Sporadic Creutzfeldt-Jakob disease: Real-Time Quaking-Induced Conversion (RT-QuIC) assay represents a major diagnostic advance

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ABSTRACT

Sporadic Creutzfeldt-Jakob disease (sCJD) is a rare and fatal neurodegenerative disorder with an incidence of 1.5 to 2 cases per million population/year. The disease is caused by a proteinaceous infectious agent, named prion (or PrP^{Sc}), which arises from the conformational conversion of the cellular prion protein (Pr^{PC}). Once formed, PrP^{Sc} interacts with the normally folded Pr^{PC} coercing it to undergo similar structural rearrangement. The disease is highly heterogeneous from a clinical and neuropathological point of view. The origin of this variability lies in the aberrant structures acquired by PrP^{Sc}. At least six different sCJD phenotypes have been described and each of them is thought to be caused by a peculiar PrP^{Sc} strain. Definitive sCJD diagnosis requires brain analysis with the aim of identifying intracerebral accumulation of PrP^{Sc} which currently represents the only reliable biomarker of the disease. Clinical diagnosis of sCJD is very challenging and is based on the combination of several clinical, instrumental and laboratory tests representing surrogate disease biomarkers. Thanks to the advent of the ultrasensitive Real-Time Quaking-Induced Conversion (RT-QuIC) assay, PrP^{Sc} was found in several peripheral tissues of sCJD patients, sometimes even before the clinical onset of the disease. This discovery represents an important step forward for the clinical diagnosis of sCJD. In this manuscript, we present an overview of the current applications and future perspectives of RT-QuIC in the field of sCJD diagnosis.

Key words: Sporadic Creutzfeldt-Jakob disease; olfactory mucosa; cerebrospinal fluid; neurodegeneration; peripheral biomarkers; prion; seeding aggregation assays.

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Molecular and neuropathological classification of sCJD subtypes

Among human prion diseases, sporadic Creutzfeldt-Jakob disease (sCJD) is the most common form affecting 1-2 individuals/million per year with similar distribution in males and females.[1] The age at onset is most frequently between 55 and 75 years.[2] sCJD presents with variable disease subtypes characterized by peculiar clinical and neuropathological features. In the past, other than the classical and more common subtypes, some clinical variants such as the Heidenhain, the myoclonic, the thalamic, the cerebellar or ataxic, and the panencephalopathic forms were reported.[3–6] In general, sCJD cases present as multifocal and rapidly progressive encephalopathies with dementia, cerebellar ataxia, myoclonus while the progression of the disease results in an akinetic and mute state and the death occurs generally within 6 months after the disease onset.[1] The common mechanism underlying these pathologies is the spontaneous conformational conversion of the cellular prion protein (PrP^{C}) into an abnormally folded conformer named prion or PrP^{Sc}. This latter propagates in an autocatalytic manner in the brain by converting the PrP^{C} into the pathological isoform.

PrP^{C} is a glycosylphosphatidylinositol (GPI) anchored protein highly expressed in the central nervous system (CNS) and encoded by the *PRNP* gene located on chromosome 20 in humans.[7,8] After its synthesis in the rough endoplasmic reticulum, PrP^{C} undergoes post-translational modifications comprising the C-terminal addition of the GPI anchor, the formation of a disulfide bridge between two C-terminal cysteine residues (Cys179-Cys214) and the N-linked glycosylation at asparagine residues (Asn181-Asn197).[9] These oligosaccharides are further modified in the Golgi apparatus to produce complex-type chains enriched in sialic acid important for the synaptic localization of PrP^{C}.[10,11] The different degrees of PrP^{C} glycosylation give rise to three isoforms of the protein: the di-glycosylated (70%), the mono-glycosylated (25%) and the un-glycosylated (5%) species.[10,12] All these isoforms are rich in α-helices structures, soluble in detergent and are sensitive to proteolytic digestion with proteinase K (PK). Conversely, PrP^{Sc} is less soluble in detergent, has higher amount of β-sheet structures and is partially resistant to PK digestion. The limited proteolysis leads to the generation of N-terminal truncated fragments of di-, mono- and un-glycosylated PrP^{Sc} that migrate at lower molecular weights compared to those of PrP^{C}.[13] Moreover, the un-glycosylated band of PrP^{Sc} can acquire two distinct molecular weights: 21 or 19 kDa which are referred to as type 1 or type 2 PrP^{Sc}, respectively.[14] Neuropathologically, the main hallmarks of sCJD are spongiform changes, astoglial activation, neuronal loss and accumulation of PrP^{Sc} (Figure 1 and Figure 2).[15]

At present, PrP^{Sc} is the only disease-specific biomarker for sCJD and the definite diagnosis can be formulated post-mortem by biochemical and neuropathological analyses aimed at identifying the PrP^{Sc} accumulation in the CNS (Figure 2).[16,17]

