TITLE

Preclinical Biomarkers of Prion Infection and Neurodegeneration

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HIGHLIGHTS

- Preclinical biomarkers are crucial for drug study design and timing of therapy in neurodegenerative diseases.
- Animal models of prion disease show long silent incubation period before clinical onset despite high prion titres.
- The ultrasensitive prion-seeding assay RT-QuIC is a highly sensitive and specific assay for diagnosis, but it remains unclear how useful it will be preclinically.
- Downstream protein markers of neurodegeneration are also very useful after diagnosis.
- Rate of change rather than absolute biomarker value may be a more sensitive measure.

ABSTRACT

Therapeutic strategies and study designs for neurodegeneration have started to explore the potential of preventive treatment in healthy people, emphasising characterisation of biomarkers capable of indicating proximity to clinical onset. This need is even more pressing for individuals at risk of prion disease given its rarity which virtually precludes the probability of recruiting enough numbers for well powered preventive trials based on clinical endpoints. Experimental mouse inoculation studies have revealed a rapid exponential rise in infectious titres followed by a maximal plateau of considerable duration prior to clinical onset. This clinically silent incubation period represents a potential window of opportunity for the adaptation of ultrasensitive prion seeding assays to define the onset of prion infection, and for neurodegenerative biomarker discovery through similarly sensitive digital immunoassay platforms.

INTRODUCTION

Prion diseases in humans encompass a diverse range of clinical and pathological conditions unified biologically by the misfolding of cellular prion protein (PrP) followed by its subsequent aggregation and propagation through seeded-polymerisation and fission[1,2]. Human prion diseases most commonly manifest as sporadic Creutzfeldt-Jakob disease (sCJD), a rapidly progressive dementia associated with ataxia and myoclonus, accounting for about 85% of cases, followed by inherited prion disease (IPD) due to highly penetrant dominantly inherited mutations within the prion protein gene (*PRNP*) which account for 10-15%[3]. The range of IPD clinical phenotypes is striking, even within a single pedigree[4], and include familial CJD (fCJD), fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker (GSS) disease, PrP systemic amyloidosis, and a long-duration behavioural variant frontotemporal dementia-like syndrome associated with some of the octapeptide insertions (OPRIs). Acquired prion disease is rare, and is caused by either medical or dietary exposures; these occur in epidemics or smaller outbreaks with long incubation periods, such as was seen with kuru in the Eastern Highlands Province of Papua New Guinea[5], with bovine spongiform encephalopathy (BSE) of cattle and its human form variant CJD (vCJD)[6], and with iatrogenic CJD related to the use of cadaver-derived human growth hormone (iCJD-hGH)[7] or dura mater[8].

In the UK, the number of individuals at risk of inherited prion disease is estimated to reach approximately 1000[9]; in addition, those at risk secondary to direct medical exposure number many thousands, given that in the very least, cadaver-derived hGH was administered to 1849 individuals between 1959 and 1985[7]. At present, disease onset in at-risk individuals is heralded by the emergence of typical clinical symptoms, and can be readily supported by highly characteristic investigation findings such as restricted diffusion in the cerebral cortex and deep nuclei on magnetic resonance brain (MRI) imaging in familial CJD (fCJD)[10–12] and iCJD-hGH[7]. However, diagnosis may not be straightforward in IPD clinical phenotypes of more insidious onset[4,13] and crucially, no 'proximity' fluid biomarker capable of predicting clinical onset exists. This is a pressing unmet need considering the infeasibility of adequately powering clinical trials involving a rare disease such as IPD without 'proximity' biomarkers as endpoints in the presymptomatic individuals[14]. Furthermore, candidate anti-prion therapies have been shown to possess greatest therapeutic effect when administered in the presymptomatic phase, but with diminished efficacy when given at or after disease onset[15–17]. In this review, we will explore recent research underpinning the feasibility of preclinical biomarkers, and the emerging candidate biomarkers that might be explored in at-risk populations.

