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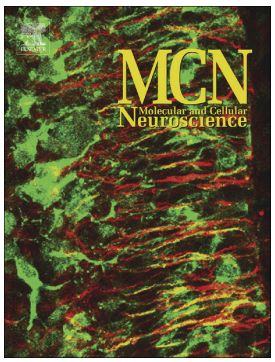

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Andrew G.B. Thompson, Simon H. Mead

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Review: Fluid biomarkers in the human prion diseases

Andrew G. B. Thompson, Simon H. Mead*

MRC Prion Unit at University College London (UCL), UCL Institute of Prion Diseases, UCL, London, W1W 7FF

*Corresponding author

s.mead@prion.ucl.ac.uk

+44 207 6795142

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Abstract

The human prion diseases are a diverse set of often rapidly progressive neurodegenerative conditions associated with abnormal forms of the prion protein. We review work to establish diagnostic biomarkers and assays that might fill other important roles, particularly those that could assist the planning and interpretation of clinical trials. The field now benefits from highly sensitive and specific diagnostic biomarkers using cerebrospinal fluid: detecting by-products of rapid neurodegeneration or specific functional properties of abnormal prion protein, with the second generation real time quaking induced conversion (RT-QuIC) assay being particularly promising. Blood has been a more challenging analyte, but has now also yielded valuable biomarkers. Blood-based assays have been developed with the potential to screen for variant Creutzfeldt-Jakob disease, although it remains uncertain whether these will ever be used in practice. The very rapid neurodegeneration of prion disease results in strong signals from surrogate protein markers in the blood that reflect neuronal, axonal, synaptic or glial pathology in the brain: notably the tau and neurofilament light chain proteins. We discuss early evidence that such tests, applied alongside robust diagnostic biomarkers, may have potential to add value as clinical trial outcome measures, predictors of future disease course (including for asymptomatic individuals at high risk of prion disease), and as rapidly accessible and sensitive markers to aid early diagnosis.

Introduction

Prion diseases, prions and the prion protein

The human prion diseases are a group of uncommon and fatal neurodegenerative disorders in which the misfolding and aggregation of a normal human protein, the prion protein (PrP), plays a central and necessary pathogenic role (1, 2). The pathogenic agent of prion diseases, the prion, is thought to comprise multi-chain assemblies of misfolded PrP and is devoid of nucleic acids. Disease aetiologies include an apparently random protein misfolding event causing sporadic Creutzfeldt-Jakob disease (sCJD) (by far the most common form), a mutation in the gene encoding the prion protein (*PRNP)* causing the inherited prion diseases (IPD), and exposure to an exogenous source of prions from another human or animal causing the acquired prion diseases: variant CJD (vCJD), iatrogenic CJD (iCJD) and kuru.

Public and scientific interest in the prion diseases was fuelled by the appearance of a novel human prion disease in the UK during the 1990s: vCJD(3). This followed a major epidemic of a prion disease amongst UK cattle, bovine spongiform encephalopathy (BSE)(4), which was soon shown to be the source of the human disease (5-7). The incidence of vCJD peaked in the year 2000, with 178 people in the UK affected to date, and only one case diagnosed in the last five years (8).

Prion strains, most likely encoded by the molecular structure of the prion, confer distinct clinical and pathological phenotypes that can be maintained on transmission to other humans or animals (9). vCJD is the human form of the prion strain that causes BSE, which is distinct from those that cause sCJD.

Concerns about risks to public health from prion disease have led to the establishment of national epidemiological surveillance centres offering diagnostic biomarkers, sometimes associated with specialist clinical teams assisting in the diagnosis and care of patients. Enabled by this infrastructure, biomarker research has focussed largely on the development and optimisation of diagnostic assays.

At present, there is no proven disease-modifying treatment for prion disease, and clinical trials are challenging. Biomarkers may be able to make several important contributions to accelerate the discovery of therapies(10).

Here we focus on fluid biomarkers for the following roles:

- Diagnosing prion disease in symptomatic individuals, including those at a very early stage of disease, and those with atypical clinical presentations
- Diagnosing prion infection by screening asymptomatic populations to identify those could pose a risk for onward transmission of disease
- Quantifying the severity or rapidity of the underlying disease process
- Stratifying patients according to the likely phenotype of their future disease course
- Whilst there is almost no directly relevant data to review, we note that predicting the timing of symptom onset in asymptomatic individuals at elevated risk of developing prion disease, such as those carrying pathogenic mutations in *PRNP,* would be a potentially valuable role.

In Table 1, we give a summary overview of the range of biomarkers and their usefulness in these different roles.

Diagnostic biomarkers

Prion diseases are defined by the presence of abnormal forms of PrP, which have a range of physical, biochemical, and functional properties. Abnormal PrP can be identified by its abundance, location and morphology (using immunohistochemistry), its relative resistance to protease digestion (when it is termed PrP^{Sc}, and typically detected by Western blot), and its functional properties (e.g. transmission of disease to laboratory animals, or seeding the misfolding of PrP *in vitro*). However, until recently it has been surrogate markers of the downstream effects of the disease process, rather than markers directly related to the pathognomonic molecular process itself, that have predominated in diagnosis of patients.

