Preparing for therapeutic trials in human prion disease: clinical and laboratory approaches to improve early diagnosis and monitoring of disease progression.

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DECLARATION

I, Andrew Geoffrey Bourne Thompson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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TABLE OF CONTENTS

ABSTRACT

Clinical and scientific understanding of the human prion diseases has advanced rapidly in recent years, and the possibility of an effective therapeutic agent being found now seems a realistic prospect. Alongside the search for an effective therapy, a number of major obstacles must be overcome to ensure that clinical trials have the best possible chance of being successful, and to maximise the benefit that can be gained from any treatment that is found. This thesis presents several projects that aim to contribute to this. Improving early diagnosis is likely to be key to making the most of any agent's therapeutic benefit. Currently most patients with prion disease are diagnosed at a late stage of disease, when there is likely to be substantial irreversible damage to the brain. Chapters 2, 3 and 4 present work using the recently developed Direct Detection Assay in blood and cerebrospinal fluid, with the aim of improving accurate early diagnosis. Past prion disease clinical trials have suffered from the lack of a validated outcome measure to monitor disease progression. Chapter 6 presents a project carried out in the context of large prospective clinical studies of prion disease in the UK, in which a bespoke clinical rating scale has been developed and validated for use in prion disease clinical trials. Chapter 5 presents a comprehensive study of the complex psychiatric and behavioural features of prion disease, including detailed clinical characterisation, observational study of symptomatic management, and investigation of factors underlying heterogeneity in these clinical features, including a genome-wide association study looking for genetic modifiers. It is hoped that these different avenues of clinical and laboratory research will all help to establish a solid groundwork for upcoming clinical trials in prion disease, maximising the chances that they will be able to demonstrate a real and meaningful benefit for patients.

CHAPTER 1. INTRODUCTION

An introduction to prion diseases

The prion diseases are a group of neurodegenerative conditions affecting humans and animals, with a number of striking and remarkable characteristics. Although the human diseases are rare, affecting around 1 or 2 people per million population each year[1], they have stimulated great scientific interest and our understanding of the diseases and the pathophysiological processes underlying them has evolved rapidly and reached a relatively advanced stage in comparison with many other neurodegenerative conditions.

The first clinical and pathological descriptions of the diseases that we now know as prion diseases were made in the 1920s, several decades prior to the discovery of the structure of DNA or the proposition of the "central dogma" of molecular biology[2]. Since then there has been a gradual process of integrating our increasingly sophisticated understanding of molecular and cellular biology with the clinically and pathologically derived classifications of diseases, and with clinical and experimental observations about them.

A number of observations about prion diseases presented major challenges to this process, as they seemed to contradict the accepted biological wisdom of the time. Foremost amongst these was the observation that prion diseases were transmissible, despite the absence of any identifiable causative agent containing nucleic acid. This is discussed in detail below. As a result, prion diseases have often been thought of as having a "unique biology"[1], and have been seen as an intriguing but esoteric field of research within biology. Interestingly in recent years this has started to change, as what had been seen as "unique" properties of prion biology are increasingly thought to be relevant to other neurodegenerative conditions[3], including some, such as Alzheimer's disease, which are

extremely common and represent major public health challenges in the context of the ageing global population[4,5].

By way of introduction I will review our current understanding of a number of key concepts in prion biology, and then describe the range of different human prion diseases.

Transmissibility of prion diseases

Scrapie, a neurodegenerative disease affecting sheep and goats which is endemic in many parts of the world, was first proven to be transmissible in the 1930s[6]. Experiments in which healthy animals were inoculated with brain tissue from affected animals showed that, after a prolonged incubation period of up to 26 months, the inoculated animals succumbed to the same disease. Experiments in which affected and unaffected flocks of sheep were alternately grazed on the same field, without any opportunity for direct contact, found that after 39 months members of the unaffected flock started to develop the disease. This suggested transmission of the disease by an agent able to survive in the environment without the need for direct animal-to-animal transmission, and helped to explain some aspects of the observed epidemiology of the disease. Further important observations were made from an "accidental experiment" in which scrapie was transmitted by subcutaneous injection of a vaccine preparation (against looping ill, an unrelated disease) derived from tissue including brain, spinal cord and spleen, some of which came from animals affected by scrapie. Despite the fact that the preparation had been treated with formalin to inactivate the looping ill virus, it was still able to transmit scrapie to some of the recipient animals.

This early experience illustrates several important observations about the transmissibility of prion disease: they may have unusually long incubation periods, they may be transmitted by multiple routes, and the infectious agent is resistant to conditions that would inactivate most previously recognised pathogens (such as formalin treatment).

Subsequently, it was established that several human neurodegenerative diseases were also transmissible, including Creutzfeldt-Jakob disease, which has become the archetypal human prion disease. The evidence for this came both from experimental studies involving deliberate transmission of disease to animals (including a large number of experiments carried out at the National Institutes of Health in the USA and reviewed by Paul Brown and colleagues[7]), and also from observations made on a number of instances of accidental transmission by a variety of routes, which are discussed below.

Strikingly, those diseases that were proven to be experimentally transmissible (through inoculation of affected brain tissue) included some that were known to be inherited in an autosomal dominant fashion, and which have subsequently been shown to be caused by specific autosomal gene mutations (all in the prion protein gene, *PRNP*)[8,9]. This raised major doubts over the hypothesis that the conditions were caused by a classical pathogen such as a virus.

A number of instances of accidental transmission of prion disease to or between humans have been reported and studied. Two patients that were implanted with intracerebral electrodes that had previously been used on a patient with an undiagnosed illness that proved to be CJD, went on to develop the same disease, despite standard decontamination procedures being used. The electrodes were subsequently implanted experimentally into the brain of a chimpanzee, who also succumbed to the disease[10,11]. As well as clearly demonstrating transmissibility, this and other examples of brain-to-brain transmission by neurosurgical instruments provided an initial clue that the infectious agent causing prion disease is able to adhere to metallic surfaces, an observation that has ultimately led to a fertile avenue of research, including the work presented below in chapters 2, 3 and 4.

Iatrogenic transmission of prion disease has also occurred on several independent occasions as a result of human-derived tissue being transplanted or inoculated into recipient individuals: this includes the injection of growth hormone harvested from pooled cadaveric pituitary gland tissue, surgical use of cadaveric dura mater tissue, corneal transplant, and more recently whole blood transfusion[12-14].

Another example of accidental human-to-human transmission of prion disease that has been very intensively studied is that of the disease kuru, which caused an epidemic amongst the Fore linguistic group in the Eastern Highlands of Papua New Guinea. It is thought that this was transmitted from person to person as a result of oral consumption of infected tissue at ritual endocannibalistic mortuary feasts [15].

A final example of accidental transmission is that of bovine spongiform encephalopathy (BSE), which caused a major epidemic amongst British cattle during the 1980s and 1990s, being transmitted to humans and causing a novel human disease that is now known as variant CJD (vCJD)[16]. This is discussed in detail below. This illustrates that the diseases can be transmitted between different species, a fact that had previously been observed both experimentally and in accidental transmissions of scrapie. Experimental transmission of prion disease to animals (particularly rodents) has become a mainstay of prion disease research, being used as a highly specific "bio-assay". The transmission of human prion diseases to experimental animals also creates uniquely robust animal models of human degenerative disease in which to test putative therapies, which is much more difficult in other conditions. For example, efforts to produce animal models of Alzheimer's disease have tended either to focus on one specific aspect of the pathophysiology (such as an amyloid precursor protein (APP) transgenic that exhibits some abnormal β-amyloid (Aβ) deposition, but no tau deposition), or elaborate double or even triple transgenics that aim to piece together a more complete range of clinical and pathological features[17]. The extent to which these

models succeed in simulating the complex pathophysiological processes in the brain of a patient with Alzheimer's disease, and to which observations made in these models (including the effects of putative treatments) will be applicable to patients is not at all clear. An animal to whom a human disease has been directly transmitted and which will go on to develop a disease with the same constellation of pathological features seems a much more satisfactory model to use from first principles.

PRNP, PrP and Prions

It has become clear that the prion protein, encoded by the prion protein gene (*PRNP*), is absolutely central to the molecular pathology of all prion diseases. Early efforts to isolate the scrapie infectious agent from brain tissue of hamsters infected with scrapie identified a 27 – 30 kDa insoluble, protease-resistant protein that appeared to be present in all infectious isolates[18]. A similar protein was also isolated from human brain tissue from patients with CJD. Sequencing part of the primary amino acid structure of this protein allowed the coding nucleic acid sequence to be inferred, and this sequence was found in an autosomal gene, with closely homologous sequences in hamster and human, encoding a protein present in normal brain: the normal or cellular prion protein(PrP^{c})[19,20]. It has subsequently been shown that transgenic animals that do not express the prion protein are entirely resistant to acquiring prion disease[21], and also in more sophisticated experiments that "turning off" host prion protein expression *after* inoculation with infectious material will prevent the disease from progressing and may even reverse some of the pathological changes[22,23]. The fact that all inherited prion diseases show linkage to the prion protein gene locus again suggests that this protein is the key molecular player in these diseases. Understanding the prion protein gene, the prion protein, and the ways in which changes in this protein occur in prion disease is therefore vital if we are to understand the diseases themselves.

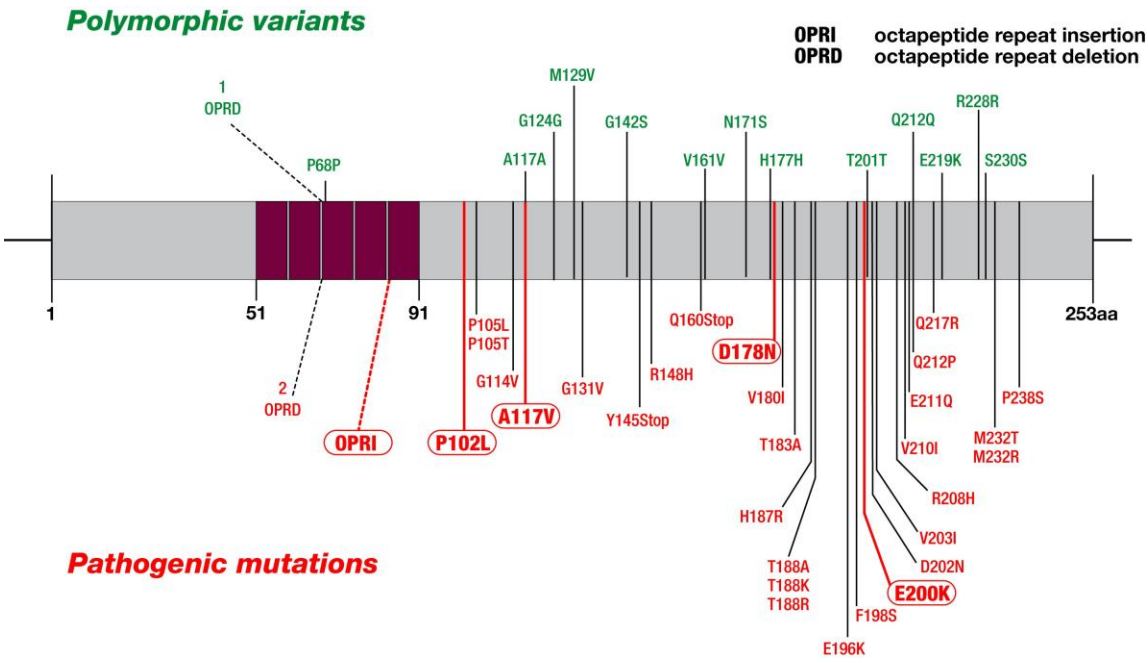


Figure 1. The prion protein gene (*PRNP*). This schematic shows the open reading frame of *PRNP*, with pathogenic mutations labelled in red, polymorphic variants labelled in green, and the octapeptide repeat section illustrated in purple.

In humans the prion protein gene, on the short arm of chromosome 20, consists of two exons, the second of which contains the entire open reading frame for the prion protein[24]. There are more than 30 known pathogenic *PRNP* mutations causing inherited prion diseases (which are reviewed below). A number of non-pathogenic polymorphisms are also found in the gene. The most important of these is the methionine/valine polymorphism at codon 129, which has been found to have a very profound modifying effect on many aspects of prion disease, including susceptibility, incubation times, rate of disease progression and clinicopathological phenotype[1]. Other polymorphisms of interest include E219K, which affects susceptibility to some specific disease types[25,26], and G127V, which has been found specifically in surviving members of the population in Papua New Guinea affected by the kuru epidemic, and appears to confer resistance to this disease[27].

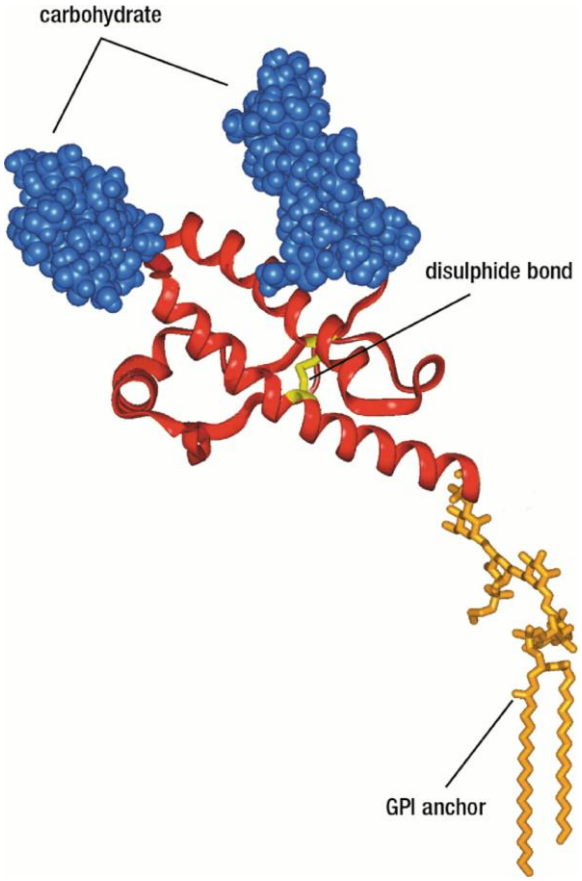


Figure 2. Structure of PrP^c. This shows the predicted structure of the C-terminal portion of PrP^c, with its associated carbohydrate molecules and GPI anchor.

The normal cellular prion protein is widely expressed, with the highest concentrations in brain and in lymphoreticular tissues[28]. It is a membrane-bound cell surface di-glycosylated protein with a glycosylphosphatidylinositol (GPI) anchor[29], and consists of two regions, each of around 100 amino acids: an unstructured N-terminal portion, and a structured C-terminal portion with several alpha-helices stabilised by a disulphide bond. This normal form of PrP is soluble, and is sensitive to degradation by proteases, including proteinase K.

In contrast, prion protein isolated from brain tissue of animals affected by scrapie, and from humans affected by prion disease (all denoted PrP^{Sc} - "PrP scrapie") is insoluble, relatively resistant to digestion by proteinase K, and has a beta-sheet-rich structure and a strong tendency to aggregate[30]. The primary amino acid structure of PrP^c and PrP^{Sc} are identical, so their strikingly different

properties are thought to be conferred by post-translational changes in the conformation of the protein.

One of the properties of the infectious agent of prion disease that has been most challenging to explain is that of prion strain. Many infectious diseases caused by micro-organisms such as bacteria or viruses demonstrate a strain phenomenon, whereby variations in the specific properties of the infectious agent and of the resulting clinicopathological disease entity are conserved on serial passage of the infection from one host to another. In these diseases these strain characteristics are thought to be encoded primarily by variation in the nucleic acid genome of the causative organism. However prion diseases, caused by an infectious agent devoid of nucleic acid, also demonstrate a strain phenomenon. On serial passage of infectious material derived from animals or humans affected by prion disease in experimental animals, such as inbred mouse lines, a number of distinct and consistent patterns (of incubation period, clinical features and pathological changes) may be seen[31]. There is also variation in the biochemical properties of different PrP^{Sc} isolates, most notably in the banding pattern seen on Western blotting after partial digestion with proteinase K[32]. There is a correlation between specific Western blotting signatures and specific clinicopathologically defined human prion strains[33-35].

It is thought that the strain characteristics of the prion disease infectious agent are encoded primarily by variation in the physical conformation of abnormally folded PrP[31]. If the conversion of normal PrP^{c} into disease-associated PrP^{Sc} involves a templating process, whereby the specific conformation of the misfolded protein is reproduced as this misfolding propagates, and this specific conformation confers specific pathogenic and biochemical properties, this could explain the conservation of prion strain characteristics. The mechanisms by which the prion protein may take on multiple different abnormal conformations, how these are transferred to newly-misfolded proteins (even if these are proteins in a new host of a different species), and how these result in the

different biochemical properties of the protein and the different clinicopathological features of the diseases have not been determined.

The human prion diseases

Classification

Based on our current understanding of the biology of prion disease as reviewed above, a rational basic scheme for classifying the human prion diseases is by aetiology. They all involve the templated misfolding of PrP^{c} into abnormal disease-associated forms, but the reason that this process begins varies between different disease types. It may occur as the result of an identifiable genetic mutation in the prion protein gene (*PRNP)* (in inherited prion disease); it may be seeded by exposure to abnormally folded prion protein from an exogenous source (in acquired prion disease); or it may occur without any obvious cause, and in what appears to be a primarily random distribution (in sporadic prion disease).

Within these groups we can sub-classify the diseases based on molecular or mechanistic factors, and on the basis of clinically-defined syndromes. Historically, the latter method has predominated, with clinicians and pathologists observing recurring patterns of clinical symptoms, signs and neuropathological changes, and defining diseases on this basis (and often lending their names to them as eponyms). More recently as our understanding of the molecular and mechanistic basis of the diseases has increased, it has been possible to use these to define disease sub-types. Although these often coincide with those defined on a clinicopathological basis, this is not always the case, and the integration of these two approaches is not always straightforward.

The classification scheme that I will use is shown in the diagram below. Particular issues relevant to each aetiological disease type will be discussed in the relevant sections below.