It is well known that PrP^{Sc} can acquire different abnormal conformations, named strains. The peculiar conformation of each strain can be faithfully transmitted to the host PrP^{C} and are believed to be responsible for the heterogeneity of prion diseases, in terms of tissue tropism, incubation period, clinical signs, neuropathological changes and interspecies transmission properties.[18-21] In 1999, Parchi and colleagues[22] classified sCJD in six major subtypes by correlating the clinical manifestations with the polymorphisms at codon 129 of the *PRNP* gene, *i.e.* methionine (M) or

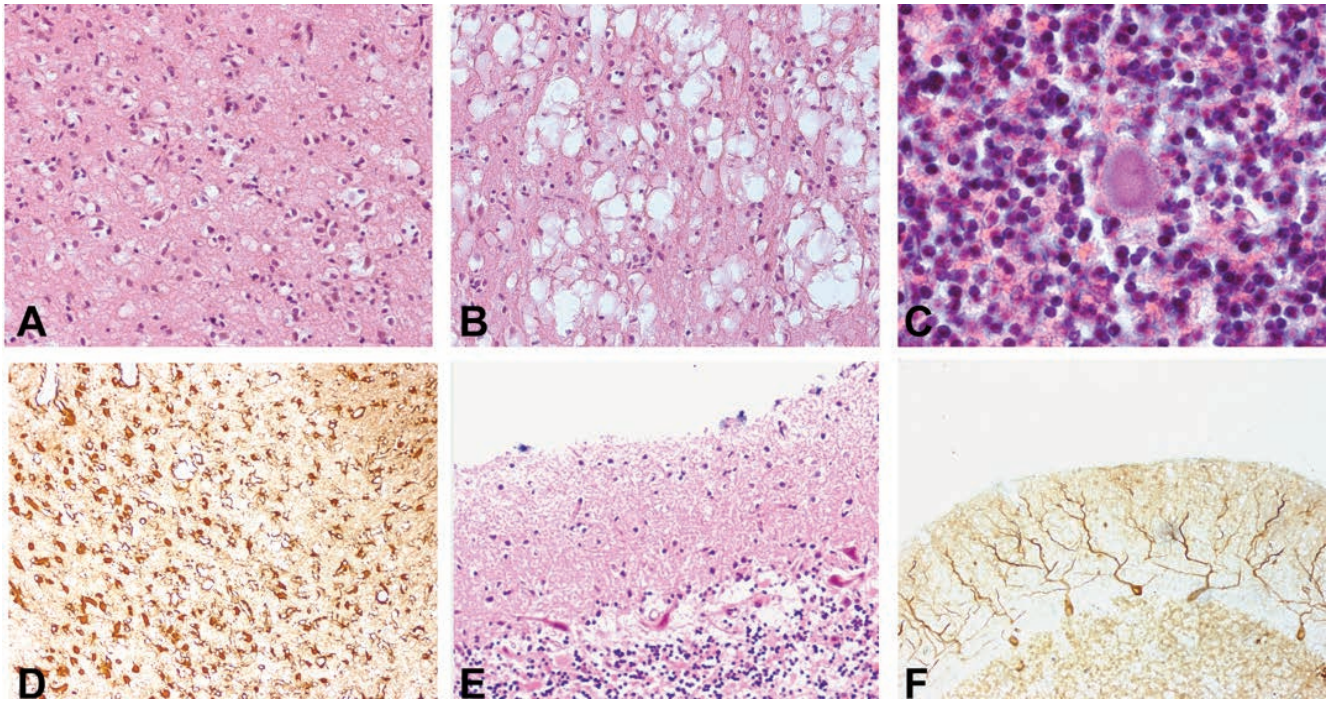

Figure 1. Creutzfeldt-Jakob disease, hallmark neuropathologic lesions. Spongiform changes may appear as small vacuoles (A) diffusely present in grey matter (H&E, cerebral cortex, 20x) or large, confluent vacuolar lesions (B) typical of the MM2-C (cortical) subtype (H&E, cerebral cortex, 20x). Kuru plaques (C) small aggregates of PrP with the tinctorial and optical properties of amyloid are typically found in the cerebellum in MV2 subtype (H&E, 60x). Astrogliosis (D) may be severe in all subtypes of Creutzfeldt-Jakob disease (glial fibrillary acidic protein immunohistochemistry, 10x). Neuronal loss (E) is usually very severe in the cerebral cortex, basal ganglia and cerebellum (H&E, cerebellum) but may be mild in some cases (F) (microtubule associated protein 2 immunohistochemistry; 10x).

valine (V), and the electrophoretic mobility of the un-glycosylated PrP^{Sc} isoform in the brain after digestion with PK (type 1 or type 2 PrP^{Sc}). These findings demonstrated that the presence of M or V at codon 129 of PrP^{C}, as well as other still unknown factors, could modulate the structural rearrangement of PrP^{C} during misfolding, thus promoting the PrP^{Sc} strains variability.[23-26] In addition, compelling evidence suggests that, in some sCJD cases, the CNS contains a mixture of PrP^{Sc} strains (*e.g.*, MM1+2, VV1+2 and MV1+2), which make the classification of the disease even more challenging (as discussed in the next paragraphs).[14,27-29]

The main pathological characteristics of each sCJD subtype are summarized in Table 1.

