 14-3-3 to discriminate within those cases with intermediate Tau levels
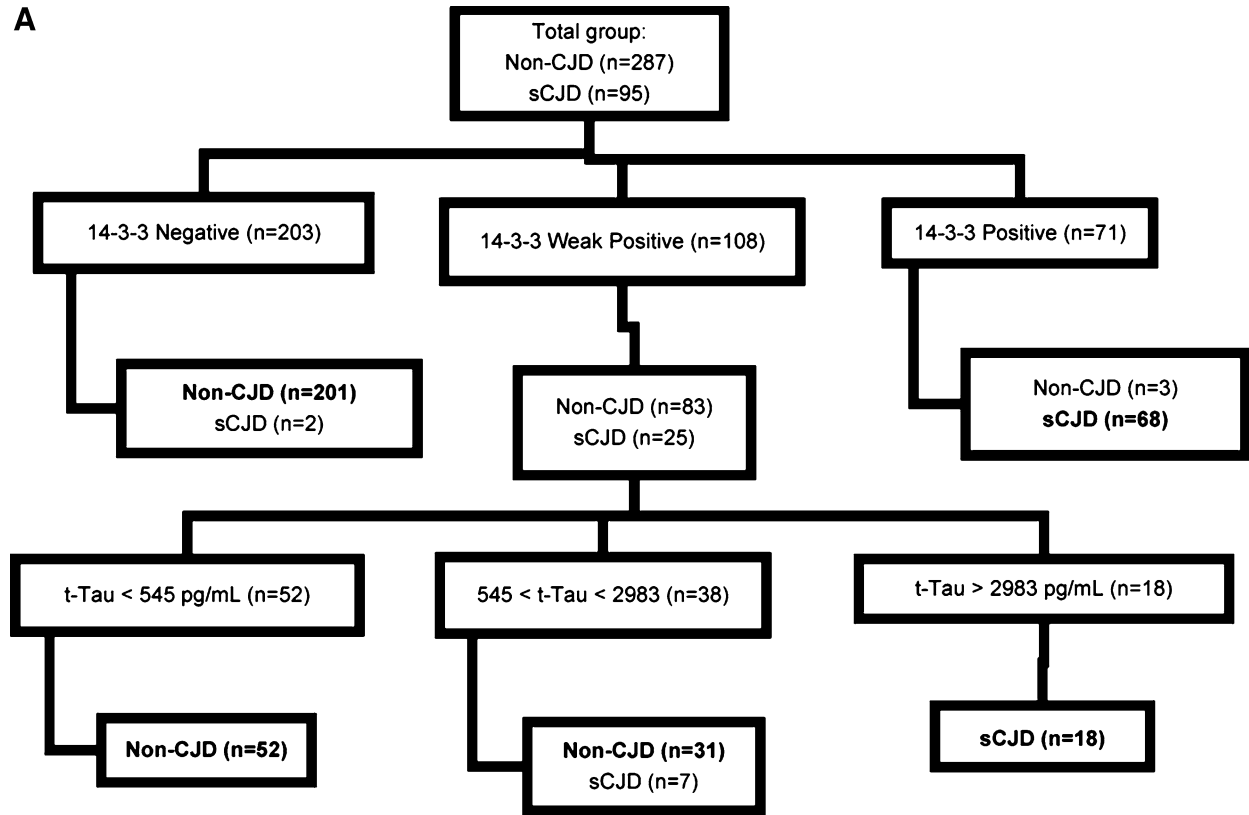
puncture (<12 vs. $\geq$12 months), a CSF 14-3-3 protein positive test in sCJD patients was more frequently associated with shorter disease duration ($p < 0.0001$; Fig. 3b). Likewise, t-Tau values were higher (<12 M = 10,307 ± 10,035 pg/mL; $\geq$12M = 6654 ± 12,298 pg/mL; $p = 0.027$) and the p-Tau/t-Tau ratio was lower (<12 M = 0.011 ± 0.023; $\geq$12 M = 0.022 ± 0.018; $p = 0.034$) in sCJD patients with shorter disease duration of disease, although no influence in overall sensitivity of these markers was observed. Age and duration of disease did not influence the levels of any of the protein markers in the non-CJD group (data not shown).