Sporadic prion disease	Sporadic CJD	*PRNP* codon 129	PrP^{Sc} Type
		MM	1
			2
			3
		MV	2
			3
		VV	2
			3
	VPSPr	*PRNP* codon 129	
		MM	
		MV	
		VV	
Inherited prion disease	Clinical syndrome	*PRNP* mutation/haplotype	
	Familial CJD	E200K	
		D178N	
		4OPRI	
		Others…	
	GSS	P102L	
		A117V	
		P105L	
		Others…	
	FFI	D178N-129M	
	Larger OPRI IPD	6OPRI	
		Others (7-, 8-, 9-, 10OPRI)…	
	Others	Y163X and others…	
Acquired prion disease	Iatrogenic CJD	Source of infection	
		Pituitary hormones	
		Dura mater graft	
		Neurosurgical instruments	
		Others…	
	Variant CJD	*PRNP* codon 129	Source of infection
		MM	Bovine
			Blood-borne
		MV?	
	Kuru		

Table 1. Classification scheme for human prion diseases. Abbreviations: CJD = Creutzfeldt-Jakob disease; VPSPr = Variably protease sensitive prionopathy; GSS = Gerstmann-Straussler-Scheinker syndrome; FFI = fatal familial insomnia; OPRI = octapeptide repeat insertion; IPD = inherited prion disease; MM, MV and VV refer to *PRNP* codon 129 genotype; 1, 2 and 3 refer to PrP^{Sc} type as classified by Hill et al[36].

Common features of all prion diseases

Although there is remarkable clinical heterogeneity between different prion disease types, and indeed within them, there are some common features that they all share. The diseases are all inexorably progressive, and ultimately fatal. Their heterogeneity is particularly apparent in the earlier stages of the diseases, but they seem to gradually converge towards a more consistent clinical picture seen across all disease types in their final stages: one of akinetic mutism, usually with rigidity and myoclonus.

There are also neuropathological hallmarks of prion disease, which are seen to varying degrees in the post mortem brain in all disease types. These are spongiosis, neuronal loss, deposition of abnormal prion protein and gliosis[37,38]. The characteristics of these changes (e.g. the pattern of PrP deposition) vary greatly between disease types (and in fact are often used to distinguish between them[37]), but they are present in some form in all prion diseases.

Sporadic prion disease

Sporadic Creutzfeldt-Jakob Disease (sCJD)

In terms of incidence, the most common type of prion disease is sporadic Creutzfeldt-Jakob disease. It occurs in around 1-2 people per million population per year in the UK, and the incidence appears to be similar in a wide range of populations worldwide where it has been studied, taking into account the varying intensity of surveillance[39-44]. This results in between 60 and 100 new cases being diagnosed with sCJD in the UK each year (see www.cjd.ed.ac.uk for year-on-year figures).

The disease typically manifests itself with a rapidly progressive neurocognitive syndrome, with the patient's condition changing much more quickly than would be expected in any other neurodegenerative condition[45]. The order in which different neurological systems are affected varies between different patients, leading to great heterogeneity in the early stages of the disease.

The most common presentation is with a rapidly progressive dementia, with physical neurological signs such as myoclonus and ataxia becoming more apparent as the disease progresses. Some other patterns of early neurological deficit that may be seen include a predominantly visual disorder (known as the "Heidenhain variant"[46,47], and a predominantly cerebellar disorder (known as the "Oppenheimer-Brownell variant"[48]). A host of other less-frequently-encountered clinical presentations with focal neurological symptoms and signs have been reported in case reports and case series. Psychiatric and behavioural symptoms are also common and have been recognised since the disease was first described: these are considered in detail in Chapter 5 below. The average duration of the illness from symptom onset to death is just 4 to 6 months, and in the most rapidly progressive cases may be only a few weeks[33,35]. Atypical cases may have a less rapid progression and a longer duration. Survival beyond 2 years of symptom onset is unusual, but is recognised in a small minority of cases. As the rapidity of progression is often used as the "red flag" feature leading to consideration of the diagnosis (and indeed a duration of greater than 2 years rules out the diagnosis according to the WHO criteria), it is likely that atypical, more slowly progressive cases have been under-recognised. This conclusion is supported by studies such as that by Bruton et al[49] which ascertained cases by screening of archived brain tissue from patients with any history of dementia, and found that 8 of the 19 cases found to have the pathological changes of CJD had not been correctly diagnosed during life, and that most of these had an atypical clinical presentation with a duration of more than 3 years.

Some of the factors accounting for this heterogeneity in the clinical presentation of sCJD have been identified, and provide valuable insights into the pathophysiology of the disease. These include genetic variation in the prion protein gene, and variation in the biochemical properties of the abnormal disease associated prion protein (PrP^{Sc}) which can be determined by carrying out Western blotting of post mortem brain tissue after digestion with proteinase K (PK), and is thought to provide a surrogate marker for variation in prion strain[36].

The polymorphism at codon 129 of the prion protein gene (*PRNP*) is very well established as both a determinant of susceptibility to sCJD and a modifier of the clinical presentation, including rate of disease progression[1,50]. Individuals that are methionine/valine heterozygous at codon 129 are at a lower risk of developing the disease than those homozygous for either allele, and if they do then they tend to have a less rapidly progressive disease, and also have a different profile of clinical features, investigation findings and neuropathological changes[34,35]. Several distinct isoforms of PrP^{Sc} can be found on Western blotting of PK-digested brain homogenate from sCJD patients, each producing a characteristic banding pattern. Two different systems for classifying and naming these PrP^{Sc} types have been proposed, one based around there being two PrP^{Sc} types [33] and one proposed by the MRC Prion Unit[36,51] in which there are 4 principal PrP^{Sc} types (see Table 2). PsP^{Sc} type has also been shown to be significantly associated with specific clinical, investigational and neuropathological profiles[34,35].

Type as per Collinge et al.	Type as per Parchi et al.	Prion diseases in which it has been found	*PRNP* codon 129 genotypes in which it has been found
1	1	sCJD, iCJD	MM
2	1	sCJD, iCJD	MM, MV, VV
3	2A	sCJD, iCJD	MV, VV (MM rarely)
4	2B	vCJD	MM

Table 2. Basic classification schemes for PrP^{Sc} that have been suggested by Collinge et al[32,51] and by Parchi et al[33]. The correspondence between different types in the two classification schemes has been the source of some controversy and may not be exact.

There is an interaction between *PRNP* codon 129 genotype and PrP^{Sc} type, and a useful way to sub-classify sCJD is on the basis of these two factors. For example, Type 1 MM sCJD (i.e. a patient that is methionine homozygous at PRNP codon 129 and has type 1 PrP^{Sc} on Western blotting) will almost always have had a classical rapidly progressive sCJD phenotype, and is relatively likely to have had the typical investigation findings of periodic sharp wave complexes on electroencephalogram (EEG) and presence of 14-3-3 protein in the cerebrospinal fluid (CSF), compared with other sCJD patients[34]. Variation in neuropathological features also correlates with PrP^{Sc} type and codon 129 genotype: for example, Type 3 MV sCJD cases are typically found to have kuru-type plaques, particularly in the cerebellar cortex, while these are not seen in any other PrP^{Sc} type/codon 129 genotype combination of sCJD[32].

Age also represents a risk factor for development of sCJD, with most patients being diagnosed in their late fifties, sixties or seventies, and extremely few cases presenting before the age of 40[52]. Incidence appears to decline at older ages, although it is possible this is at least partly explained by lower rates of diagnosis in this age group due to less aggressive investigation and more frequent misdiagnosis as Alzheimer's disease or another common age-associated neurodegenerative disease. Age also appears to act as a modifier of the clinical presentation, with a number of clinical features

being more common in patients with onset of disease at a younger age, and also a tendency to longer disease duration in younger patients[53].

sCJD has historically been thought of as a diagnosis of exclusion, with a focus on investigations looking for evidence of an alternative, reversible cause of rapidly progressive neurocognitive decline (such as an inflammatory, metabolic, or neoplastic process). While it is the case that there are treatable conditions that may mimic the presentation of sCJD (which itself remains untreatable and invariably fatal) it is now usually possible to reach a positive diagnosis on the basis of clinical presentation and investigation findings. Even in the absence of a disease-modifying treatment, this is valuable as it reduces the amount of time that is taken up with medical investigations (time that is extremely precious in the context of a rapidly progressive and fatal diagnosis), as well as the risks associated with these investigations. For example, brain biopsy should rarely be necessary in the diagnosis of sCJD if patients are investigated appropriately[54]. Making a timely positive diagnosis may also allow patients to play a more active role in planning their end-of-life care, and will allow patients to be enrolled into clinical studies, including therapeutic trials at an earlier stage: issues related to this are discussed in detail below in Chapter 6.

The criteria for diagnosis of sCJD have been formally set out by the World Health Organisation (WHO), and modifications have been introduced on the basis of new evidence and improved diagnostic technologies. The most recently updated version of the criteria is shown in Table 3 below. The major change in this version was the inclusion of MRI scanning of the brain as a supportive investigation, which has substantial benefits for sensitivity of the criteria.

A. Clinical signs
1. Dementia 2. Cerebellar or visual dysfunction 3. Pyramidal or extrapyramidal dysfunction 4. Akinetic mutism
B. Tests
1. Periodic sharp wave complexes on EEG 2. Presence of 14-3-3 protein in CSF (in patients with a duration of less than 2 years) 3. High signal abnormalities in caudate nucleus and putamen or at least two cortical regions (temporal, parietal, occipital) on DWI or FLAIR MR brain imaging sequences
Probable CJD
Two from A and at least one from B
Possible CJD
Two from A and duration less than 2 years

Table 3. Clinical diagnostic criteria for sporadic CJD, updated to include MRI brain scanning results. Adapted from Zerr et al, 2009[34].

The specific role of CSF in the diagnosis of sCJD is discussed in detail below in Chapter 3, and the development of new diagnostic techniques, which are not yet incorporated into the formal criteria, is discussed in Chapters 2 and 3.

Variably protease sensitive prionopathy (VPSPr)

In 2008, Gambetti et al[55] reported 8 patients with what appeared to be a novel disease involving the prion protein, which they called Protease Sensitive Prionopathy. The patients had a progressive and

fatal neurodegenerative disease, and on neuropathological examination of the brain were found to have an unusual pattern of abnormal PrP deposition. This abnormal PrP was also found to be predominantly protease-sensitive, in contrast to that found in sCJD, and produced a quite distinct pattern on Western blotting from that seen in any subtype of sCJD. Interestingly, all 8 of these patients were homozygous for valine at the *PRNP* codon 129 polymorphism (129VV). Subsequently, however, further patients have been reported, including individuals with all codon 129 genotypes[56,57]. The non-129VV individuals reported were found to have relatively protease-resistant abnormal PrP, although still with the same distinct banding pattern on Western blotting. The disease was therefore renamed Variably Protease-sensitive Prionopathy (VPSPr).

The clinical presentation in these patients was with a combination of psychiatric symptoms (particularly affective symptoms and alteration in behaviour), a frontal-predominant cognitive decline, language dysfunction, parkinsonism and ataxia[56,57]. Based on the 26 cases so far reported, there does appear to be some effect of codon 129 on the clinical presentation, with psychiatric and cognitive symptoms more prominent in 129VV individuals, while motor signs predominate in the 129MV and 129MM individuals, who may also have myoclonus, which does not seem to occur in 129VV patients. In comparison with sCJD, this disease tends to have a longer duration, and a somewhat different (though overlapping) pattern of symptoms and signs. It has not yet been reported whether this disease is transmissible to experimental animals, and so it is not clear whether it represents a true *prion* disease.

There are no patients with this novel disease included in the clinical studies presented in this thesis.

Inherited prion diseases

Around 10 to 15% of patients presenting with prion disease are found to have a pathogenic mutation in the prion protein gene (*PRNP*), and in most cases will have a family history of a similar illness consistent with autosomal dominant inheritance. More than 30 pathogenic mutations have been identified[24], although a relatively small number of these account for the majority of cases of inherited prion disease seen in practice. The pathogenic mutations are either nonsense point mutations causing a single amino acid substitution (or rarely a premature stop codon resulting in a truncated protein), or insertion mutations causing expansion of the octapeptide repeat section of the protein. The mutations are fully penetrant, apart from a few exceptions that are discussed below.

The clinical phenotype of inherited prion disease is remarkably variable, and several distinct clinicopathological diseases were described prior to the demonstration that they were all caused by mutations in the same gene. These canonical IPD syndromes are familial CJD, Gerstmann-Straussler-Scheinker syndrome (GSS), and Fatal Familial Insomnia (FFI). In addition it is now clear that some of the relatively common PRNP mutations (the octapeptide repeat insertions with 5 or more additional repeats) typically cause a relatively slowly progressive disease dominated by cognitive decline, which is quite distinct from the 3 canonical clinical syndromes[58,59]. There are clear genotype/phenotype relationships, with particular *PRNP* mutations tending to cause particular clinical syndromes, although these relationships are not absolute and quite distinct clinical presentations are sometimes seen in different affected members of a single pedigree. As in sCJD, the codon 129 polymorphism of *PRNP* acts as an important modifier of the phenotype in a number of the inherited prion diseases. The major clinical phenotypes of inherited prion disease are reviewed below.

The diagnosis of inherited prion disease requires the presence of a pathogenic mutation in *PRNP* to be demonstrated in a patient with a compatible clinical presentation. The clinical investigations

used to diagnose sCJD (presence of signal change on brain MRI, periodic sharp wave complexes on EEG, 14-3-3 protein in the CSF) may be abnormal in IPD, particularly familial CJD, but are less sensitive than in sCJD, and are largely made redundant by the highly sensitive and specific molecular test.

As the genetics of inherited prion disease are now well understood, and a reliable molecular test is available to make the diagnosis, patients are often diagnosed at an earlier stage of their disease, and there is the potential for relatives that are at risk of carrying a pathogenic mutation to receive genetic counselling and to consider having a predictive genetic test.

Familial CJD

Some patients with a clinical presentation indistinguishable from that of sCJD have a pathogenic mutation in *PRNP* causing their illness. The most common pathogenic PRNP mutation worldwide, E200K, usually causes a familial CJD phenotype. It is unusual in that the age of disease onset can vary widely within a single pedigree, and some individuals carrying the mutation may reach old age (>80 years) without developing the disease[60]. Another relatively common mutation, D178N, is subject to phenotype modification by the codon 129 polymorphism: individuals with a 129V-178N haplotype will usually present with familial CJD, while a 129M-178N haplotype was found in the Italian pedigree in which FFI was first described[61]: this is reviewed below. The smaller octapeptide repeat insertion mutations (most commonly 4-OPRI), also cause a familial CJD phenotype, although their inheritance is more complex as it appears that the mutation is only penetrant in the presence of a susceptibility haplotype on the wild type allele[62]. Other mutations causing a CJD phenotype that are less commonly encountered in the UK include V210I[63] and E211Q[64].

If compared on a group basis, there are differences between the clinical presentation of sCJD and familial CJD: familial cases are younger, have longer disease duration, and are more likely to have

seizures, but on an individual basis it is not possible to distinguish on purely clinical grounds[65]. Although a convincing family history will often be given, the lack of full penetrance in some of the mutations causing familial CJD, combined with the commonly encountered issues of lack of an adequate family history or non-paternity, mean that it is not a rare occurrence to find a pathogenic *PRNP* mutation in a patient with apparently sporadic disease. This is one reason why *PRNP* genotyping tends to be routinely offered to all patients with prion disease.

Gerstmann-Straussler-Scheinker syndrome (GSS)

The most common *PRNP* mutation in the UK is P102L, which causes GSS, a syndrome characterised by progressive ataxia and cerebellar dysfunction with relative preservation of cognitive function during the early stages of the disease. Patients with GSS will often become so physically disabled by ataxia and dysarthria that cognitive testing becomes very difficult to perform before they have developed a marked dementia, although this does occur as the disease progresses. There is a very large UK pedigree affected by the P102L mutation which has been extensively studied[66]. Other mutations that cause GSS and which are encountered in the UK include P105L[67] and A117V[68]. The onset of GSS is typically at an earlier age than sCJD, and is influenced by the codon 129 polymorphism: MM patients typically develop symptoms between 35 and 50, while MV patients typically do so between 45 and 60[66]. The disease duration in GSS from onset of symptoms to death is variable, but the average is around 5 years[66,69].

Fatal Familial Insomnia

Fatal Familial Insomnia was first described in an Italian pedigree with a D178N mutation in *PRNP*[61,70]. The clinical syndrome in these patients consisted of intractable insomnia as the predominant early symptom, followed by autonomic dysfunction, myoclonus, spasticity and cerebellar dysfunction, and appeared to be quite distinct from the CJD-like illness of previously described carriers of this mutation. They were also found to have more dramatic and focal pathological changes (neuronal loss and gliosis) in the thalamus. In contrast with previously described D178N cases, it was found that the affected individuals in this pedigree had a D178N mutation sharing a *PRNP* allele with methionine at codon 129, and it was suggested that the codon 129 polymorphism was having a dramatic modifying effect on the phenotype of the mutation[61]. Since then the same mutation has been found in other patients and pedigrees, including in the UK, and the haplotype-genotype relationship seems to be less clear than initially thought. Many patients with the 129M-178N haplotype have presented with a more CJD-like illness[71-74]. Insomnia has also been increasingly recognised in other prion disease types (both sporadic and inherited), so that most patients with sleep disturbance as a presenting symptom of prion disease in fact have sporadic disease and do not carry the FFI mutation haplotype[75]. Some have therefore suggested the existence of a "Sporadic Fatal Insomnia" syndrome, mimicking that of FFI but in the absence of a causative mutation. It seems likely that there are unknown phenotypic modifiers accounting for these observations, and that the phenotypic spectra of the different pathogenic mutations/haplotypes overlap.

Large octapeptide repeat insertion inherited prion disease (>4-OPRI)

Whereas insertion of 4 additional octapeptide repeats results in a CJD-like illness as described above, larger insertions tend to produce a more slowly progressive disease with cortical dementia as its major manifestation, although again there is considerable phenotypic variability. The best characterised of these is the 6-OPRI mutation: this affects a large kindred in the UK that has been extensively studied[58,76], and has also been identified in a number of other independent kindreds[77-80].

5-OPRI, 7-OPRI, 8-OPRI and 9-OPRI mutations have also been identified in patients with a broadly similar clinical phenotype[81-84]. The most common clinical phenotype in these patients is of a slowly progressive dementia with prominent apraxia and early behavioural and psychiatric features, combined with relatively mild physical manifestations which may include ataxia, myoclonus, pyramidal and extrapyramidal features, dystonia and chorea. In clear contrast with the typical GSS phenotype, patients may remain independently mobile and physically able until a stage when they have very marked cognitive impairment. Disease durations may be up to 20 years or more, and age of onset is younger than in other forms of IPD, often in the third or fourth decade of life. There is a clear effect of the codon 129 polymorphism on the age of onset in the large UK 6-OPRI kindred, with 129MV individuals developing symptoms on average around 10 years later than 129MM individuals[58].

