Original research

Evaluation of the impact of CSF prion RT-QuIC and amended criteria on the clinical diagnosis of Creutzfeldt-Jakob disease: a 10-year study in Italy

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ABSTRACT

Background The introduction of the prion Real-Time Quaking-Induced Conversion assay (RT-QuIC) has led to a revision of the diagnostic criteria for sporadic Creutzfeldt-Jakob disease (sCJD).
Validation studies are needed for the amended criteria, especially for their diagnostic value in the clinical setting.

Methods We studied 1250 patients with suspected CJD referred for diagnosis to two Italian reference centres between 2010 and 2020. Focusing on the first diagnostic assessment, we compared the diagnostic value of the old and the amended criteria and that of different combinations of clinical variables and biomarker results.

Results The studied cohort comprised 850 participants with CJD (297 definite sCJD, 151 genetic CJD, 402 probable sCJD) and 400 with non-CJD (61 with neuropathology). At first clinical evaluation, the sensitivity of the old criteria (76.8%) was significantly lower than that of the amended criteria (97.8%) in the definite CJD cohort with no difference between definite and probable sCJD cases. Specificity was ~94% for both criteria against the non-CJD cohort (82.0% against definite non-CJD group). Cerebrospinal fluid (CSF) RT-QuIC was highly sensitive (93.9%) and fully specific against definite non-CJD patients. Limiting the criteria to a positive RT-QuIC or/and the combination of a clinical course compatible with possible CJD with a positive MRI (Q-CM criteria) provided higher diagnostic accuracy than both the old and amended criteria, overcoming the suboptimal specificity of ancillary test results (ie, CSF protein 14-3-3).

Conclusions CSF RT-QuIC is highly sensitive and specific for diagnosing CJD in vitam. The Q-CM criteria provide a high diagnostic value for CJD.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The introduction of prion real-time quaking-induced conversion assay (RT-QuIC) to the diagnostic criteria for sporadic Creutzfeldt-Jakob disease (CJD) has enhanced epidemiological surveillance. However, less is known about the impact of amended criteria on the diagnostic accuracy in the clinical setting.

WHAT THIS STUDY ADDS

⇒ Focusing on the first diagnostic assessment, our study shows that the cerebrospinal fluid (CSF) prion RT-QuIC alone and the amended criteria significantly improve accuracy compared with previous criteria. The combination of CSF RT-QuIC and the association of clinical variables with brain MRI provides an additional diagnostic value.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ A wider diffusion of CSF RT-QuIC, and its combination with brain MRI, would significantly improve diagnostic accuracy for sporadic CJD. Limiting the use of surrogate CSF biomarkers to initial screening would reduce false-positive clinical diagnosis.

INTRODUCTION

Creutzfeldt-Jakob disease (CJD), the most common human prion disease, is a neurodegenerative disorder caused by brain accumulation of a misfolded form (PrP^{Sc}) of the cellular prion protein. CJD comprises a prevalent sporadic form (sCJD) of unknown aetiology, a genetic form (gCJD) linked to mutations in the prion protein gene (*PRNP*) and an acquired form caused by prion transmission mainly through medical procedures.[1 2] CJD manifests as a heterogeneous, rapidly progressive neurological syndrome characterised by a variable combination of dementia, motor signs, visual disturbances and myoclonus.[3] Current sCJD classification recognises six major subtypes with distinctive clinicopathological features, mainly defined by the patient genotype at *PRNP* polymorphic codon 129 (methionine, M; valine, V) and two PrP^{Sc} types (types 1 and 2) with different sizes of their proteinase-resistant core (eg, MM1, VV2, VV1).[4 5] Notably, this classification also applies to the prevalent gCJD subtypes.[6]

The early discrimination of CJD patients from those with other rapidly progressive dementias (RPDs) is hindered by the broad clinical heterogeneity at onset and the variable disease progression, sometimes overlapping with other more prevalent neurodegenerative dementias.[7] Nonetheless, formulating an early diagnosis is crucial to rule out

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other potentially treatable neurological syndromes and reduce the risk of iatrogenic transmission.[8 9]

The primary support for the clinical diagnosis of sCJD has been provided for several years by electroencephalography,[10] cerebrospinal fluid (CSF) surrogate biomarkers of neurodegeneration[11–14] and brain MRI.[15–19] However, these biomarkers demonstrated a suboptimal diagnostic accuracy and a significant variability across sCJD subtypes.[3 12 20]

The long-standing search for a disease-specific biomarker eventually led to the development of the prion real-time quaking-induced conversion (RT-QuIC), an ultrasensitive assay indirectly revealing minute amounts of PrP^{Sc} in CSF and other tissues through an amplification strategy.[21–23] The high accuracy of RT-QuIC in discriminating sCJD from non-CJD cases prompted the update of sCJD diagnostic criteria in 2017.[24] As the main change, the previous stringent clinical criteria for labelling a patient possible CJD (ie, the association of dementia and multifocal neurological signs), an uncommon scenario in the early disease stages, especially in the 'atypical' sCJD subtypes, are no longer necessary in front of an RT-QuIC positivity in patients with a rapidly progressive neurological syndrome.