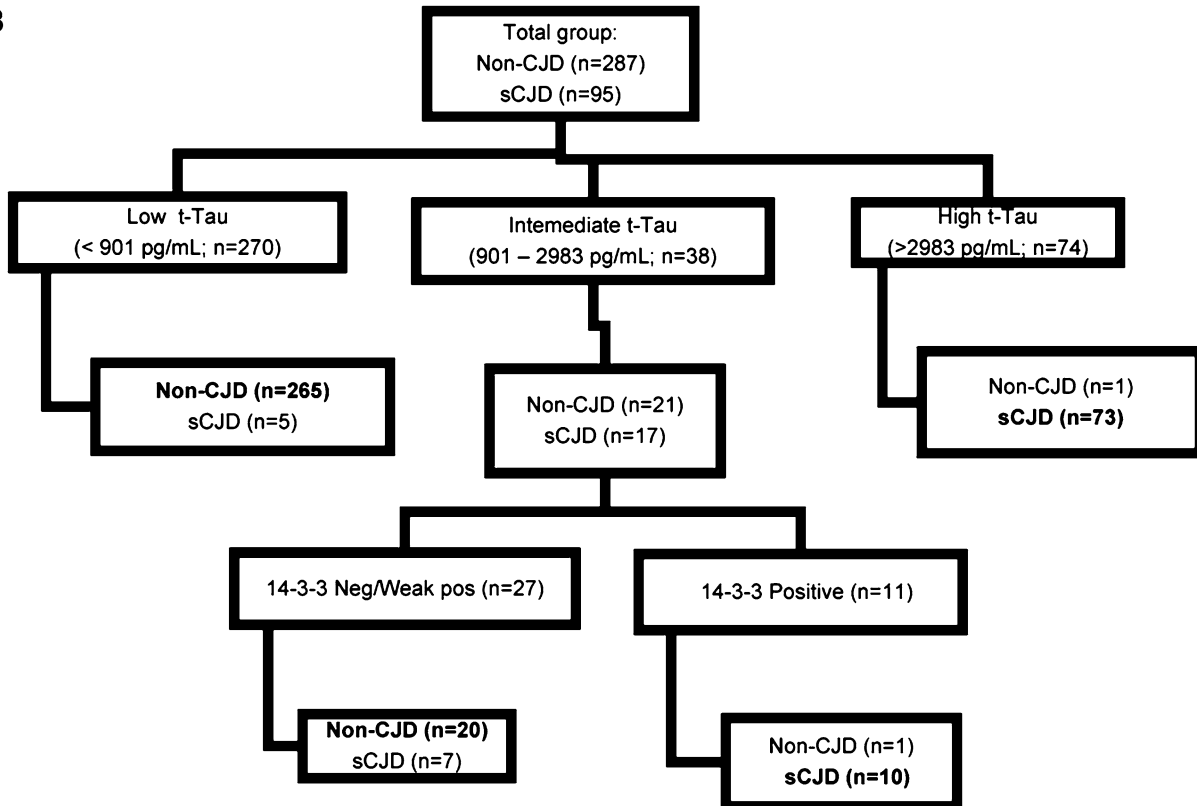## Influence of *APOE* and *PRNP* genotypes on protein markers