BASIS OF PRECLINICAL BIOMARKER DETECTION

Prion propagation can be modelled by experimental intracerebral inoculation of prions into wild-type mouse brain. Infectious prion titres rise exponentially over weeks to a plateau, subsequently remaining relatively stable until clinical onset; the length of the asymptomatic plateau phase in prion disease is inversely proportional to the amount of expressed PrP[18]. If a similar pattern is seen in IPD, prion

infectivity may be detectable in biofluids substantially before markers of neurodegeneration (Figure 1).

Similar to AD, prospective longitudinal cohorts of at-risk individuals afford the best opportunity to identify potential proximity biomarkers[19,20]; in prion disease the study population comprises asymptomatic carriers of disease-causing *PRNP* mutations, untested blood relatives of *PRNP* mutation carriers and those with iatrogenic exposure e.g. recipients of cadaveric h-GH or vCJD-infected blood transfusion. While participant visits (usually on an annual basis) can capture a diverse biomarker profile from neuroimaging, neuropsychology, clinical assessments, neurophysiological tests, etc., biofluid sampling has emerged as an exciting avenue most likely to yield success in identifying such a proximity marker. The serial sampling of a diverse biomarker profile not only creates a valuable resource that researchers can interrogate repeatedly, it also allows the determination of rate of change of biomarker values against that of controls, which may prove to be a more valuable measurement[21].

Determination of expected age of onset remains one of the trickiest aspects of interpreting longitudinal data derived from these prospective cohorts. It was found that age of symptom onset in autosomal dominant familial AD cohorts is reliably similar across generations thereby enabling determination of expected age of onset in individuals at risk of familial AD with a high degree of confidence[22]. However, the considerable variation in ages of onset of individual IPD mutations (large standard deviations) render this method less helpful[14,23].

CELL-FREE CONVERSION (PRION SEEDING) ASSAYS

Cell-free conversion assays appeal greatly as methods of detecting disease-associated PrP chiefly because they seek to replicate the fundamental sequence of prion propagation *in vitro*. The assays in use are the real-time quaking conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA) assay[24]; both revolve around putative disease-associated PrP in biological samples capable of seeding conversion of recombinant PrP (rPrP) or cellular PrP (for PMCA) to aggregates of prion amyloid when incubated together in a reaction mix, and accelerated by disruption of aggregates through intermittent exposure to kinetic energy, with some differences between them (Table 1).

RT-QuIC has proved to be excellent *reporting* assay (it does not generate infectious prions) capable of detecting disease-associated PrP down to the attogram (10^{-18}) range in some circumstances[25], making it the candidate with most potential for defining the onset of prion infection in the at-risk population. Orru *et al.* 2012 showed high levels of seeding activity by RT-QuIC in CSF and brain of hamsters experimentally inoculated with 263K scrapie prions during the clinically silent incubation period[26]. While RT-QuIC is primarily a qualitative assay, certain parameters such as length of lag phase, area under the curve, and serial dilutions allow for some quantitative measures of longitudinal samples to determine rate of

change, if indeed seeding activity is present for during the clinically silent incubation period[27–29]. Such is its versatility that RT-QuIC has been successfully applied to various biological tissues[24], the most immediately pertinent of which are CSF and olfactory mucosa (OM) for the purposes of this review[30–33] given their proximity to neural tissue. The application of classical PMCA, while able to amplify infectious prions with strain fidelity, has been largely confined to vCJD prions to date with one instance of disease-associated PrP detection in blood in an individual during the presymptomatic phase[34,35].

