Advanced tests for early and accurate diagnosis of Creutzfeldt–Jakob disease

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Abstract | Early and accurate diagnosis of Creutzfeldt–Jakob disease (CJD) is a necessary to distinguish this untreatable disease from treatable rapidly progressive dementias, and to prevent iatrogenic transmission. Currently, definitive diagnosis of CJD requires detection of the abnormally folded, CJD-specific form of protease-resistant prion protein (PrP^{CJD}) in brain tissue obtained postmortem or via biopsy; therefore, diagnosis of sporadic CJD in clinical practice is often challenging. Supporting investigations, including MRI, EEG and conventional analyses of cerebrospinal fluid (CSF) biomarkers, are helpful in the diagnostic work-up, but do not allow definitive diagnosis. Recently, novel ultrasensitive seeding assays, based on the amplified detection of PrP^{CJD}, have improved the diagnostic process; for example, real-time quaking-induced conversion (RT-QuIC) is a sensitive method to detect prion-seeding activity in brain homogenate from humans with any subtype of sporadic CJD. RT-QuIC can also be used for *in vivo* diagnosis of CJD: its diagnostic sensitivity in detecting PrP^{CJD} in CSF samples is 96%, and its specificity is 100%. Recently, we provided evidence that RT-QuIC of olfactory mucosa brushings is a 97% sensitive and 100% specific for sporadic CJD. These assays provide a basis for definitive antemortem diagnosis of prion diseases and, in doing so, improve prospects for reducing the risk of prion transmission. Moreover, they can be used to evaluate outcome measures in therapeutic trials for these as yet untreatable infections.

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Creutzfeldt–Jakob disease (CJD) is a neurodegenerative illness caused by the misfolding of the host prion protein (PrP; BOX 1). Once formed, the initial pathological prion protein (PrP^{CJD}) oligomers can act as seeds for further misfolding of normal cellular PrP (PrP^{C}) in a cascade that results in neuronal death and the clinical manifestations of the disease, such as cognitive decline, hallucinations, incoordination and involuntary movements. The 'prion paradigm' of PrP misfolding and seeded polymerization into disease-causing PrP^{CJD} has provided the basis for understanding the molecular mechanism of the misfolding of other disease-associated host proteins (such as amyloid-β (Aβ), α-synuclein and tau) that are associated with a variety of neurodegenerative disorders[1,2].

CJD and other rare prion diseases, such as variably protease-sensitive prionopathy, sporadic and fatal familial insomnias, Gerstmann–Sträussler–Scheinker syndrome and PrP systemic amyloidosis (TABLE 1), have a variety of clinical and pathological phenotypes; the host PrP genotype and the conformational pattern of the pathological PrP^{CJD} partially contribute to the phenotype seen in a given patient[3–8].

Early diagnosis of CJD remains challenging because the clinical manifestations of prion diseases at onset are variable and nonspecific. In patients presenting with rapidly evolving dementia characterized by progressive impairment in multiple cognitive domains, sporadic (sCJD) should be considered only after potentially treatable nonprion conditions — including autoimmune, neoplastic, paraneoplastic, and toxic or metabolic illnesses (such as heavy metal toxicity or Wernicke encephalopathy) — have been excluded[9–11].

In the absence of a positive result for PrP^{CJD} in the brain tissue, which is strictly required for a definitive diagnosis of CJD, only supporting investigations, such as typical diffusion patterns on MRI, periodic sharp and slow waves complexes (PSWCs) on EEG and the detection of 14-3-3 protein in the cerebrospinal fluid (CSF) are helpful in the diagnostic process, although they do not enable a definitive diagnosis to be made[12]. Recently,

Key points

- Early and accurate diagnosis of Creutzfeldt–Jakob disease (CJD) is essential to avoid iatrogenic transmission and to distinguish CJD from potentially treatable dementias
- Diagnosis of CJD in living patients is challenging, mainly because the disease phenotypes are highly heterogeneous, and detection of the misfolded protein in the brain tissue is often not feasible
- Supportive investigations such as EEG, MRI and cerebrospinal fluid biomarkers have a relatively low diagnostic sensitivity and specificity in CJD
- Diagnosis of CJD has been markedly improved by novel ultrasensitive seeding assays, such as real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA), which are based on amplified prion detection
- RT-QuIC is specific and highly sensitive for sporadic CJD, whereas PMCA is extremely sensitive for detecting variant CJD prions in biological fluids and in extraneural or lymphatic tissues
- In the future, novel assays analogous to RT-QuIC or PMCA might provide a protein-seeding-based diagnosis in other neurodegenerative diseases in which prion-like neurodegenerative processes are implicated

however, marked improvements in the diagnostic process have been provided by novel ultrasensitive seeding assays that are based on amplified detection of PrP^{CJD}. These assays provide a basis for accurate antemortem diagnosis of prion diseases, reduction of iatrogenic prion transmission, and biomarker-based evaluation for future therapeutic trials[13]. Moreover, related tests might aid diagnosis of neurodegenerative diseases in which prion-like neurodegenerative processes are implicated. Here, we review the state of the art in the diagnosis of CJD and other human prion diseases in light of these new assays for PrP^{CJD} seeding activity.