We looked for an influence of the *APOE* and *PRNP* genotype on the levels of the protein markers assessed (data not shown). This analysis is obviously limited by the very low frequency of some of the genotypes. We did not find any influence of the *APOE* genotype on the levels of t-Tau and p-Tau in any of the diagnostic groups. Concerning *PRNP* genotype, homozygoty for valine was associated with the highest t-Tau levels in sCJD patients (21,392 ± 18,240 pg/mL vs. 6368 ± 7361 pg/mL in MV, $p < 0.01$), and heterozygous with the lowest (vs. MM 10,102 ± 8567 pg/mL $p < 0.05$). The same effect of the valine allele was seen for p-Tau values, with the VV subjects having the highest levels (72 ± 22.5 pg/mL vs. MV 38.3 ± 16.5 and MM 35 ± 18 pg/mL, $p = 0.001$), but not for the p-Tau/t-Tau ratio, although MV genotype still presented significantly higher ratios than the MM group (1.6 ± 1.6 vs. MV 0.53 ± 0.45 and MM 0.91 ± 1.8, $p = 0.032$). Among the non-CJD group, *PRNP* genotype only influenced p-Tau/t-Tau ratio, where VV patients had the lowest ratios facing others (0.106 ± 0.103 vs. MV 0.14 ± 0.07 and MM 0.13 ± 0.06, $p = 0.023$).

In the sCJD group, we also examined the influence of *PRNP* codon 129 polymorphism on the sensitivity of the different markers. A positive 14-3-3 result was found in all homozygotes (MM and VV), while the sensitivity of this test was tendentiously lower for the MV genotype ($p = 0.05$, Fig. 3c). Neither for t-Tau or p-Tau/t-Tau ratio was the sensitivity influenced by the *PRNP* codon 129 polymorphism ($p > 0.05$; Fig. 3c).
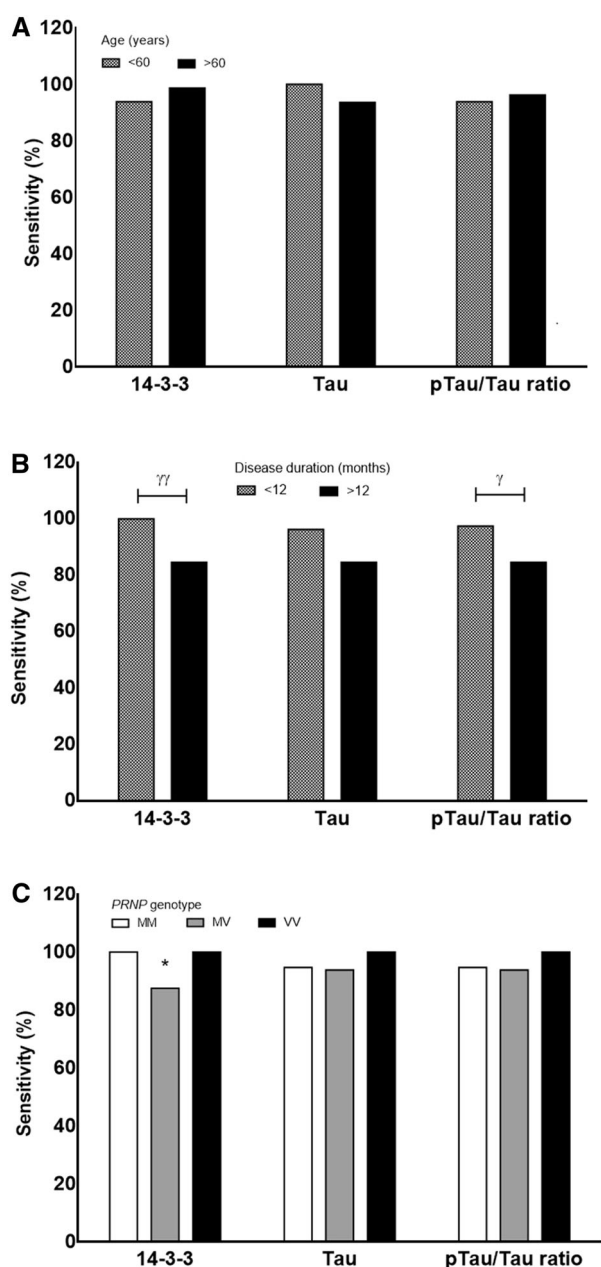
A

Total group:
Non-CJD (n=287)
sCJD (n=95)

14-3-3 Negative (n=203)

Non-CJD (n=201)
sCJD (n=2)

14-3-3 Weak Positive (n=108)

Non-CJD (n=83)
sCJD (n=25)

14-3-3 Positive (n=71)