Y163X Inherited Prion Disease

A very unusual familial disease affecting a kindred in Eastern England has recently been shown to be caused by a truncation mutation of *PRNP*: Y163X[85]. The clinical phenotype is characterised by a very long "prodrome" of chronic diarrhoea, followed by development of a predominantly sensory peripheral polyneuropathy, autonomic dysfunction, and a slowly progressive dementia with seizures. Pathologically there is remarkably widespread deposition of PrP amyloid, including in the gastrointestinal tract and peripheral nerves. This truncation mutation results in loss of the GPI anchor that usually attaches PrP to the cell surface, which may account for the very unusual distribution of pathological amyloid deposits in this disease, and for its very unusual clinical phenotype.

Acquired prion diseases

Kuru

During the 1950s, it was observed that specific tribes living in the highlands of Papua New Guinea (the Fore linguistic group) were affected by an epidemic of a progressive and fatal neurological

disease, particularly affecting females and young males[86]. This disease was characterised by a progressive cerebellar syndrome, associated with tremor, choreiform and athetoid movements, and affective symptoms including emotional lability, but without a marked dementia until the late stages of disease. It was observed that the practice of endocannibalistic ritual mortuary feasts, which was common among the Fore, might provide a route of transmission of this disease through the population. In particular the specific customs regarding which individuals would consume specific body parts seemed to provide a plausible explanation for the distribution of the disease in the population. This disease, Kuru, was found to be transmissible by inoculation of brain homogenate from affected individuals into primates[87], and similarities between the neuropathological changes seen in kuru and in scrapie, as well as in various aspects of their clinical and epidemiological features, were noted[88].

The incidence of kuru has declined, with no cases occurring in younger individuals, consistent with cessation of cannibalism by 1960[89]. However, older individuals that were presumably exposed prior to this, did continue to develop the disease, with some new cases arising even after 2000, suggesting incubation periods of more than 40 years (and, taking into account the age at which male children stopped participating in endocannibalism, probably more than 50 years)[90]. Study of this epidemic human prion disease has been extremely informative, with potentially great relevance to the vCJD epidemic in the UK which is discussed below. In particular, comparing individuals that developed kuru with those that did not develop the disease despite being exposed, and comparing those with short and very long incubation periods has led to various important observations, particularly related to the genetics of this disease. It appears that codon 129MM homozygous individuals were most susceptible to kuru, to the extent that the part of the Fore population with this genotype were almost “exhausted” by the epidemic, with few 129MM adults found amongst groups who had lived through the epidemic[86]. Some 129MV and 129VV individuals did also develop kuru, but they appeared to be less susceptible and to have longer incubation periods, with the majority of very late

cases being heterozygous[91]. *PRNP* genotyping in the population of Fore that had survived the kuru epidemic also identified a novel genetic variation, G127V, which has not been found in any other population[92]. This appears to be protective against infection, and as a result has been subject to strong natural selection in areas affected by kuru and become much more common in those areas than in unaffected areas nearby.

Iatrogenic CJD

Transmission of a prion disease from one person to another as a result of medical intervention was first reported in 1974 in a patient who had received a cadaveric corneal graft from a patient that had died of pathologically confirmed CJD: the recipient developed symptoms of CJD 18 months after the transplant[93]. Subsequently, small numbers of cases of transmission by implanted intracerebral electrodes and neurosurgical instruments have been reported[10]. In these cases the clinical disease in the recipient seemed to closely resemble classical sporadic CJD, as described above.

Much more numerous have been cases resulting from injection of cadaveric pituitary hormones, or surgical grafting of cadaveric dura mater tissue. In both cases the pooling of cadaveric tissues from multiple different donors (thousands in the case of pituitary hormones), presumably including one or a very small number derived from individuals with a sporadic or inherited prion disease, seems to have resulted in many individuals being infected.

Dura mater related iatrogenic CJD has been particularly common in Japan, and the vast majority of cases have occurred in patients receiving grafts from a single manufacturer[94]. Again the clinical presentation of these patients seems to be very similar to those with sporadic CJD.

In the case of recipients of cadaveric human growth hormone injections, approximately 1 in 100 individuals receiving the relevant product are thought to have developed clinical CJD[95], although

calculating the numbers exposed to the specific products known to pose a risk is not simple, and the risk may be as much as 6%[96]. This is clearly far more than would be expected in the general population, providing compelling circumstantial evidence that the hormone injections are responsible. Most cases of human growth hormone related iatrogenic CJD have occurred in France, the UK and the USA[12], and new cases have continued to arise in the UK in the last few years (such as those included in the clinical data presented in this thesis). This suggests incubation periods for this disease of 20 years or more, as use of the implicated cadaveric hormone preparations ceased in the mid 1980s[12]. The clinical presentation of these cases often consists of a predominantly cerebellar syndrome initially, with dementia as a relatively late feature compared with classical CJD. Some have observed that this has similarities with the clinical course of kuru (see above), and speculated that this may relate to the fact that both diseases result from peripheral exposure to infectious material of human origin, rather than direct inoculation into or onto the brain.

Variant CJD

During the 1980s an epidemic of bovine spongiform encephalopathy, a prion disease of cattle, developed in the UK[97]. As it was known that this disease was transmissible, that prion diseases could be transmitted from one species to another, and that a very large number of people had potentially been exposed to material from cattle incubating the disease, this raised concerns that the disease might be transmitted to humans. This prompted enhanced surveillance of clinical cases of prion disease in the UK. In 1995 and 1996 the first cases of what appeared to be a new variant of prion disease were reported[98-100], and in the following years increasing numbers of patients with the same novel clinicopathological presentation were identified.

What became known as variant CJD (vCJD) was distinguished from sCJD in a number of ways. Patients were younger, with an average age at death of 26, and in subsequent years a clear bimodal age distribution of prion disease emerged. Disease duration was longer, with an average of 14

months. The clinical features and progression also appeared to be different, with patients often presenting with prominent psychiatric symptoms in the early stages of disease, before developing frank cognitive decline, neurological impairments and involuntary movements[101,102]. The pathological features of this new disease were also distinct from those of sporadic CJD, with characteristic "florid plaques" of abnormal PrP in brain tissue[99], and also a wider distribution of abnormal PrP deposition in the body, including lymphoreticular tissues such as the palatine tonsils, spleen and appendix[103].

vCJD also has a different profile from the other prion diseases in its typical clinical investigation findings. A characteristic pattern of abnormality on brain MRI, with T2/FLAIR hyperintensity in the posteromedial thalamus (known as the pulvinar sign), has proved to be a useful diagnostic marker with a high level of specificity in the appropriate clinical context[104]. In contrast with sCJD, the presence of 14-3-3 protein in the CSF is less commonly seen, and periodic sharp waves on EEG generally do not occur in vCJD[105]. The presence of abnormal PrP in peripheral lymphoreticular tissues allows tonsil biopsy to be used to make a tissue diagnosis without the need for brain biopsy: both PrP immunohistochemistry and Western blotting after protease K digestion can be applied to tonsillar tissue, with highly specific appearances in vCJD[106].

Diagnostic criteria based on these distinctive clinical and pathological features were established, and have been used for ongoing surveillance[105,107]. These stipulate criteria for diagnosing "probable variant CJD" and "definite variant CJD", the latter requiring neuropathological examination of brain tissue (either from biopsy or post mortem).

The question of whether this newly identified clinicopathological syndrome was the result of transmission of BSE to humans was addressed in a number of ways. Western blotting of brain tissue from individuals that had died of variant CJD, after digestion with protease K, produced a banding

pattern different from any previously described cases of sCJD, but similar to that produced by BSE brain tissue[51]. It was found that macaque monkeys inoculated with BSE tissue developed florid plaques[108]. Transmission studies in transgenic mice expressing human or bovine PrP found that innocula derived from BSE and vCJD brain tissues had very similar transmission properties, in terms of incubation period, and the nature and distribution of pathological changes, and that these were distinct from any sCJD-derived innocula[109]. This suggested that BSE and vCJD were both caused by the same prion strain. Taken together, all of this has provided widely accepted evidence that vCJD is caused by cross-species transmission of BSE.

The number of patients diagnosed with vCJD in the UK continued to increase each year until 2000, and since then has gradually decreased. In total, 177 cases of probable or definite vCJD have been diagnosed in the UK (www.cjd.ed.ac.uk), as well as small numbers in 11 other countries[110]. In total around 200 individuals are thought to have reached criteria for a diagnosis of probable or definite vCJD worldwide.

All of those cases formally classified as probable or definite vCJD have been homozygous for methionine at the codon 129 polymorphism of *PRNP*[110]. This suggests that codon 129 plays a very strong role in determining susceptibility to vCJD. However, it is possible that, as seen in kuru, non-MM vCJD cases have substantially longer incubation periods and have not yet presented. Alternatively, codon 129 might have a significant phenotype-modifying effect, as seen in some of the inherited prion diseases, so that non-MM vCJD would not present with clinicopathological features that would meet the current diagnostic criteria. A single case has been reported of an individual heterozygous at codon 129 in whom vCJD was the likely clinical diagnosis[111], and in fact the clinical presentation and investigation findings were quite typical, as was his age. This patient did not undergo tonsil or brain biopsy or post mortem examination, so a pathological diagnosis could not be made.

3 individuals with clinical vCJD had previously received non-leucodepleted red cell blood transfusions from donors who had themselves gone on to develop clinical vCJD, suggesting that they may have been infected with blood-borne prions. These 3 patients were all MM homozygotes at codon 129. One further implicated blood transfusion recipient, who was heterozygous at codon 129, died of non-neurological disease but was found *post mortem* to have vCJD-type PrP^{Sc} deposition in the spleen and in one lymph node, but not in the brain[112]. Spleen tissue from this individual has subsequently been shown to be infectious, transmitting disease to susceptible mice with similar transmission characteristics and neuropathology to equivalent tissue from 129MM "primary" vCJD patients[113]. Another codon 129 heterozygote who was a haemophiliac and had received a large amount of plasma products, as well as some blood transfusions, was also found to have PrP^{Sc} deposition in the spleen after dying of an unrelated non-neurological disease, but the brain was not available for study[114]. Issues related to blood-borne infectivity in vCJD are discussed in further detail in Chapter 2 below.

In light of the potentially widespread exposure to BSE-infected material, and the possibility of very long incubation periods or chronic asymptomatic carrier states, attempts have been made to estimate the prevalence of asymptomatic vCJD infection in the UK population. As PrP^{Sc} is known to be present in tonsils and appendix in vCJD patients[103], including in appendix tissue removed prior to the onset of symptoms[115,116], large archives of these tissues that have been surgically removed and stored have been examined to look for evidence of PrP^{Sc}. Several studies of this type have been performed. The largest and most recent, based on 32,441 appendix samples, found evidence of abnormal PrP deposition on immunohistochemistry in around 1 in 2000 (95% confidence interval 1248 to 3546)[117]. The 16 positive samples were genotyped and found to include all codon 129 genotypes. If correct, it is not clear how this prevalence can be reconciled with the very low prevalence of clinical vCJD: it may be that there is a relatively prevalent asymptomatic carrier state

with only a very small minority going on to develop neurological disease. The possibility of a latent epidemic of individuals with much longer incubation periods cannot be entirely discounted, and experience from the study of kuru provides a precedent for longer incubation periods in non-MM individuals[118]. However, the results of the appendix archive prevalence study would suggest that there are more than 10,000 MM homozygous individuals in the UK asymptomatically carrying the disease, and as this is the only established strong susceptibility factor for developing symptomatic disease, codon 129 certainly cannot explain this discrepancy. A critical complimentary study, examining a similar archive of appendix samples that were removed prior to the appearance of BSE in the UK in order to show that the abnormalities seen are likely to be related to BSE exposure, is currently underway.

The NHS National Prion Clinic

The NHS National Prion Clinic offers specialist clinical assessment and follow-up for all cases of suspected prion disease in the UK. In 2004, a national referral system for prion diseases was set up. All UK neurologists were asked by the Chief Medical Officer to refer all patients with suspected prion disease jointly to the National CJD Research and Surveillance Unit (based in Edinburgh) and to the NHS National Prion Clinic. In addition to increasing access to specialist clinical care and improving surveillance, this also aimed to provide opportunities for large-scale clinical research. This is one of the major roles of the National Prion Clinic. The two major clinical research studies that have been carried out at the National Prion Clinic over the last 10 years are described in the following section. In addition to being an NHS specialist clinic, it forms part of the Medical Research Council (MRC) Prion Unit. This allows the clinical research carried out through the National Prion Clinic to be closely integrated with the basic science research carried out at the MRC Prion Unit.

The PRION-1 trial and the National Prion Monitoring Cohort study

Following promising results from work on inhibition of prion replication *in vitro*, the widely used anti-malarial drug quinacrine was proposed as a potential therapeutic agent in human prion disease[119]. As its safety profile was already well established, it was felt that a clinical trial could be planned rapidly, and the Department of Health asked the MRC Prion Unit and National Prion Clinic to do this. The resulting clinical trial, PRION-1, was an open-label, patient preference trial of quinacrine for all types of human prion disease, which recruited patients from 2001 to 2008 [120]. In total the trial enrolled 107 patients with prion disease. 40 patients received quinacrine during the trial follow-up period. Although an option for randomisation to immediate or delayed quinacrine was included in the trial design, only 2 patients chose to be randomised, and so the trial was essentially an observational study comparing patients who chose to take quinacrine with those that chose not to. Patients with moderately advanced disease at enrolment proved to be more likely to choose quinacrine than those in the early or late stages of disease, confounding the effect of quinacrine treatment on mortality, which was the primary endpoint. When disease severity at enrolment was adjusted for, there was no significant effect of quinacrine treatment on survival. Subsequent analysis of data collected using a battery of rating scales also showed no significant effect of quinacrine when adjusted for severity of disease at enrolment[121]. This rating scales data is discussed in detail in Chapter 6 below.

Following on from the PRION-1 trial, the National Prion Clinic has been running a large scale observational study which aims to enrol all patients with prion disease in the UK: the National Prion Monitoring Cohort. This includes all cases of probable or definite sporadic Creutzfeldt-Jakob disease (sCJD), variant CJD (vCJD), iatrogenic CJD (iCJD), and inherited prion disease (IPD). Also eligible for enrolment are asymptomatic individuals known to be at risk of inherited prion disease (tested asymptomatic gene mutation carriers or untested first degree relatives of those with a confirmed pathogenic *PRNP* mutation), or vCJD (recipients of implicated whole or leucodepleted blood

transfusion that have been notified of their risk by the Health Protection Agency). A small group of healthy controls were also recruited (friends or relatives without pathogenic *PRNP* mutations or other known risk factors).

The over-arching aim of the Cohort study is to prepare for future clinical trials in prion disease, and to maximise the chances that a trial will be able to demonstrate any beneficial effect from putative therapeutic agents. By building up a large, detailed natural history data set from a cohort of patients followed up using a "trial-like" study protocol, the Cohort aims to provide a historical control group that will help to overcome the challenge of recruiting sufficient numbers of patients with these rare diseases to achieve the necessary statistical power for a robust clinical trial. Another major aim is to develop and validate good clinical outcome measures for use in future prion disease clinical trials, as it was clear from the experience of PRION-1 that neither mortality, nor any of the rating scales commonly used in clinical trials for other neurological conditions performed well in a prion disease population. I took primary responsibility for this aspect of the Cohort study and this project is presented in Chapter 6 below, where these issues are discussed in detail.

In addition to these primary aims, the National Prion Monitoring Cohort also provides unprecedented opportunities for prospectively studying a variety of clinical aspects of the prion diseases in a large cohort of patients across the range of different types of prion disease. I took advantage of this to carry out a comprehensive study of the behavioural and psychiatric features of the prion diseases, which is presented below in Chapter 5.

CHAPTER 2. DEVELOPING NEW APPLICATIONS FOR THE DIRECT DETECTION ASSAY AS A BLOOD TEST

Introduction

The need for new non-invasive diagnostic tests for prion disease

Diagnosis of the sporadic and acquired human prion diseases, as formalised in diagnostic criteria used internationally for surveillance purposes[34,105] relies on identifying typical patterns of clinical features combined with supportive investigation findings (presence of 14-3-3 protein in the cerebrospinal fluid (CSF), periodic sharp wave complexes on electroencephalogram (EEG) or typical patterns of signal change on magnetic resonance imaging (MRI) of the brain) to reach a "Probable" diagnosis. Large scale studies have shown that this results in a diagnostic specificity of only 71% for sporadic CJD [34], while for variant CJD specificity seems to be much higher[105]. A "Definite" diagnosis of sporadic CJD can only be reached by confirming the presence of typical neuropathological changes (spongiosis, astrogliosis and deposition of abnormal prion protein (PrP) in a characteristic pattern) in brain tissue obtained either at autopsy or biopsy. In the case of variant CJD (vCJD) it is possible to demonstrate the presence of abnormal protease-resistant PrP in lymphoid tissue from the palatine tonsils obtained at biopsy[106].

In practice, the current diagnostic process results in most patients being diagnosed at a late stage of disease, and the overwhelming majority of patients die without a "Definite" diagnosis being made. This delay in diagnosis has a major impact on patients and carers as most of the disease course is taken up with diagnostic uncertainty and multiple investigations, and also represents a major barrier to the design of therapeutic trials in prion disease. None of the non-invasive investigation findings listed above are entirely specific for prion disease, and the development of a non-invasive test allowing accurate, early diagnosis of prion disease would have a major clinical impact.

Blood-borne prion infectivity

Following the emergence of vCJD in the UK during the 1990s, efforts were made to identify recipients of transfused blood that had been donated by individuals who subsequently went on to develop clinical vCJD. By 2006, 66 such recipients had been identified, of whom 26 were still alive. 2 of these recipients had *already* been diagnosed with vCJD, while a third was found to have abnormal prion protein in lymphoid tissue at autopsy but did not have symptoms of the disease when they died[13]. Since then, one further member of this at risk group has also developed vCJD[14]. This strongly suggests that these individuals have been infected with blood-borne vCJD prions, present in blood donated by individuals that were asymptomatic at the time of donation. This raises major public health concerns about the safety of blood transfusion in the UK, where the number of people asymptomatically incubating vCJD remains unknown but may be as high as 1 in 2000 based on recent studies examining archived appendix tissue[117].