Current pre-mortem diagnostic criteria for sCJD combine clinical features with several biomarkers: derived from magnetic resonance imaging (MRI) of the brain (abnormal signal on diffusion weighted or FLAIR images in the caudate/putamen and/or two cortical regions), generalised periodic complexes on electroencephalography (EEG), and laboratory analysis of cerebrospinal fluid (CSF) (11). sCJD typically causes a remarkably rapid clinical progression: the average time from symptom onset to death from sCJD is around 4 months (12). In clinical practice, the important differential diagnosis is often with non-degenerative, and potentially treatable, processes such as encephalitis or vasculitis, or systemic conditions such as hepatic encephalopathy. These alternative diagnoses can almost always be distinguished from sCJD by clinical assessment combined with MRI of the brain and examination of CSF (13, 14), although by the time this has taken place patients are often at a relatively advanced stage of disease.

There are ways in which diagnosis of sCJD needs to improve, and where biomarkers are likely to play a vital role. Improving early diagnosis is a key objective, to allow patients and their families to plan for the end of life unburdened by the ongoing search for a diagnosis, and to enable participation in clinical trials before too much irreversible neurological damage has occurred. Atypical cases are undoubtedly under-ascertained (particularly sCJD in the elderly and those with phenotypes that are more slowly progressive and less easy to distinguish from common neurodegenerative disorders), exemplified by the steady and striking increase in incidence of sCJD over recent decades (8, 15). In typical cases with established disease, diagnostic biomarkers often serve to reinforce an already high level of clinical suspicion. In atypical cases and at very early stages they may have a more decisive role to play, but this is largely unexplored at present.

The inherited prion diseases (IPD) are caused by autosomal dominant, usually highly penetrant mutations in the prion protein gene (*PRNP*). They are diagnosed based on a consistent clinical syndrome in a patient carrying one of these mutations. Their clinical phenotypes are highly variable between different mutations, and to a lesser extent between different individuals carrying the same mutation and even within affected families (16-19).

The primary diagnostic biomarker for IPD is sequencing of the *PRNP* gene, sometimes combined with clinical investigations to rule out common conditions that might mimic the presentation.

The acquired prion diseases may be strongly suspected when there is a history of a specific iatrogenic exposure, and they can have distinct investigation features. vCJD diagnosis is based on clinical features combined with biomarkers: the presence of a characteristic pattern of abnormality ("pulvinar" sign) on MRI brain scan, and in cases where diagnostic uncertainty persists by tonsil biopsy demonstrating the presence of abnormal prion protein with specific biochemical properties (20, 21).

Tissue biopsy

Examination of brain tissue (using standard histological methods, immunohistochemistry, and Western blotting of PrP after partial protease digestion) is the gold standard method for confirming a diagnosis of sporadic or acquired prion disease. This allows the classical neuropathological hallmarks of prion disease to be demonstrated (spongiosis, neuronal loss, PrP deposition and gliosis) (22). It also allows strain-typing of the accumulated protease-resistant PrP, which distinguishes vCJD from sCJD (5) and the different molecular sub-types of sCJD from each other (in combination with *PRNP* codon 129 genotyping) (23, 24). However, brain biopsy in patients suspected of having prion disease is increasingly rare in practice as the accuracy of less invasive tests has improved. It may still be considered if there is reason to suspect an alternative treatable condition which has not been confirmed by less invasive testing (such as a primary CNS vasculitis or lymphoma) (25, 26).

The distribution of abnormal PrP deposition in vCJD is more widespread than in sCJD, and it can be identified and analysed from lymphoreticular tissues, including palatine tonsil biopsy (21, 27, 28).

Cerebrospinal fluid (CSF)

CSF has been the primary fluid analyte for identifying biomarkers in human prion diseases. Although methods for detecting biomarkers in blood have recently started to show real promise (as they have in many neurodegenerative diseases), those in CSF remain the most well-established, and the only ones which are currently applied in clinical practice. In addition to allowing analysis of biomarkers associated with prion disease, lumbar puncture allows vital CSF tests necessary to rule out alternative treatable diagnoses that might mimic prion disease (for example, a raised CSF white blood cell count, which would suggest an inflammatory or infective aetiology)(13, 29).

Approaches to identifying CSF biomarkers of prion disease have fallen broadly into 2 categories: detecting by-products of the very rapid neurodegeneration of prion disease ("surrogate markers" below), and detecting specific molecular species related to the fundamental disease process.

Surrogate markers in CSF

14-3-3 proteins

These are a family of highly conserved intra-neuronal proteins, which together constitute around 1% of the total cytosolic protein content of neurons (30). The presence of 14-3-3 proteins in the CSF of patients with CJD was first identified by carrying out 2D gel electrophoresis and silver staining on CSF samples from patients with CJD and a range of controls, and identifying bands that appeared to be specific to CJD (31). When the proteins in two CJD-specific protein spots were identified, they proved to be members of the 14-3-3 family of proteins. This represents an early success for using an unbiased proteomic approach to identify a clinically applicable biomarker.

Since they were first described, it has been clear that their presence in CSF is not intrinsically specific to the disease process of CJD: patients with viral encephalitis and those with recent cerebral infarction, for example, were often found to have 14-3-3 protein present in their CSF, as did occasional patients with other more common causes of dementia (31, 32). Conversely, it has been shown that CSF 14-3-3 is less likely to be detectable in atypical, more slowly progressive forms of sCJD (33-35). It seems that the presence of 14-3-3 proteins at detectable levels in the CSF is an indicator of recent or ongoing rapid neuronal damage from any cause. As such, its sensitivity and specificity as a test for sCJD varies depending on how highly selected a patient group is being studied.