MM1 and MV1 subtype

MM1 is the most common form of sCJD (67% of all cases) while MV1 cases are rare (3%). Western blot analysis shows, for both subtypes, type 1 PrP^{Sc} and a glycoform pattern characterized by the predominance of the mono-glycosylated band. Despite the difference at codon 129 of *PRNP*, MM1 and MV1 cases share many pathological features. MM1/MV1 CJD patients present with the myoclonic (or classic CJD) and the Heidenhain's variant.[22] The mean age at onset of the disease is 66 years with an average clinical duration of 4 months. Clinical manifestations include cognitive impairment with memory loss and confusion/disorientation, depression, anxiety, psychosis and gait or limb ataxia.[30] Neuropathologically, the brain of these patients shows spongiosis with fine vacuoles. The basal ganglia, thalamus and cerebellum are less affected than the cerebral neocortex. The hippocampal cortex and brain stem are largely spared. The pattern of PrP^{Sc} deposition is synaptic and mainly affects the cerebral cortex while the cerebellum, the basal ganglia and thalamus are less involved (Figure 2 A,B).[31] Moreover, the amount of PrP^{Sc} signal directly correlates with the severity of spongiosis.

VV2 subtype

The VV2 subtype corresponds to the cerebellar or ataxic variant and occurs in 15% of sCJD cases. The Western blot profile shows type 2 PrP^{Sc} with a preponderance of the mono-glycosylated isoform. The mean age at onset is 64 years (with a range of 40-83 years) and the clinical duration is about 7 months.[32] Ataxia is the commonest early clinical feature accompanied by cognitive impairment and oculomotor signs while myoclonus is less frequent. In the late stages of the disease patients exhibit dementia, myoclonus and pyramidal signs. Neuropathologically, the spongiosis preferentially affects the deep layers of the frontal and occipital cortex, the entorhinal cortex and the hippocampus.[31] Cerebral neocortex may be relatively spared particularly in cases with rapid courses. The cerebellar cortex is atrophic, with abundant PrP^{Sc} deposits characterized by a focal and plaque-like pattern that are negative for Congo Red and Thioflavin-S (amyloid stains). In addition, strong PrP^{Sc} deposition often occurs around neuronal perikarya in the cerebral cortex (Figure 2 C,D). The distribution of PrP^{Sc} immunostaining is affected by the disease duration. In cases with shorter disease duration, PrP^{Sc} involve diffusely the gray-matter region except for the neocortex which is affected only in patients with longer disease duration.[33]

MV2 subtype

MV2 sCJD subtype is phenotypically and biochemically similar to VV2 cases (type 2 PrP^{Sc} and predominance of the mono-glycosylated form) and accounts for 10% of all sCJD. The mean age at onset is 65 years with a range of 36-83 years while the disease