The presence of blood-borne infectivity in vCJD also raises the possibility of a blood-based test for the condition, if this infectivity and/or associated disease-specific factors could be directly detected. In addition to providing a non-invasive diagnostic test, this might have the potential to allow detection of infectivity in donated blood from individuals in the asymptomatic incubation period of vCJD: the reported cases of blood-borne transmission involved blood donated several years prior to symptom onset, implying that circulating prions are already present at this stage.

Efforts to detect disease-associated PrP in blood

Existing laboratory diagnostic techniques (such as high sensitivity Western Blotting after digestion with protease K) failed to demonstrate the presence of disease-specific PrP species in blood from patients with vCJD[103], so a new technique was needed. As the concentration of prion/abnormal PrP in blood is likely to be very low based on estimates derived from rodent transmission studies[122,123],

while normal PrP is relatively abundant in blood, a major challenge was to find ways of detecting these extremely small amounts, or of concentrating them to allow detection.

In recent years a number of novel molecular techniques for the detection and/or amplification of disease-associated PrP species have been developed, and efforts have been made to adapt and apply these as blood tests.

Approaches based on the Protein Misfolding Cyclic Amplification (PMCA) technique[124], whereby the templated misfolding of PrP^{c} is seeded by a test sample containing misfolded PrP and the accumulated misfolded protein is then detected, have shown some promise. Quaking Induced Conversion (QuIC), a modified form of PMCA, combined with an immunoprecipitation step has been shown to detect extremely small amounts of brain-derived disease-associated PrP when vCJD brain homogenate is diluted into human plasma in work published by Orrú et al[125]. Up to 10^{14}-fold dilutions (that would be estimated to contain only a few attograms of abnormal PrP per ml), could be differentiated from dilutions of non-prion disease brain homogenate. The technique was also able to distinguish between serum and plasma taken from scrapie-infected hamsters (including one in the "preclinical" stage between inoculation and the onset of manifest neurological dysfunction) and that taken from uninfected hamsters. However, at the time of publishing this work the authors had not had access to vCJD patient samples and so had not been able to assess the functioning of this assay in the actual analyte in which it might be applied as a clinical diagnostic test.

Other techniques have been evaluated and shown some usefulness in identifying non-human animals infected with prion disease. For example, using an anti-PrP capture antibody bound to a glass surface, a fluorescent-tagged detection antibody and then a scanning laser microscopy method to detect intensely-fluorescing targets, Bannach et al[126]were able to differentiate between plasma taken from scrapie-infected sheep and that from control sheep in a small study. However, again no

application of this technique to human vCJD patient samples has been published.

A major challenge to this area of research is the scarcity of blood samples from patients with vCJD. Validation of a diagnostic assay would usually involve testing of a very large panel of disease and control samples, but this is not possible for vCJD, of which there have only ever been around 200 clinical cases.

The UK National Institute for Biological Standards and Control (NIBSC) CJD Resource Centre has proposed a step-wise framework by which putative blood-based diagnostic tests for vCJD may be evaluated: this is made available at http://www.nibsc.ac.uk/pdf/CJDtest-draft1.pdf (link active as of 8/11/13). It is based on the assumption that a successful assay would be able to detect, firstly, infected human brain and spleen diluted into plasma; secondly, scrapie-infected sheep plasma; and finally, human vCJD patient plasma. They have also assembled an archive of relevant biological samples, including vCJD patient plasma samples, and access to these is granted by an expert committee based on an initial submission from the developers and the assay's performance in each step of the process.

Publications arising from this process have not given full details of the assay methodologies being evaluated, because of "commercial-in-confidence" issues (the possibility of large scale applications to blood transfusion safety brings with it potential commercial value). One paper presented the performance of 6 different assays in testing the same panel of plasma samples, some spiked with varying dilutions of vCJD and control human brain and spleen, but not including any real vCJD patient samples[127]. It concluded that some of these showed potential as blood-based diagnostic tests, as they detected vCJD tissue homogenates diluted down to concentrations thought to be similar to, or lower than, that of infectivity in patient blood. A subsequent paper presented the results from evaluation of a single assay in all stages of the process, showing that it performed well in detecting

diluted brain and spleen homogenate in plasma, but had poor sensitivity for scrapie-infected sheep plasma and failed entirely to detect real vCJD patient plasma[128].

While the thinking behind this strict step-wise approach is understandable, and the aim of protecting the scarce resource of patient samples is entirely appropriate, the assumption that a single assay will be able to perform in the three different stages of the process, without any opportunity for empirical re-optimisation in each new analyte, may be questionable. In particular, it may be that disease-associated PrP species present in brain or spleen homogenate will behave quite differently from those present endogenously in patient plasma samples (for example, as a result of differences in their conformation, their immediate biochemical environment and associated molecular species, or interactions with other molecular and cellular constituents of these analytes, or any combination of these).

Through its close association with the National Prion Clinic, which has been involved in the clinical care of many patients with vCJD in the UK, the MRC Prion Unit has built up an archive of blood samples from patients with vCJD (as well as other prion disease types), which provides unrivalled opportunities for the development of diagnostic assays. In the following sections, I will describe the development of the Direct Detection Assay at the MRC Prion Unit. This is the only putative blood-based vCJD diagnostic assay that has been shown to successfully detect vCJD patient samples in a masked sample panel[129], and it forms the basis of the work presented in this chapter and in chapter 3.

Binding of prions to steel

An early clue that human prion diseases were transmissible came from the observation that several patients exposed to metallic neurosurgical instruments or intracerebral electrodes previously used on patients with CJD went on to develop CJD themselves[10,11]. This led to the observation that prions

bind avidly to metal surfaces, including that of surgical steel[130]. This property of prions has subsequently been used as the basis for a variety of novel laboratory techniques.

These include the "Standard Steel Binding Assay" developed by members of the Molecular Diagnostics group at the MRC Prion Unit[131], in which steel wires are used to transfer infectivity from mouse brain homogenate to a prion-susceptible cell culture.
This prompted the investigation of steel as a capture matrix to bind and concentrate the abnormal PrP species in vCJD patient blood, which in turn led to the development of the Direct Detection Assay.

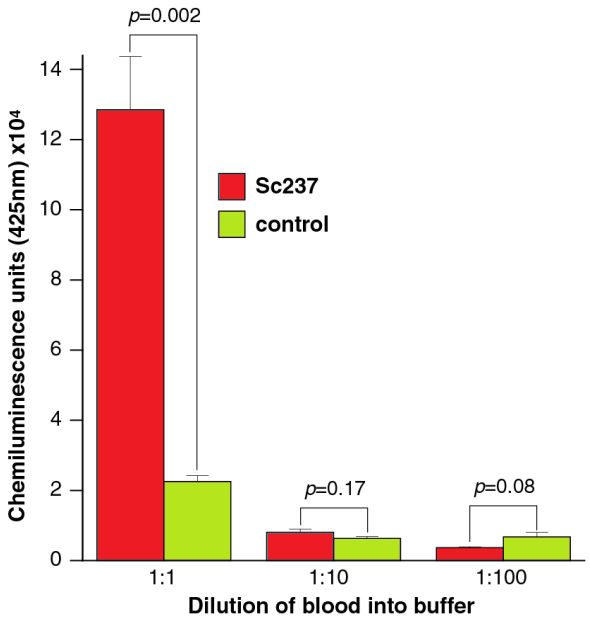
The Direct Detection Assay

The Direct Detection Assay (DDA) uses steel powder to capture disease-associated PrP-containing species from whole blood. These are then detected using an adapted enzyme-linked immunosorbent assay (ELISA) process. The weak ferromagnetism of steel powder allows a magnet to be used to capture the steel during wash steps. The assay also includes a heat shock step prior to incubation with the primary (PrP-binding) antibody, which aims to increase epitope availability. The full details of the method are described under *Materials and Methods* below.

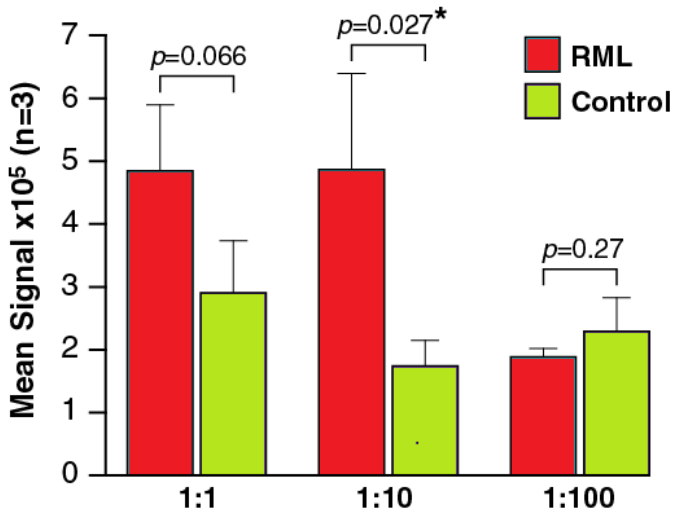
Significantly, the assay does not include any protease treatment of the samples, so does not rely on the protease-resistance of some disease-related abnormal PrP in the same way as other established diagnostic assays. As the anti-PrP antibody used in the assay does not bind preferentially to abnormally-folded PrP relative to normal cellular PrP[132], the disease-specificity of the assay seems to be the result primarily of the avid steel-binding properties of the disease-associated PrP-containing species relative to normal cellular PrP.

A number of observations from the development of the DDA are of particular relevance to understanding its performance in different circumstances, and therefore to its development for further applications.

Early work on the assay used blood from experimental animals (mice and hamsters) and human blood from normal healthy UK blood donors 'spiked' with brain homogenate from patients. It became clear from this work that the optimum conditions for performance of the assay varied considerably in different circumstances. Blood from mice infected with the RML prion strain could be differentiated from control mouse blood when diluted 1:10 prior to steel capture (but not when diluted 1:1 or 1:100), while blood from hamster infected with Sc237 prion strain could be detected at 1:1 dilution (but not 1:10 or 1:100) (unpublished data - see Figure 3).



A)



B)

Figure 3. A) Differentiation of Sc237-infected hamster blood from controls at clinical end point of disease using DDA. Primary antibody ICSM 18B. B) Differentiation of RML-infected Tg20 mouse blood from controls at clinical end point of disease. Primary antibody ICSM 10B. Assay conditions and reagents in both experiments as per the standard protocol described in the "Materials and methods" section of this chapter below except for primary antibody in (B). Data and images kindly provided by Dr Graham Jackson and Dr Julie Edgeworth, who carried out these experiments.

In experiments using normal human blood spiked with brain homogenate, a spiked-blood:DDA capture buffer dilution of 1:1 produced results showing differentiation between a dilution of 10^{-10} vCJD homogenate and 10^{-6} normal homogenate (see Figure 4), suggesting that the assay was successfully detecting <40fg of abnormal PrP.

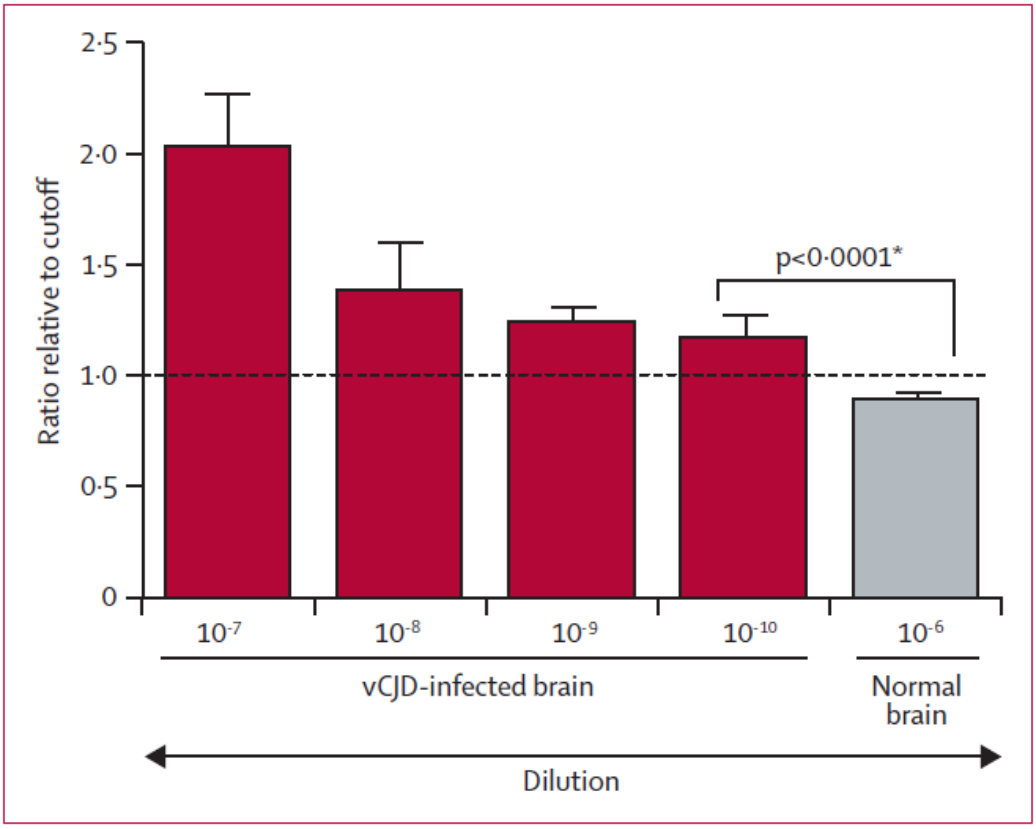


Figure 4. Reproduced from Edgeworth et al, 2011[129]. Differentiation of vCJD brain homogenate diluted into normal human whole blood. Bars show the mean of six replicates and are expressed relative to the cutoff value (three SDs greater than the mean chemiluminescent signal for a normal brain sample; dashed line). Error bars show SDs. *Two-tailed, unpaired t test. The "spiked" blood was diluted 1:1 into the DDA capture buffer. Conditions otherwise as per the standard DDA protocol described below.

However, when the assay was subsequently applied to real patient samples, it was found that differentiation of vCJD patients from controls could only be achieved with a 1:100 dilution prior to capture (see Figure 5).

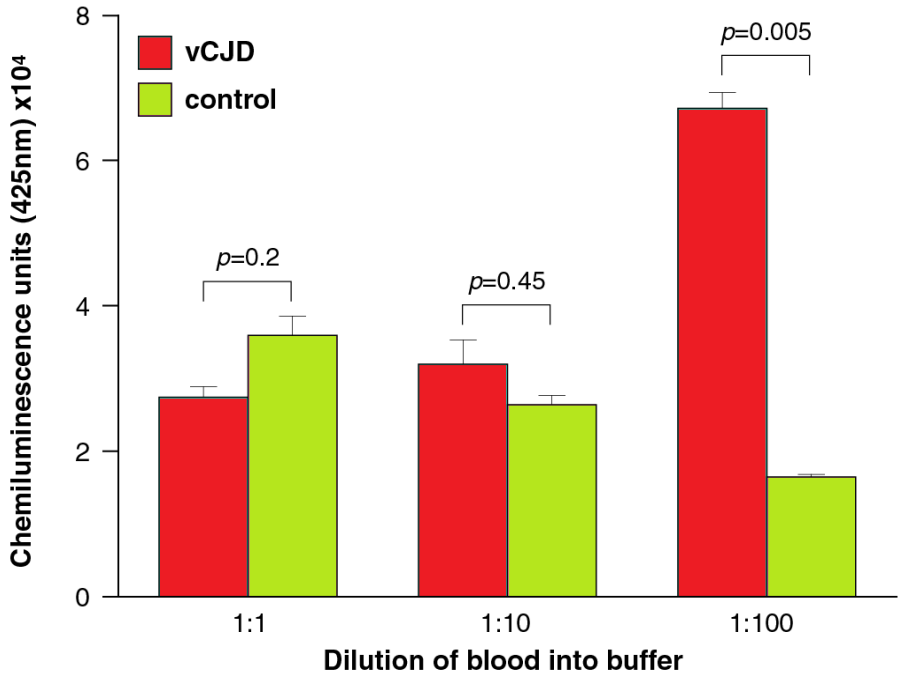


Figure 5. Effect of dilution of blood into DDA capture buffer on differentiation between vCJD patient and normal control human whole blood samples. Standard DDA protocol as described below in "Materials and methods" section used, apart from variations in dilution.

Potential reasons for this variation in performance of the assay in different circumstances are likely to fall into two categories: factors relating directly to the disease-specific PrP-containing species itself, and factors relating to other constituents of the analyte. The former category would include prion strain characteristics, PrP conformation and epitope availability, aggregate size, characteristics of non-PrP constituents of the disease-specific PrP-containing species; the latter would include the presence of other metal-binding molecules in the analyte that could compete with the disease-specific PrP-containing species, and the presence of components that would impair effective washing of the steel powder or other technical steps of the assay. It is useful to keep these possibilities in mind while investigating the application of the assay to different disease types, and to different analytes.

Validation of the Direct Detection Assay as a diagnostic blood test for vCJD

Following extensive optimisation of the DDA protocol for use in human whole blood, its validity as a diagnostic test for vCJD was tested using a masked panel of samples, consisting of 21 patients with vCJD, 27 with sporadic CJD, 42 with other (non-prion) neurological diseases, and 100 normal controls (anonymized samples from healthy blood donors). The protocol used for this experiment is that documented in detail below in the *Materials and methods* section. Masked panel samples were tested alongside a quality control panel consisting of 6 known normal control samples and 2 samples from vCJD patients. Samples were classified as reactive if the mean luminescent signal from three replicate wells exceeded a cut-off value, which was defined as the mean + 3 standard deviations of the normal samples in the quality control panel tested on the same plate. Each sample in the masked panel was tested on two separate occasions, and was only classified as positive if it was reactive on both occasions.

The results of this experiment have been published by Edgeworth et al[129], and are shown in Figure 6. 15 samples were classified as positive, and all of these were from patients with vCJD. This suggests an assay sensitivity for vCJD of 71·4% (95% CI 47·8% to 88·7%) and a specificity of 100% (95% CI97·8% to 100%). 3 of the 6 vCJD patient samples that were classified as negative were reactive in one of the assay runs but not the other. Amongst the non-vCJD samples, 8 of the normal control samples were reactive in one assay run but not the other, and all other samples were non-reactive in both assay runs.