The prion RT-QuIC assay raised great expectations for improving the accuracy of early CJD diagnosis, especially in cases with 'unusual' clinical presentation and/or a slow clinical progression.[25] Indeed, recent studies involving European and USA CJD Surveillance Centres reported a significantly improved in-life clinical diagnosis and a raised estimation of disease incidence with the amended criteria.[26–28] However, these studies focused on cohorts of individuals with postmortem neuropathological evaluation, which are only partially representative of the actual clinical scenario and are associated with a selection bias. Moreover, they compared the diagnostic values of the different criteria from the perspective of epidemiological surveillance, considering the clinical data collected over the entire disease course, not only at the time of the first diagnostic evaluation.

In this study, we assessed the impact of the prion RT-QuIC on the clinical diagnosis of CJD at the first assessment in an extensive series of consecutive patients referred to two major Italian CJD Reference Centres over ten years. Moreover, we compared the diagnostic value of RT-QuIC associated with different combinations of clinical features and surrogate biomarkers.

METHODS

Inclusion criteria and clinical definitions

We investigated patients with suspected CJD referred between January 2010 and December 2020 to the neuropathology laboratory at the Institute of Neurological Sciences of Bologna (ISNB) and the National CJD Surveillance Unit at Istituto Superiore di Sanità (ISS) in Rome.

The total number of referred patients was 3347. Medical records, including electroencephalogram (EEG), result of protein 14-3-3 test, brain imaging and follow-up information were reviewed up to May 2022. When available, the result of the t-tau assay was also collected. From the initial group, we excluded patients lacking medical records and those with an extremely low probability of being affected by CJD by applying broad inclusion criteria (figure 1). They included at least one of the following: (1) positive or uncertain 14-3-3 assay; (2) CSF t-tau>600 pg/mL; (3) positive MRI (according to diagnostic criteria) and (4) clinical course (including follow-up) compatible with the possible CJD diagnosis according to the diagnostic criteria. The selected cohort included 1250 patients.

All selected patients received two diagnostic formulations according to (1) the updated WHO criteria[19] and (2) the 2017 European Union (EU) criteria[24] (figure 2A), both based on the data collected at the first diagnostic evaluation. Moreover, each patient received a final diagnosis based on neuropathological examination, *PRNP* sequencing and/or all clinical and laboratory data available at the last follow-up. We applied the 2017 EU criteria at final diagnosis to define probable CJD patients lacking neuropathological examination or pathogenic mutation in *PRNP*.

A categorisation of 'probable sCJD', 'possible sCJD' and 'non-CJD' was attributed to each participant according to the different criteria (figure 2A).

sCJD participants with a neuropathological assessment were given a subtype classification according to Parchi *et al*.[5 29] Patients with a mixed subtype were classified based on the dominant histotype (eg, MM1+2C classified as MM1 when the MM2C features were focal) and then merged into the corresponding pure subtype.

Alternative diagnoses to prion disease were given either by neuropathological examination (definite non-CJD) or clinical criteria (probable non-CJD). Specifically, we defined probable non-CJD patients showing improvement or stabilisation at follow-up or receiving an alternative clinical diagnosis supported by genetic, neuroradiological and/or laboratory findings (see online supplemental material 1 (methods section) for details). Clinical information was too scanty to reach a reliable classification in 24 cases.

CSF biomarker analyses

Samples obtained by lumbar puncture with standard procedure were centrifuged in case of blood contamination, divided into

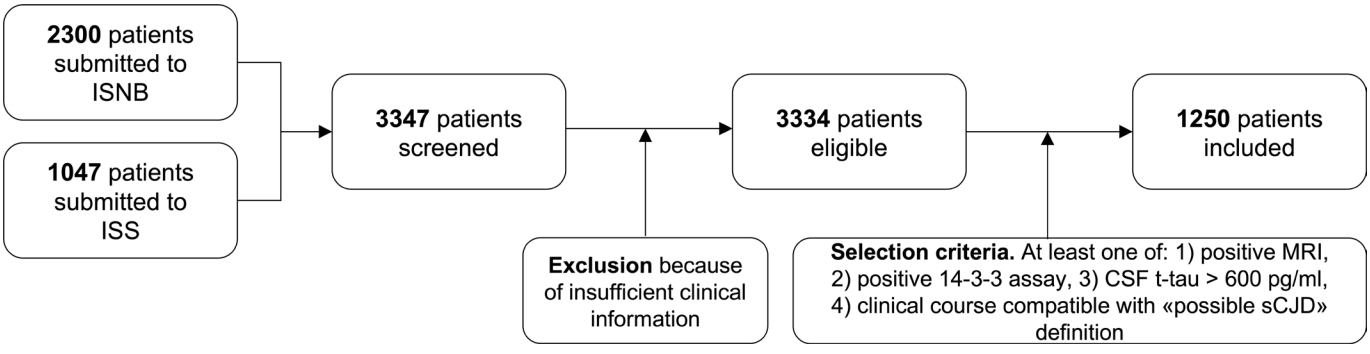

Figure 1 Study flow chart. CSF, cerebrospinal fluid; ISNB, Institute of Neurological Sciences of Bologna; ISS, Istituto Superiore di Sanità (Rome); sCJD, sporadic Creutzfeldt-Jakob disease; t-tau, total tau.