A number of large studies assessing the diagnostic performance of CSF 14-3-3 protein have been carried out in the context of national CJD surveillance and CSF testing centres (36-44). Sensitivity and specificity as a diagnostic test for sCJD in these individual studies ranged from 89 to 94% and from 67 to 98% respectively, and a meta-analysis concluded an overall sensitivity of 92% and specificity of 80% (45). Even in the last few years, further technical refinements of the methods for detecting 14-3-3 protein continue to be published (46-48), and it remains part of the diagnostic criteria for sCJD.

Tau

The microtubule-associated protein tau is implicated in the pathophysiology of a range of neurodegenerative diseases, but also has a role as a non-specific biomarker of neuronal damage. In a misfolded and phosphorylated state it is a principal component of disease-associated protein aggregates in a range of so-called “tauopathies” including Alzheimer’s disease, corticobasal degeneration, progressive supranuclear palsy and some forms of frontotemporal dementia (49). Although not a classical pathological hallmark of prion disease, it has been shown that phosphorylated tau deposits are a prominent autopsy finding in some inherited prion diseases (50). In sCJD, the prevalence of tau deposition does not appear to be markedly increased above age-related expectations, but some distinct and

atypical patterns of deposition are observed suggesting that it may not proceed entirely independently of the co-existing prion disease pathology(51).

Similar to 14-3-3 protein, total tau concentration in CSF appears to increase as a result of neuronal damage from any cause. Patients with rapidly progressive sCJD typically have very high levels, and if a high cut-off value is used to classify the test as positive, CSF tau has good diagnostic performance for sCJD amongst patients with rapidly progressive dementia. Different studies have shown sensitivities ranging from 75% to 98% and specificities from 67% to 99% (52-58). Direct comparison suggests that total tau is superior to 14-3-3 protein when the latter is tested using Western blot, particularly in terms of specificity (56) and amongst those with an equivocal "weak-positive" 14-3-3 result (59), although probably no better than newer ELISA-based methods for quantifying 14-3-3(47, 60).

The diagnostic performance of CSF tau is also dramatically affected by the selection of population in which it is studied. In a test population with a low prevalence of sCJD cases (e.g. less than 10%), the likelihood that a positive CSF tau result indicates a diagnosis of sCJD drops dramatically (56). As such, neither 14-3-3 nor total tau are appropriate for use in isolation to screen for CJD cases amongst unselected dementia cases or other patient populations where prevalence will be low. Confirming this, one study looked at 200 cases with high CSF tau identified through 3 memory clinics in France. The majority had either AD (73.8%) or mixed vascular/AD (18.8%) while only 4 patients (2%) had a final diagnosis of CJD (61).

Comparing total tau concentration with that of phosphorylated tau addresses this lack of specificity to some extent. In Alzheimer's disease the ratio of p-tau:t-tau is typically increased, whereas in sCJD this is reversed (with high levels of total tau without a commensurate increase in p-tau). This was studied in a large cohort and an optimal ratio cut-off <0.0713 for distinguishing CJD from AD (including both rapid and typical slower cases) was derived, giving a sensitivity of 99% and specificity of 94%. In the memory-clinic-based study mentioned above, taking a similar ratio of <0.075 as indicative of sCJD correctly identified all 4 sCJD cases, and incorrectly classified only 2 of 148 AD cases (61). Another study has also shown good performance of specifically measuring non-phosphorylated tau in CSF in the differential diagnosis of AD and CJD (62) (although this compared CJD with an unselected AD group with whom there would be little clinical overlap).

Neurofilament light chain (NfL)

NfL is a neuronal cytoskeleton component, and is released when there is neuronal damage in a wide range of conditions. CSF NfL concentration is substantially elevated in patients with sCJD, including those with more slowly progressive, atypical disease courses where the more established diagnostic biomarkers (14-3-3, tau and even RT-QuIC) have a lower sensitivity. However it is also raised in a range of other conditions causing dementia: as a result it appears to have a very high level of sensitivity but relatively limited specificity for

sCJD when a clinically relevant control group is used to evaluate its diagnostic performance (63). Although this is likely to limit its value as a diagnostic test applied in isolation, it may still have useful roles to play. CSF NfL concentrations are also raised in several types of inherited prion disease where other established CSF biomarkers have tended to have lower sensitivity, and it may have greater value in distinguishing atypical slower cases of sCJD from particular alternative diagnoses such as rapidly progressing AD (64, 65). It might also have potential as a highly sensitive, inclusive initial step in a diagnostic algorithm aiming to increase ascertainment of atypical cases, but would need to be combined with other more specific tests, and this would need to be evaluated directly in the relevant patient populations.

α-synuclein

α-synuclein, a key protein in the pathogenesis of several other neurodegenerative conditions, also appears to act as a surrogate marker of neuronal damage in prion disease. Measured in CSF, it appears to have remarkable diagnostic value for sCJD (with a sensitivity of 98% and specificity of 97% in one study), and also to be elevated in rapid CJD-like forms of IPD (66, 67). This recent discovery shows considerable promise for use as a diagnostic assay, and will need to be replicated in other large patient cohorts.

Other surrogate CSF biomarkers

Various other markers of downstream aspects of prion disease pathology have been identified and evaluated as potential diagnostic markers, notably the S100b protein, Glial Fibrillary Acidic Protein (GFAP) and neuron-specific enolase (NSE). While these do have some sensitivity and specificity for CJD, they have not really challenged 14-3-3 or the other markers discussed above in terms of diagnostic performance, or for other purposes. For example, in one direct comparative study the sensitivity of S100b in the diagnosis of sCJD was 65% compared with 86% for 14-3-3(53).