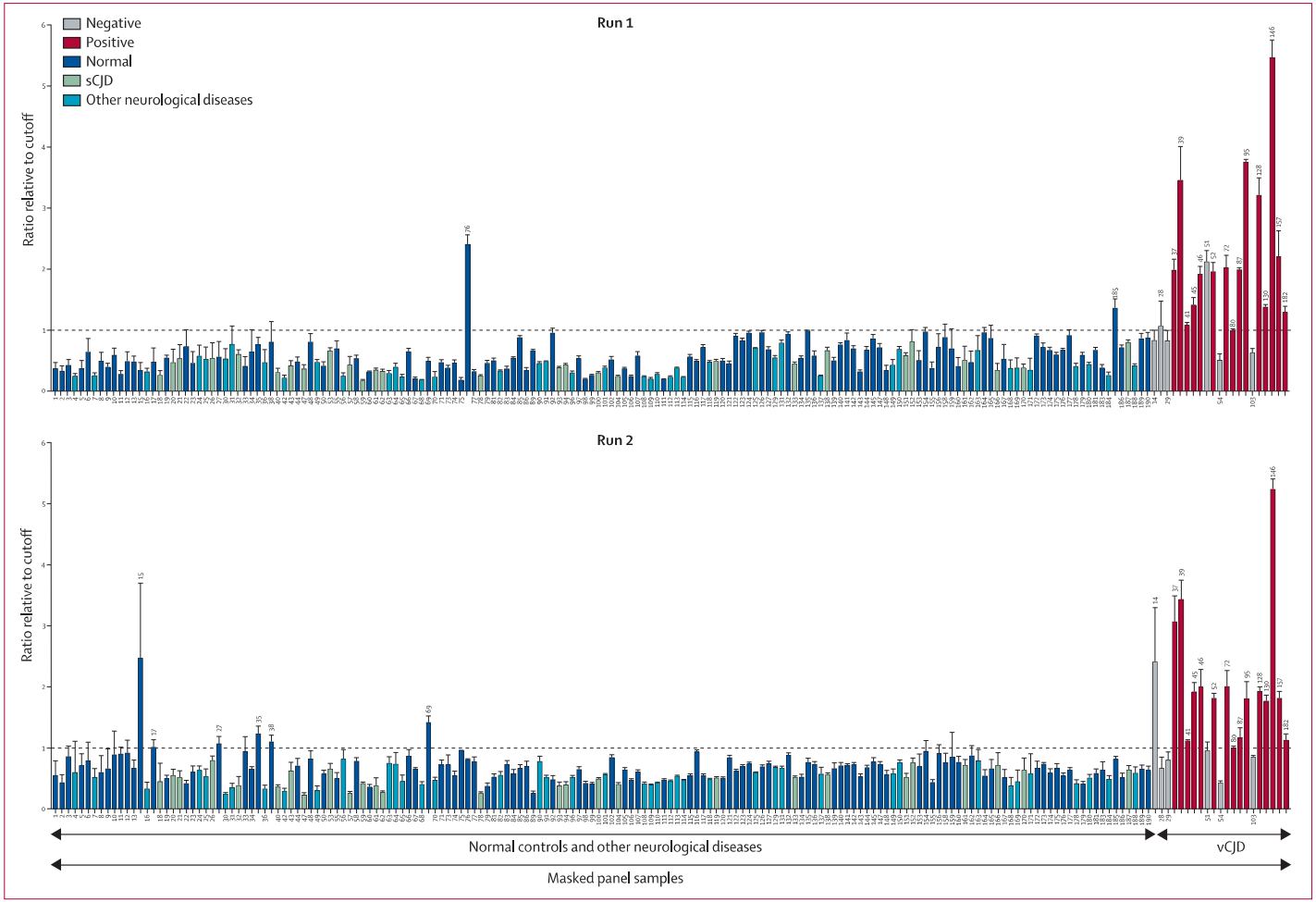


Figure 6. Results of the masked panel experiment, reproduced from Edgeworth et al, 2011[129]. Dark blue bars = healthy normal controls; turquoise bars = controls with other neurological diseases; green bars = patients with sCJD; red bars = samples testing positive in both assay runs (all vCJD); grey bars = vCJD patient samples testing negative in at least one assay run.

New applications for the DDA

The primary objective in developing the DDA was to produce a blood-based diagnostic assay for vCJD, and a major secondary aim is to develop a screening test that can identify asymptomatic individuals during the incubation period of vCJD. However, the DDA may also have the potential to be applied to a range of other prion disease types and other analytes, both for use as a diagnostic test and as a biomarker for monitoring progression of disease or response to experimental therapeutic agents in clinical trials. I have investigated several of these possible applications.

Further work (that has been done since the projects presented here were completed) aiming to establish whether the DDA has sufficiently high levels of specificity to be applied as a screening test is discussed below in Chapter 7.

The DDA in inherited prion disease

Inherited prion diseases (IPD) can be reliably and rapidly diagnosed by demonstrating the presence of a pathogenic mutation on sequencing of the *PRNP* gene, in an individual with an appropriate clinical presentation. There is therefore no need for a novel molecular diagnostic test for these conditions. However, they present an ideal opportunity to investigate possible biomarkers for prion disease, for a number of reasons.

As patients are often aware of their familial risk before symptoms develop, and disease progression can be relatively slow, diagnosis of IPD is often made at a much earlier stage than that of the sporadic or acquired prion diseases. Indeed some individuals choose to undergo predictive genetic testing, so that they are "diagnosed" years or even decades before the onset of symptoms. As a result of this, an effective therapeutic agent for prion disease would have the potential to be of very great benefit in IPD: the most appealing way to treat a degenerative condition would be to initiate

treatment in the very early stages of disease, or even before symptom onset, so that the disease process could be slowed or halted before much irreversible damage has been done, and IPD presents the best opportunity for this. However, to achieve this goal it would be extremely valuable to have a method for monitoring the disease (and any response to treatment) at a stage when there are minimal symptoms, or even no symptoms at all. Therapeutic trials aiming to use clinical outcome measures to prove efficacy of a preventive treatment, or one that modifies a slowly progressive disease, are likely to require very long timescales and large numbers of patients. A biomarker that could provide early evidence that the experimental therapeutic agent was indeed engaging with and modifying biological pathways relevant to the pathogenesis of the disease would be extremely valuable as an adjunct to clinical outcome measures. Ultimately, if an effective therapeutic agent were found, such a biomarker might also help with deciding when to initiate treatment in "presymptomatic" individuals.

As the DDA starts to be used as a clinical diagnostic test for vCJD, it is important to fully establish its specificity for this particular disease type, so that results can be appropriately interpreted. Although samples from sCJD patients were included in the published masked panel experiment, there were no samples from patients with IPD. Testing of IPD samples therefore also plays an important part in establishing the clinical role of the DDA as a test for vCJD. I therefore investigated the performance of the DDA in inherited prion disease.

Testing fractionated blood samples using the DDA

Even before the accidental transmission of vCJD by blood transfusion there had been concern that prion diseases might be transmissible by this route, prompting research using animal bio-assays to assess the level of infectivity in whole blood and also in individual blood fractions[122,123]. This informed the decision to institute the leucodepletion of red cell fractions and the discarding of plasma fractions from all UK blood donations since 1999[133]. More recent work has used BSE-infected

sheep as a model for vCJD, and shown that all fractions of blood taken from infected sheep during the incubation period are able to transmit disease[134]: this included whole blood, red cells, buffy coat, plasma, platelets, and leucoreduced red cells, leucoreduced plasma and leucoreduced platelets.

Although, DDA positivity certainly cannot be seen as equating directly with infectivity, it may have a role as a surrogate marker indicating the presence of disease-specific abnormal PrP which may well include or co-exist with infective species.

The counterintuitive need to dilute human blood samples 100-fold prior to steel capture in the DDA raises the possibility that there is an inhibitory factor present in whole blood that needs to be diluted for the assay to function. If this factor could be separated from the disease-specific PrP-containing species detected by the assay by simple fractionation of blood this might allow the amount of analyte used in the assay to be increased and consequently improve the signal to noise ratio of the assay. I therefore investigated the performance of the DDA in individual fractions of blood.

Materials and methods

General methods for all molecular diagnostics work using Direct Detection Assay (DDA)

As part of the Molecular Diagnostics team I contributed to the development of a standard protocol for the Direct Detection Assay as a diagnostic test for vCJD, which was used for the masked panel testing that was reported in Edgeworth et al, 2011[129]. This was used for all experiments described below, except where modifications to the protocol are specifically noted.

Standard Direct Detection Assay protocol for experiments with blood

Stainless steel powder (<45µm, *Goodfellow*) is washed thoroughly using 2% triton-x-100 (*Sigma*), 70% ethanol and sterile double-distilled water (ddH_2O). It is then separated into individual aliquots of 23mg of steel powder, each in 115µL ddH_2O as a suspension and allowed to settle. The supernatant liquid is removed immediately prior to use.

DDA capture buffer is prepared with the following constituents: 101mM tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 8.4, 2.04% bovine serum albumin (BSA), 2.04% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) detergent, 1 complete protease inhibitor tablet (*Roche*)/50ml, and 10µL benzonase (Grade II, *Merck*)/50ml.

All subsequent steps are carried out in a Containment Level III laboratory, adhering to all relevant local operating procedures.

Blood samples to be tested are thoroughly thawed from storage at -70°C. 8µL of blood is mixed with 792µL of DDA capture buffer, and the mixture added to a single 23mg steel powder aliquot. This is then incubated overnight (17 +/- 2 hours) on an Eppendorf Thermomixer at a temperature of 18°C shaking at 650rpm.

Wash solution is prepared, consisting of phosphate-buffered saline and 0.05% Tween detergent. The supernatant is removed from each steel powder sample, and these are then each washed 5 times using 1mL of wash solution for each wash. After the fifth wash is removed, dry steel samples are "heat-shocked" for 5 minutes at 110°C, and then allowed to cool.

Biotinylated anti-PrP antibody ICSM18B (*D-Gen Ltd*) is prepared at a concentration of 0.6µg/ml, and 50µL added to each steel powder sample. These are then incubated for 1 hour on a Thermomixer at 37°C shaking at 750rpm.

Supernatant is removed and samples are washed a further 3 times using 1mL of wash solution for each wash. Horseradish peroxidase conjugated NeutrAvidin-HRP (*Pierce*) is prepared at 1:100 000 dilution, and 50µL added to each dry steel powder sample. These are then incubated for 45 minutes on a Thermomixer at 37°C shaking at 750rpm.

Supernatant is removed and samples are washed a further 3 times using 1mL of wash solution for each wash. *Femto* chemiluminescent SuperSignal reagent (*Pierce*) is prepared by mixing equal volumes of the 2 constituent parts as per the manufacturer's instructions. 60µL is added to each steel powder sample. Each steel powder sample is then resuspended in this liquid and separated equally between 3 wells of a black, 96-well, flat-bottomed ELISA plate (Greiner), so that each contains 20µL of *Femto* reagent and approximately 7.67mg of steel powder.

A dilution series of NeutrAvidin-HRP giving dilutions of $1:1x10^6$, $1:1x10^7$ and $1:1x10^8$ is also prepared. 20µL of each of these is added to 3 wells.

A further 80µL of Femto reagent is then added to each well, and the plate is immediately read using a M100 plate reader (*Tecan*), using luminescence settings. The mean luminescent signal measured from the 3 wells for each sample is taken as the DDA result for that sample.

Standard quality control panel for DDA experiments on blood

Unless stated otherwise, in all DDA experiments described below test samples were tested alongside a standard quality control (QC) panel of at least 4 known normal control samples, and at least 1 known DDA-positive vCJD sample. A reference value, the "DDA Cut Off", was calculated for each assay run, by calculating the Mean + 3 Standard Deviations of the luminescent signal from the normal QC samples. The QC panel was deemed satisfactory if the mean luminescence reading for the vCJD sample(s) was greater than 1.4 times the DDA Cut Off (based on previous experiments using the same vCJD samples). If this was the case, the results from the test samples in the same assay run were included in the analysis. If the QC panel results were not satisfactory, then the test sample results were disregarded and the whole assay run was repeated.

DDA in inherited prion disease.

I used the standard DDA protocol to test a panel of blood samples from patients with inherited prion disease. Some of these assay runs were kindly performed by laboratory technicians Ms Justine Korteweg, Ms Claire Grant and Ms Samantha Jones, under my supervision.

A large number of EDTA whole blood samples from patients with inherited prion disease have been received and stored in the MRC Prion Unit, with written consent for their use in research studies provided by patients or next-of-kin. These include sets of samples taken at different times from the same individuals, sometimes over the course of many years, and also samples taken from asymptomatic individuals who are known to carry a pathogenic *PRNP* mutation. I identified all symptomatic patients that had at least 1 suitable EDTA sample stored, and identified the most

recently taken sample from each of these patients (i.e. the sample taken at the latest stage of their disease). This yielded a total of 52 samples, from a range of inherited prion disease types, as documented below.

***PRNP* mutation**	**Number of patients/samples**
4-OPRI	3
5-OPRI	4
6-OPRI	15
A117V	3
D178N	4
E200K	4
G54S*	1
P102L	14
Q212P	2
Y163X	2
Total	52

*G54S has been found in one patient with an atypical clinical neurodegenerative syndrome, and abnormal prion protein deposition at autopsy, but also in a number of healthy control individuals, so it is not conclusively known whether this is a pathogenic mutation or a polymorphism.

Each of these samples was tested on at least 2 separate occasions using the DDA protocol above. Test samples were classified "DDA positive" if their mean luminescence reading was greater than the DDA Cut Off on both occasions that they were tested. This was the same criterion used to classify samples as positive in the published masked panel experiment into the use of the DDA as a diagnostic test for vCJD[129]. I also planned to test any other, earlier samples that had been taken from the same patients as those producing a positive result in the initial set of 52 samples, in order to investigate the timecourse of DDA positivity through the disease course.

Analysis for factors interacting with diagnosis/disease type to affect DDA result

Information relating to each sample, including the date the sample was obtained, the date of onset of the patient's illness, the patient's date of birth and the date of the patient's death (if deceased), was obtained from the records of the MRC Prion Unit and/or the NHS National Prion Clinic. Exploratory analyses were performed to look for factors that might interact with diagnosis/disease type in influencing the DDA result. For example, were samples taken at a very late stage of disease more likely to produce a positive DDA result than samples taken from patients at an earlier stage of the same disease type? This could provide clues as to the potential of the DDA as a biomarker of disease progression, and might also highlight possible confounding factors affecting the sensitivity of the test.

The putative interacting factors examined were:

1) Age of patient at time of sampling
2) Time from onset of disease to sampling
3) Time from sampling to death
4) Time from sampling until testing (i.e. age of the sample itself when tested).

DDA in fractionated variant CJD bloods

In order to investigate the distribution of disease-associated abnormal PrP in vCJD patient blood fractions using the DDA, I identified blood sample sets stored at the MRC Prion Unit with appropriate research consent that had been taken from vCJD patients and healthy controls where there was whole EDTA blood and fractionated EDTA blood stored from samples taken on the same occasion. Fractionation of blood had been performed shortly after receipt of the samples at the MRC Prion Unit, using local operating procedures that have been standardized to ensure consistency of sample quality. Fractionated blood sets consisted of (1) red cell/platelet fraction, (2) plasma fraction (where

"plasma 1" (upper part of plasma fraction after centrifugation) and "plasma 2" (lower part of plasma fraction) were stored separately, "plasma 1" was used), and (3) mononuclear cell fraction.

The suitable sample sets identified included some vCJD sample sets from which the whole blood sample was known to produce a consistent strong positive DDA result in previous experiments, and also some from which the whole blood sample was known to be DDA-negative. This provided the opportunity to assess whether testing any individual blood fraction might have advantages over testing whole blood with respect to sensitivity.

Only a limited number of sample sets meeting the necessary criteria could be identified. The samples sets included in this experiment are summarised below.

Diagnosis	Number of whole blood & fraction sample sets
Normal healthy control	9
vCJD	8 (NB: 1 set without plasma)

Initially, each set of samples was tested using the standard DDA protocol, treating each blood fraction as if it were whole blood, with the exception of the very small MNC "pellet" which was resuspended in phosphate buffered saline (PBS) back to its original concentration in the blood sample from which it was derived ("1x" concentration). Further experiments also tested a subset of MNC samples at a range of concentrations (1x, 2x, 5x, 20x). Subsequently, some adaptations to the protocol were made in order to optimise the performance of the assay in fractions that showed some evidence of differentiation of vCJD from control samples.

Plasma and RBC/platelet fractions were also tested after being diluted in PBS so that their final concentration while being incubated with steel powder and capture buffer would be as close as possible to their concentration in the assay using whole blood: plasma fractions and red cell/platelet

fractions were diluted 5:7 in PBS. This ratio was based on the typical proportions of each fraction produced from each whole blood sample.

The standard QC panel as described above was used for each assay run.

In addition to testing blood fractions individually, “reconstituted whole blood” was tested for a subset of samples, by mixing plasma, red cell/platelet and mononuclear cell fractions in a ratio of 5:5:1 (based on the proportions usually obtained at fractionation), and then testing this mixture alongside the corresponding whole blood sample, using the standard DDA protocol.

Results

DDA in inherited prion disease (IPD)

The results obtained from the testing of the IPD sample panel are shown in Figure 7.

3 of the 52 samples were classified DDA positive (ratio to Cut Off > 1 on 2 separate assay runs). 2 were from patients with a P102L mutation, while one was from a patient with a 4-OPRI mutation. Unfortunately, the samples that tested positive were the only samples taken from these patients: there were no corresponding samples taken at earlier stages of disease available. It was therefore not possible to pursue the plan to test a timecourse of earlier samples from the same patients at this stage.

Illustrative scatter plots showing the relationship of the possible interacting factors (see *Materials and Methods*, above) to the mean DDA ratio to Cut Off for each sample are shown in Figure 8.