Recent biochemical work predicts that the success of RT-QuIC in detecting disease-associated PrP during the preclinical stage is likely to hinge on 2 key factors - concentration of seed in biofluid samples (sensitivity) and seed-substrate compatibility. RT-QuIC sensitivity can be enhanced by altering microplate reader conditions (raising shaking temperature, changing shaking parameters) or components of the reaction mix (NaCl concentration, sodium dodecyl sulphate, Hofmeister effects), all of which must be balanced against erosion of assay specificity[36,37]. As for seed-substrate compatibility, so far, it appears that rPrPs used in RT-QuIC protocols designed for sCJD diagnosis (full-length or truncated hamster, sheep-hamster chimera) can be readily seeded clinical samples from symptomatic individuals with IPD mutations which cause the fCJD phenotype (E200K, V210I, V180, etc.)[32,38–40]. However, this remains unresolved in GSS (P102L and A117V), FFI (D178N-129M), systemic peripheral amyloidosis (Y163X), and OPRIs as they continue to be either resistant or simply unexplored. To complicate matters, differential co-propagation of distinct PrP species within and between individuals is known to occur with the same mutation such as that seen in P102L (wild type vs. mutant P102L prions), each potentially with its own unique seed-substrate compatibility[41]. In this regard, bank vole rPrP seems to show a wide range of seed compatibility[42] while full-length human rPrP has been successful for CSF across a small number of E200K, D178N-129M and P102L patients[43]. It is also possible that rPrP sequence homology may be useful for certain mutations such as P102L and A117V i.e. using human P102L or A117V rPrP, drawing on experience from mouse transmission studies[44,45]. Thus, it is clear that an exhaustive, methodical and iterative interrogation approach still needs to be undertaken to determine the optimum seed-substrate pairing for non-fCJD IPD mutations, before CSF or OM RT-QuIC can realise its full potential in defining the onset of prion infection in the at-risk population.

FLUID BIOMARKERS OF PRION DISEASE NEURODEGENRATION

Neurodegenerative biomarkers in prion disease are by-products of neuronal death or distress, or markers of astrogliosis, none of which are discriminatory for one disease over another. Regardless of their lack of specificity for prion disease, serial sampling of biomarkers in a defined cohort such as asymptomatic carriers of *PRNP* mutations confers the advantage of rate of change measurement, eschewing dependence on individual values within an expected 'normal range'[21]. Furthermore, the low mean ages of clinical onset across IPD makes it unlikely to be

confounded by elevation or markers attributable to co-existence of common neurodegenerative diseases such as AD and dementia with Lewy body.

Neurofilament Light (NfL)

Chief amongst candidate proximity biomarkers is the neurofilament light subunit from the category of intermediate filaments, found with greatest abundance in the neuronal axoplasm; hence, its elevation reflects the degree of axonal injury[46]. Its pre-eminence derives from assorted studies in animal models[47] and humans demonstrating elevated blood and/ or CSF NfL levels in the preclinical phase, compelling elevation over controls in the disease phase, its prognostic value, and response (lowering of NfL levels) to effective treatment, across a number of neurological diseases[21,47–49]. Crucially, Jucker *et al.* 2019 demonstrated that rate of change of NfL segregates mutation and non-mutation carriers in a cohort of dominantly inherited AD up to a decade before onset, predicts clinical conversion, and is corroborated by imaging and functional measures of neurodegeneration[21]. In prion disease, NfL is elevated in excess over other neurodegenerative diseases[50,51]; in one serendipitous instance, NfL was found to be elevated in serum and CSF 2 years prior to purported symptom onset in a single individual carrying the P102L mutation[52].

Tau

Microtubule associated protein tau, like NfL, is excessively elevated in CSF and blood of sCJD patients through neuronal destruction rather than deposition[50,53], though neurofibrillary tangles containing hyperphosphorylated tau have been observed pathologically in GSS-causing mutations (P102L, A117V, P105L) and OPRIs[54]. In a small series of symptomatic individuals with assorted IPD mutations, serum and CSF tau levels were comparable to controls in some cases where NfL was highly elevated suggesting that the latter may be a more sensitive preclinical biomarker[52]. However, it should be pointed out that it is often misconstrued that tau in conventional studies like those above represents full-length tau. In fact, the capture antibodies used in the studies above generally recognise only short amino acid sequences in the protein mid-region and thus may fail to capture the entire gamut of tau fragments, some of which may be highly elevated in excess of other fragments in neurodegeneration and worth exploring in prion disease[55].