Diversity of CJD

CJD is the most common human prion disease. It is usually sporadic, but about 10–15% of cases are familial (genetic CJD (gCJD)); this form of CJD is inherited in an autosomal dominant fashion. In a small minority of patients, prion diseases are acquired via iatrogenic transmission (iCJD) or exposure to the bovine spongiform encephalopathy (BSE) agent (variant CJD (vCJD)). The number of iCJD cases has dramatically decreased over the past 15 years after elimination of therapeutic misadventures, and no new cases of vCJD have been reported since 2012, presumably because of measures taken to reduce the consumption of high-risk bovine tissues[14,15].

Sporadic CJD

The mean age at onset of sCJD is around 70 years[14]. The main clinical characteristics of sCJD include rapidly progressive cognitive decline, myoclonus, cerebellar ataxia, visual symptoms, and pyramidal and extrapyramidal signs[16]. In some patients, psychiatric symptoms, behavioural changes and/or insomnia are also present[16,17]. The main factors affecting the disease course are age at onset, sex, prion protein gene (*PRNP*) codon 129 genotype, and PrP^{CJD} glycotype. The time from diagnosis to death is substantially shorter in patients older than 80 years (mean survival 3 months) than in those aged around 50 years (mean survival 7 months). Female patients live about 1 month longer than male patients[18]. Moreover, *PRNP* codon 129 genotype and PrP^{CJD} glycotype affect disease susceptibility, severity and prognosis, as described below.

PRNP Met/Val polymorphism. The PRNP methionine/valine (MV) polymorphism at codon 129 can influence clinicopathological phenotypes and length of survival[18,19]. About 50% of the general population has codon 129 heterozygosity (MV), probably because ancestral prion disease epidemics have favoured the MV genotype during the evolution of modern humans[20,21]. By contrast, more than 80% of patients with sCJD are homozygous for either methionine or valine, and the disease course in these patients is much shorter than in heterozygous patients[17–19].

PrP^{CJD} glycotype. Another factor that influences disease severity and survival time is the glycotype of PrP^{CJD} that accumulates in the brain in CJD. Two major PrP^{CJD} glycotypes — type 1 and type 2A — have been internationally validated for sCJD[17,22] The glycotypes are distinguished mainly by the electrophoretic migration of the unglycosylated fragments: the unglycosylated PrP^{CJD} type 1 fragment migrates at 19 kDa, whereas type 2A migrates at 21 kDa. These PrP^{CJD} types are presumed to reflect differences in the abilities of distinct CJD strains or conformers to accommodate differentially glycosylated PrP monomers into their multimeric assemblies[22]. The different sCJD glycotypes have preferential associations with codon 129 MV polymorphisms: MM genotypes have a high propensity to combine with type 1 PrP^{CJD} glycotype (MM1), whereas VV and MV genotypes tend to be associated with type 2 PrP^{CJD} (VV2 and MV2, respectively). Overall, MM1, VV2, MV1 and MV2 groups comprise more than 90% of all sCJD cases, whereas MM2 and VV1 are rarely observed[17,22] (TABLE 2). Thus, codon 129 genotype and PrP^{CJD} type, together with age at disease onset and sex, act as prognostic factors in sCJD[18].

Variant CJD

Phenotypically, vCJD is relatively homogeneous. The mean age at onset is 28 years (range 12–74 years), and the median survival after diagnosis is 14 months (range 6–39 months)[23]. Clinical symptoms at onset include psychiatric, behavioural and/or sensory symptoms, which are followed by cognitive decline, cerebellar signs and involuntary movements as the disease progresses. MRI reveals high bilateral pulvinar signals that are distinct from those observed in sCJD[15,23]. The CSF level of 14-3-3 is not a useful marker in vCJD, and the PSWC EEG pattern manifests only in the late stages of disease, if at all[24]. The presence of PrP^{CJD} in the tonsil, detected by immunoblotting and immunohistochemistry, helps to distinguish vCJD from other prion diseases[25] (FIG. 1).

A third PrP^{CJD} glycotype, type 2B, is associated with vCJD but, unlike with sCJD, the overall profile of PrP^{CJD} is dominated by diglycosylated PrP molecules. The vCJD infectious agent provides a key example of a prion strain's propensity to affect only hosts with a specific polymorphism: all clinically affected patients

PrP^{CJD}
The infectious, often partially protease-resistant form of prion protein that is associated with Creutzfeldt–Jakob disease.

Prion paradigm
According to the prion paradigm, a misfolded, usually pathological, form of a host protein acts as an infectious agent by servingas a folding template, inducing the conversion of its normal counterpart into more of the misfolded form; the misfolded proteins then propagate within and/or between hosts to cause phenotypic changes, such as neurodegenerative disease.

14-3-3 protein
A protein biomarker of neuronal damage that is not disease-specific for Creutzfeldt–Jakob disease. However, elevated cerebrospinal fluid levels of 14-3-3 can serve as a useful diagnostic aid for for distinguishing Creutzfeldt–Jakob disease from other dementias.

PRNP methionine/valine (MV) polymorphism
The normal polymorphism at codon 129 of the human prion protein (*PRNP*) gene that codes for the prion protein.

are MM at codon 129, except for a report in 2016 that described one affected patient with MV (http://www.cjd.ed.ac.uk/). Although vCJD prions have been identified in the spleen and appendix in MV individuals who received blood or factor VIII plasma derivatives from donors who subsequently developed vCJD, these individuals have not, to date, developed clinical symptoms of vCJD[26–28]. Moreover, no reports of clinical vCJD in VV individuals have been observed to date. However, anonymous appendix screening of appendix tissue in the UK has shown that vCJD PrP^{CJD} is present in ~1 in 2,000 asymptomatic individuals independent of codon 129 genotype[29]. Collectively, these findings raise public health concerns that MV and VV individuals could act as asymptomatic carriers and perpetuate prion infections among the population via blood or plasma donations[30].