A

probable sCJD	RPCD and: • at least two of: I. myoclonus II. pyramidal/extrapyramidal signs III. cerebellar/visual signs IV. akinetic mutism • at least one positivity among: EEG, CSF 14-3-3 assay, brain MRI ***(both updated WHO and 2017 EU criteria)*** or Progressive neurological syndrome and a positive prion RT-QuIC assay ***(2017 EU criteria only)***
possible sCJD	RPCD and: • at least two of (I-IV) • disease duration ≤ 24 months ***(both updated WHO and 2017 EU criteria)***
non-CJD	• no RPCD, *or* • RPCD/psychiatric symptoms alone, *or* • RPCD + only one of (I-IV) regardless of biomarker positivity (EEG, CSF 14-3-3 assay, brain MRI) ***(both updated WHO and 2017 EU criteria)***

B

A progressive neurological syndrome and a positive prion RT-QuIC assay						
OR						
positive brain MRI	RPCD and clinical signs in at least two domains among: • myoclonus • pyramidal/extrapyramidal • cerebellar/visual • akinetic mutism					
	AND					
	positive brain MRI	positive CSF 14-3-3 assay	CSF t-tau > 1250 pg/ml	positive brain MRI	**OR**	CSF t-tau > 1250 pg/ml
Q-M	**Q-CM**	**Q-C14**	**Q-CT**	**Q-CMT**		

Figure 2 Definition of the diagnostic criteria and combinations of different biomarkers/clinical features evaluated for their diagnostic accuracy. (A) Definitions of the 'probable sCJD', 'possible sCJD' and 'non-CJD' diagnoses according to the updated WHO criteria and the 2017 EU criteria. (B) We combined the novel criterion introduced by the 2017 EU criteria (RT-QuIC+ progressive neurological syndrome) to different associations of clinical variables and other biomarker results at the time of first diagnostic assessment, obtaining five different combination criteria: Q-M, Q-CM, Q-C14, Q-CT, Q-CMT (Q,RT-QuIC; M, MRI; C, clinical features; 14, 14-3-3 protein; T, tau protein). Brain MRI was considered 'positive' with high DWI or FLAIR signal in caudate/caudate-putamen/caudate-putamen-thalamus or at least two cortical areas (parietal, temporal or occipital). EU, European Union; CSF, cerebrospinal fluid; RPCD, rapidly progressive cognitive decline; RT-QuIC, real-time quaking-induced conversion assay; sCJD, sporadic Creutzfeldt-Jakob disease; t-tau, total tau; DWI, diffusion-weighted image

aliquots and stored in polypropylene tubes at −80°C until analysis. At ISNB, t-tau and protein 14-3-3 gamma isoform were analysed by immunoassays using commercially available laboratory kits. At ISS, the 14-3-3 test was performed by western blotting (for details, see online supplemental material 1 (methods section)).

CSF RT-QuIC was performed as previously described,[12 30] using either a full-length (PQ-CSF) or a truncated (IQ-CSF) hamster recombinant prion protein as substrate. We tested 571 patients by PQ-CSF (274 at ISNB, 297 at ISS), 181 by IQ-CSF (all at ISNB) and 498 by both protocols. Moreover, we retested by IQ-CSF (at ISNB) all PQ-RT-QuIC negative samples from patients with either probable or definite CJD with sufficient CSF volume (88 of 101).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, California, USA) and MedCalc (MedCalc Software, Ostend, Belgium). Data were expressed as median and IQR. For continuous variables, depending on the number of groups, the Mann-Whitney U test or the Kruskal-Wallis test (followed by Dunn-Bonferroni post hoc test) were used. Differences were considered statistically significant at $p<0.05$. The Fisher's exact test and χ^2 test were used for categorical variables. McNemar's test was used to compare the diagnostic sensitivity and specificity of different criteria in the same clinical group. ROC curves were compared by the DeLong test.

For calculating the sensitivity of the diagnostic criteria, all patients fulfilling the 'probable sCJD' diagnosis were regarded as true positive. Specificity was calculated considering all patients not responding to the 'probable sCJD' diagnosis as true negative.

Finally, we compared the diagnostic accuracy of 2017 EU criteria to that of RT-QuIC alone and of different biomarkers and clinical features combinations at the time of CSF collection. For this purpose, we analysed 223 definite CJD (164 definite sCJD and 59 gCJD) and 220 non-CJD (38 definite non-CJD) with all biomarkers available. The definitions of the combination criteria (Q-CM, Q-M, Q-C14, Q-CT, Q-CMT) are reported in figure 2B.

RESULTS

Demographic and clinical data and final case classification

A total of 850 participants received a final diagnosis of either definite or probable CJD according to 2017 EU criteria and *PRNP* sequencing and 400 of non-CJD. The former group included 297 definite sCJD, 151 gCJD and 402 probable sCJD, whereas the non-CJD comprised 61 subjects with alternative neuropathological diagnoses, 315 with alternative clinical diagnoses, and 24 lacking an alternative diagnosis (see online supplemental material 1 (tables 1 and 2) for details).

There was no significant difference in sex distribution ($p=0.95$), time from onset to diagnostic evaluation ($p=0.32$) and disease duration ($p=0.06$) between CJD and non-CJD patients. CJD patients were significantly younger at disease onset than the non-CJD participants ($p=0.014$) (table 1).

Diagnostic performance of the updated WHO criteria at the time of CSF collection

In the definite sCJD group, 224 out of 297 participants fulfilled the diagnosis of probable CJD at CSF collection according to the revised WHO criteria, yielding a sensitivity of 75.4%. A similar diagnostic sensitivity characterised the total CJD cohort (74.8%) (table 2).