CSF Total PrP (t-PrP)

CSF t-PrP has attracted attention as a potential pharmacodynamic marker because of recent interest in development of endogenous PrP-depleting therapies, but it also harbours some promise as a proximity marker for a subgroup of at-risk individuals, provided precautions are taken during CSF handling to avoid PrP loss[56]. CSF t-PrP has long known to be reduced in CJD[57] but a recent study showed this was also the case in symptomatic individuals with E200K, V210I and D178N-129M mutations[58]; values for P102L patients in this study are similar to the 'non-primarily neurodegenerative diseases' individuals but they cannot be considered as strictly normal healthy individuals, even if age-matched. Curiously, asymptomatic D178N-mutation carriers (n=3 with 6 CSF samples) at least 20 years from onset had low levels of t-PrP similar to symptomatic ones. Thankfully, ELISA-based detection of CSF

t-PrP does not suffer the similar pitfall of missing tau fragments as its accuracy has been corroborated by mass spectrometry measurement of various PrP peptides[59].

CSF α-synuclein Like tau, α-synuclein is presumably another surrogate of neuronal destruction in the CSF, but one with in which highly elevated levels provide good distinction between CJD and other neurodegenerative diseases[60]. As for its potential as a proximity marker, CSF α-synuclein might be more applicable to *PRNP* mutations causing the fCJD phenotype given that its levels in P102L and D178N are indistinguishable from controls in this study; CSF α-synuclein has not been measured in any preclinical cases so far.

CSF YKL-40

Astrogliosis is a key feature of prion neuropathology along with spongiform change and PrPSc deposition, and thus it is no surprise that CSF YKL-40 levels are found to be elevated in sCJD, E200K and D178N patients relative to controls[61]; CSF S100 beta is another marker of astrogliosis long established in the diagnostic work-up of sCJD[62] (not discussed here). YKL-40 is of some interest as increased YKL-40 mRNA expression is detectible at the preclinical stage in brains of mice inoculated intracerebrally with human CJD prions, indicating possible detection in CSF before clinical onset.

CSF Neurogranin

Neurogranin is a post-synaptic protein concentrated on the dendritic spines of neurons almost exclusively in the telencephalon, mediating calcium influxes associated with long-term potentiation and thus seen as a marker of synaptic function; elevated CSF levels distinguish both CJD and AD patients (but not other neurodegenerative diseases) from controls[63]. Since synaptic dysfunction has been shown to precede neuronal loss in the early phase of disease in experimental prion-inoculated mice[64], measurement of CSF neurogranin is perceived to possess some potential as a proximity marker.

CONCLUSION

Focus has recently shifted to identifying the proximity of presymptomatic individuals to clinical onset not only in prion disease but also in other neurodegenerative diseases, as treatment administered in the presymptomatic phase is likely to be more beneficial compared to at or after disease onset. Indeed, recent advances in development of prion seeding assays and digital immunoassays with detection limits down to single molecules appear to have the requisite ultrasensitivity for biomarker characterisation before clinical onset. As such, it is now left to several prospective prion disease cohorts worldwide to continue longitudinal biofluid sample accrual, and interrogate the candidate biomarkers in both presymptomatic individuals and in those who have converted during follow-up to identify reliable proximity markers. Reports from these may have a profound effect on the clinical management of individuals at risk of prion disease and ultimately on the feasibility and design of preventative therapeutic trials.