In the UK, the costs of importing blood from abroad to prevent the potential spread of prion through blood or blood derivatives is enormous, and a simple and effective screening test is, therefore, urgently needed[31].

In vivo diagnosis of sporadic CJD

Changes in cortical and subcortical grey matter detected by conventional fluid-attenuated inversion recovery (FLAIR) or diffusion-weighted MRI sequences, PSWCs seen on EEG, and detection of 14-3-3 protein in the CSF are helpful in distinguishing sCJD from other rapidly progressive dementias[32–35]. However, acute neuronal damage in other neurological diseases, such as encephalitis, vascular disorders, malignancies (for example, primary CNS lymphoma, intravascular lymphoma or infiltrative diffuse astrocytosis) and metabolic disorders, can occasionally cause biomarker changes similar to those seen in CJD[9,36,37]. Diagnostic accuracy for sCJD is increased by the combination of 14-3-3 protein positivity and elevated tau protein levels in the CSF, although tau has never been included in the international diagnostic criteria[38].

The lack of a reliable intravital test for sCJD was a challenge for many years. Early evidence for prion infectivity of peripheral tissues and biological fluids was obtained from transmission studies in nonhuman primates and transgenic mice expressing humanized PrP^{CJD} (REFS 39,40). These studies indicated that kidney, lung, eye, spleen, lymph nodes and blood are potentially infectious, albeit with a relatively low efficiency. Accordingly, PrP^{CJD} deposition was occasionally detected by immunoblotting and immunohistochemistry in the spleen, lymph nodes and muscles. However, samples from these tissues are difficult to obtain in living patients, and the chance of detecting PrP^{CJD} from such samples is relatively low: sensitivity of immunoblotting and immunohistochemistry to detect PrP^{CJD} is 55% in the spleen and 27–33% in the muscle, and the sensitivity of this technique to detect PrP^{CJD} in the lymph nodes has not been determined[41–44] (FIG. 1).

More recently, involvement of the olfactory pathway, including the olfactory neuroepithelium, was reported on the basis of findings in deceased individuals with sCJD and in a biopsy specimen of olfactory mucosa obtained from a patient with sCJD, suggesting that olfactory mucosa is potentially useful for the *in vivo* diagnosis of CJD[45,46] (FIG. 1). Although the detection of PrP^{CJD} by immunoblotting and immunohistochemistry is highly specific, the diagnostic sensitivity of this method is relatively low; moreover, biopsy of olfactory mucosa is too invasive a procedure for widespread use, leading to the dismissal of this method as a future diagnostic strategy[46].

According to current guidelines, definitive diagnosis of CJD requires neuropathology or the detection of PrP^{CJD} in brain tissue either by immunohistochemical staining or immunoblotting. Usually, such tissue is collected postmortem, but it can also be obtained from brain biopsies. The rarity of the latter procedure means that most definitive diagnoses are made postmortem, in part because conventional immunochemical tests lack

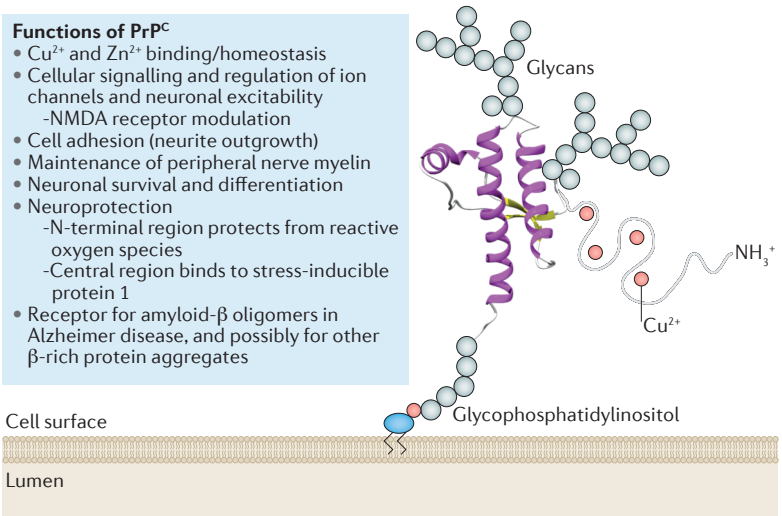
Box 1 | **Structure and function of the cellular prion protein**

The normal cellular prion protein (PrP^C) is a glycophosphatidylinositol-anchored glycoprotein with a largely α-helical (magenta) C-terminal domain and an intrinsically disordered N-terminal domain that binds Cu^{2+} and Zn^{2+}. Typically, PrP^C is exposed on the cell surface, but it can also be located on the lumenal side of intracellular organelles or vesicles.

PrP^C is expressed in several cell types, both in the nervous system and peripheral tissues, although it is most abundant in neurons. The vast majority of PrP^C molecules are synthesized in the endoplasmic reticulum and Golgi apparatus as glycoproteins that are bound to cellular membranes by a glycophosphatidylinositol anchor. Typically, PrP^C molecules follow the secretory pathway to the cell surface, where they are exposed to the extracellular milieu. PrP^C can then be re-internalized into endocytic vesicles and recycled to the cell surface. PrP^C can also be cleaved internally at two different sites by endogenous proteases to generate N-terminal and C-terminal fragments.