In definite sCJD, the sensitivity of the updated WHO criteria did not significantly differ among subjects carrying different genotypes at codon 129. However, in the total CJD cohort, the sensitivity was significantly higher in patients carrying MM than in those with MV or VV (online supplemental material 1 (table

Table 1 Demographic variables and distribution of biomarker results in the diagnostic groups

	CJD			Non-CJD		
	Definite sCJD (n=297)	gCJD (n=151)	Total (n=850)	Definite (n=61)	Total (n=400)	P value
Demographic values						
Age at onset, years	68 (62–76) (297)	65 (58–72) (151)	69 (62–74) (850)	76 (66–81) (61)	70 (61–77) (400)	0.01*, <0.001†‡
Female, %	50.5 (297)	53.6 (151)	52.8 (850)	52.4 (61)	52.5 (400)	–
Time onset-diagnostic evaluation, months	2.2 (1.4–3.9) (297)	2.2 (1.5–4.2) (151)	2.5 (1.5–4.8) (850)	1.5 (0.8–3.9) (61)	3.0 (0.9–10.3) (400)	0.02‡, 0.03†
Disease duration, months	4 (2–7) (289)	4 (2–6) (132)	4 (2–10) (750)	2 (1–6) (51)	6 (1.5–24.1) (134)	0.003‡, 0.01†
Biomarker results						
EEG positive, %	125, 44.3 (282)	77, 55.4 (139)	332, 41.2 (806)	8, 14.8 (54)	21, 7.0 (299)	<0.001*†‡
CSF 14-3-3 positive, %	244, 82.7 (295)	114, 77.0 (148)	645, 76.6 (842)	34, 60.7 (56)	113, 28.9 (391)	<0.001*‡, 0.02†
CSF t-tau >1250 pg/mL, %	192, 93.6 (205)	66, 89.2 (74)	437, 89.9 (486)	31, 50.8 (61)	128, 34.7 (369)	<0.001*†‡
MRI positive, %	194, 76.7 (253)	94, 73.4 (128)	562, 75.5 (744)	5, 12.5 (40)	18, 7.2 (251)	<0.001*†‡
RT-QuIC positive, %	281, 94.6 (297)	136, 90.1 (151)	798, 93.9 (850)	0, 0 (61)	1, 0.25 (400)	<0.001*†‡

Continuous values are expressed as median (IQR). Only p values of statistically significant comparisons are shown. Numbers in brackets refer to patients with available demographic data/biomarker results. Data of the definite sCJD and gCJD cohorts were compared with those of the non-CJD group with neuropathological evaluation (definite); data of the whole CJD cohort were compared with those of the whole non-CJD cohort.
*Comparison between total CJD and total non-CJD.
†Comparison between gCJD and definite non-CJD.
‡Comparison between definite sCJD and definite non-CJD.
CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; EEG, electroencephalogram; gCJD, genetic CJD; RT-QuIC, real-time quaking-induced conversion; sCJD, sporadic CJD.

3)). When comparing the sCJD subtypes, we found a lower sensitivity in MV2K than in MM(V)1 participants (table 3).

MV2K patients were more often classified as possible sCJD (p=0.01) and showed positive EEG or 14-3-3 assay less frequently (p<0.001) compared with those belonging to the MM(V)1 subtype.

Of the 214 definite or probable CJD participants not correctly identified as probable sCJD by the revised WHO criteria, 33 were classified as possible sCJD and 181 as non-CJD. Clinical and biomarker characteristics of patients not classified as 'probable sCJD' by the revised WHO criteria are provided in table 4 and online supplemental material 1 (table 4) .

At the first diagnostic evaluation, the WHO updated criteria falsely classified 24/400 non-CJD patients as probable sCJD in the total cohort and 11/61 in the neuropathological cohort, yielding a specificity of 94.0% and 82.0%, respectively (table 2). The clinical

Table 2 Sensitivity and specificity of different CJD diagnostic criteria at first diagnostic evaluation

		Updated WHO criteria				2017 EU criteria			
Final diagnosis	n	sCJD probable	sCJD possible	Non-CJD	Sensitivity (95% CI)	sCJD probable	sCJD possible	Non-CJD	Sensitivity (95% CI)
Definite sCJD	297	224	10	63	75.4* (70.2 to 80.0)	291	1	5	98.0 (95.7 to 99.1)
gCJD	151	120	7	24	79.5* (72.3 to 85.1)	147	2	2	97.3 (93.4 to 99.0)
Definite CJD	**448**	**344**	**17**	**87**	**76.8* (72.7 to 80.5)**	**438**	**3**	**7**	**97.8 (95.9 to 98.8)**
Probable sCJD	402	292	16	94	72.6* (68.1 to 76.8)	402	0	0	100 (99.0 to 100)
Probable or definite CJD	**850**	**636**	**33**	**181**	**74.8* (71.8 to 77.6)**	**840**	**3**	**7**	**98.8 (97.8 to 99.4)**
		sCJD probable	sCJD possible	Non-CJD	Specificity (95% CI)	sCJD probable	sCJD possible	Non-CJD	Specificity (95% CI)
Definite non- CJD	61	11	8	42	82.0† (70.5 to 89.6)	11	8	42	82.0 (70.5 to 89.6)
Probable non-CJD	339	13	61	265	96.2† (93.5 to 97.7)	14	61	264	95.9 (93.2 to 97.5)
Total non-CJD	**400**	**24**	**69**	**307**	**94.0† (91.2 to 95.9)**	**25**	**69**	**306**	**93.7 (90.9 to 95.7)**

Data referring to total definite CJD, total (probable+definite) CJD, and total non-CJD are shown in bold.
*Compared with the 2017 EU criteria, p<0.001 (McNemar's test).
†Compared with the 2017 EU criteria, p>0.99 (McNemar's test).
CJD, Creutzfeldt-Jakob disease; EU, European Union; gCJD, genetic CJD; sCJD, sporadic CJD.