Non-CJD (n=3)
sCJD (n=68)

t-Tau < 545 pg/mL (n=52)

Non-CJD (n=52)

545 < t-Tau < 2983 (n=38)

Non-CJD (n=31)
sCJD (n=7)

t-Tau > 2983 pg/mL (n=18)

sCJD (n=18)

B

Total group:
Non-CJD (n=287)
sCJD (n=95)

Low t-Tau
(< 901 pg/mL; n=270)

Non-CJD (n=265)
sCJD (n=5)

Intemediate t-Tau
(901 – 2983 pg/mL; n=38)

Non-CJD (n=21)
sCJD (n=17)

High t-Tau
(>2983 pg/mL; n=74)

Non-CJD (n=1)
sCJD (n=73)

14-3-3 Neg/Weak pos (n=27)

Non-CJD (n=20)
sCJD (n=7)

14-3-3 Positive (n=11)

Non-CJD (n=1)
sCJD (n=10)

Fig. 3 Effect of age, disease duration, *PRNP* codon 129, and *APOE* genotype in the sensitivity of CSF markers for sCJD. Age and disease duration were assessed at the time of lumbar puncture. 14-3-3(+): all bands considered; t-Tau (+): >1039 pg/mL; p-Tau/t-Tau (+): <4.36. *$p = 0.05$ vs. MM/VV; $^{\gamma}p < 0.05$ vs. >12 months disease duration; $^{\gamma\gamma}p < 0.0001$ vs. >12 months disease duration

Discussion

In this work, we assessed the added value of t-Tau and the p-Tau/t-Tau ratio, on the diagnostic accuracy of patients with a clinical suspicion of sCJD. Overall, our results showed that Tau protein levels and p-Tau/t-Tau ratio add diagnostic value to 14-3-3 results, particularly in weak-positive cases, where 65 % of the cases could be further

clarified. Besides, *PRNP* and *APOE* genotype showed no impact on Tau sensitivity, contrary to 14-3-3.

Our study group consisted of 382 patients: 95 definite sCJD patients, presenting with a rapidly progressive dementia, associated in most cases with myoclonus and ataxia; and a control group of 287 patients, with a clinical suspicion of sCJD, but in whom an alternative diagnosis was reached (non-CJD). All non-CJD patients presented with dementia, and in most cases, another symptom (most often psychiatric problems), could be found. In accordance to what has been reported by others [7, 20, 35], roughly two-thirds of non-CJD patients suffered from non-reversible disorders, with AD being the single most prevalent group, and one-third had a potentially treatable disease. Our study group was selected amongst a total of 872 patients that were referred to our laboratory for CSF 14-3-3 determination between 2000 and 2012 and for whom a final diagnosis was reached. Clearly, the percentage of patients that had an available confirmatory diagnosis is low (44 % of all samples received), and this is a limitation of this study. In addition, the low autopsy rate, in relation to the total number of samples that tested positive or weak-positive for 14-3-3, restricted the number of samples included and probably introduced some bias to the study. In fact, from all patients that had a clear positive 14-3-3 result during this timeframe ($n = 147$), only 68 (46 %) were submitted to autopsy and could be included in this study, as we decided to include only definite sCJD patients, whereas all patients that remained with a clinical diagnosis of probable or possible sCJD were excluded from the analysis. For the non-CJD group, neuropathological confirmation of clinical diagnosis was not mandatory, allowing the inclusion of a larger number of non-CJD patients. This resulted in an over-representation of the non-CJD group in comparison with the sCJD group, which is also a statistical limitation of this study. This unbalance is also related to the increasing number of referrals that we and other reference laboratories for human prion diseases, have been experiencing over the years [20, 21], resulting in higher number of CSF samples tested that do not fulfil the WHO diagnostic criteria for possible sCJD, and, therefore, should not be tested in the first place.