Although it is clear that the conversion of PrP^C to pathological forms underpins the prion diseases, describing an overarching physiological role for PrP^C in healthy individuals has remained difficult. Manipulation of PrP^C levels has been reported to influence a variety of cellular functions and to result in altered host phenotypes, including impairments in metal homeostasis, development, synaptic plasticity, circadian rhythm, and stress responses[68–71]. However, some of the reported PrP^C knockout phenotypes might be attributed to flanking genes[72]. In prion diseases, most, if not all, of the α-helical structure PrP^C is refolded to β sheets and loops concurrent with assembly into disease-associated PrP multimers, such as amyloid fibrils, with as yet unresolved tertiary and quaternary structures. Besides its involvement in prion diseases, one the more renowned pathophysiological roles of PrP^C is mediation of some of the neurotoxic effects of amyloid–β oligomers in Alzheimer disease models[71].

Functions of PrP^C
- Cu^{2+} and Zn^{2+} binding/homeostasis
- Cellular signalling and regulation of ion channels and neuronal excitability
  - -NMDA receptor modulation
- Cell adhesion (neurite outgrowth)
- Maintenance of peripheral nerve myelin
- Neuronal survival and differentiation
- Neuroprotection
  - -N-terminal region protects from reactive oxygen species
  - -Central region binds to stress-inducible protein 1
- Receptor for amyloid-β oligomers in Alzheimer disease, and possibly for other β-rich protein aggregates



NMDA, *N*-methyl-D-aspartate.

Table 1 | **Human prion diseases: incidence, genetics and clinical characteristics**

Disease	Aetiology	Overall prevalence	*PRNP* pathogenic mutations	Clinical features
CJD	Sporadic	90% of all CJD	None	Cognitive decline, behavioural, visual, motor and ataxic dysfunctions, myoclonus and akinetic mutism
	Genetic	~10% of all CJD	Puntiform or octapeptide insertions	Usually similar to sporadic CJD
	Iatrogenic	Rare	None	Usually similar to sporadic CJD
	Variant	Rare	None	Psychiatric and sensory symptoms, ataxia, involuntary movements with a mean duration of more than 6 months
Fatal insomnia	Sporadic	Rare	None	Cognitive decline, ataxia, psychiatric signs, insomnia
	Genetic	Rare	Asp178Asn in association with 129M	Insomnia, dysautonomia, ataxia, myoclonus, epileptic seizures
Variably protease-sensitive prionopathy	Sporadic	Rare	None	Cognitive decline, psychiatric symptoms, ataxia
Gerstmann–Sträussler–Scheinker syndrome	Genetic	Rare	Puntiform or octapeptide insertions	Progressive dementia, ataxia, extrapyramidal and pyramidal signs (Pro102Leu mutation might result in a phenotype similar to sporadic CJD)
PrP systemic amyloidosis	Genetic	Three families	Stop-codon mutation at codon 163 or 195	Sensory and/or sensorimotor autonomic neuropathy

CJD, Creutzfeldt–Jakob disease; M, methionine; *PRNP*, prion protein gene.

sufficient sensitivity to detect the minute amounts of PrP^{CJD} that are present in tissues other than the brain, including the CSF.

The development of an ultrasensitive ELISA in which PrP^{CJD} detection is improved by a steel powder capture technique provides a promising alternative approach to conventional testing. This test detects very low concentrations of PrP^{CJD}, with 10^{-10} dilutions of vCJD-affected brain giving positive results. This test has a sensitivity of 71% and a specificity of 100% for diagnosing vCJD from blood samples, but it failed to detect PrP^{CJD} in the blood of patients with sCJD or other human prion diseases[47,48].

Seeding assays for PrP^{CJD}

Protein misfolding cyclic amplification. Considerable progress has been made in establishing more-sensitive tests for PrP^{CJD} by exploiting its self-propagation activity. Following initial demonstrations that PrP^{CJD} itself can induce the conversion of PrP^{C} to a PrP^{CJD}-like state *in vitro*[49], highly sensitive PrP^{CJD} amplification reactions known as protein misfolding cyclic amplification (PMCA) reactions were developed[50–53]. PMCA assays take advantage of the fundamental replication mechanism of prions, namely, the prion-induced seeded conversion of PrP^{C} into more prions[54] (FIG. 2). Test samples are combined with homogenates of brain tissue or cells that contain PrP^{C}, and are subjected to cycles of sonication and rest[50–54]. PrP^{CJD} in the sample induces the conversion of PrP^{C} into much greater quantities of PrP^{CJD}, which can then be detected by immunoblotting or surround optical fibre immunoassay (SOFIA)[43]. SOFIA by itself is ~10^{8}-fold more sensitive than capture ELISA for detection of sCJD and vCJD in brain tissue. In experimental animal models, PMCA and SOFIA can be much more sensitive for infectivity than are bioassays, and can detect as little as 10^{10}–10^{12}-fold dilutions of sCJD and/or vCJD brain homogenate[43].