Table 3 Comparison of diagnostic sensitivity of the WHO and 2017 EU criteria and RT-QuIC alone among sCJD subtypes

	MM(V)1 (n=205)	VV2 (n=40)	MV2K (n=30)	MM(V)2C (n=11)
Updated WHO criteria	163/205 79.5%*	30/40 75%	17/30 56.7%	7/11 63.6%
2017 EU criteria	201/205 98.0%	39/40 97.5%	30/30 100%	11/11 100%
RT-QuIC alone, overall	193/205 94.1%	38/40 95.0%	30/30 100%	10/11 90.9%
RT-QuIC alone, PQ-CSF	151/175 86.3%	28/34 82.3%	22/26 84.6%	8/10 80%
RT-QuIC alone, IQ-CSF	109/118 92.4%	32/32 100%	21/21 100%	7/7 100%

Eleven patients were not included because the available brain tissue was insufficient to complete histotyping. Only p values of statistically significant comparisons are shown. Patients classified as 'probable sCJD' are considered 'true positive' in the different criteria' sensitivity calculation.
*Compared with MV2K, p=0.01.
CSF, cerebrospinal fluid; EU, European Union; RT-QuIC, real-time quaking-induced conversion assay; sCJD, sporadic Creutzfeldt-Jakob disease.

and biomarker features of non-CJD patients falsely classified as probable sCJD by the updated WHO criteria are shown in table 5.

Diagnostic performance of the 2017 EU criteria at the time of CSF collection

At CSF collection, the 2017 EU criteria identified as probable sCJD 291 of 297 definite sCJD patients, yielding a sensitivity of 98.0%. The diagnostic sensitivity was similar in the total CJD cohort (98.8%).

There were no statistically significant differences in the diagnostic sensitivity of 2017 EU criteria among CJD patients carrying different genotypes at *PRNP* codon 129 or those belonging to different subtypes (table 3, online supplemental material 1 (table 3)). The 2017 EU criteria showed significantly higher sensitivity than the updated WHO criteria in all CJD groups (table 2).

The six definite sCJD patients who tested negative by the 2017 EU criteria belonged to the MM1 (n=4), VV2 (n=1) and VV1 (n=1) subtypes.

According to the 2017 EU criteria, 25 of 400 non-CJD participants were diagnosed as probable sCJD, bringing the specificity to 93.7%. The specificity was 82.0% against the definite non-CJD patients. The specificity of both WHO and EU 2017 criteria was not statistically different in both definite and probable non-CJD groups (table 2).

For the 2017 EU and WHO criteria, area under the curve (AUC) values were lower in the analyses involving definite non-CJD patients than in the whole non-CJD cohort (online supplemental material 1). In all group combinations, the AUC values of the 2017 EU criteria were significantly higher than those of the WHO updated criteria (online supplemental material 1 (table 5)).

Performance of diagnostic criteria for sCJD in gCJD

The 2017 EU criteria showed greater diagnostic sensitivity than the WHO criteria in the gCJD group (97.3% vs 79.5%, p<0.001) (table 2). Sensitivity values were either comparable to those for definite sCJD (2017 EU criteria, 97.3% vs 98.0%, p=0.74) or slightly higher (WHO criteria, 79.5% vs 75.4%, p=0.41).

Diagnostic value of CSF RT-QuIC

In the whole CJD cohort, CSF RT-QuIC gave a positive result in 798 of 850 participants, yielding a diagnostic sensitivity of 93.9%. Sensitivity was similar in the cohorts of the definite

Table 4 Clinical variables and biomarker results in the non-CJD group defined by WHO criteria at the time of diagnostic evaluation

Clinical variable	CJD* (n=181)	Non-CJD* (n=307)	P value	Biomarker results	CJD* (n=181)	Non-CJD* (n=307)	P value
No RPCD %	20 11.0	65 21.2	**0.004**	EEG positive (n) %	44 (172) 25.6	16 (222) 7.2	**<0.001**
RPCD±psychiatric symptoms %	28 15.5	129 42.0	**<0.001**	CSF 14-3-3 positive (n) %	128 (179) 71.5	95 (298) 31.9	**<0.001**
RPCD+1 clinical feature %	133 73.5	113 36.8	**<0.001**	CSF t-tau >1250 pg/mL (n) %	113 (138) 81.9	106 (301) 35.2	**<0.001**
Myoclonus %	9 5.0	24 7.8	0.27	MRI positive (n) %	123 (156) 78.8	14 (195) 7.2	**<0.001**
Pyramidal/extrapyramidal %	29 16.0	68 22.1	0.13	Striatum high signal %	67 (156) 42.9	8 (195) 4.1	**<0.001**
Pyramidal %	18 9.9	27 8.8	0.75	Cortical high signal† %	91 (155) 58.7	7 (195) 3.6	**<0.001**
Extrapyramidal %	19 10.5	48 15.6	0.13				
Cerebellar/visual %	90 49.7	17 5.5	**<0.001**				
Cerebellar %	83 45.8	15 4.9	**<0.001**				
Visual %	28 15.5	5 1.6	**<0.001**				
Akinetic mutism %	5 2.8	4 1.3	0.30				