PrP-based markers in CSF

Total PrP concentration

Total PrP levels in the CSF of patients with prion disease tend to be reduced relative to controls (68). It has been speculated that this may result from sequestration of soluble monomeric protein into aggregates in the brain (analogous to the proposed mechanism for reduction of CSF $A\beta_{1-42}$ in Alzheimer's disease). This has been investigated in more detail in a large cohort recently and shown to have moderate diagnostic value, with reductions in sCJD, iCJD and some IPD (69). However, findings on the specificity of reduced total PrP in CSF to prion disease have varied between studies (68, 70). Overall it seems unlikely that it will have a role in differential diagnosis, but it may have some other useful properties (see below).

Protease resistant and/or aggregated PrP

The unusual physical properties of disease-related prion protein aggregates, particularly their resistance to protease digestion, have been exploited for prion disease diagnosis in solid tissues. However attempts to apply the same methods to CSF have failed (71). Some novel methods have shown that there are detectable aggregates in CSF (e.g. dual colour scanning for intensely fluorescent targets (72)), but these are methodologically involved and have been largely superseded by those taking advantage of the specific property of PrP aggregates to seed PrP misfolding and aggregation.

PrP seeding activity and infectivity (RT-QuIC, PMCA)

The defining property of prion diseases is infectivity, which appears to result from the ability of abnormal forms of PrP to "seed" the misfolding and aggregation of other PrP molecules. Testing for evidence of infectivity using animal bio-assays (e.g. intracerebral inoculation of human tissue homogenates or fluid into laboratory animals) represents the gold standard for demonstrating the presence of prion infectivity, and therefore prion disease. However, primate transmission experiments carried out at NIH found that only 4 of 27 CSF samples from patients with CJD transmitted disease (73), and it is not practical or ethical to apply animal bio-assay for routine clinical diagnosis.

Replicating this process *in vitro* has theoretical appeal as the basis for a biomarker assay, as it lends itself to the detection of very small amounts of disease-associated protein aggregate through repeated cycles of seeded misfolding (analogous to the polymerase chain reaction used to amplify tiny amounts of DNA), and as it exploits the fundamental biological mechanism of prions it would be hoped it could provide high specificity. This process also has the potential to maintain the molecular phenotype of the prion strain through conformational templating, so could convey other valuable information about aetiology or likely phenotype.

Simulating the seeded misfolding process *in vitro* has been used as the basis for a range of assays with applications for both research and as clinical biomarkers. Protein misfolding cyclic amplification (PMCA) uses a normal mouse brain homogenate (or alternative) substrate to provide normal PrP and any other cofactors needed for seeded misfolding to proceed. This is mixed with a test sample and subjected to repeated cycles of sonication and incubation: if the test sample contains a misfolded prion seed, it converts the normal PrP in the substrate, so that β-sheet-rich, fibrillar, protease-resistant material composed largely (but not exclusively) of PrP gradually accumulates, and can be detected by Western blotting after protease digestion. This process produces material that is infective (causing disease if inoculated into susceptible animals), and preserves some of the biochemical properties of the misfolded prion protein in the seed, including those associated with strain (74, 75). PMCA can detect seeding activity in CSF in sCJD (76), while a more recent modified PMCA

method showed positive results from 40 of the 41 vCJD cases tested but not from any sCJD cases or controls tested (77).

The most fruitful adaptation of *in vitro* seeded misfolding for clinical application has been the assay known as real-time quaking induced conversion, RT-QuIC. This advantageously uses recombinant PrP as a substrate, and cycles of vigorous shaking in place of sonication. The accumulation of misfolded PrP is detected using thioflavin T which fluoresces on binding to amyloid fibrils as they form (78). This has proved to be a very valuable diagnostic CSF test for sCJD, with a sensitivity of between 80 and 91% and specificity of between 98 and 100% (79-81). It is now becoming established as the main laboratory diagnostic test for sCJD at referral/surveillance centres, taking over from 14-3-3 and tau, and it has been shown to have excellent inter-laboratory reproducibility in formal ring-trials (79). In its established form it gives a binary positive or negative result. It may be somewhat less sensitive to atypical, more slowly progressive strains of sCJD, and its performance in other prion disease types is variable(82). CSF from patients with rapid, CJD-like IPD reliably produces positive RT-QuIC reactions, while CSF from patients with less rapidly progressive forms of IPD and with vCJD appears to less readily seed the reaction(83).

A number of modifications and improvements to the RT-QuIC method have been proposed, both to improve its diagnostic performance and to provide a quantitative (rather than a binary positive/negative) result, which might allow exploration of further roles as a biomarker beyond diagnosis. A second-generation RT-QuIC method (using a truncated Syrian hamster recombinant PrP substrate and some modifications to incubation conditions) appears to have improved sensitivity without loss of specificity (giving a positive result in 80% of samples testing negative with the original assay method)(84-86). This method also demonstrates some strain-specific differences in the kinetics of amplification.

Using a process of end-point dilution, RT-QuIC can be used to determine the concentration of “seeds” in a test sample in a quantitative way (87), although any clinical significance of applying this approach to human CSF as a quantitative biomarker has not yet been explored. Some other approaches such as quantifying aspects of the reaction kinetics (e.g. duration of lag phase, area under the curve, maximal fluorescence) have been explored with some promise (88).