The aforementioned studies suggested that, in principle, diagnosis of CJD should be possible on the basis of samples that contain much lower levels of PrP^{CJD} than are observed in brain tissue. Indeed, in a study that combined PMCA with SOFIA detection, PrP^{CJD} was detected in the CSF of all 10 patients with sCJD but none of the 10 non-CJD controls[43]. PMCA with immunoblotting can also detect PrP^{CJD} in the urine of patients with vCJD[43,51]. In one study, positive PMCA reactions were observed in 13 of 14 urine samples from patients with vCJD, but not in any samples from 224 non-CJD controls, giving an estimated sensitivity of 93% and a specificity of 100%[51]. As seen with the blood sample assay with PMCA followed by SOFIA[43], however, PMCA followed by immunoblotting did not detect PrP^{CJD} in the urine of patients with sCJD. Unfortunately, from a practical diagnostic perspective, the PMCA assays have typically been hindered by the following requirements: brain or cell homogenates as sources of PrP^{C} substrate; technically challenging sonications; read-outs that are time-consuming and low-throughput (immunoblotting); and extended assay times (for example, 4–5 days in the case of the urine test for vCJD)[51]. Moreover, PMCA reactions, in faithfully duplicating prion propagation, generate large amounts of CJD infectivity.

RT-QuIC. To address the practical shortcomings discussed above, researchers have pursued alternative methods for detecting PrP^{CJD}-associated seeding activity. These collective efforts have culminated in real-time quaking-induced conversion (RT-QuIC) assays[55–65] (FIG. 2). Like PMCA, RT-QuIC assays are based on prion-seeded conversion of PrP^{C} into abnormal, self-propagating aggregates. However, the RT-QuIC products have structures that are somewhat different from *bona fide* PrP^{CJD}, and have not caused any clinical manifestations of disease on intracerebral inoculation into rodents (B. Caughey *et al.*,

Protein misfolding cyclic amplification (PMCA)
A highly sensitive and specific *in vitro* prion amplification reaction in which a test sample is mixed with suitable sources of PrP^{C} (usually brain homogenates) and subjected to cycles of sonication and rest; amplified protease-resistant, infectious prions are typically detected by western blot after proteinase K treatment.

RT-QuIC
Real-time quaking-induced conversion (RT-QuIC) is a highly sensitive and specific *in vitro* test for prion-associated seeding activity in which a test sample is mixed with recombinant PrP^{C} in multiwell plates that are subjected to cycles of shaking and rest. As the reaction progresses, prion-seeded, but apparently non-infectious recombinant PrP amyloid fibrils are detected by enhanced fluorescence of an amyloid-sensitive dye.

unpublished work). RT-QuIC reactions can be performed in 96-well plates, and the prion-seeded products can be detected with fluorescence plate readers, using the amyloid-sensitive dye thioflavin T. RT-QuIC assays are often as sensitive as PMCA, but have the advantages of using bacterially expressed recombinant PrP^{C} as a substrate, easily replicable shaking and fluorescence as a read-out, all of which facilitate high-throughput analyses.

Several research groups have conducted large studies in which CSF samples collected from sCJD patients and controls were evaluated using RT-QuIC assays[55–61]. Together, these studies comprise analyses of hundreds of sCJD cases and an even larger number of controls. Diagnostic sensitivities observed in these studies range from 77–97%, and specificities range from 99–100%[55,56]. These data provide strong evidence that RT-QuIC analysis of CSF can serve as a highly accurate and practical antemortem diagnostic test for sCJD. The specificity values of <100% that have been reported in some of the aforementioned studies are attributed to rare patients who were PrP^{CJD}-positive on RT-QuIC reactions, but were not diagnosed clinically with a prion disease. It remains unclear whether these cases might represent actual false positives arising from prion-free individuals, or true positives whose prion infection was disguised clinically by other concurrent diseases.

The diagnostic validity of RT-QuIC testing seems robust in all sCJD subtypes, and does not seem to be affected by the timing of lumbar puncture. It is unlikely, therefore, that samples tested early in the clinical phase of sCJD would be particularly prone to yield false-negative results[56]. However, a patient's codon 129 *PRNP* genotype or PrP^{CJD} glycotype might influence the performance of the test[56,59,60]. Whether PrP^{CJD} seeding activity can be detected in the CSF of healthy individuals carrying *PRNP* mutations, or of those exposed to potentially prion-contaminated medical or surgical procedures, remains to be explored.

From a more technical perspective, the various laboratories performing CSF analyses have each employed somewhat different RT-QuIC assay protocols with respect to reaction buffer composition, temperature, shaking motion and speed, and recombinant PrP^{C} substrate. Given that the results reported by different groups are fairly similar, at least some variations in RT-QuIC protocol can clearly be tolerated. The CJD-associated RT-QuIC seeding activity in CSF specimens is stable under a variety of storage conditions[58]. However, blood contamination can interfere with the assay, so removal of erythrocytes within 3 days of collection has been recommended[58]. In our experience, the most demanding aspect of RT-QuIC assays is the preparation of suitable recombinant PrP^{C} substrate, that is, one that is readily convertible to amyloid in the presence of prion seeds, but is very slow to convert spontaneously in their absence. Recently, a new PrP^{C} substrate (Syrian golden hamster residues 90–231) was described. This substrate, when combined with adjustments to RT-QuIC reaction conditions, improved the diagnostic sensitivity to ~96% without sacrificing specificity[60]. These conditions also markedly shortened assay times and allowed the detection of extremely dilute, subinfectious levels of PrP^{CJD} in a matter of hours. However, more-extensive multicentre testing of these new protocols is warranted before their diagnostic performance can be fully established.