From the commonly employed routine laboratory investigations for the diagnosis of sCJD (EEG, CSF analysis, and MRI), CSF 14-3-3 protein immunodetection was the most sensitive (98 %), followed by EEG (55 %) and MRI (40 %). This is in agreement with data from international collaborative studies [10, 13], and with the majority of the literature reporting the sensitivity of the 14-3-3 test [7, 11, 12]. However, the specificity of the 14-3-3 assay, in our population, was clearly sub-optimal (70 %), lower than the reported in some previous studies [11–13, 20], but similar to recent prospective data from the

UK and Canada [19, 36]. These data are very similar to what we had previously reported in a much smaller population (30 sCJD vs. 40 non-CJD patients) [14]. The poor specificity of the 14-3-3 assay could be related to several factors: the wide range of pathologies included in our control population that can lead to rapid and massive neuronal destruction; the tendency for a non-discriminatory application of the 14-3-3 assay as screening test for RPD in our country; and to the lack of standardization in the definition of positive results. The concern of declining specificity due to higher number of CSF 14-3-3 tests performed in various neurological conditions has been recently explored in a large multicenter longitudinal study [20], and this did not seem to be the case, with protein 14-3-3 test specificity remaining high and stable in the diagnosis of CJD during 10 years across centers. However, test specificity varied with respect to differential diagnosis and in line with our own study, a tendency for the 14-3-3 test to become more often false positive in acute neurological events (CNS tumors and encephalopathies) than in neurodegenerative and non-neurological forms of dementia was observed. The issue of standardization is an inherent limitation of the qualitative nature of the WB method used in the 14-3-3 assay, and as no standard approach exists, some authors consider all bands [11], while others only consider strong bands as positive [13, 19, 20]. When we reanalyze our data, considering only clear 14-3-3 bands as positive, an increase in specificity to 99 %, and with a decrease in sensitivity to 71 %, is observed. To avoid person-to-person bias of visual inspection and the difficult interpretation of weak bands, digital analysis of the 14-3-3 immunoblots with inclusion of recombinant 14-3-3 protein as standard has been employed by others [36, 37]. Also to overcome the difficulties of the WB method, several quantitative methods, such as ELISA and protein capture assays, have been developed [38, 39], resulting in a similar specificity, but also a lower sensitivity. Until now, such assays have not been used in clinical routine setting for various reasons: sub-optimal results, optimal conditions for the test unknown, comparative value not determined. Recently, a commercial ELISA kit for the gamma isoform of 14-3-3 protein has been made available [40] and has been validated in a large population of sCJD and non-CJD patients against the conventional WB method [41]. In our recently published work, we demonstrated, in a smaller cohort of definite sCJD and non-CJD, that this quantitative 14-3-3 ELISA is extremely promising, since it alone correctly classified 93 % of cases [42].

t-Tau assay, on the contrary, proved to be extremely specific and only slightly less sensitive than the 14-3-3 assay for the differentiation between sCJD and non-CJD patients, as previously reported [11, 12, 19, 25, 36]. In the BIOMARKAPD European Project, many efforts were made in the last years to standardize t-Tau and p-Tau ELISA assays and improve pre-analytical handling practices [43–45]. Those trials made possible their broad use in biochemical diagnosis with high utility during the last few years. However, recent data from a large multicenter study [20] did not find t-Tau specificity overall increased in relation to 14-3-3, but only when considering certain differential diagnostic groups, as potentially treatable inflammatory diseases. In our study, t-Tau higher specificity (94 %) was observed for the comparison between sCJD patients and almost all the differential diagnostic groups included in the non-CJD patients, even in patients that are known to suffer from taupathies, such as AD and FTLD, associated with modest increases in CSF t-Tau levels [46, 47]. It is unclear why CSF Tau protein should be of greater specificity than 14-3-3, as the factors influencing the release of neuronal proteins in sCJD and other conditions are not fully understood. Moreover, while the sensitivity of 14-3-3 seemed to be influenced by the duration of disease at the time of lumbar puncture, as previously reported [10, 12–14, 19], t-Tau did not. Conflicting results exist regarding this issue, with some studies failing to find any influence of age and/or disease duration on t-Tau sensitivity [12, 14, 22], but other doing so [13, 19]. Another small study showed that t-Tau could also be a useful marker for sCJD in serum samples [48]. In a recent report, it was observed that in CJD individuals, t-Tau levels and t-Tau to p-Tau ratios increased over time, and the combination of increased t-Tau levels and increased t-Tau to p-Tau ratios in CJD patients has a very high specificity against important differential diagnoses [49].