Blood

Since the start of the BSE/vCJD public health crisis there were concerns about the onward (human-to-human) transmission of vCJD by blood transfusion. These concerns were realised as iatrogenic vCJD was diagnosed in three recipients of blood from donors who themselves went on to die from vCJD, and asymptomatic infection was seen in a fourth transfusion recipient at post-mortem (89-91). These circumstances have been extensively reviewed elsewhere (92). This transmission of vCJD from human to human through transfusion of blood that was donated during the asymptomatic incubation phase of the disease,

combined with the fact that there is likely to have been very widespread dietary exposure of the UK population to BSE prions, raised major public health concerns around the safety of blood products from UK blood donors. This prompted a number of changes in transfusion practice (including routine leucodepletion of donated red cells, and disposal of plasma fractions from UK donors), and also drove efforts to develop assays that could detect vCJD infection in blood and be applied for screening. Prion infectivity (determined by transmission to laboratory mouse models) has been demonstrated in both vCJD and sCJD plasma (93), but no clinical cases of prion disease attributable to blood-borne transmission from a patient with sCJD have been identified.

One of the striking characteristics of the acquired prion diseases is that the incubation period – the time between exposure to exogenous prion-infected material and symptom onset – can be extremely long. In iatrogenic CJD related to cadaveric human growth hormone and in kuru, it is thought that incubation periods can be 40 years or even longer (94-96). In the case of vCJD, the incidence of the human disease peaked around 8 years after the peak incidence of BSE amongst UK cattle, and has subsequently fallen steadily. However, prior to 2009, all patients diagnosed with vCJD and tested were found to be *PRNP* codon 129 methionine homozygotes, raising the possibility that there might be a genetically-determined variation in incubation period (as appears to be the case for both iCJD and kuru). Subsequently two patients heterozygous at codon 129 have been diagnosed with vCJD, and it remains to be seen whether this represents the beginning of a further disease outbreak amongst this genetic group (97, 98).

It should be remembered that while the compelling motivations regarding public health have led to a focus on blood biomarkers for diagnosis of vCJD infection and for screening blood donations, blood biomarkers for sCJD could also be very useful and could have advantages over CSF in several respects. Blood samples can be taken easily, without involving specialist neurological clinicians, and so would be ideal for an early diagnostic test. Repeated blood sampling to track disease progression (and possibly response to experimental therapeutics) is much more feasible and acceptable to patients than repeated lumbar puncture.

PrP-based markers in blood

Exploiting the peculiar physical, chemical and biological properties of disease-related PrP aggregates has been the mainstay of many assays for prion disease. This is a particularly appealing approach for blood based assays, where there is a significant challenge of detecting very small amounts of disease-specific PrP isoforms against a background of the substantial amounts of normal PrP present in blood. These properties include protease resistance, adherence to steel surfaces, the ability to seed the misfolding and aggregation of monomeric PrP *in vitro*, and the ability to infect susceptible animals with prion disease.

PrP concentration and protease resistant/aggregated PrP

The prion protein is normally expressed in blood, so detecting small variations in its concentration related to CNS disease is challenging. Using highly sensitive methods (DELFIA) in a small set of patients with sCJD, vCJD and healthy and neurological controls showed that normal PrP levels in whole blood appear to be reduced in vCJD but also in the neurological control group, while levels were increased in plasma of patients with sCJD relative to both healthy and neurological controls. There was substantial overlap between groups.(99)

Classical methods for detecting protease-resistant PrP, as used to demonstrate and characterise disease-related PrP aggregates in solid tissues, have not been able to do so in blood.

Immunoassay of steel-binding PrP species ("Direct Detection Assay")

An approach based on the avid adherence of prions to steel surfaces was pursued to develop a blood test for vCJD, which uses stainless steel powder to capture disease-associated PrP for immunodetection(100, 101). In the initial report this was studied in 21 whole blood samples from patients with vCJD, 27 from patients with sCJD, 42 other neurological diseases and 100 healthy controls (UK blood donors). 15 (71%) of the vCJD cases were classified as positive using the assay while no other samples were, suggesting a high level of specificity. This was further investigated by testing 5000 blood samples from healthy volunteers from a population not exposed to BSE (USA), from which there were no false positives, suggesting extremely high specificity (95% CI of 99.93 – 100%). 2 patients with sCJD have subsequently tested positive, showing that the test is not entirely specific to vCJD. This assay has not been used to test blood samples taken during the pre-symptomatic incubation phase of vCJD so there is no direct evidence that it can identify these, although the same assay methodology has been shown to identify blood samples from mice in the very early incubation period of prion infection (102).

In vitro PrP seeding methods

The clinical transmission of vCJD by transfusion of blood donated prior to symptom onset clearly implies that prion infectivity can be present in the blood of affected individuals during the pre-symptomatic phase. A large number of transmission studies in small and large animals also suggest that prion infectivity is present in the blood of asymptomatic infection carriers (summarised in (103)). Assay methods based on detecting PrP seeding activity in blood as a surrogate for infectivity therefore have a promising theoretical basis.

In 2016, two groups published methods based on PMCA that were able to identify small sets of vCJD blood samples (14 and 18 in each study) with almost perfect sensitivity and specificity against mixed control groups including patients with sCJD, various non-prion neurological diagnoses and healthy controls (104, 105). These appear to be the most promising blood-based diagnostic tests for symptomatic vCJD currently available. However, the necessarily small numbers of patients included in these studies, and the lack of any

opportunity for prospective validation given the thankfully minimal incidence of vCJD in recent years, should be borne in mind.