Detection of PrP^{CJD} in the olfactory mucosa

On the basis of presences of PrP^{CJD} in the olfactory neuroepithelium[45], samples from the olfactory mucosa provide another promising strategy for antemortem diagnosis of CJD. Brushings from the olfactory mucosa can be collected by a simple, rapid and gentle brushing procedure (see Supplementary information S1 (video))[61]. The olfactory mucosa sampling procedure involves visualizing the nasal vault with a rigid, sheathed fibroscope and brushing the mucosal surface with a narrow swab.

Table 2 | **Codon 129 genotype and PrP^{CJD} glycotype influence disease phenotype in CJD**

PrP^{CJD} glycotype	Overall occurrence of PrP^{CJD} glycotypes (%)	Codon 129 genotype	Occurrence in different subtypes (%)	Common clinical signs at onset	Survival (months)	Sensitivity of supportive tests (%)		
						EEG (PSWCs)[12,23]	MRI[32,34]	Protein 14-3-3 (REFS 12,33)
Sporadic CJD								
Type 1	70	MM	90	Cognitive or ataxic	4	73	70	91
		MV	7	Cognitive or ataxic	5	70	70	86
		VV	3	Cognitive	11	42	60	90
Type 2A	30	MM	15	Cognitive	12.5	44	60	79
		MV	36	Ataxic	12	17	79	100
		VV	49	Ataxic	6	13	77	100
Variant CJD								
Type 2B	100	MM	100	Psychiatric or sensory	14	0*	90	50
		MV	<1‡	NA	NA	NA	NA	NA
		VV	0	NA	NA	NA	NA	NA

CJD, Creutzfeldt–Jakob disease; M, methionine; NA, not applicable; PSWCs, periodic sharp and slow waves complexes; PrP^{CJD}, protease-resistant prion protein; V, valine. *Detected only in the late stages of the disease. ‡One reported case.

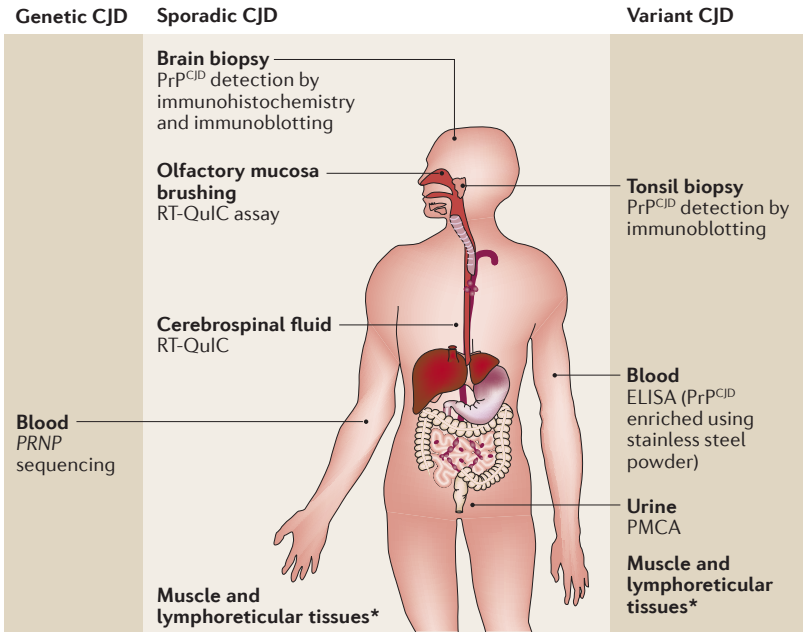


Figure 1 | **Tissue and body fluid samples for prion protein detection in patients with CJD.** Diagnosis of the different forms of Creutzfeldt–Jakob disease (CJD) requires samples from different tissues. In genetic CJD, DNA sequencing of blood samples detects prion protein gene (*PRNP*) mutations or insertions. In sporadic CJD, CJD-specific misfolded prion protein (PrP^{CJD}) detected in the brain biopsy samples provides a definitive diagnosis of the disease. Real-time quaking-induced conversion (RT-QuIC) assay of the cerebrospinal fluid (CSF) and olfactory mucosa can demonstrate the presence of PrP^{CJD} with a sensitivity and specificity of nearly 100%. In peripheral tissues, PrP^{CJD} has occasionally been detected postmortem in muscle, spleen and lymph nodes by immunoblotting and/or immunohistochemistry. In variant CJD, PrP^{CJD} can be detected in tonsil biopsies by immunoblotting and/or immunohistochemistry, and in blood and urine by ELISA and protein misfolding cyclic amplification (PMCA). Postmortem, PrP^{CJD} has also been detected in muscle and lymporeticular tissues. *PrP^{CJD} detected from postmortem samples by immunohistochemistry and immunoblotting.

To maximize the likelihood of contacting the olfactory mucosa and olfactory sensory neurons, which contain PrP^{CJD}, a series of three or four brushings is obtained from both nostrils, because respiratory mucosal surfaces adjacent to the olfactory mucosa seem to lack detectable amount of prion-seeding activity[61,62].