In fact, a slight increase in CSF p-Tau levels in the sCJD group was observed in relation to the total non-CJD group. When we compared the p-Tau levels in sCJD patients, specifically with differential diagnostic groups associated with neurofibrillary pathology, namely AD and FTLD, no differences could be found (not shown). The reason for this small increase in p-Tau in the CSF of sCJD patients is not completely understood, as Tau hyperphosphorylation and neurofibrillary tangles are usually absent in sCJD. By contrast, the rapid neurodegenerative process, including axonal degeneration, in sCJD leads to an excessive liberation of Tau proteins, which causes the low p-Tau/t-Tau ratio. In Llorens et al. [50], p-Tau/t-Tau ratio discriminating power was higher in the differential diagnosis of sCJD when compared with other dementias than Tau alone. In our study, the combination of t-Tau with p-Tau in the p-Tau/t-Tau ratio did not improve diagnostic accuracy in relation to t-Tau alone, but resulted in a similar improvement in specificity and overall accuracy in relation to 14-3-3 ($p < 0.0001$), when considering all cases. This result is in line with a very recent study, reporting that the combined use of the 14-3-3 protein assay, t-Tau levels, and p-Tau/t-

Tau ratio improved the specificity of diagnosis compared with the use of the 14-3-3 protein assay alone (47 % for 14-3-3 alone; 86 % for 14-3-3 combined with t-Tau; and 91 % for 14-3-3 combined with the p-Tau/t-Tau ratio) [31]. However, in the same study, very few patients had autopsy-proven diagnosis, in contrast with our study, in which all sCJD patients had neuropathological confirmation. Besides, in our study, we have taken a new approach regarding the analysis of results, where we specifically addressed the 14-3-3 weak-positives (inconclusive) results in terms of biomarker's sensitivity and genotype influence.

The results from our logistic regression models show that the addition of t-Tau to 14-3-3 is significantly useful in the differentiation between sCJD and non-CJD patients ($p < 0.0001$), but further inclusion of p-Tau does not bring any additional discriminatory accuracy to the 14-3-3 model. This added value is particularly seen in terms of the specificity of the model and of the LR+, but not of sensitivity, which is already optimal for the model that only includes 14-3-3. In fact, the two sCJD patients that had a negative 14-3-3 result also tested negative for t-Tau and for the p-Tau/t-Tau ratio. Furthermore, when we isolated 14-3-3 positive from weak-positive cases, we could see that t-Tau added value in the specificity and in the LR+ was only seen in the weak-positive cases. Again, even just for weak-positive cases, addition of p-Tau/t-Tau ratio to 14-3-3 had no impact on sensitivity but specificity and overall accuracy improved although not significantly ($p = 0.0595$). Worth mentioning, the only three cases of non-CJD patients that had a clear positive 14-3-3 result also tested positive for t-Tau and p-Tau/t-Tau ratio.

These results were then corroborated by decision tree models, where in the model that forced 14-3-3 as the first variable, t-Tau was only useful in reducing the uncertainty of 14-3-3 weak-positive cases. From 108 patients with a weak-positive 14-3-3 test (28 % of the study population), the addition of t-Tau cleared the results of 70 patients (that were either positive or negative for t-Tau), leaving only 38 patients (10 % of the study cohort; 31 non-CJD and 7 sCJD patients) with an inconclusive final classification. Interestingly, when no variables were forced into the model, t-Tau was selected as the first decision variable, that alone only left 38 patients (10 % of the population) with intermediate results. This small group was then separated according to their 14-3-3 result, with weak-positive results treated as negative, and leaving no patients with an inconclusive result. Regardless of this experimental result, 14-3-3 is thus far still the only CSF marker included in the international guidelines for CJD. Our main focus still relies on demonstrating that t-Tau can be of added value in clinical routine diagnosis (especially on weak-positive cases) rather than testing its replacement feasibility.