Comparison between patients diagnosed as CJD at final assessment and those confirmed as non-CJD.
*Refer to the final diagnosis.
†At least two cortical areas. P values of statistically significant comparisons are shown in bold.
CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; EEG, electroencephalogram; RPCD, rapidly progressive cognitive decline; t-tau, total tau.

Table 5 Clinical and biomarker characteristics of non-CJD patients falsely classified as CJD by the updated WHO criteria

Definite non-CJD (n=11)					
	CF+MRI (n)	CF+14-3-3 (n)	CF+EEG (n)	Total	List of final diagnoses
Degenerative	0 (2)	2 (3)	1 (3)	**3**	AD (2), LBD (1)
Inflammatory/infectious	1 (1)	3 (3)	0 (2)	**3**	IRIS encephalitis (2), PML (1)
Neoplastic	0 (0)	2 (2)	0 (2)	**2**	CNS lymphoma (1), metastatic carcinoma (1)
Vascular	0 (1)	1 (1)	1 (1)	**1**	lacunar disease (1)
Other	0 (2)	2 (2)	0 (2)	**2**	AD+WE (1), non-significant neuropathological findings (1)
Total, %	**1 (6), 16.7**	**10 (11), 90.9**	**2 (10), 20**	**11**	
Non-CJD with clinical diagnosis (n=13)					
	CF+MRI (n)	CF+14-3-3 (n)	CF+EEG (n)	Total	List of final diagnoses
Degenerative	0 (0)	2 (3)	1 (3)	**3**	AD (2), spastic paraplegia (1)
Inflammatory/infectious	1 (2)	4 (5)	1 (4)	**5**	infectious (3), autoimmune (1) or actinic (1) encephalitis
Vascular	2 (4)	2 (5)	1 (5)	**5**	Small vessel ischaemic disease (4), PRES (1)
Total, %	**3 (6), 50**	**8 (13), 61.5**	**3 (12), 25**	**13**	
Total (both definite and non-definite non-CJD), %	**4 (12), 33.3**	**18 (24), 75**	**5 (22), 22.7**	**24**	

Numbers in brackets refer to the number of patients with available biomarker results (MRI, 14-3-3 assay or EEG). According to diagnostic criteria, clinical findings are considered 'positive' when the association of rapidly progressive cognitive impairment and signs/symptoms in at least two neurological domains is met.
AD, Alzheimer's disease; CF, clinical findings; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; EEG, electroencephalogram; IRIS, immune reconstitution inflammatory syndrome; LBD, Lewy body disease; PML, progressive multifocal leukoencephalopathy; PRES, posterior reversible encephalopathy syndrome; t-tau, total tau; WE, Wernicke encephalopathy.

and probable sCJD (94.6% vs 94.8%). There was no statistically significant difference in the sensitivity of RT-QuIC among patients carrying different genotypes at *PRNP* codon 129 (online supplemental material 1 (table 3)). Despite not being statistically significant, RT-QuIC sensitivity was higher in definite sCJD patients belonging to the MV2K, VV2 and MM(V)1 subtypes than those of the MM(V)2C group (table 3) and in E200K carriers compared to those carrying V210I (online supplemental material 1). The definite sCJD subjects with a negative RT-QuIC (n=16) comprised 12 MM1, 2 VV2 and 1 each of the MM2C and VV1 subtypes.

In definite CJD participants, IQ-CSF yielded an overall higher sensitivity (92.0%) than PQ-CSF (86.9%); in the subgroup of patients undergoing both examinations, the comparison was statistically significant ($p<0.001$). Twenty-five out of 39 patients undergoing both analyses and testing negative at PQ-CSF obtained a positive result at the IQ-CSF. Finally, there were no significant differences in the rate of CSF RT-QuIC positivity between definite sCJD and gCJD cases ($p=0.08$).

A single probable non-CJD subject tested positive at RT-QuIC, bringing the assay specificity to 99.7%. Based on the clinical history and CSF Alzheimer's disease (AD) biomarkers, this patient was diagnosed with AD; available medical records indicated a disease duration of more than 84 months. There were no positive RT-QuIC tests in the definite non-CJD patients.

The diagnostic values of brain MRI, EEG and CSF 14-3-3 and t-tau proteins are reported in table 1 and online supplemental material 1 (result section).

Comparison of the accuracy of different clinical biomarkers and RT-QuIC combinations at diagnostic evaluation

When considering either the definite sCJD or the definite CJD group against the total non-CJD group, there were no significant differences in diagnostic accuracy between the Q-CM, Q-CMT, or EU 2017 criteria and the RT-QuIC alone (table 6 and online supplemental material 1 (table 7)).