These PMCA methods (104, 105) have not yet been applied to large sample sets to establish their specificity in this context as far as we are aware, and as they both rely on substrate derived from transgenic mouse brain, prolonged rounds of incubation and sonication, and on Western blotting, there would be major practical challenges to overcome before they could be applied for large-scale screening. However their reported levels of sensitivity and specificity are very promising.

Uniquely, the method published by Bougard *et al* has also been applied to blood samples taken from patients with vCJD prior to the onset of symptoms. This was made possible by the practice of the French Blood Service storing aliquots of all blood donations, including those from two vCJD patients who were regular blood donors in the years prior to their illnesses. For both patients, multiple pre-symptomatic samples were available for testing and it was found that the earliest samples tested negative but those closer to symptom onset were positive. The earliest positive sample was taken 31 months prior to symptom onset (104).

Surrogate markers in blood

Tau

Quantifying total tau in serum using standard ELISA methods showed promise that this may provide a useful marker, but most controls and some CJD cases were below the limit of detection of this sort of assay, limiting its potential (106). Using new ultra-sensitive immuno-assay methods (*Simoa*) several groups have now shown that tau is often markedly increased in the serum and plasma of patients with sCJD and some other prion disease types relative to healthy controls and a number of neurodegenerative and other conditions (107-109). Significantly this includes conditions that may have an overlapping clinical presentation with less typical cases of prion disease. Correlation of plasma tau with CSF tau is strong (Spearman R=0.59) (108). Comparing plasma tau in sCJD with other non-prion diagnoses causing rapidly progressive dementia, the areas under ROC curves were 0.722 for non-AD pathologies and 0.756 for rapidly progressive AD (108).

Neurofilament light chain

NfL, measured in serum or plasma using the same ultrasensitive immunoassay platforms, has recently shown great promise as a biomarker across a range of neurological diseases (neurodegenerative and otherwise). Several studies have shown that NfL concentration is very substantially increased in the blood of patients with sCJD, with little or no overlap with levels found in healthy controls (107-109). Correlation of plasma NfL with CSF NfL is also strong (Spearman R=0.69) (108). However, as expected, there does seem to be an overlap with clinically relevant control groups. In Kovacs *et al*'s paper comparing plasma NfL in sCJD

with groups of rapidly progressive dementia caused by Alzheimer's disease or a mixture of other pathologies, the areas under ROC curves were 0.657 and 0.497 respectively. This means plasma NfL is unlikely to have a role in distinguishing established CJD (i.e. at the stage that patients are typically referred to specialist clinical services currently) from other conditions that can cause a similar clinical presentation, but it may nevertheless be very useful. For example, it might have a role as a screening or "triaging" test for patients with early or mild symptoms, providing evidence that a patient has a destructive neurological condition of some kind, and prompting further definitive and disease-specific investigations. Work to directly evaluate this potential application will be useful.

Other analytes

The potential use of olfactory mucosa and skin as relatively accessible tissues to sample for the diagnosis of sCJD has recently been explored. Olfactory mucosa can be sampled with a relatively non-invasive brush or swab procedure under fibre-optic guidance, and when tested using RT-QuIC appears to have high sensitivity and specificity comparable to that of CSF (110-112). A single study using RT-QuIC to test skin samples, albeit mostly obtained at autopsy, showed promising results in a small group of patients including sCJD and vCJD(112), and would warrant further evaluation in *in vivo* samples.

Urine may also have potential as a minimally-invasive analyte. While no protease-resistant PrP can be identified in urine, a PMCA assay was able to detect PrP seeding activity in 13 out of 14 tested samples from patients with vCJD, with no positive results from 68 sCJD patients and 152 mixed controls(113). Interestingly, a study using an adapted Direct Detection Assay (immunodetection of steel-binding PrP) found positive results in 40% of sCJD and only 8% of vCJD cases tested, with no false positives amongst 125 mixed controls(114).

Fluid biomarkers of disease severity and/or rapidity

There are considerable challenges for clinical trials in a rare, rapidly progressive and highly disabling condition like sCJD (10, 115). Clinical symptoms and signs in sCJD can be highly variable between different patients, particularly in the early stages, as can their rate of progression over time. Consequently, comparing or combining clinical parameters derived from different patients in a statistically robust way can be challenging(116, 117). Biomarkers that provide objective measures of severity (meaning the degree of disease progression, on a continuum from onset of neurodegeneration to death) or rapidity of the underlying disease process could be very helpful as outcome measures both in the symptomatic and asymptomatic phases of illness. However, development of biomarkers for these purposes itself faces some of the same difficulties. Suggestive conclusions can be drawn from studying cross-sectional data from many patients studied at different stages of disease, but this requires caution in the knowledge of the make-up of these patient populations and potential confounding factors. For example, patients with rapidly progressive disease will tend to be enrolled to clinical and biomarker studies at a later stage of disease than those

with more slowly progressive disease, so disease severity and rate of decline will be strongly associated and may lead to misleading results if only one is considered. On the other hand, longitudinal data will inevitably be enriched for patients with more slowly progressive disease, as those with the most rapidly progressive symptoms will have died or become too disabled to participate in research studies.

Israel has a particularly high prevalence of IPD related to the E200K mutation of *PRNP*. These patients have been studied in respect of the role of tau as a CSF biomarker of disease severity. Correlation was seen between CSF tau and disease severity measured by multiple rating scales, and also with extent of cortical involvement on diffusion-weighted MRI sequences (118, 119). Serial measurement of CSF tau suggests it may increase from early to mid-stage, and possibly tail off at late stage disease (42), but larger studies would be useful.