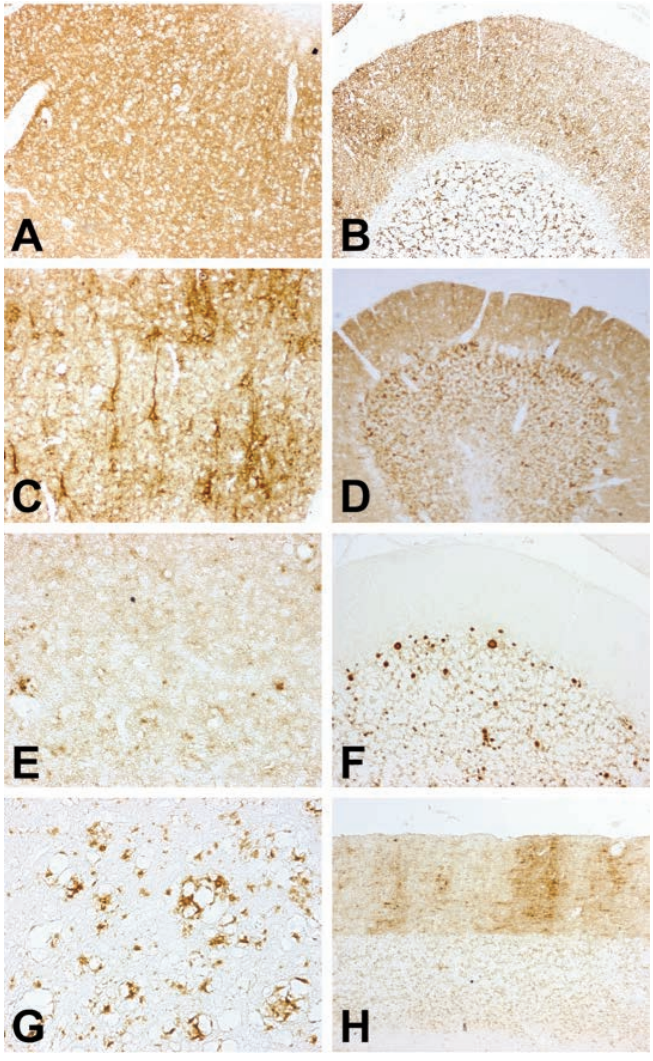

Figure 2. Creutzfeldt-Jakob disease, patterns of pathological PrP deposition (PrP immunohistochemistry using 3F4 monoclonal antibody). In MM/MV1 subtype PrP deposition is mainly of synaptic type and appears as homogeneous, finely granular immunoreactivity in the neuropil of the cerebral cortex (A) (10x), while in the cerebellum is finely granular in the molecular layer while forming coarser deposits in the granular layer (B) (10x). In VV2 subtype PrP deposition often decorates the boundaries of pyramidal neurons of the cerebral cortex (C) (10x), while in the cerebellum plaque-like deposition takes is common in the granular layer (D) (10x). In most of the patients with the MM2 subtype PrP deposition takes variable aspects in the cerebral cortex (E) (20x), while the typical feature is the presence of Kuru plaques, small aggregates of PrP with the tinctorial and optical properties of amyloid, in the cerebellum (F) (10x). MM2-C (cortical) subtype is characterized by PrP immunoreactivity more intense at the rims of the large vacuoles of spongiosis in the cerebral cortex (G) (20x), while the cerebellum is usually relatively spared (H) (10x).

duration is significantly longer than VV2 with a mean of 17 months (range of 5-72 months). Early manifestations of the disease include oculomotor abnormalities, memory loss, behavioral disturbances and signs of the peripheral nervous system or medullary involvement. In MV2 cases ataxia is the most common presenting sign and cognitive deterioration, myoclonus and pyramidal signs, aphasia and apraxia arise during disease progression. The main hallmark that distinguishes MV2 cases from VV2 is the presence of cerebellar Kuru-type amyloid plaques in the Purkinje cell layer (positive for Congo Red and Thioflavin-S) (Figure 2 E,F).

MM2-thalamic subtype

MM2-thalamic (MM2T) sCJD is a rare subtype (2% of all the cases) known also as sporadic fatal insomnia (sFI).[34,35] Biochemical characterization shows type 2 PrP^{Sc} with a predominant mono-glycosylated isoform. The mean age at onset is around 52 years (range 26-71 years) with a mean duration of 16 months (range 8-36 months). Besides insomnia, other common symptoms include dementia, and motor signs as ataxia, dysarthria, tremor, myoclonus and spasticity.[34,36] Thalamus is the most affected brain region especially in the medial dorsal and anterior ventral nuclei where marked atrophy (observed also in the inferior olivary nucleus) and severe astrogliosis is accompanied by prominent neuronal loss. Spongiform changes and faint PrP^{Sc} deposition may be present in the cerebral cortex.

MM2-cortical subtype

This rare cortical variant of MM2 subtype (type 2 PrP^{Sc} with a prevalence of the mono-glycosylated band) represents 2% of all sCJD cases and is characterized by progressive dementia and disturbances of higher cognitive functions, high-frequency aphasia and apraxia and late myoclonus or epileptic seizures.[37] The average age at onset is 64 years with a 49-77 year range and the disease duration is approximately 16 months. Brain lesions are similar to that of MM1 or MV1 subtypes but, despite the relatively long disease duration, the cerebellum is almost spared. Large vacuoles are present in the cerebral cortex, basal ganglia and thalamus which might be confluent. Immunodetection of PrP^{Sc} reveals a coarse pattern of staining which occasionally localizes at the rim of the vacuoles (perivacuolar PrP^{Sc} deposition) (Figure 2 G,H).[31]

VV1 subtype

VV1 is the rarest subtype of sCJD representing 1% of the total cases. The Western blot analysis shows type 1 PrP^{Sc} with a prevalence of the mono-glycosylated isoform. Patients are relatively younger (mean age at onset 44 years) compared to other sCJD subtypes with a mean duration of 21 months (range 17-42 months). Early symptoms include psychiatric or cognitive abnormalities that evolve in extrapyramidal signs and ataxia while myoclonus was observed only in few patients. Massive spongiform lesions affect the cortico-striatal regions while other subcortical regions and cerebellum are almost spared. Although the severe spongiform changes observed in VV1 patients, PrP^{Sc} immunochemistry shows faint punctate staining confined in the cerebral cortex.[31]

Mixed subtypes

Type 1 and type 2 PrP^{Sc} have been found to co-exist in about 35% of sCJD cases and may be present in the same or distinct anatomical regions of the same patient.[29] This finding is more frequent in MM (43%) than MV (23%) and VV (15%) cases.[14] The predominance of PrP^{Sc} type 1 or 2 influences the clinical and neuropathological phenotype of the diseases. The MM1+2 cases mimic the clinical phenotype of MM1 while the PrP^{Sc} deposition is a combination of the typical neuropathological features of MM1 and MM2 (synaptic and perivacuolar patterns, respectively). Conversely, VV1+2 subjects are similar to VV2 sCJD cases in terms of clinical and neuropathological features.[14]

Table 1. Pathological features of sCJD molecular subtypes.