However, when evaluated against the definite non-CJD group, Q-CM criteria showed higher accuracy than the 2017 EU criteria in both groups. Similarly, the diagnostic accuracy of RT-QuIC alone was also superior to that of the 2017 EU criteria, although only in the definite sCJD group (table 6). In contrast, in both groups (definite sCJD and definite CJD), there were no statistically significant differences between the Q-C14, Q-CT and Q-CMT criteria and the 2017 EU criteria. The higher specificity of RT-QuIC alone and Q-CM criteria compared with all other criteria explained the better accuracy for the most part. The specificity of RT-QuIC and Q-CM criteria was not statistically different in both cohorts (online supplemental material 1). Details about the specificity and sensitivity of the combination criteria are provided in online supplemental material 1 (tables 8 and 9).

DISCUSSION

The diagnostic criteria for sCJD were initially introduced for epidemiological purposes to monitor disease incidence and the possible appearance of novel disease variants.[9] Without a pathology-specific biomarker, the WHO criteria required stringent clinical features to reach a diagnosis of CJD to limit the false positive clinical diagnosis. However, with improved knowledge of the sCJD spectrum, it has become clear that the chosen clinical criteria and supportive biomarkers have reduced sensitivity for the atypical sCJD subtypes.[12 20] Furthermore, they showed significant limitations for the clinical practice since they often require waiting for the full manifestation of neurological symptoms and signs to formulate the clinical diagnosis of probable sCJD. At the same time, the early recognition of a potentially treatable form of an RPD is an absolute priority in the clinical setting. Accordingly, our evaluation of the sensitivity of WHO criteria at the time of CSF collection yielded a suboptimal performance (74.8% in the whole CJD cohort, 75.4% and 79.5% in

Table 6 Comparison of diagnostic accuracy of 2017 EU criteria, RT-QuIC alone or combined with clinical variables and other biomarkers in the definite sCJD cohort

Total non-CJD (n=220)							
		RT-QuIC alone	**Q-CM**	**Q-M**	**Q-C14**	**Q-CT**	**Q-CMT**
	AUC values (95% CI)	**P value**					
2017 EU criteria	**0.960** (0.935 to 0.977)	0.66	0.27	0.53	0.77	0.70	0.28
RT-QuIC alone	**0.964** (0.940 to 0.980)	–	0.45	0.27	0.49	0.82	0.93
Q-CM	**0.969** (0.946 to 0.984)	–	–	0.08	0.20	0.45	0.66
Q-M	**0.951** (0.925 to 0.971)	–	–	–	0.61	0.44	0.31
Q-C14	**0.958** (0.933 to 0.976)	–	–	–	–	0.56	0.34
Q-CT	**0.962** (0.938 to 0.979)	–	–	–	–	–	0.31
Q-CMT	**0.965** (0.941 to 0.981)	–	–	–	–	–	–
Definite non-CJD (n=38)							
		RT-QuIC alone	**Q-CM**	**Q-M**	**Q-C14**	**Q-CT**	**Q-CMT**
	AUC values (95% CI)	**P value**					
2017 EU criteria	**0.906** (0.857 to 0.942)	**0.05**	**0.04**	0.69	0.61	0.16	0.11
RT-QuIC alone	**0.966** (0.931 to 0.987)	–	0.77	0.12	0.06	0.19	0.24
Q-CM	**0.962** (0.926 to 0.984)	–	–	0.12	0.054	0.19	0.23
Q-M	**0.922** (0.876 to 0.955)	–	–	–	0.81	0.78	0.72
Q-C14	**0.913** (0.865 to 0.948)	–	–	–	–	0.41	0.34
Q-CT	**0.932** (0.888 to 0.963)	–	–	–	–	–	0.32
Q-CMT	**0.935** (0.892 to 0.965)	–	–	–	–	–	–

p values of statistically significant comparisons are shown in bold.
For the definition of the combination criteria see Figure 2B. AUC values are calculated using the definite CJD group (n=164) as "cases". In the upper part of the table the "control" group includes the total non-CJD cohort, while in the lower part only the definite non-CJD cases have been included.
Data referring to the total CJD and non-CJD cohorts are shown in bold.
Abbreviations: AUC, the area under the curve; CI, confidence interval, CJD, Creutzfeldt-Jakob disease
AUC, area under the curve; EU, European Union; RT-QuIC, real-time quaking-induced conversion assay; sCJD, sporadic Creutzfeldt-Jakob disease.

the definite sCJD and gCJD subgroups, respectively). Most of the patients (84.6%) not fulfilling the definition of probable sCJD at this time were classified as non-CJD, confirming that the main reason for the limited sensitivity of these criteria is the lack of suggestive clinical features at an early disease stage. Regarding specificity, the updated WHO criteria yielded an overall value of 94.0%. However, the specificity was only 82.0% in the neuropathological cohort, which is notoriously enriched with patients in which CJD is highly suspected based on clinical course and/or biomarker profile.

In line with previous studies,[26–28] in our definite CJD and sCJD cohorts, the 2017 EU criteria yielded a sensitivity of 97.8% and 98.0%, respectively, significantly higher than those obtained by the updated WHO criteria. Notably, CSF RT-QuIC allowed reclassifying 94 out of 104 (90.4%) definite CJD patients not fulfilling the updated WHO criteria at the time of diagnostic evaluation. Moreover, 110 out of 402 (27.4%) probable sCJD of our cohort lacking a suggestive clinical course and/or other supportive biomarkers at the time of CSF collection received this diagnosis only due to the CSF RT-QuIC. Of note, in patients who underwent both PQ-CSF and IQ-CSF, the latter showed a significantly higher sensitivity with unchanged specificity, confirming that a wider diffusion of second-generation RT-QuIC assays may improve diagnostic performances.