A study looking at several brain-derived proteins measured in CSF (total tau, p-tau, 14-3-3, NSE) and correlating these with different aspects of brain pathology assessed at post mortem concluded that different markers showed different patterns (120). Interestingly, this suggested that there was a negative correlation between tau and 14-3-3 and the extent of cortical neuropathology. However this careful study, with CSF markers assessed at a single in vivo time-point and pattern of brain pathology assessed at the single post mortem time-point, is open to multiple interpretations.

Both CSF α-synuclein and NfL appear to show some association with total disease duration, with higher concentrations in patients with shorter disease duration (attributed to their having more rapid disease progression, although the caveats above should be borne in mind). α-synuclein showed relative stability in individual patients across different disease stages, and NfL showed some tendency to gradual increase (in the small number of patients in each study with serial CSF samples) (63, 121). Comparing lumbar punctures done at different disease stages (defined by dividing each patient's total disease duration into 3 equal parts), one large study showed that CSF 14-3-3, tau, S100b and NSE had higher sensitivity at later disease stages, suggesting that these may become progressively more abnormal through individual disease courses, and this appeared to be corroborated by the subset of individual patients with serial samples tested (122).

Total PrP concentration in CSF measured on more than one occasion in a small number of patients with sCJD showed a trend towards progressive reduction through the disease course, although this was not consistent across all individual patients (69).

The MRC Prion Unit/National Prion Clinic have recently published data showing that serum tau correlates with rate of clinical progression in sCJD when this is quantified using a validated CJD-specific rating scale, and also measured serum tau and NfL in serial samples from a small number of individual patients (109, 123). We and others have also shown that tau concentration in blood varies with *PRNP* codon 129 genotype in sCJD. This mirrors, and may well result from, the marked difference in rate of disease progression (and presumably neuronal loss) between these groups, but might also indicate a strain-specific phenomenon. The considerable practical advantages of a readily accessible blood biomarker for monitoring disease activity warrant further study of this area.

Stratification – predictors of disease phenotype

Rate of clinical progression (disease rapidity) is highly variable both between and within the different prion diseases, as is the specific constellation of symptoms that predominate (particularly early in the disease course). The most rapidly progressing sCJD patients survive for only a few weeks after symptom onset, whereas atypical slowly progressing patients can have illnesses lasting several years. This represents a substantial challenge to the design of therapeutic trials with clinical end-points, as any 'signal' of a treatment effect must be detected against the 'noise' of this natural variation. If aspects of subsequent clinical progression can be predicted at the point of enrolment to therapeutic trials, allowing stratified analysis of more homogenous patient groups, statistical power might be substantially improved – making it more likely that the conclusions of the trial will be correct (124). Giving patients and families more detailed and accurate predictions of the likely course of their illness is also of value in itself. Several established and emerging biomarkers may contribute to this.

The most well-established single modifier of prion disease phenotype is *PRNP* codon 129 genotype (124, 125). As a stratification biomarker this has the major strength of being unchanging in each patient, with the same result obtained whether the patient is tested prior to symptom onset or at the end stage of disease, as well as being readily testable from a standard blood sample.

In IPD, different pathogenic *PRNP* mutations are associated with vastly different clinical phenotypes, some of which progress slowly over the course of many years, while others have very rapid clinical progression indistinguishable from that of sCJD (126). Some present with gradual cognitive decline as the predominant symptom (18), while others present with a progressive cerebellar syndrome with relatively preserved cognition (Gerstmann-Straussler-Scheinker syndrome). Clearly, stratifying patients according to *PRNP* mutation

will be essential in therapeutic trials for mixed inherited prion diseases, to allow appropriate choice of outcome measures and meaningful analysis.

Prion strain type, defined according to the pattern seen on Western blot of disease-related PrP after partial protease digestion, interacts with codon 129 genotype as a phenotype modifier of sCJD. In practice, this can usually be established only by *post mortem* analysis of brain tissue, and as such cannot act as a predictor of phenotype in practice. A biomarker that determines strain type during life, without resorting to brain biopsy, would be useful. Some efforts have been made to identify specific patterns of surrogate markers in CSF in different sCJD subtypes (82), and there is some indication that CSF total tau and NfL are differentially altered in different sCJD strains (64). There are promising indications that RT-QuIC, particularly in its second-generation form, may allow differentiation of prion strains (85, 86). However none of these CSF-based markers have yet been studied specifically as predictors of subsequent clinical progression, and it is unclear how much additional predictive value they will add to the strong effect of *PRNP* codon 129 genotype.

Conclusions and directions for future work

The current repertoire of fluid biomarkers for human prion diseases has some major strengths but there are also important unmet needs, and there is great promise for the future.

We are fortunate to have highly sensitive and specific assays available to diagnose sCJD once it is suspected in a patient. Whilst CSF is relatively invasive to obtain, for the foreseeable future it will be essential in the urgent diagnostic work-up of patients to exclude mimicking treatable disorders. The RT-QuIC CSF assay represents a significant advance in the molecular diagnosis of prion disease during life, and may prove to be the prototype for a novel class of biomarker techniques across a range of neurodegenerative diseases (127-129). It continues to be developed and refined, and may have roles to play beyond diagnosis.