When brushings of the olfactory mucosa were subjected to RT-QuIC analysis, levels of PrP^{CJD} seeding activity that were orders of magnitude higher than those in the CSF were detected rapidly in most samples from patients with sCJD. To date, nasal brushings from a total of 43 patients with sCJD and 43 non-CJD controls have been assessed, and have indicated an overall diagnostic sensitivity of 97.5% and a specificity of 100%. This sensitivity is superior to that achieved by testing CSF samples from the same patients (77%).

Despite the encouraging results, further evaluation of olfactory mucosa testing is needed to better establish its diagnostic performance and to determine how early in the course of CJD infection seeding activity can be detected. RT-QuIC seeding activity has also been detected in olfactory mucosa samples from 10 patients with genetic prion disease attributed to E200K, V210I, V180I or P102L *PRNP* mutations, but much more work will be needed to determine the extent to which the nasal brushing approach is useful for monitoring disease progression in the many genetic prion diseases of humans (Bongianni, M., Zanusso, G. *et al.*,unpublished work). The results obtained to date suggest that RT-QuIC testing of nasal brushings could become a viable alternative or a confirmatory adjunct to CSF testing for antemortem diagnosis of human prion diseases. In addition to its potential utility in antemortem testing, the nasal brushing approach might also be helpful for postmortem analyses to confirm or rule out sCJD diagnosis without the need for autopsy. However, unlike brain specimens from autopsies, postmortem olfactory mucosa samples would not provide information on the PrP^{CJD} glycotype, the distribution of lesions, the pattern of PrP deposition, or associated pathologies, and cannot, therefore, fully replace neuropathological studies.

Finally, although most of the RT-QuIC studies of human prion disease to date have focused on sCJD, other studies have shown that RT-QuIC can also detect vCJD very sensitively in brain tissue or in a small amount of homogenized brain tissue diluted in plasma[63]. Moreover, through use of a recombinant PrP^{C} substrate derived from bank voles, all 28 of the different prion strains seen in humans and animals that have been tested so far have been detectable by RT-QuIC[64]. From a practical perspective, this finding means that a single assay can be used to detect most, if not all, prion diseases.

Conclusions

Preventive health measures have dramatically reduced the incidence of iatrogenic transmission of CJD and exposures to BSE-infected materials that have caused vCJD in humans. However, such measures do not apply to sCJD, which is likely to arise spontaneously. Thus, this most common form of CJD will persist as a source of prion infectivity and mortality.

RT-QuIC assays of CSF and olfactory mucosa brushings can provide sCJD diagnosis with a specificity and sensitivity of nearly 100% in less than a day. However, samples from a large group of patients with other diagnoses, including treatable, rapidly progressive dementias, should be tested to further confirm these promising initial results.

Brown and Farrell suggested that RT-QuIC testing of nasal brushings and the CSF should be applicable to all patients admitted with symptoms of dementia or cerebellar signs; such a procedure would virtually eliminate the risk of iatrogenic CJD transmission[13]. These authors profiled three recent surgical patients who were given non-CJD diagnoses and were then operated on with instruments that had not been subjected to specialized prion sterilization[13]. Later diagnoses of CJD in these patients led to the realization that many patients who had undergone surgery with potentially CJD-contaminated instruments might have been at risk of iatrogenic exposures. Total prevention of accidental prion exposure of instruments during surgery or endoscopy may not be feasible, because some patients might undergo surgery a few weeks or months before the clinical onset of CJD. Therefore, systematic prion inactivation on surgical instruments remains strongly recommended[13].