We also found that the 2017 EU criteria for sCJD allow the accurate diagnosis of gCJD cases. The finding is relevant since many gCJD patients lack a family history of prion disease.[31] Therefore, the amended criteria could help physicians identifying CJD without *PRNP* sequencing results.

Notably, this is the first study comparing the diagnostic sensitivity of 'old' and 'amended' CJD criteria in a significant group of patients belonging to different clinicopathological subtypes. We found no significant difference in the diagnostic sensitivity of the 'amended' criteria and the RT-QuIC assay alone among patients with different alleles at codon 129 or belonging to the most common clinicopathological subtypes. This result likely reflects the high sensitivity of the IQ-CSF RT-QuIC for the VV2 and MV2K[30 32 33], the second and third most prevalent sCJD subtypes, which we confirmed in this more extensive study.

Regarding the diagnostic performance of the surrogate biomarkers, our data confirm their significantly lower specificity compared with the RT-QuIC, even in association with stringent clinical criteria. However, MRI specificity was higher than that of CSF surrogate markers, in line with previous reports (92.8% in the whole non-CJD cohort).[34 35] However, the MRI sensitivity was suboptimal (75.5%) and either significantly lower or comparable to those reported in previous studies,[35–38] a result probably related to the fact that most MRI images were evaluated in general hospitals. Indeed, the experience of neuroradiologists interpreting the images, besides the technical characteristics of the scanners, is a well-known source of variability in the diagnostic performance of MRI in CJD.[39] Moreover, EEG also has a limited diagnostic value because of its low sensitivity. Our data, especially those from patients with neuropathological confirmation, clearly demonstrate that the association of positive clinical features and MRI represents the best single biomarker to combine with RT-QuIC. Therefore, we propose the Q-CM criteria be considered the reference diagnostic criteria for sCJD. At the same time, physicians should be increasingly aware of the diagnostic limitations of EEG and surrogate CSF markers.

The results of our 'combination' criteria for the diagnosis of CJD deserve further comment. We found that, compared

with the 2017 EU criteria, a positive RT-QuIC assay alone or an association of positive clinical features and a positive brain MRI (Q-CM criteria) showed a significantly higher specificity. Notably, the diagnostic specificity of Q-CM criteria was not substantially different from that of RT-QuIC alone. Accordingly, the Q-CM criteria and the RT-QuIC alone yielded the highest accuracy among the evaluated tests in all examined subcohorts. These results call for a reevaluation of the role of EEG and CSF surrogate markers in the clinical diagnosis of CJD. Indeed, RT-QuIC and MRI provided a positive result in the large majority of CJD patients. Classifying as probable CJD a patient with a positive surrogate CSF marker (ie, 14-3-3 or t-tau) in front of negative RT-QuIC and MRI evaluations, as the current criteria recommend, will contribute more false-positive than accurate diagnosis, given the suboptimal specificity of these markers.

The main strength of our study is the inclusion of a large cohort of CJD patients with a definite neuropathological diagnosis, allowing their accurate classification into specific clinicopathological subtypes. Including two independent patient cohorts representing the majority of the Italian CJD surveillance network is another strength. The focus on the diagnostic value at the time of the first diagnostic assessment aimed to describe at best the real-world impact of CSF RT-QuIC on diagnosis, distinguish this study from previous ones, and represent an innovative aspect. Finally, the performance of both PQ-CSF and IQ-CSF in a large patient group is also an added value.

Our study is not free of limitations. First, it included a low number of definite non-CJD patients, with a selection bias towards those in whom CJD was highly suspected according to clinical course and biomarker results. A second limitation is the lack of a systematic revision of brain MRI by experienced neuroradiologists. Moreover, the limited rate of patients with available CSF t-tau results in one of the two cohorts (ISS) could have influenced this biomarker's overall diagnostic performance.

In conclusion, our study confirms, in a large well-characterized cohort, that CSF RT-QuIC is a highly sensitive and specific biomarker for diagnosing CJD in vitam and that its introduction to current criteria improved diagnostic accuracy and epidemiological surveillance. Moreover, focusing on the time of first diagnostic assessment, our results show that criteria considering either a positive second-generation RT-QuIC assay alone in a progressive neurological syndrome and/or a positive brain MRI in an appropriate clinical context provide higher accuracy than the 2017 EU criteria. The latter result encourages a further revision of the criteria limiting the use of 'surrogate' CSF markers for diagnosis only when the RT-QuIC assay is unavailable. However, protein 14-3-3 and t-tau at low cut-off values might remain valid biomarkers for preliminary extended screening,[40] given the current limitations of applying the RT-QuIC for such purposes.

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Contributors PP designed the study. AMam, MR, ME and AP performed the RT-QuIC assay. ME and BP performed CSF surrogate markers analyses. AMam, AP and SC performed genetic analyses. PP and SB performed neuropathological examinations. DT, LV, EC, AL, AMas and SB collected clinical data, and AMas, SB and PP analysed the data and interpreted the results. AMas, SB and PP wrote the manuscript draft. All authors critically revised the manuscript and approved its content before submission. PP acted as guarantor.

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