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FIGURE 1

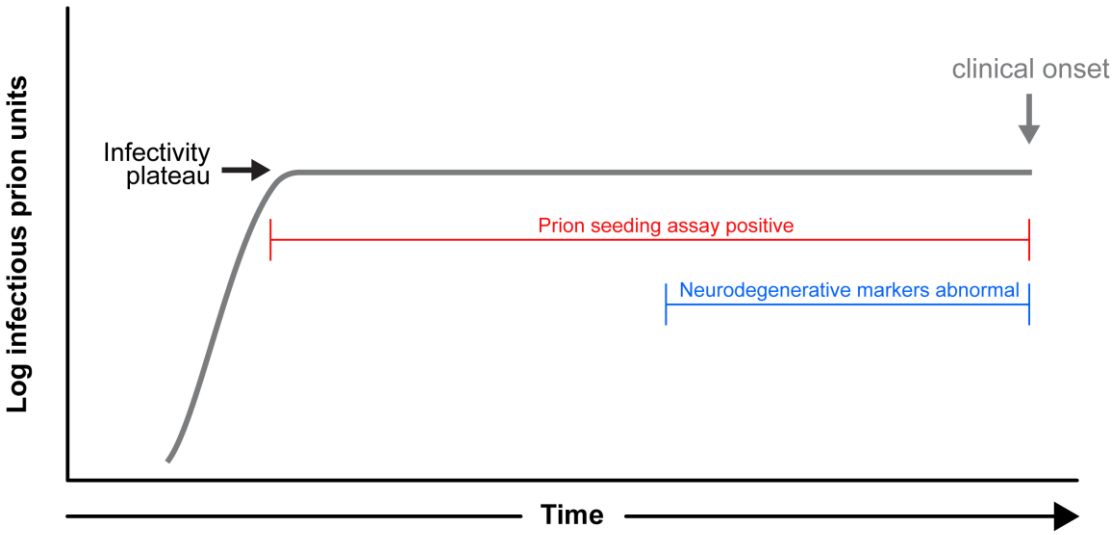

FIGURE 1 Expected timeline of prion infection and neurodegeneration according to the Sandberg-Collinge model of prion propagation

The Sandberg-Collinge model proposed that prion titres (grey line) rise exponentially shortly after prion infection to a maximal plateau on which its remains for a considerable time before clinical onset. If this holds true in humans, ultrasensitive prion seeding assays (red) may become positive (CSF ± OM) given the high prion titres. At some point later in the incubation period cumulative toxicity is expected to occur with increasingly abnormal biomarker measures (blue), culminating in symptom onset. Figure adapted from Sandberg *et al*. 2011 Nature Comm[65].

Table 1 Key comparisons between cell-free conversion assays: real-time quaking conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA) assays

	RT-QuIC	**PMCA**
Conversion substrate	Recombinant PrP	Normal brain homogenate
Kinetic energy source	Intermittent shaking	Sonication
Read-out	Thioflavin T fluorescence	Western blot
Products	Non-infectious prion amyloid	Infectious prions with strain fidelity
Application in human prion disease	Wide including sporadic CJD, iatrogenic CJD and some types of IPD	vCJD
Applicable human biofluid samples	CSF, olfactory mucosa, skin	CSF, blood and urine

REFERENCE HIGHLIGHTS

**This worldwide collaborative study demonstrated that conventional prevention trials in IPD are not feasible due to the rarity of the illness and the low annual conversion rate, thereby necessitating characterisation of biomarkers of clinical endpoint in the at risk population[14].

**This study introduced the concept of rate of change measurement in a biomarker (NfL) may be superior to reliance on cross-sectional absolute levels. The rate of change of NfL in the dominantly inherited AD cohort segregated mutation carriers from non-mutation carriers up to 10 years before absolute NfL levels[21].

*This large study of CJD CSF validates the earlier iterative work in optimisation of the RT-QuIC assay for greater sensitivity without erosion of specificity. The 2nd generation assay, named IQ-RT-QuIC, improved RT-QuIC sensitivity by over 20%[38].

*This study uncovered the abundance of smaller tau fragments and dispelled the assumption that the tau species in AD is full-length tau. The approach of delineating the relative abundance of precise protein fragments may reverberate throughout the neurodegenerative biomarker pool[55].

*Though this article is older than 2 years, it remains the only published instance where a biomarker (NfL) was shown to be elevated for an extended period (2 years) prion to clinical onset in a carrier of the P102L mutation[52].

*Using ultrasensitive digital immunoassay platforms, the authors demonstrated elevation of tau and NfL in blood of a large cohort of sCJD patients (n=45), some of which contain serial sampling from individuals patients (16 samples from 6 patients). NfL showed 100% sensitivity and specificity over normal controls. Tau levels correlated with disease progression[50].