Noteworthy, when we compared the AUC of the ROC curve obtained with t-Tau alone (0.983; 95 % CI 0.972–0.994) with either the AUCs of the ROC curves derived from the logistic regression model that included 14-3-3 combined with t-Tau or 14-3-3 combined with p-Tau/t-Tau ratio, no differences were seen ($p = 0.202$ and $p = 0.3306$, respectively). This further indicates that t-Tau alone is a very good CSF marker for sCJD, and is not inferior to a combination of markers, as had been suggested by Hamlin and colleagues [25]. This is also in line with a 6-year prospective study for sCJD in Canada, where the authors did not observe any evidence for diagnostic value of 14-3-3 beyond that available from Tau [36]. Given the current environment of enormous pressure for economical restraint, one has to consider the cost/benefit relation when faced the decision of choosing between the CSF markers (14-3-3 or Tau proteins). Logistic regression analysis and comparison of combined models performed in this study show that the input of p-Tau protein as a third marker is not relevant (even in weak-positives only) facing the effect of t-Tau combined with 14-3-3, which already maximize diagnostic accuracy. With this in mind, our second decision model (combining 14-3-3 with t-Tau) would be preferable, as it would only require double marker testing in a much lower number of individuals, despite the fact that it happens at the expenses of specificity and not the sensitivity, which remains stable.

Moreover, due to the nature of the methodology employed in the t-Tau assay, a much larger number of samples can be tested in the same period of time than with the 14-3-3 WB assay and potentially reducing the diagnosis timeframe for weak-positive cases.

Regarding the molecular characteristics of the sCJD group, the frequency of the various *PRNP* genotypes was in accordance to the frequency distributions generally reported in the literature [51, 52]. *APOE* genotyping was performed in a sub-set of the population, showing no differences in the distribution of the different genotypes between the two diagnostic groups. *PRNP* codon 129 polymorphism is known to influence the sensitivity of CSF protein 14-3-3 testing for sCJD. Overall, 14-3-3 was the most influenced marker by the patients characteristics, showing lower sensitivity for sCJD patients with longer disease duration ($\geq$12 M), ($p < 0.0001$), and heterozygous for *PRNP* codon 129 ($p = 0.05$). On the contrary, the sensitivity of t-Tau towards sCJD remained unchanged by these patient characteristics. Despite the fact that the relatively small number of patients might limit the conclusions taken from this analysis, these are in general agreement with the results from large population studies [50, 53]. This difference in the protein markers sensitivity between sCJD sub-types might be accounted by the modulation of clinical phenotype by molecular characteristics. A host genotype

effect has been reported, with codon 129 heterozygosity increasing the duration of illness, [52]. In fact, in our study, MV patients presented a longer disease course, when compared with the other genotypes ($p < 0.05$ vs. VV and MM).

In summary, this study has shown that a combination of tests clearly improved sensitivity and specificity of sCJD diagnosis to optimal levels. Therefore, we propose that, besides 14-3-3 protein detection, CSF t-Tau should also be used in clinical practice. This would be specially advised in cases, where 14-3-3 gives a weak-positive result, or in cases with an atypical presentation, like patients with long disease duration and MV heterozygous for the *PRNP* codon 129. True *pre-mortem* laboratory diagnosis of human prion diseases may eventually be achieved with new approaches based for example on PrPSc [2, 3]. In the meantime, optimized application of known surrogate markers of neurodegeneration will continue to be at the forefront of sCJD diagnosis. In this context, our study indicates that in subjects, where a 14-3-3 result is already available, further assessment of Tau proteins will only be of added value in case of a weak-positive result. Nevertheless, additional studies exploring larger populations also with definite sCJD diagnosis are required to reproduce our current findings.

Compliance with ethical standards

Ethical standards The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. This study was approved by the Ethics Board of Coimbra University Hospital and all subjects or responsible caregivers, whichever appropriate, gave their informed consent.

Conflicts of interest The authors declare that they have no conflict of interest.

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