Nevertheless, epidemiological data over time makes a strong case for continued under-ascertainment of prion diseases, and making a diagnosis at an earlier stage of disease is an important goal that remains difficult to achieve. A sensitive but less specific test using a readily accessible biofluid like blood could encourage and accelerate the consideration of sCJD in patients with possible early symptoms and in less selected patient populations (e.g. all patients with dementia), if this is then followed by more specific tests. Some of the surrogate blood biomarkers show potential for this role, but will require direct evaluation in the relevant clinical populations.

Novel analytes such as olfactory mucosa (sampled using a brush or swab), skin biopsy and urine are intriguing and potentially important avenues for future biomarker research.

Unsurprisingly, in the past the focus for blood biomarker development has been on the concern that vCJD infection is being transmitted by blood transfusion. Promising tests have been developed that may have the potential to be adapted and applied for screening donors, but as the number of primary vCJD cases have dwindled, and there have been no further cases of secondary (iatrogenic) vCJD, the investment and prospective samples required to develop these tests further is in question.

For potential use in clinical trials, blood biomarkers of neurodegeneration show promise but need to be evaluated in larger cohorts that have been well studied, both clinically and longitudinally, and we anticipate examples of use in a clinical trial setting.

There are good reasons to expect that treatments for human prion diseases will be more effective when started as early as possible in the disease course, or even before the onset of symptoms in individuals identified as being at high risk of prion disease. In this context a "proximity marker", indicating that a high-risk individual (e.g. a *PRNP* mutation carrier) is likely to be close to the onset of symptoms would be valuable. Recent work shows some potentially interesting properties of total prion protein concentration in CSF, with low

concentrations before the onset of symptoms in a few of the small number of asymptomatic *PRNP* mutation carriers tested(69). Another study included testing of several biomarkers (including tau and NfL in both CSF and blood) in a single patient with IPD before and after the onset of symptoms, providing a hint that several of these may be abnormal in the presymptomatic phase (107). However neither of these studies demonstrated any dynamic change in these biomarkers relative to timing of symptom onset, so they do not provide any direct evidence relevant to predicting this. Future work needs to address when these and other blood and CSF biomarkers change by following individuals from the asymptomatic into the symptomatic phase of the disease.

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Analyte	Biomarker	*Diagnosis : Sensitive?*	*Diagnosis : Specific?*	*Abnormal in pre-symptomatic / carriers?*	*Predicts phenotype ?*	*Marker of severity / rapidity ?*	*Predicts timing of onset in individuals ?*
Brain biopsy	*Histopathology and Western Blot*	**++++**	**++++**		**++?**		
Lymphoid biopsy (e.g. Tonsil)	*Histopathology and Western Blot*	**+++**	**++++**	**++?**			
PRNP genotype	*Codon 129 polymorphism*	**n/a**	**n/a**	**n/a**	**+++** sCJD	**n/a**	**+** iCJD/some IPD
	Pathogenic mutations	**++++** IPD	**++++**	**++++**	**+++**	**n/a**	**+** IPD
CSF	*14-3-3*	**+++** sCJD	**++**			**+?**	
	RT-QuIC, including "2nd generation"	**++++** sCJD/rapid IPD **++** vCJD/slow IPD	**++++**	**-??**	**++?**		
	Total tau (high cut-off)	**+++** sCJD	**++**	**+??**		**+++?**	
	pTau:tTau ratio	**+++** sCJD	**+++?**				
	NfL	**++++** sCJD	**+++**	**+??**		**+?**	
	α-synuclein	**++++?** sCJD	**++++?**			**++?**	
	Total PrP	**++** sCJD, iCJD, IPD	**+?**	**++?**		**++?**	
	Others, inc S100b, NSE…	**++** sCJD, vCJD	**+**				

Blood	*Direct Detection Assay*	**+++?** vCJD	**++++**				
	PMCA+ methods	**++++?** vCJD	**++++?**	**+++??**			**++??**
	Tau	**+++** sCJD	**++**	**-??**		**+++?** sCJD	
	NfL	**++++** sCJD	**++**	**+??**			
Olfactory mucosa	*RT-QuIC*	**+++** sCJD	**++++**				
Skin	*RT-QuIC*	**+++?** sCJD **++?** vCJD	**+++?**				
Urine	*PMCA*	**+++?** vCJD **-?** sCJD	**+++?**				
	DDA	**++?** sCJD **+?** vCJD	**+++?**				

KEY			
Usefulness in this biomarker role		**Quality of evidence flags**	
++++	**Excellent**	**?**	**Unconfirmed: including when evidence is from a single methodologically sound but unreplicated study, different studies are conflicting, or potential to fill this role is extrapolated from indirect evidence not direct evaluation**
+++	**Good**		
++	**Moderate**		
+	**Weak**		
-	**Some evidence that it is not useful**		
	No relevant evidence available	**??**	**Published data from only 1 or 2 cases**

<u>Table 1.</u> This table gives an overview of the available fluid and tissue-derived biomarkers for human prion disease, and gives a global impression of their usefulness for a range of biomarker roles based on the available evidence. This is based on our review of the literature, as discussed in the text. We also indicate where the evidence is limited, conflicting or indirect. Inevitably this is a simplification and should be considered in conjunction with the full discussion in the text.

Fluid biomarkers in the human prion diseases.

Andrew Thompson and Simon Mead

Highlights:

- There are highly sensitive and specific CSF biomarkers for diagnosis of sporadic CJD
- The RT-QuIC assay has been an important advance in molecular diagnosis
- Several tests that can detect variant CJD infection in blood have now been reported
- Brain-derived proteins measured in blood, such as tau and NfL, show great potential
- Beyond diagnosis, these and other biomarkers may be valuable for clinical trials