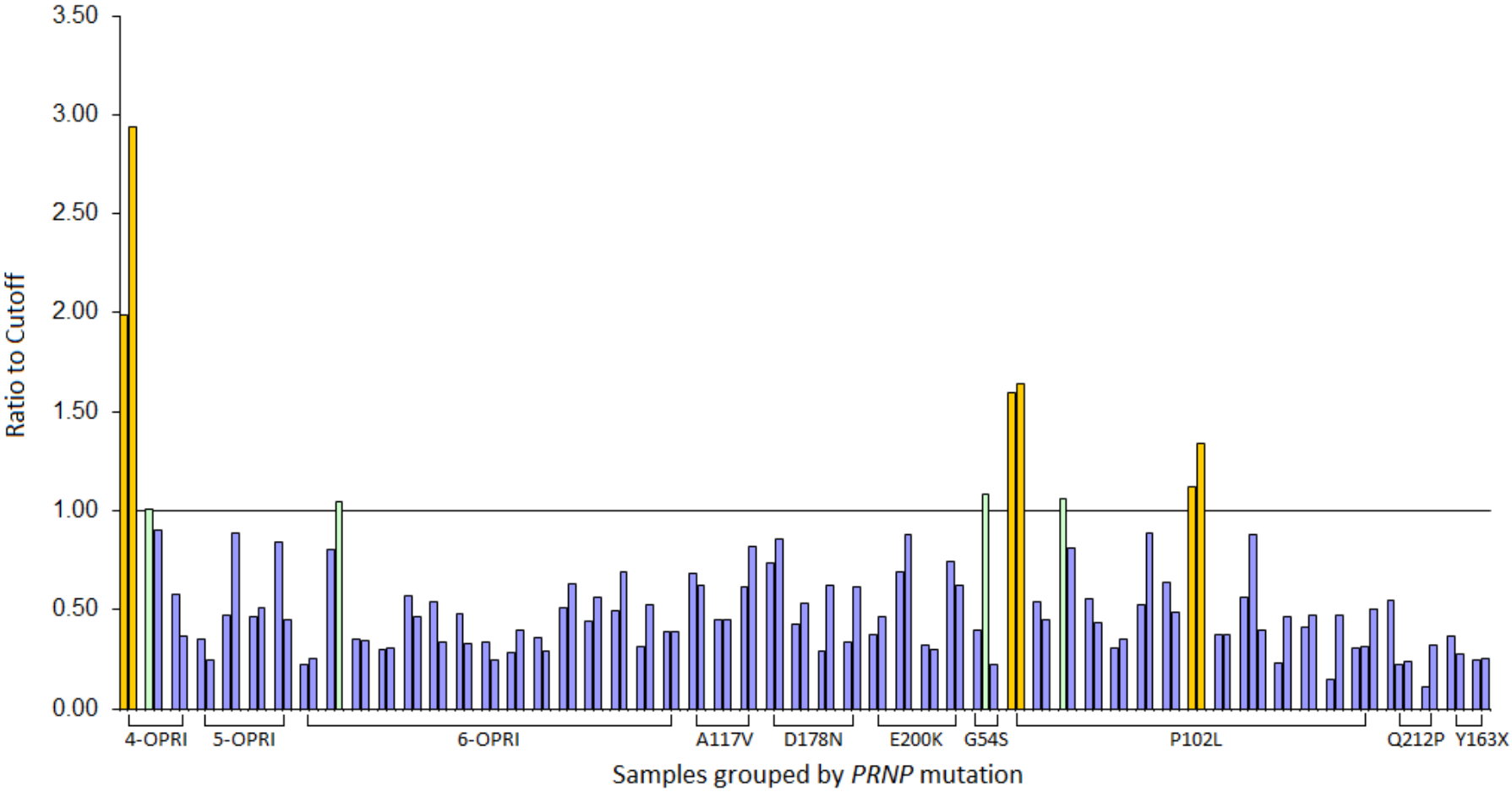


Figure 7. Direct detection assay results for panel of 52 inherited prion disease whole blood samples, each tested on ≥2 occasions, grouped by *PRNP* mutation. Yellow bars indicate samples where both assay runs produced a Ratio to Cut Off value > 1 (see *Materials and Methods)*, and the sample was deemed "DDA positive"

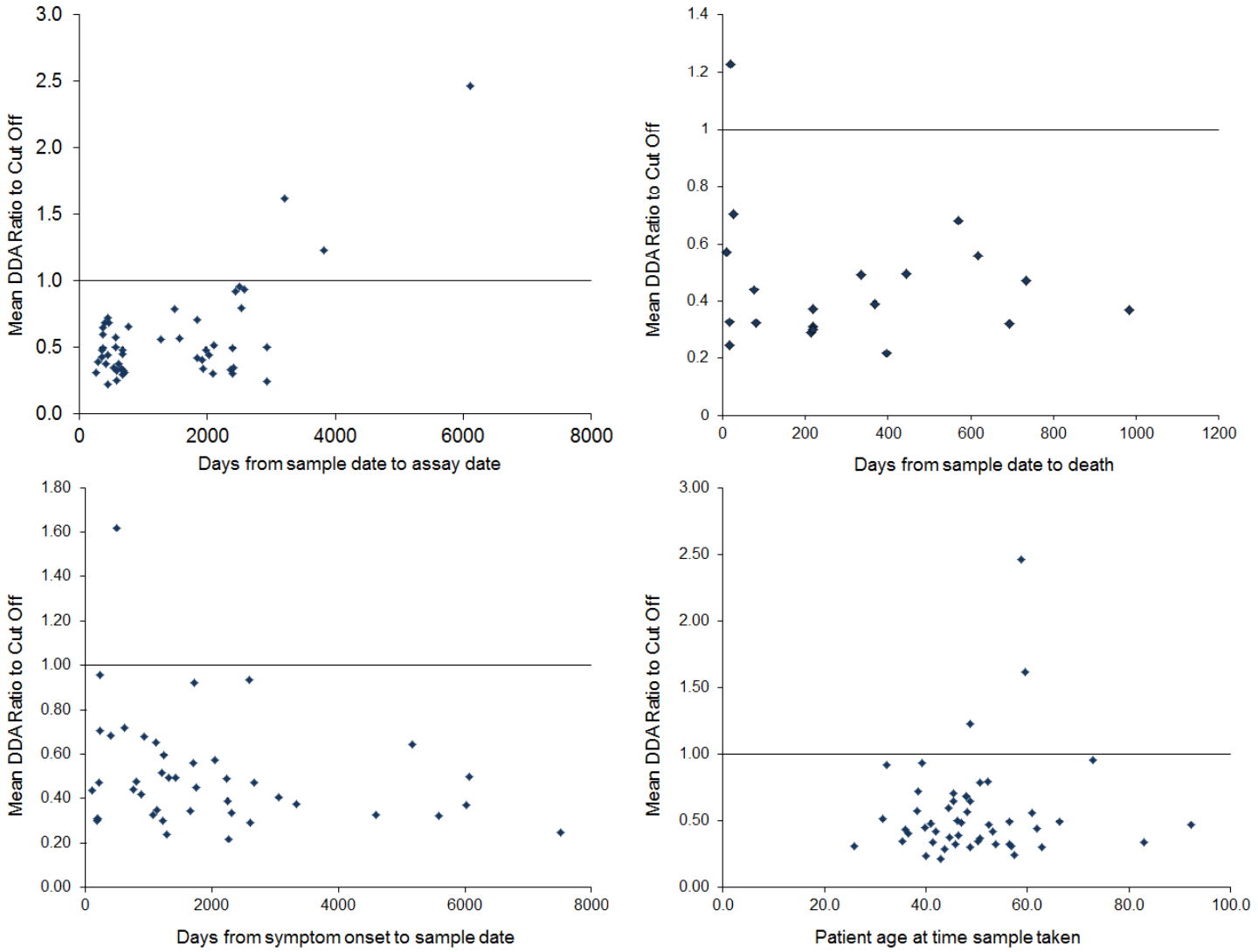


Figure 8. Scatter plots showing relationship of mean DDA Ratio to Cut Off in the inherited prion disease panel to 4 putative interacting factors.

All of the information could not be established for all of the patients (e.g. some patients still alive so time from sample to death not available, clinical information on date of symptom onset not available for some patients). From these it is apparent that the 3 DDA-positive samples were the 3 oldest samples at the time of testing, suggesting a possible interaction between the age of a sample and DDA result. This exploratory *post hoc* analysis is useful for identifying potential interactions and generating hypotheses, but these cannot be rigorously tested without an independent data set.

To test the hypothesis that older samples were more likely to be DDA positive in an entirely independent data set, the data that had been obtained using an identical DDA protocol for the masked panel experiment published in Edgeworth et al, 2011[129] was used. This consisted of testing a large panel of samples including those from 21 vCJD patients, 35 sCJD patients, 42 patients with other non-prion neurological diseases, and 100 healthy controls (UK blood donors). These experiments were carried out by my colleagues in the Molecular Diagnostics team, Dr Julie Edgeworth and Mr Mike Farmer.

The relationship of sample age to DDA result (expressed as ratio to Cut Off) in all masked panel samples for which information on date of sampling was available is illustrated in Figure 9. These consisted of 21 vCJD, 27 sCJD, 25 AD, 6 familial AD, 4 FTLD and 7 "prion-mimic" samples (from patients referred to the National Prion Clinic with clinically suspected prion disease who ultimately proved to have an alternative diagnosis). As healthy control samples were anonymized it was not possible to establish the date of sampling.

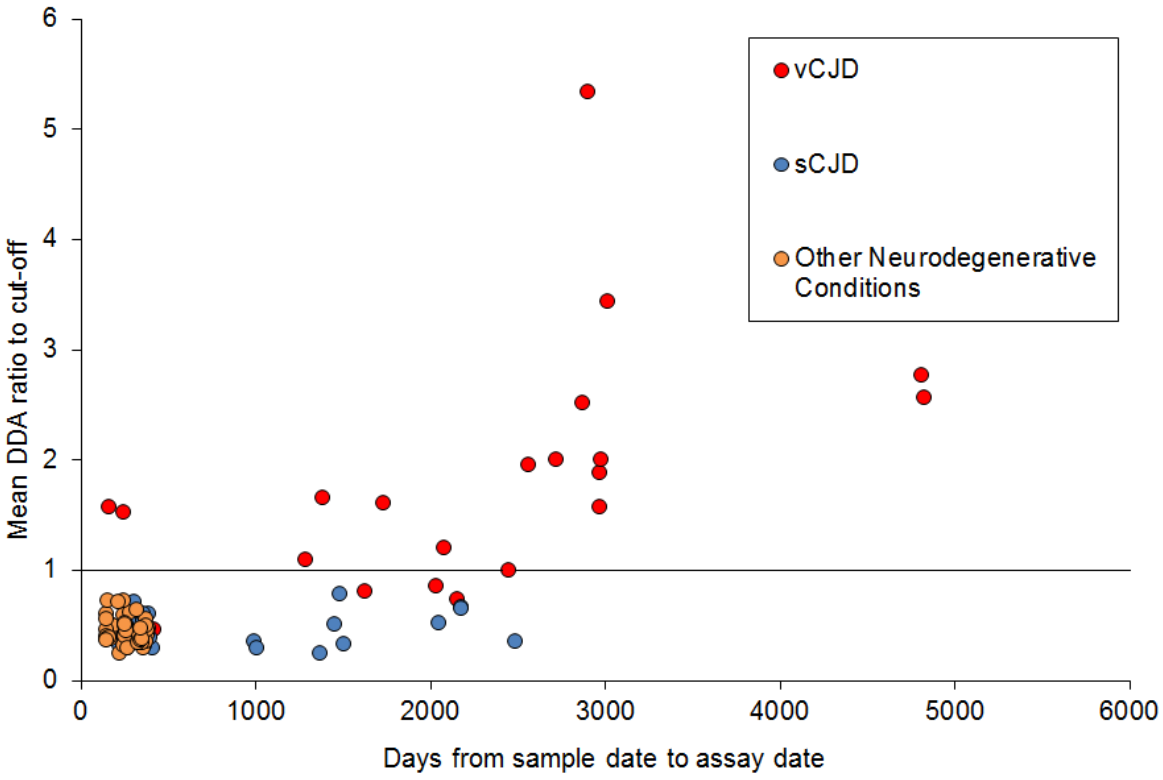


Figure 9. Relationship between DDA ratio to Cut Off and sample age in the samples tested in the masked panel experiment published in Edgeworth et al, 2011[129].

Linear regression analysis was performed on this data set with DDA ratio to Cut Off as the dependent variable, and age of sample and diagnostic category (dichotomised as vCJD or non-vCJD) as the independent variables. This is summarised below:

	Unstandardized Coefficients		Standardized Coefficients		
Model	**B**	**Std Error**	**Beta**	**t**	**p value**
Constant	2.053	0.361		5.687	<0.001
Age of sample (per year)	0.104	0.025	0.389	4.115	<0.001
Diagnosis (vCJD vs non-vCJD)	-0.860	0.176	-0.463	-4.898	<0.001

Linear regression with DDA Ratio as dependent variable (IBM SPSS Statistics).

This shows that, even when adjusted for sample age, vCJD diagnosis remains a strong independent predictor of a higher DDA result, but also suggests that the age of a sample does indeed have a strongly significant effect on the DDA result. If the non-vCJD samples are taken in isolation, there is no significant evidence of an association between sample age and DDA ratio to Cut Off (p = 0.46), so it is clearly the effect *within* the set of vCJD samples that is driving this association. It is important to note, however, that the only 10 samples tested in this experiment which were older than 2500 days were all from vCJD patients, and that these included the samples producing the 9 highest DDA ratios. It is not possible to make a like-for-like comparison with any non-vCJD samples of this age from this data, and the possibility that the specificity of the test is reduced in very old samples cannot be ruled out. Thankfully this is unlikely to be relevant to the clinical use of the DDA, where it will be employed to test very recently acquired samples.

DDA in fractionated vCJD bloods

The results of the initial testing of individual blood fractions and corresponding whole bloods are illustrated in Figure 10.

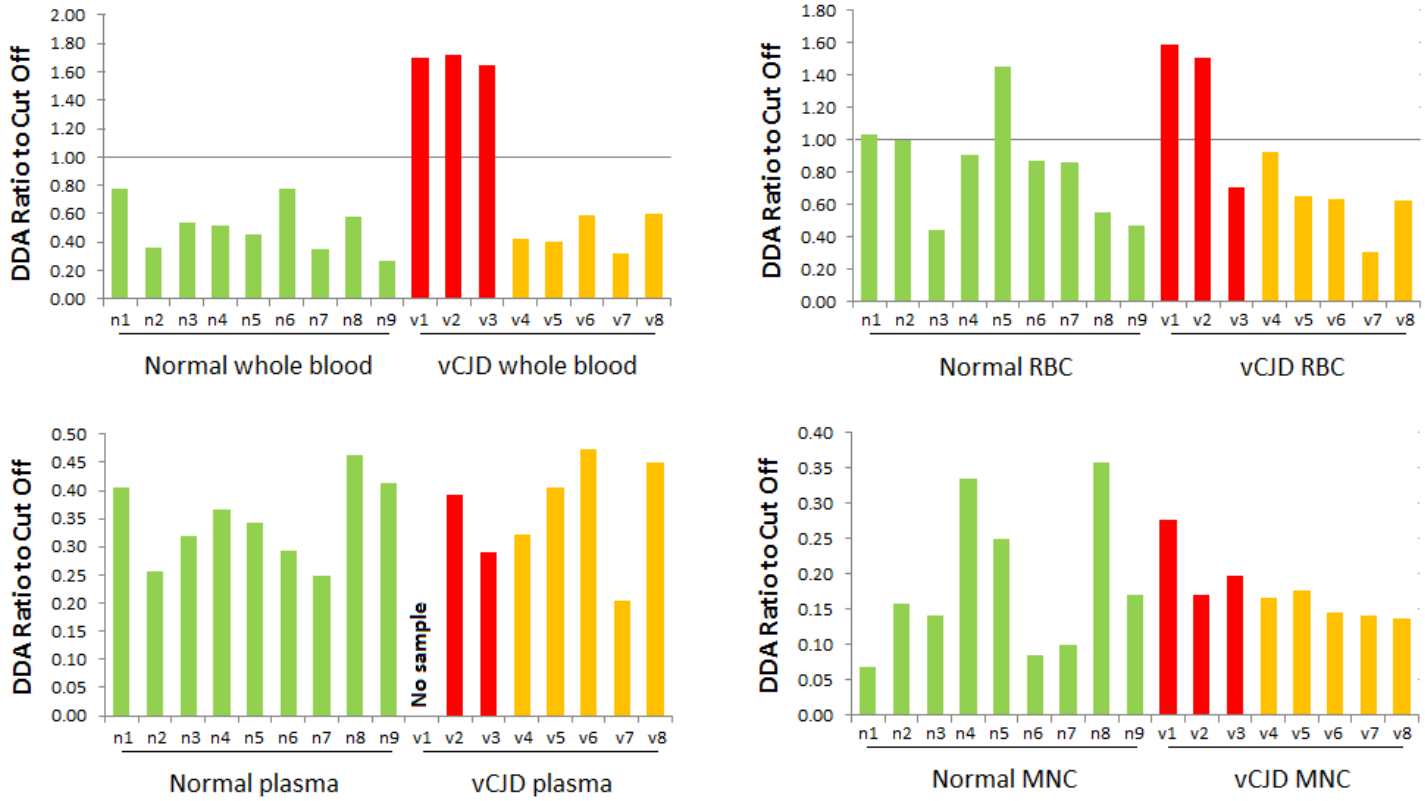


Figure 10. DDA results from initial screen of individual blood fractions and corresponding whole blood samples from normal controls and vCJD patients. RBC/platelet fractions and plasma fractions were undiluted, MNC pellet was resuspended to original concentration ("1x"). vCJD samples are divided into those for which the paired whole blood tested in the same assay runs was DDA positive (red bars) and those for which it was DDA negative (orange bars). n1-n8 and v1-v8 denote different individuals from whom blood samples were taken; normal controls and vCJD patients respectively.

There was greater variability in the luminescent signal from normal control RBC/platelet fractions than from normal control whole bloods, with some normal control RBC/platelet fractions producing a DDA ratio to Cut Off of > 1. However, as can be seen from Figure 10, 2 of the 3 vCJD RBC/platelet fraction samples tested that were paired with DDA-positive whole blood samples produced a DDA ratio to Cut Off > 1, while the same was not true of plasma or MNC fractions, where there seemed to be very little difference between normal control and vCJD samples. This suggested that it may be the RBC/platelet fraction that harboured the majority of the PrP-containing species detected by the DDA, and prompted further investigation of this.

In an attempt to reduce the high "background" signal from normal control RBC/platelet fractions, and also to allow more direct comparison of the relative contribution of each fraction to the overall DDA signal obtained from corresponding whole blood, I tested red cell/platelet fractions after diluting them back to their original concentration prior to fractionation (as described in *Materials and Methods)*. Each available vCJD sample and a panel of normal control samples were each tested on at least 2 separate occasions. Results are shown in Figure 11 .