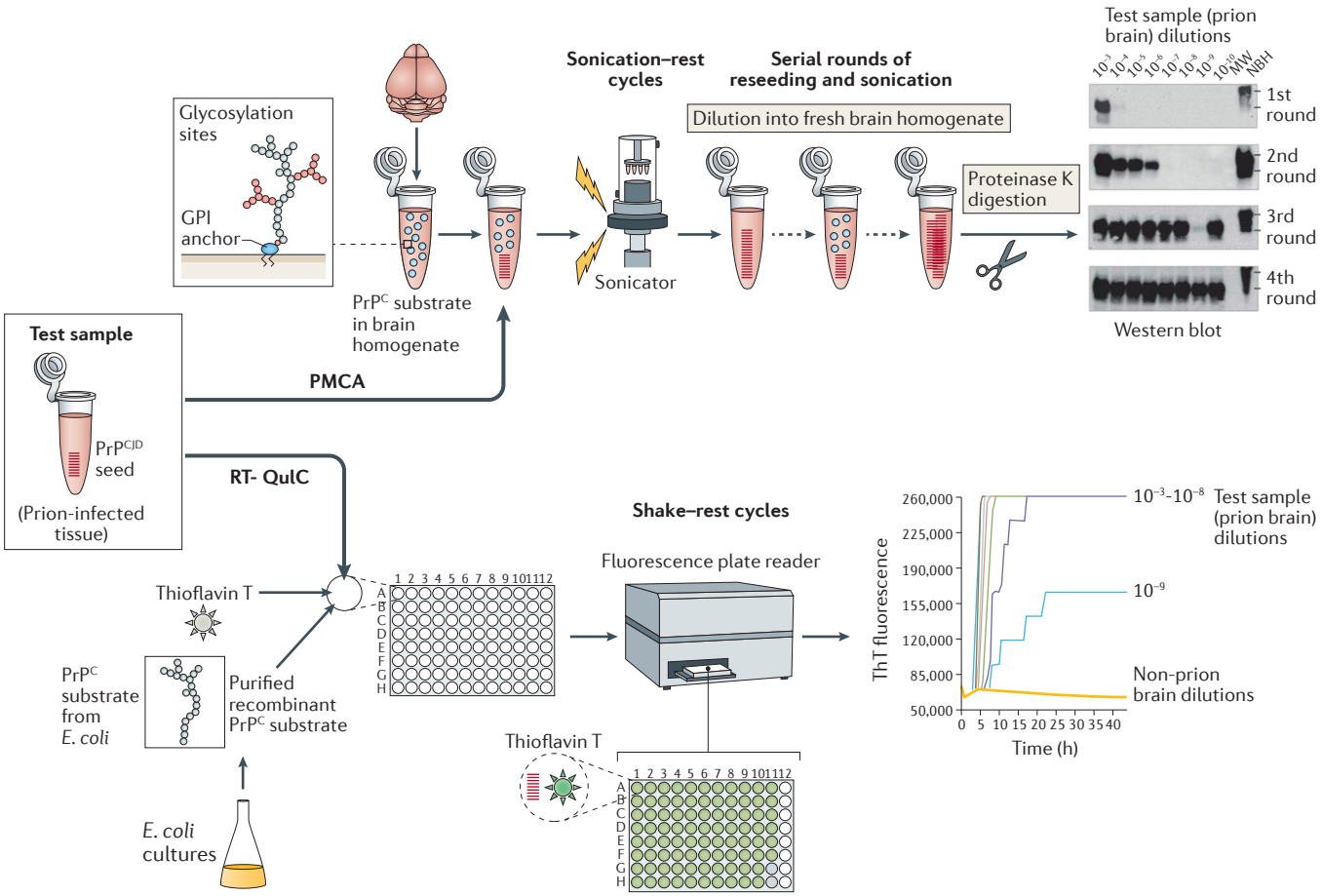


Figure 2 | **Diagnostic testing for Creutzfeldt–Jakob disease: PMCA versus RT-QuIC.** The principles of protein misfolding cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC) are illustrated. In PMCA, the test sample is mixed with a suitable source of normal prion protein (PrP^{C}), usually uninfected brain homogenate, and subjected to cycles of sonication and rest, typically for 48 h. Additional rounds of PMCA are performed by diluting products of the previous round into fresh brain homogenate, followed again by sonication cycles. The typical readout is the detection of any prion-seeded, protease-resistant PrP reaction products by digestion with proteinase K (to eliminate any remaining PrP^{C} substrate in the uninfected brain homogenate) and western blotting. The western blot shows that the number of rounds required to generate a detectable band correlates inversely with the prion concentration in the test sample. In the example shown, reactions were seeded with serial 10-fold dilutions of brain homogenate from an individual with prion disease. In RT-QuIC, the test sample is mixed with recombinant PrP^{C} in multiwell plates and subjected to cycles of shaking and rest. As the reaction progresses, prion-seeded recombinant PrP amyloid fibrils are detected by the enhanced fluorescence of thioflavin T (ThT), an amyloid-sensitive dye. The graph provided shows the cumulative ThT fluorescence from eight replicate wells seeded with serial 10-fold dilutions of prion brain homogenate. The stepwise increases in fluorescence are due to rapid growth of prion-seeded amyloid fibrils in individual wells after different lag phases, which is often more evident in reactions seeded with extreme dilutions of prion-containing samples. Permission obtained from Macmillan Publishers Ltd © Morales, R. *et al. Nat. Protoc.* **7**, 1397–1409 (2012). GPI, glycophosphatidylinositol; PrP^{Sc}, scrapie prion protein.

The robustness of the RT-QuIC assay suggests that this test should be included as a key diagnostic criterion for sCJD. We anticipate that in most cases, the initial testing would involve analysis of a CSF sample. However, if a CSF sample is unavailable, or if initial RT-QuIC testing of the CSF is negative, RT-QuIC testing of an olfactory mucosa brushing is indicated.

Aggregation of misfolded proteins is also implicated in other neurodegenerative disorders, such as Alzheimer disease (misfolded Aβ and tau) and Parkinson disease (α-synuclein). *In vitro* amplification studies have shown that, like PrP^{CJD}, these misfolded protein aggregates can seed polymerization of the normal protein isoforms via prion-like mechanisms of replication[66]. In addition, the olfactory bulb is known to be an early site of involvement in both Alzheimer disease and synucleinopathies, and anosmia is an early symptom in synucleinopathies[67]. Thus, it seems plausible that the development of highly sensitive seeding assays of olfactory mucosa brushings, analogous to the RT-QuIC test for prion diseases, might assist intravital diagnosis of other brain proteinopathies.

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Author contributions
G.Z. and B.C. researched literature for the article and provided substantial contributions to discussion of content. All authors participated in writing of the article and in reviewing/editing the manuscript before submission.

Competing interests statement
B.C. is an inventor on patents and patent applications related to RT-QuIC assays. The other authors declare no competing interests.

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ERRATUM

Advanced tests for early and accurate diagnosis of Creutzfeldt–Jakob disease

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In the version of this article initially published online and in print, the Figure 2 erroneously depicted recombinant prion protein with attached glycans, and Table 2 listed an erroneous glycotype for sporadic Creutzfeldt–Jakob disease. The errors have been corrected for the PDF and HTML versions of the article.