sCJD molecular subtypes	% of cases	Median age at onset (years)	Duration (months)	Main neuropathological alterations
MM1	67	66	~ 4	Diffuse spongiosis with small vacuoles affecting the neocortex, striatum and cerebellar cortex. Synaptic pattern of PrP^{Sc} deposition
MV1	3	66	~ 4	
VV1	1	44	~ 21	Severe spongiosis with fine vacuoles in the cerebral cortex and striatum. Punctate pattern of PrP^{Sc} deposition
MM2 - thalamic	2	52	~16	Atrophy of the thalamus and inferior olivary nuclei with spongiform alterations confined to the cerebral cortex. Weak and synaptic pattern of PrP^{Sc} deposition.
MM2 - cortical	2	64	~ 16	Severe spongiosis with large confluent vacuoles predominantly in cerebral cortex and striatum. Perivacuolar and coarse pattern of PrP^{Sc} deposition.
MV2	10	65	~ 17	Diffuse and confluent spongiosis similar to VV2 subtype. Amyloid Kuru plaques in the molecular and granular layer of the cerebellum.
VV2	15	64	~ 7	Spongiform changes found in the cerebellum, striatum, thalamus and brainstem. Plaque-like and perineuronal pattern of PrP^{Sc} deposition.

MM1, Methionine/Methionine – PrP^{Sc} type 1; MV1, Methionine/Valine - PrP^{Sc} type 1; VV1, Valine/Valine - PrP^{Sc} type 1; MM2-T, Methionine/Methionine – Thalamic PrP^{Sc} type 2; MV2-C, Methionine/Valine – Cortical - PrP^{Sc} type 2; MV2, Methionine/Valine PrP^{Sc} type 2; VV2, Valine/Valine PrP^{Sc} type 2.

Clinical challenges

The clinical diagnosis of sCJD is particularly challenging especially in the early stages of the disease.[38] It relies on defined criteria that classify the disease as possible or probable.[39] Several clinical, instrumental and laboratory tests are commonly used to formulate an *in vivo* diagnosis of sCJD: electroencephalogram (EEG),[40] magnetic resonance imaging (MRI)[41] and cerebrospinal fluid (CSF) biomarkers analysis. Several CSF biomarkers have been investigated including the 14-3-3 protein, total tau (t-tau) and phosphorylated tau (p-tau) proteins, neurofilament light chain (NfL), neuron-specific enolase and α-synuclein. The most reliable and commonly used are 14-3-3 and t-tau.[42,43]

14-3-3 protein is a biomarker of neuronal cell death and therefore it is not specific for prion diseases. It is commonly reported to possess an average sensitivity of 85-95% and specificity of 40-100%[32,44-48] for sCJD. However, the main issue in using the 14-3-3 as a biomarker for prion diseases lies in the fact that its elevation is common in some neurologic and neurodegenerative diseases including herpes simplex encephalitis, other encephalitis, intracerebral metastases, metabolic encephalopathy, hypoxic brain damage, dementia with Lewy bodies (DLB) and Alzheimer's disease (AD).[33,49-51] Therefore, 14-3-3 analysis may increase the probability of CJD when other clinical features are suggestive of prion disease but it cannot be assumed as a specific biomarker.[52]

Increased levels of t-tau (cut off >1300 pg/mL) may identify sCJD patients with a sensitivity of 67–91% and a specificity of 67–95%.[44,46,47,53-56] This measurement helps to differentiate sCJD from AD. Indeed, t-tau was 3.1 times higher in sCJD compared to AD and 41 times higher than in healthy subjects.[57] Recently, the ratio t-tau/p-tau was found elevated in sCJD patients with a specificity of 94–97% and a sensitivity ranging from 75–94%.[46,58-60]

Among other CSF biomarkers proposed for prion disease diagnosis, NfL has been reported to be significantly elevated in sCJD compared to other neurodegenerative disorders like AD, DLB, frontotemporal dementia and vascular dementia. However, despite increased NfL levels enable discrimination of sCJD from normal controls,[61-64] they do not consent accurate discrimination between sCJD and other rapidly progressive dementias,[65] neurodegenerative dementia[64] and neurological diseases with dementia syndromes.[62] Recently, serum NfL analysis has been suggested as a diagnostic marker for prion diseases showing similar sensitivity and specificity to CSF markers in differentiating sCJD from healthy subjects.[66,67]

α-synuclein (α-syn) is commonly used as a biomarker for a group of diseases known as α-synucleinopathies, which includes, among the others, Parkinson's disease (PD)[68] and dementia with Lewy bodies (DLB),[69] but its usefulness for CJD diagnosis has been recently investigated. Two studies reported that total α-syn (t-α-syn) was specifically elevated in CSF of sCJD patients compared to control subjects.[70,71] Similarly, the phospho-serine-129 α-synuclein (p-α-syn) was found elevated in the CSF of sCJD patients compared to PD, DLB and neurological controls. A combined analysis of both markers, showed 90.5% sensitivity and 97.6% specificity for sCJD diagnosis.[72] Other CSF and serum biomarkers of prion diseases, including the neuron specific enolase (NSE),[73,74] the S100B protein,[75] SERPINA3[76] and thymosin β4[77] are currently under investigation. Unfortunately, although useful for the clinical diagnosis of CJD, CSF biomarkers are not disease-specific.