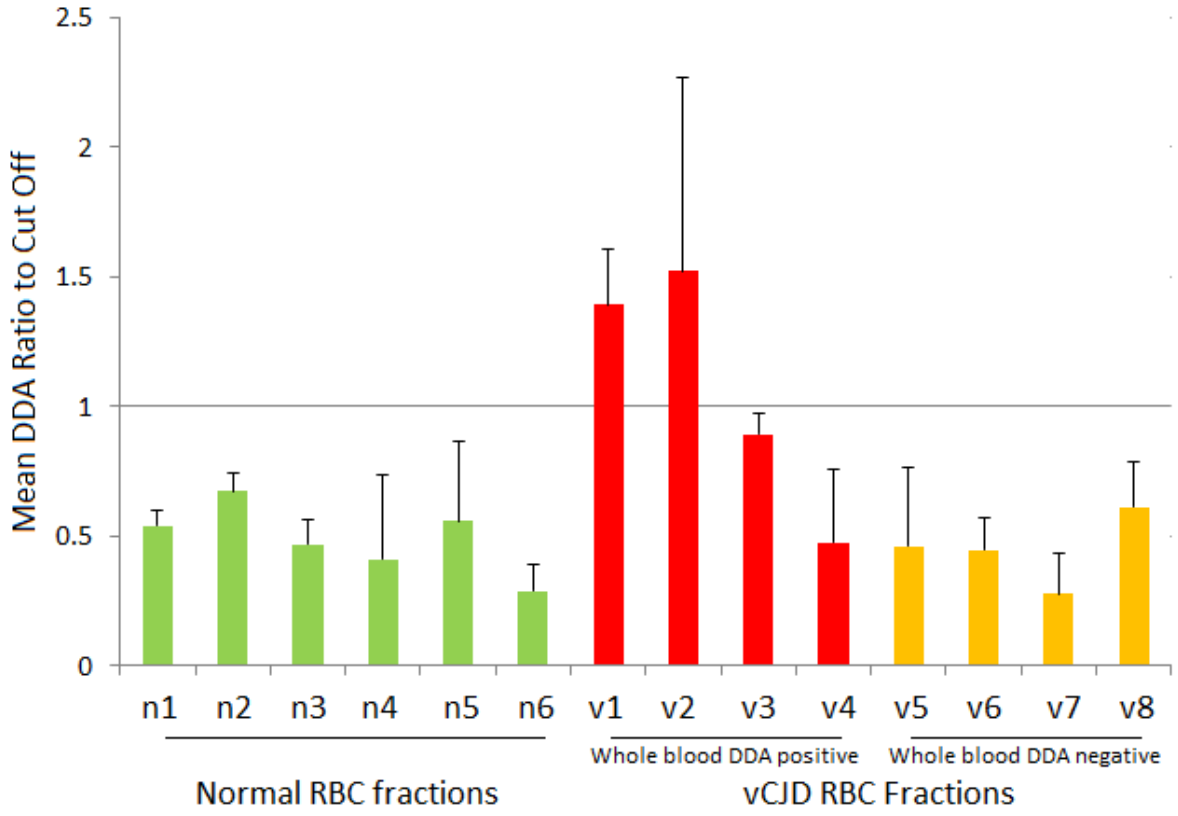


Figure 11. DDA results for pre-diluted RBC/platelet fractions from normal controls and vCJD patients (Mean + SD of Ratio to Cut Off from at least 2 independent assay runs for each sample). vCJD samples are divided into those for which the paired whole blood tested in the same assay run was DDA positive (red bars) and those for which it was DDA negative (orange bars). n1-n6 and v1-v8 denote different individuals from whom blood samples were taken; normal controls and vCJD patients respectively.

The overall mean DDA ratio to Cut Off for normal control RBC/platelet fractions was 0.48 (SD 0.2), while for vCJD RBC/platelet fractions paired with DDA positive whole bloods it was 1.33 (SD 0.57). A 2 tailed *t* test comparing these 2 groups gives a *p* value of 0.088. To further investigate whether the PrP-containing species causing vCJD whole bloods to produce a positive DDA result is present in RBC/platelet fractions I directly compared the signal from the paired samples in this experiment, shown in Figure 12. The correlation coefficient between these was 0.894, and Pearson's R^2 was

0.798 (p = 0.018), suggesting that 80% of the variation in signal from RBC/platelet fractions can be accounted for by variation in the corresponding whole blood sample.

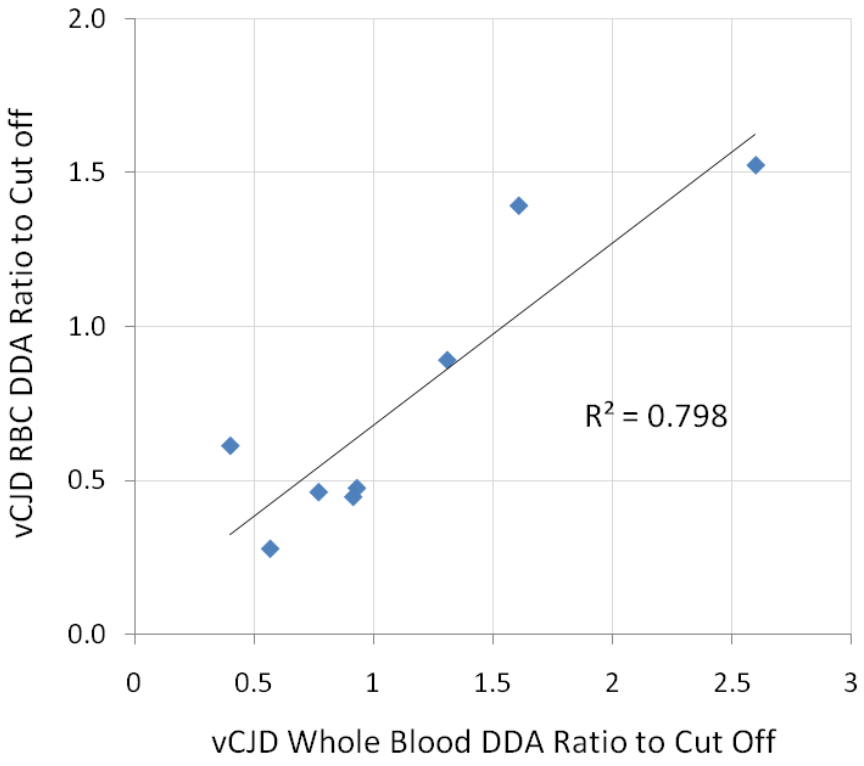


Figure 12. Correlation of DDA ratio to Cut Off from vCJD whole bloods with corresponding RBC/platelet fractions.

The results obtained from testing recombined blood fractions and corresponding whole bloods are shown in Figure 13.

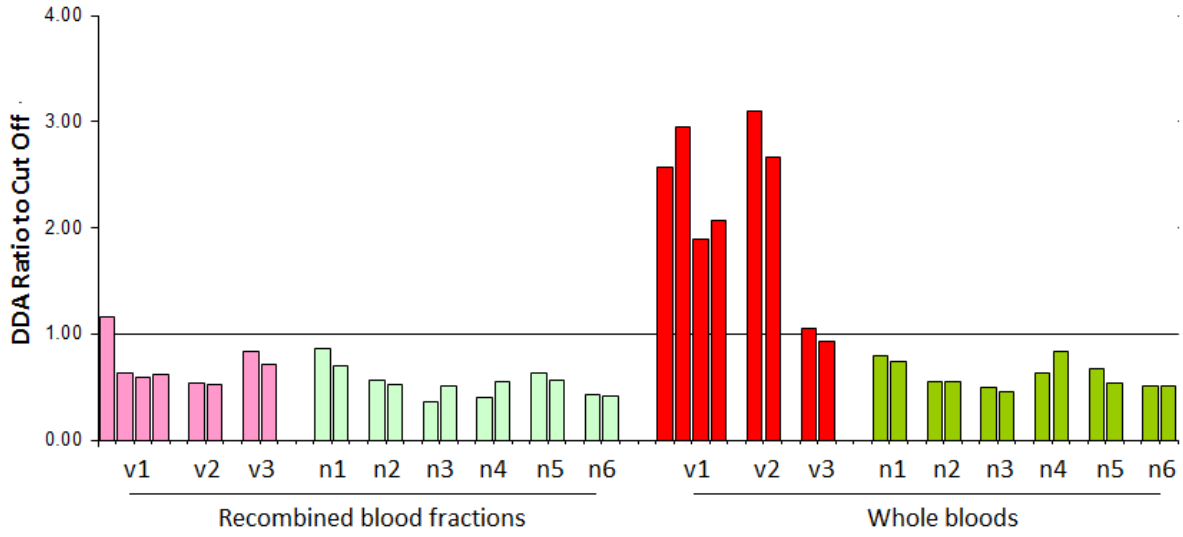


Figure 13. DDA results for recombined blood fractions (RBC/platelet + plasma + MNC fractions mixed in proportions matching those obtained at fractionation) and corresponding whole bloods. v1-v3 and n1-n6 denote different individuals from whom blood samples were taken; vCJD patients and normal controls respectively.

None of the recombined blood fractions produced a DDA ratio to Cut Off consistently greater than 1, despite some being derived from whole blood consistently producing a strongly positive signal.

Discussion

DDA in inherited prion disease

The results documented above from testing of a panel of inherited prion disease blood samples have several important implications.

At the time of testing, the 3 samples that were deemed DDA-positive were the first non-vCJD samples to have produced a positive result in the assay using the standard protocol as published in Edgeworth, 2011[129], which is now being used as a diagnostic test for vCJD through the NHS National Prion Clinic. This has clear implications for the specificity of the DDA as a test for vCJD. This needs to be borne in mind when interpreting the result of the DDA in clinical practice, and ideally *PRNP* genotyping should be performed alongside the DDA as a definitive way to rule out IPD. This is particularly pertinent as IPD can affect patients of a similar age to those typically affected by vCJD, and there is substantial overlap in the clinical features. Since these experiments were carried out, further work has been done to establish the specificity of the DDA[135]. This demonstrated a positive DDA result in 2 patients with sporadic CJD, but no false positive results from testing 5000 US blood donors, 200 UK blood donors, and 352 patients with non-prion neurodegenerative diseases. This suggests that the assay is very highly specific to prion disease, but not entirely specific to variant CJD, and so lends further weight to the conclusion that the positive results from IPD patients in the experiments presented here were true positives.

The large archive of blood samples from patients with inherited prion diseases held by the MRC Prion Unit presents a valuable opportunity to study the potential value of a blood-based biomarker for the progression of prion disease. Blood samples have been taken at many different stages of progression of disease, often in the same individual patients. This includes samples taken from carriers of pathogenic *PRNP* mutations at a time before they have developed symptoms.

Unfortunately, none of the IPD samples that were DDA-positive in this study were from patients with other earlier samples stored, so the immediate plan to test an associated time course of samples could not be pursued. However, the fact that some IPD blood samples do appear to contain disease-associated PrP that can be detected using the DDA suggests that it will be worth exploring this further: efforts are ongoing to improve the sensitivity of the DDA in general, and if this is successful it will be possible to revisit this panel of samples.

The IPD results also provided a clue to identifying a factor that interacts with diagnosis in affecting the DDA result: sample age. The further analysis using data from the masked panel experiment suggests that *in diagnostic groups associated with a positive DDA* (i.e. vCJD and now IPD); older samples tend to produce higher DDA results. There is no evidence that increasing sample age in other diagnostic groups can lead to "false positive" results (at least within the age-range of non-vCJD samples tested) if we conclude that the positive results in 3 IPD samples are "true positives". This will have clear implications for interpreting the results of other experiments using older archived samples, but may also be relevant to the clinical use of the assay. Samples being tested for clinical purposes using the DDA are likely to be days or weeks old, while the published sensitivity of the assay was established in a masked panel of samples including a number of very old samples. It is therefore possible that sensitivity in new clinical samples will be less than that demonstrated in the masked panel, and this will need to be re-assessed prospectively by comparison with final clinical and/or neuropathological diagnoses as the clinical use of the DDA continues.

In light of these observations, it is useful to reflect on some fundamental challenges and potential pitfalls in the development of diagnostic assays for very rare diseases. In an ideal situation, the sample set in which a diagnostic assay is developed and optimised should be entirely independent from the sample set in which it is then validated once an optimised protocol has been established. This was not possible in the development process of the DDA, as samples from patients with vCJD

are so rare: only 21 patients had suitable samples available and could be included in the masked panel experiment. Many of the same vCJD patient samples, or different samples from the same patients, were included in both the empirical, iterative process of developing and optimising the assay, and the masked panel experiment by which its apparent diagnostic accuracy was demonstrated. A potential problem with this approach is that the optimised assay may inadvertently rely on "off-target" differences between the disease and control sample sets in which it has been optimised (any variables other than diagnosis which happen to differ between the disease and control samples, such as sample age in this case, but also potentially random variation in other unknown variables), and that this confounding effect will not be eliminated at the validation stage because the same samples are used. At the very least, one would expect this to result in an apparent reduction in the assay's performance when it is subsequently used in an independent sample set, including its prospective use in clinical practice - simply because it has not been optimised to work well in these specific samples. This likely over-estimation of the assay's future performance is also relevant to our expectations when it is applied to different diagnostic groups and different analytes, as in the work presented in this chapter and the next.

Although not ideal, the methodology used for the development and validation of the DDA was borne out of necessity; there is no way around the fact that the samples are very scarce. The results of the masked panel experiment remain impressive and promising, but the methodological issues discussed here and the resulting caveats to the results wider applicability must be kept in mind when interpreting further work using the assay.

DDA in fractionated variant CJD blood samples

The study of the DDA's performance in fractionated bloods from vCJD patients and controls did not show any clear advantage in testing any individual blood fraction over whole blood in terms of sensitivity or specificity for vCJD.

The results suggest that, in some patients, the red cell/platelet fraction contains disease-associated PrP that can be detected using the DDA. The DDA signal from these samples correlates well with the signal from corresponding whole bloods, which would be consistent with the PrP-containing species in whole blood which is detected in a positive DDA localising to the red cell/platelet fraction. However, the number of vCJD and normal control fractions available for testing was small and therefore the results must be interpreted with caution.

We must also be very cautious in interpreting the *absence* of disease specific signal from the other blood fractions in these experiments, and should certainly not conclude that this represents evidence that they do not contain abnormal/disease-associated PrP, let alone infectivity. The contribution made by other constituents of whole blood to the performance of the assay remains poorly understood, and it is possible that a "co-factor" present in whole blood and in the red-cell platelet fraction may allow disease-associated PrP in these analytes to be detected, while that in the other fractions is not detectable by the assay in its current form.

The methods used for fractionation of bloods for laboratory analysis are not the same as those used for separation of red cells and plasma for blood transfusion purposes. This represents a further major caveat to drawing any conclusions from this work with respect to the possible application of the DDA to screening blood products after fractionation, or to the likelihood of specific blood products used in clinical transfusion practice carrying infectivity. Studies aimed specifically at this question, such as that by McCutcheon et al using BSE-infected sheep have used blood fractionation techniques that closely mimic those used in clinical practice[134].

Ideally, this study would be followed up with a larger study using a larger panel of independent samples, and at least some of these should be fractionated using methods similar to those used in

clinical practice to prepare therapeutic blood products. However, this is not currently possible as the necessary samples do not exist in archive and recent trends in vCJD epidemiology mean there is no immediate prospect of obtaining new vCJD samples.

General discussion and future plans

The Direct Detection Assay is the first blood-based assay that has been shown to distinguish vCJD patient blood samples from control samples in a rigorous, blinded experiment[129]. As such it represents a very promising new technique which may have many applications across the field of prion disease research and clinical practice, and possibly even in other related protein-misfolding diseases.

However, as a novel technique that has been developed using a necessarily empirical approach and for a very specific application, it remains poorly understood in terms of the biochemical and biophysical processes that underpin it. As a result, the process of adapting and applying the technique in different circumstances is challenging and requires a careful and rigorous approach.

Alongside this work, efforts were ongoing to find ways to improve the performance of the DDA as a diagnostic test for vCJD, and to improve our understanding of how the assay works. One observation from the early work on the DDA as reviewed above that has prompted further investigation is the surprising need to dilute a very small blood input volume (8µL) 100-fold in order for the assay to function adequately. This seems somewhat paradoxical, since the concentration of abnormal PrP in patient blood is likely to be extremely low. If we could find a way to increase the amount of blood input to the assay, or simply scale up the assay as a whole, then perhaps the performance of the assay could be improved. The experiments presented below in chapter 4 constitute part of this project.

If it does prove possible to significantly increase the sensitivity and overall performance of the DDA, then it will be worth revisiting the sample sets tested in this chapter: for example, it may prove possible to find abnormal PrP in more cases of IPD if the sensitivity of the assay is improved, and to complete the abandoned plan described above to investigate a timecourse of samples from before and after clinical onset of disease.

CHAPTER 3. DIRECT DETECTION ASSAY IN CEREBROSPINAL FLUID

Introduction

CSF in the diagnosis of neurodegenerative disease

Examination of cerebrospinal fluid (CSF), sampled by lumbar puncture, forms part of the diagnostic process for many central nervous system disorders. CSF is typically analysed for the presence of cells (blood cells or atypical/malignant cells), protein and glucose levels, and a wide variety of more specialised tests can be used depending on the clinical context and the diagnoses being considered.

In neurodegenerative disorders it has become clear that the CSF can provide a "window" onto changes occurring in the brain: particularly by measuring the levels of specific disease-related proteins.

This has been demonstrated most clearly, and studied most thoroughly, in Alzheimer's disease. The neuropathological hallmarks of Alzheimer's disease are senile plaques: extracellular aggregations of β amyloid (Aβ), a protein encoded by the amyloid precursor protein (*APP*) gene; and neurofibrillary tangles: intracellular aggregations of hyper-phosphorylated tau, a protein encoded by the microtubule-associated protein tau (*MAPT)* gene[136]. The aggregation of these proteins into insoluble, polymeric forms is thought to play a fundamental role in the pathogenesis of the disease. The levels of a particularly "amyloidogenic" isoform of β-amyloid, $A\beta_{42}$, and of both phosphorylated tau (P-tau) and total tau (T-tau) can be measured in the CSF, and have been found to change in a characteristic way in patients with AD.