The definite diagnosis depends on *post-mortem* examination of the brain aimed at identifying and characterizing the disease-specific biomarker of prion diseases, the PrP^{Sc}. Through a combination of biochemical (*e.g.,* Western blot after PK digestion), immunohistochemical and genetic analyses it is possible to identify the specific sCJD subtype. Thanks to the recent development of the ultrasensitive seeding aggregation assays, named Real-Time Quaking Induced Conversion (RT-QuIC) and Protein Misfolding Cyclic Amplification (PMCA) the diagnostic accuracy of prion diseases has been significantly increased. In particular, the PMCA enabled efficient detection of traces of PrP^{Sc} in the CSF, urine and blood of patients with variant CJD (vCJD), which is related to the consumption of foodstuff obtained from cattle affected by bovine spongiform encephalopathy. However, this technique, has never been able to efficiently detect PrP^{Sc} associated with sCJD.[78-81] In contrast, the RT-QuIC has been optimized to efficiently detect low amounts of sCJD prions in the CSF, olfactory mucosa and skin samples in a more rapid and safe manner (with respect to PMCA) while requiring a limited handling of the specimens and reducing the risk of their contamination.[82-85] For this reason, the RT-QuIC has been adopted by several specialized centers for the analysis of biological samples collected from patients with suspected sCJD, as detailed in the next section.

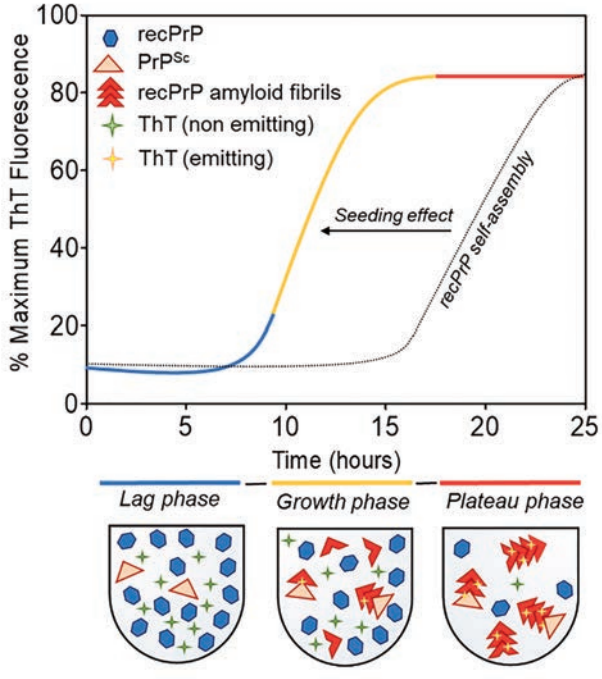

Figure 3. Schematic illustration of the RT-QuIC reaction. The RT-QuIC process is divided into three phases: (1) the lag phase, (2) the growth phase and (3) the plateau phase. The reaction mix is composed by recombinant PrP (recPrP) and Thioflavin T (ThT) which are dissolved in common buffers. The addition of PrP^{Sc} (pink triangle) to the reaction induces the convertion of recPrP (blue hexagon) into a misfolded form (red arrow) which starts to aggregate and form recPrP amyloid fibrils. In the absence of PrP^{Sc}, recPrP can aggregate (dotted line) following a well-defined kinetics. The formation of the aggregates induces the emission of a ThT fluorescence signal (yellow star). In the presence of PrP^{Sc}, the kinetics of recPrP aggregation is significantly accelerated (solid line). The increased kinetics of recPrP aggregation is known as seeding effect.

RT-QuIC assay

RT-QuIC is an ultrasensitive technique developed by Atarashi *et al.* in 2011 in the field of prion diseases. This assay exploits the intrinsic ability of PrP^{Sc} to promote the conformational rearrangement of PrP^{C} that can aggregate into amyloid fibrils.[86] The assay mimics *in vitro* the process of PrP^{C} misfolding and aggregation which occurs *in vivo*. Recombinant PrP^{C} (recPrP) with the amino acid sequence of different species can be used as a reaction substrate. The addition of traces of PrP^{Sc} to the reaction substrate induces its aggregation and the kinetics of this process can be monitored in real-time by using a fluorescent dye, named Thioflavin-T (ThT).[87] In general, each sample is analyzed in quadruplicates using a multi-well plate.[88] The samples are subjected to cyclic phases of incubation and shaking using a dedicated fluorescence microplate reader.[89] In the presence of PrP^{Sc}, the incubation phase stimulates the formation of recPrP amyloid fibrils, while the shaking phase permits the fragmentation of the aggregates into smaller units capable to recruit and convert further recPrP into new amyloid fibrils.[90]

The *in vitro* aggregation process can be represented on a cartesian plane where fluorescence is plotted against time generating a kinetic curve characterized by three phases: i) a lag phase, where PrP^{Sc} interacts with recPrP and induces this latter to misfold ii) a growth phase, where misfolded recPrP aggregate to form oligomers and small amyloid fibrils sensitive to ThT (exponential increase of fluorescence) and iii) a plateau phase, where almost all recPrP is incorporated into fibrils. Under normal reaction conditions, recPrP spontaneously aggregates while the addition of PrP^{Sc} (even in traces) to the substrate significantly accelerates the kinetic of recPrP aggregation (seeding effect) (Figure 3). A samples is considered positive when at least 2 out of 4 replicates show a seeding effect. The RT-QuIC end-products are partially resistant to PK digestion.[91]

RT-QuIC enabled PrP^{Sc} detection in CSF, olfactory mucosa (OM), skin, eye, peripheral nerve, and digestive system of patients with different forms of prion diseases (Table 2).

The assay developed in 2011 was considered the "first generation RT-QuIC" since the analyses were performed at 42°C using the recombinant full-length Syrian Hamster prion protein ($recSHa_{(23-231)}$) as reaction substrate. With this experimental setting it was possible to detect PrP^{Sc} in the CSF of a series of Japanese subjects with sCJD and 30 Australian sCJD patients with 80% sensitivity and 100% specificity.[92]

One year later, the analyses of 123 patients with neuropathologically confirmed sCJD showed that RT-QuIC was able to identify PrP^{Sc} in CSF with a sensitivity of 91% and specificity of 98%.[84]

In 2014, Orrù and colleagues[84] performed RT-QuIC analysis of OM and CSF collected from living patients with possible or probable clinical diagnosis of CJD. The RT-QuIC analysis of OM identified 30 out of 31 sCJD patients with a sensitivity of 97% and specificity of 100% while the analysis of CSF showed less sensi-

Table 2. Specificity and sensitivity of 1st and 2nd generation of RT-QuIC.

Samples	Year	Reference	Substrate recPrP	Sensitivity %	Specificity %
CSF	2011	Atarashi *et al.*[82]	recSHa (23-231)	91.5	100.0
	2012	McGuire *et al.*[88]	recSHa (23-231)	89.0	99.0
	2014	Orrù *et al.*[84]	recSHa (23-231)	77.0	100.0
	2015	Cramm *et al.*[114]	recSHa (23-231)	Not reported	Not reported
	2015	Orrù *et al.*[115]	recSHa (90–231)	95.8	100.0
	2016	Cramm *et al.*[93]	recSHa (23-231)	85.0	99.0
	2016	Groveman *et al.*[94]	recSHa (23-231)	72.5	100.0
	2016	Groveman *et al.*[94]	recSHa (90–231)	93.8	100.0
	2016	Park *et al.*[95]	recSHa (23-231)	76.5	100.0
	2016	McGuire *et al.*[92]	recSHa (23-231)	100.0	100.0
	2017	Franceschini *et al.*[83]	recSHa (90–231)	97.2	100.0
	2017	Bongianni *et al.*[96]	recSHa (23-231)	71.4	100.0
	2017	Bongianni *et al.*[96]	recSHa (90–231)	82.6	100.0
	2017	Lattanzio *et al.*[56]	recSHa (23-231)	82.1	99.4
	2017	Foutz *et al.*[107]	recSHa (90-231)	92.0	98.5
	2017	Foutz *et al.*[107]	recSHa (90–231)	95.0	100.0
	2018	Rudge *et al.*[110]	recSHa (23-231)	89.0	100.0
	2018	Hermann *et al.*[109]	recSHa (23-231)	97.0	99.0
	2019	Abu-Rumeileh *et al.*[55]	recSHa (23-231)	82.5	100.0
	2019	Abu-Rumeileh *et al.*[55]	recSHa (90–231)	97.4	100.0
	2020	Fiorini *et al.*[100]	recSHa (90–231)	96.0	100.0
	2020	Rhoads *et al.*[97]	recSHa (90–231)	90.3	98.5
	2020	Xiao *et al.*[98]	recSHa (90–231)	96.7	100.0
OM	2014	Orrù *et al.*[84]	recSHa (23-231)	97.0	100.0
	2017	Bongianni *et al.*[96]	recSHa (90–231)	92.0	100.0
	2020	Fiorini *et al.*[100]	recSHa (90–231)	91.4	100.0
Skin	2017	Orrù *et al.*[105]	recSHa (23-231)	100.0	100.0
	2020	Mammana *et al.*[85]	recSHa (23-231)	89.0	100.0
Eye	2018	Orrù *et al.*[106]	recSHa (90–231)	100.0	100.0
PN	2019	Baiardi *et al.*[116]	recSHa (90–231)	100.0%	100.0%
DS	2019	Satoh *et al.*[117]	not reported	100.0%	not reported

CSF, cerebrospinal fluid; OM, olfactory mucosa; PN, peripheral nerve; DS, digestive system; $recSHa_{(23-231)}$, recombinant full-length syrian hamster prion protein; $recSHa_{(90-231)}$, recombinant N-terminally truncated syrian hamster prion protein.

tivity (77%) but similar specificity (100%). In 2016, the first multicenter studies demonstrated the reproducibility, reliability, and robustness of the first generation of CSF RT-QuIC (PQ-CSF) in clinical practice.[92,93]

In 2015, the group of Caughey optimized the RT-QuIC protocol and developed the "second generation RT-QuIC" by introducing two modifications: i) the use of N-terminally truncated $recSHa_{(90–231)}$ as a reaction substrate and ii) the increasing of temperature from 42°C to 55°C. The use of a different substrate and a higher temperature improved the RT-QuIC performances by identifying positive CSF samples in shorter time (4-14 hours) compared to those required by the first generation assay (2,5-5 days). In particular, the RT-QuIC analysis identified PrP^{Sc} in 46 out of 48 CSF samples collected from sCJD individuals yielding 95.8% diagnostic sensitivity and 100% specificity. Another retrospective study of Groveman *et al.*[94] confirmed the high sensitivity and specificity (94% and 100%, respectively) of the CSF RT-QuIC test in a large cohort of patients (n=113) with probable or definitive sCJD diagnosis. Further investigations were then performed with the aim of improving the sensitivity of the assay in detecting PrP^{Sc} in CSF and OM.[55,56,83,92,95-98] In 2020, an international trial confirmed the robustness and reliability of the second generation RT-QuIC for the diagnosis of sCJD.[99] Furthermore, a recent article by Fiorini *et al*.[100] demonstrated that through the combined RT-QuIC analysis of the CSF and OM collected from the same sCJD patient it is possible to reach a diagnostic accuracy of 100%. For these reasons, the RT-QuIC has been introduced among the diagnostic criteria of some surveillance centers.[101] Recent evidence showed that recPrP with the bank vole amino acid sequence can detect almost all PrP strains (from human or animal origin), but it has not been introduced in the diagnostic field yet.[102-104]

From 2017, the RT-QuIC assay has been extended to the analysis of other peripheral tissues. In particular, Orrù *et al*. explored the potential prion seeding activity and infectivity of skin collected from 21 sCJD patients and eye (retina, sclera and cornea) collected *post-mortem* from 11 sCJD cases.[105,106] Similarly, Mammama *et al*. analyzed skin biopsies of sCJD patients and identified PrP^{Sc} with 89% sensitivity and 100% specificity.[85]

Correlations of RT-QuIC results with neuropathological findings

To date, only few studies have investigated whether there is a correlation between the RT-QuIC results and the phenotypes of sCJD.[107,108] In the case of CSF samples, the sensitivity of RT-QuIC was found to be high in the most common MM1/MV1 and VV2 sCJD cases, while it was lower in MV2 cases (75–93%).[55,56,83,97,107,109] In other rare subtypes, including VV1 and MM2, the sensitivity was found to range between 0-100% and 44-78%, respectively.[56,97,109-111] In these latter cases, the limited amount of CSF, hampered the possibility to properly evaluate the diagnostic accuracy of the assay. In 2016, Foutz *et al.* observed a correlation between RT-QuIC kinetics and sCJD subtypes. In particular, they observed that MM1 cases had significantly shorter lag phase and higher fluorescence values compared to MM2 cases, and these findings enabled discrimination of both phenotypes with an accuracy of 95%. At the same time, the extended lag phase and lower intensity of fluorescence allowed to differentiate VV1 to VV2 individuals with an accuracy of 80%. MV1, MV2, and mixed type cases did not show significant differences in terms of lag phases or fluorescence intensities.[107] Recently, Piconi *et al*. subjected to PK digestion the RT-QuIC products obtained from the analysis of brain homogenates (BH) and CSF of patients with the six phenotypes of sCJD. In this case, regardless of the sCJD subtype, all samples displayed PK-resistant signal characterized by similar electrophoretic mobility and banding profile, even when challenged with several anti-PrP antibodies.[108] Thus, in contrast to the work of Foutz, they could not identify peculiar features useful to distinguish the six sCJD subtypes. For this reason, the possibility to identify sCJD subtypes by RT-QuIC remains to be clearly elucidated. Very recent findings show that formalin fixed brains are capable to exert an efficient seeding activity by RT-QuIC, using both animal[112] and human specimens (*personal communication*).

Conclusions

Currently, the RT-QuIC test represents the most reliable and powerful tool for the early detection of PrP^{Sc} in peripheral tissues of patients with a suspected clinical diagnosis of sCJD.[90] The reason for the rapid growth of RT-QuIC use in the clinical practice, although still confined to specialized laboratories, lays in the fact that it is not invasive for the patients, has a relatively low cost and a high predictive value. Among the advantages, the method is not time-consuming and enables the analysis of a huge number of samples in a relatively short period of time.[113] Overall, these characteristics support the choice by WHO to include the CSF RT-QuIC test in the diagnostic criteria for sCJD.[39,99,101] As previously mentioned, only few specialized laboratories have adopted the RT-QuIC technology. However, the assay is relatively easy to learn and can be rapidly used by trained personnel, thus consenting its widening in other centers specialized in the diagnosis of neurodegenerative diseases associated with protein misfolding. Future multi-center trials will consent to verify the robustness of the RT-QuIC for the analysis of new peripheral tissues (*e.g.*,. OM, skin) and to further explore the potential of this assay to stratify patients in their early disease stage.

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