Levels of CSF $A\beta_{42}$ are reduced in patients with AD, typically to about 50% of normal levels. The reasons for this are not clear, but it has been suggested that there may be a "sink effect" as the $A\beta_{42}$ is increasingly incorporated into senile plaques in the brain parenchyma and is pulled out of the CSF[137]. Levels of CSF tau tend to be increased, with a particular increase in P-tau, and an increase in

the ratio of P-tau : T-tau. These CSF parameters have been included in recent diagnostic criteria for Alzheimer's disease[138].

They have also been extensively investigated in patients that might be in the very early stages of AD but would not yet reach the clinical diagnostic criteria (patients with mild cognitive impairment that is not affecting day-to-day functioning), and have been found to have value in predicting which of these patients will go on to develop Alzheimer's disease[139]. Along with other techniques such as *in vivo* imaging of amyloid deposition (using positron emission tomography (PET) with ligands that bind to amyloid)[140] in patients with mild cognitive impairment, or even normal healthy people, this has led to a re-thinking of the definition of Alzheimer's disease and the lexicon used to describe different stages of the disease, to include "prodromal" or "pre-dementia" Alzheimer's disease to describe individuals in whom there is evidence of an ongoing Alzheimer's type amyloid disease process, but who have not yet reached the clinical criteria for a diagnosis[141]. This is part of an important re-conceptualisation of neurodegenerative disease, acknowledging that the underlying molecular pathological process begins some time before the onset of clinical symptoms and that it may be possible to identify patients in the very early stages of disease, or even before the onset of clinical disease. As efforts to find disease-modifying treatments progress, this possibility of achieving very early diagnosis becomes an important goal: the benefit gained from any treatment that can stop or slow down the disease process is likely to be greatest if it can be started as early as possible.

Efforts have been made to apply a similar approach to finding diagnostic markers of other neurodegenerative diseases, including Parkinson's disease[142], frontotemporal lobar degeneration[143] and Motor Neuron Disease[144], by examining the CSF levels of a range of disease-related proteins. Although some promising candidates have been identified, these have not been found to have such clear diagnostic value (often showing significant differences on group-to-group comparisons, but with considerable overlap between groups), and no CSF parameters are currently included in the clinical diagnostic criteria for any of these conditions.

CSF in prion disease

Current use of CSF in the diagnosis of prion disease

In all types of prion disease the CSF typically has a normal cell count of 0-2 cells/µL[145]. A pleocytosis (presence of increased numbers of white blood cells) suggests an alternative diagnosis, particularly an inflammatory disorder. Total protein level is usually normal or only modestly elevated, and there is rarely evidence of intrathecal immunoglobulin synthesis. Measurement of these "routine" CSF parameters, as well as other disease-specific tests such as polymerase chain reaction for viral DNA, play a vital role in ruling out alternative diagnoses that may produce a similar clinical presentation[45]. As these other conditions may be treatable (e.g. with antiviral or immunomodulatory agents), the initial phase of investigation of patients with prion disease tends to focus on ruling out these alternative diagnoses. As a result of this, there is a tendency for prion disease to be thought of as a diagnosis of exclusion, exacerbating the problem of late diagnosis, but some CSF tests can provide positive support for a diagnosis of prion disease, particularly sporadic CJD.

14-3-3 Proteins

The 14-3-3 proteins comprise a large family of intracellular proteins found in all eukaryotic cells, and constitute about 1% of the total protein content of brain neurons [146]. They are found in the CSF in a variety of conditions where there is rapid and extensive neuronal destruction. They are detected using a qualitative assay, giving a positive, negative or "weak positive" result.

The 14-3-3 assay is included in the World Health Organization's diagnostic criteria for sporadic CJD (sCJD)[34,147].

The assay is typically positive in classical, rapidly progressive sCJD, with a sensitivity of 90-95% for the MM1 subtype [34,148]. However, it is less sensitive for longer duration cases, for younger patients, and for the acquired and the more slowly progressive inherited prion diseases [149]. The test is positive in only about 40% of cases of variant CJD [148,150].

Interestingly, successive studies over the years have tended to show a reducing sensitivity of the 14-3-3 assay for sCJD [34,148,151-154]. This may well be related to the increasing recognition and inclusion of cases with atypical, more slowly progressive clinical features leading to more "false-negative" results in recent studies.

There is some evidence that the 14-3-3 assay is more sensitive when performed at later stages of disease. It has therefore been suggested that in cases where there is continuing diagnostic uncertainty there may be a role for repeating the assay at a later stage if the first is negative [148]. However, in practice this is often superseded by a decision either to obtain a definitive tissue diagnosis (with brain biopsy), or that further investigations are no longer appropriate.

The specificity of the 14-3-3 assay for prion disease is dependent on the population of patients being tested, but in many studies has been quite low, at around 70-80% [34,148]. A large multicentre, multinational study including 29,000 samples sent to national CSF testing centres suggested that its specificity for distinguishing sCJD from other neurodegenerative diseases was as high as 95 – 97%, but for distinguishing it from acute neurological conditions it was reduced to 82 – 87%[155]. In patients with clinically suspected CJD, false positive results occur most commonly when the final diagnosis is inflammatory or malignant (including CNS tumours and paraneoplastic syndromes)[148].

Other causes of a positive result include recent stroke, infective encephalitis and subacute sclerosing panencephalitis (SSPE), but these diagnoses can usually be ruled out clinically or on the basis of other tests. A raised CSF cell count has been shown to be highly significantly associated with an increased false positive rate[148], and should always prompt investigation for other causes.

The specificity is not high enough for the test to have a role in screening unselected patients with dementia for prion disease [154].

S100b Proteins

These are a large family of calcium binding cytoplasmic proteins found in glia in the CNS, as well as widely outside the CNS. They are detected using a quantitative assay. Their levels are raised in the CSF in a large number of destructive diseases of the nervous system where there is extensive gliosis, including CJD. As with the 14-3-3 proteins, they are more likely to be raised in rapidly progressive disease, where sensitivity is around 90%[148]. However, the specificity is even lower than for 14-3-3[156], and in practice they rarely add any further useful diagnostic information.

Tau (τ)

Tau is an intracellular micro-tubule associated protein, which is found in increased levels in the CSF when there is destruction of neurons. In some disease states the Tau protein becomes hyperphosphorylated. The levels of total Tau and hyperphosphorylated Tau can be measured using quantitative assays.

The total Tau (T-Tau) level in the CSF is elevated in a wide variety of degenerative CNS disorders, including Alzheimer's disease. It is increasingly used, in combination with CSF $A\beta_{42}$, as a diagnostic marker for Alzheimer's disease, as discussed above[137].

In sCJD it is often elevated to a much higher level than in the more common slowly evolving degenerative disorders. If a high threshold is used, the sensitivity is again high for rapidly progressive CJD, and the specificity seems to be similar to that of the 14-3-3 assay [148,157].

It has been suggested that CSF levels of hyperphosphorylated Tau (P-Tau) are particularly elevated in variant CJD (vCJD), such that the ratio of P-Tau to T- Tau is higher in vCJD than sCJD [158]. This may have a role in helping to distinguish between these two conditions.

A more recent study compared levels of P-Tau and T-Tau in CSF from patients with CJD and in those with other non-CJD dementias, and suggests that a high ratio of T-Tau : P-Tau may have a role as a marker to distinguish these two groups[159]. The study also looked at the relationship between the levels of CSF tau and the duration of survival after the sample was taken, and found that levels were higher in samples taken closer to the date of death. One explanation for these results would be that T-Tau levels rise as an individual patient's disease progresses, raising the possibility that this might be used as a biomarker of disease progression. However, the results might also be explained by T-Tau levels being higher in individuals with more rapidly progressive disease, who will tend to have their investigations including CSF examination closer to their date of death. The study also found in a subset of patients that had more than one CSF sample taken, that the levels of T-Tau and the T-Tau : P-Tau ratio tended to be higher in the later sample, so it may be that these CSF parameters will have a role as a marker of disease progression, although this will require further investigation.

Other Proteins

Various other proteins have been considered as CSF markers for CJD, including Neuron-Specific Enolase, prostaglandins and interleukins. However, these are less sensitive and specific than those above, and have little clinical utility. The limited data available on $A\beta_{42}$ in CJD suggest that it has little diagnostic significance[157].

Prion protein in CSF

Prion protein (PrP^c) is present in normal cerebrospinal fluid, detectable by Western blotting and ELISA techniques[160].

As discussed above, it is known that diseases characterized by accumulation of a misfolded, aggregated form of a specific protein in the brain may result in changes in the measurable concentration of the monomeric form of this protein in the CSF (e.g. levels of $A\beta_{42}$ are reduced in the CSF of patients with Alzheimer's disease[137]).

A study using ELISA to quantify the levels of PrP^c in CSF from patients with sporadic CJD, as well as from those with a range of other degenerative and non-degenerative neurological conditions and from healthy controls showed that there was a significant reduction in the detectable concentration in CJD, but also in the other neurodegenerative conditions studied[161]. The study also showed some correlation between disease severity and CSF PrP^c concentration in CJD, Alzheimer's disease and Lewy Body Dementia. As the range of PrP^c concentrations in CJD largely overlapped that in the other degenerative diseases, the potential for this parameter being used as a diagnostic test is limited, but the observation may be useful in understanding changes in CSF PrP in CJD and other disease states.

The possibility of detecting abnormal, disease-associated forms of PrP in CSF from patients with prion disease has also been explored. Efforts to detect protease-resistant PrP in CSF using Western blotting have failed [160]. Primate bio-assay experiments, carried out at the National Institutes of Health in the USA, assessed the infectivity of a range of different tissues and fluids including CSF from patients with CJD (both sporadic and familial) [7]. From 27 CSF samples inoculated into primates, 4 transmitted the disease. For comparison, inoculation of brain tissue from patients transmitted disease in 100% of cases, and inoculation of spinal cord tissue transmitted disease in 4 out of 6 cases. This suggests that in at least some patients, the infectious agent is present in CSF (assuming there has not been contamination with brain or other tissue). As the infectious agent is thought to consist primarily of aggregated PrP, with its disease-causing and infective properties conferred by

conformational properties of the PrP aggregates, this suggests that it may be feasible to detect disease-specific forms of PrP in patient CSF.

More recently, assays based on *in vitro* seeded conversion of PrP^c into an aggregated form have been applied to CSF with some success. These approaches are conceptually appealing, as they make use of a biochemical property thought to be very highly specific and central to the pathophysiology of prion diseases: the templated conversion of normally folded PrP into a misfolded amyloid-rich form. It would be hoped that this should lend them much greater specificity than measurement of the non-specific markers of neuronal damage currently used in clinical practice.

The most promising of these, known as real-time quaking induced conversion (RT-QuIC)[162], has been evaluated as a diagnostic CSF test. This was developed from earlier techniques using protein misfolding cyclic amplification (PMCA), which were able to detect seeding activity in CSF from prion disease infected animals, but required laborious repeated rounds of amplification and Western blotting of the products of these reactions[163,164]. RT-QuIC involves adding Thioflavin and a test sample to a preparation of recombinant PrP, which is then shaken repeatedly. If the test sample is able to "seed" the conversion of the recombinant PrP into an aggregated amyloid-rich form, this will bind to the Thioflavin and emit fluorescence, which can be detected in real time.

In a UK-based study, RT-QuIC was used to test CSF samples from 123 patients with neuropathologically confirmed sCJD, alongside a control group of 103 samples from patients that were clinically suspected to have CJD but subsequently found to have an alternative diagnosis[165]. The test was positive in 103/123 sCJD cases, and in 1/103 control cases, giving a sensitivity of 89% and a specificity of 99%. A smaller study from Japan suggested sensitivity of greater than 80% and specificity of 100% [166]. These suggest that this technique may be superior to the established CSF assays reviewed above (14-3-3, Tau etc.) as a diagnostic test for sCJD. A study testing dilute patient

brain homogenate using RT-QuIC tested samples from sCJD and vCJD patients: while sCJD produced strong positive results, the assay was less efficient in detecting the abnormal PrP from vCJD brain[165]. None of the published studies have reported testing of vCJD patient CSF using RT-QuIC.

These experiments suggest that CSF from patients with sporadic CJD contains molecular species capable of seeding the conversion of recombinant PrP^c to an aggregated amyloid-rich form, which are not present in CSF from controls. An "amplification" process ensues, with the misfolded recombinant PrP itself seeding further conversion. Assuming that the seeding species is itself primarily composed of PrP, with this property conferred by its conformation, this again suggests that there are abnormal disease-associated PrP species in sCJD patient CSF.

A number of other novel techniques for detecting disease-associated PrP in CSF have been published: these include combining PMCA with "SOFIA" ("surround optical fibre immunoassay"- a technique using capture with specific monoclonal antibodies and a novel laser-based detection method)[167,168]; and "SIFT" ("scanning for intensely fluorescent targets" after capture with monoclonal antibodies)[169]. While they have not reached the same stage of validation as a diagnostic test as RT-QuIC, they have produced some promising results in small scale experiments, lending further support to the conclusion that detectable disease-associated PrP is present in the CSF of patients with sCJD.

As the earlier work developing the Direct Detection Assay (DDA) for use in blood (as discussed in detail above, and culminating in the publication of Edgeworth et al, 2011[129]) suggested that this technique could detect very low levels of disease-associated PrP in the presence of significant levels of PrP^c without the use of protease, it provides a promising candidate as a potential novel diagnostic CSF test.

Most of the evidence reviewed above for the presence of abnormal disease-associated PrP and/or infectivity in CSF comes from sCJD, in contrast with blood-borne infectivity for which the only evidence in human prion diseases is in vCJD. It may therefore be more likely that the DDA will have a role as a CSF test in sCJD. The distribution of infectivity in the tissues of patients with these 2 conditions is known to differ greatly, with that in sCJD almost entirely restricted to the central nervous system (with very low levels detectable in the spleen), while that in vCJD is also very widely distributed in peripheral tissues[103]. CSF may therefore be a more useful diagnostic analyte than blood in sCJD. However, the DDA had only previously been proven to detect vCJD-associated abnormal PrP, and it is possible that there are vCJD strain-specific properties of the abnormal PrP species that allow their detection in the DDA, and might preclude its use as a test for sCJD.

I investigated these issues, with the aim of adapting and validating the DDA as a diagnostic CSF test for prion disease.

Relevance of previous work using DDA in blood

From the earlier work on the DDA in blood, including that published in Edgeworth et al 2011[129], there is evidence that the DDA can differentiate between blood samples from patients with vCJD and those from controls, by detecting vCJD-specific molecular species that bind to steel powder and are bound by a PrP-specific antibody. As blood contains relatively large amounts of PrP^c and the antibody used binds to both normal PrP^c and misfolded PrP, the disease-specificity of the DDA seems likely to be conferred by differing steel-binding properties of the PrP-containing species in vCJD patient blood compared with control blood.

It is not known, based on the previous work, to what extent the properties of the blood-borne vCJD-specific PrP-containing species that allow their detection in the DDA are shared by PrP-containing species present in other tissues or fluids, or in other disease types. It is known that different prion

strains may lead to accumulation of abnormal PrP with very different biochemical properties[170,171], so it cannot be assumed that abnormal PrP in sCJD will behave in the same way as that in vCJD in the assay. In addition it is not known what contribution is made to the performance of the assay by other factors present in blood that may interact with the disease-specific PrP-containing species, for example by affecting its steel-binding properties.

In preparation for testing patient CSF samples it was therefore important to address several fundamental unanswered questions:

Key questions to address

Can normal PrP^c produce high signal in the DDA?

The detection of very low levels of abnormal PrP in the context of high background level of PrP^c in patient blood suggests that PrP^c does not in itself produce high signal in the DDA when it is used in blood, but this needs to be investigated in other analytes, as it may have a major impact on the sensitivity and specificity of the test (by affecting its signal-to-noise ratio). To investigate this I planned to test an analyte containing large amounts of PrP^c (wild type FVB mouse brain homogenate)alongside an analyte differing only in the absence of PrP^c (PrP-knockout FVB mouse brain homogenate) at a range of dilutions.

Can the DDA detect vCJD-specific PrP-containing species in the absence of blood?

In order to address this question, I planned to test vCJD patient and control brain homogenate diluted into the DDA capture buffer, in the absence of blood, and if necessary re-optimise the assay conditions.

Can the DDA detect vCJD-specific PrP-containing species in the presence of CSF?

Prior to testing prion disease patient CSF samples, I planned to investigate whether any factors present in normal CSF inhibit the performance of the DDA, by testing dilute vCJD patient and normal brain homogenate in the presence of CSF at different concentrations, and comparing this with the same concentrations in the absence of CSF.

Can the DDA detect sCJD-specific PrP-containing species in the presence of CSF?

As discussed above, there are reasons to think that the DDA would have a more useful role as a CSF test in sCJD rather than vCJD, and so having shown detection of vCJD-associated abnormal PrP in the presence of CSF, I planned to carry out equivalent experiments using sCJD brain homogenate, prior to proceeding with testing of diagnostic patient samples.

Can the DDA differentiate vCJD or sCJD patient CSF samples from controls?

Finally I planned to assemble a panel of vCJD, sCJD and control diagnostic CSF samples and test these using the DDA.

Availability of samples

In comparison with working with blood, a number of particular challenges are presented by planning assay development experiments with CSF.

CSF is typically sampled (at lumbar puncture) in small quantities, and usually on only one occasion during the investigation of a patient with a neurological illness, including a suspected prion disease. Once samples have been used for diagnostic testing, the volumes available for use in research are often very small. In order to conserve these small and precious samples (particularly those taken from patients with very rare diseases), as much as possible, it is necessary to take a carefully considered strategy to the process of assay development; a “trial and error” approach is likely to quickly exhaust the available analyte.

Whereas large numbers of anonymised blood samples taken from normal healthy people can be obtained from the blood transfusion services (e.g. the UK's NHS Blood Transfusion), it is very unusual for normal healthy people to have CSF samples taken, so these are rarely available for use as control samples. A compromise is to use CSF samples that have been taken from patients with neurological symptoms or diagnoses that are unlikely to cause any major changes in the CSF constituents.

Obtaining CSF at autopsy can allow much larger samples to be taken, but may limit the applicability of findings to samples obtained at lumbar puncture during life, as there may be a risk of contamination of the fluid with brain or other tissue, or changes in the constituents of the CSF after death due to loss of active homeostatic mechanisms.

The experiments presented here represent exploratory work seeking to demonstrate proof of principle that the Direct Detection Assay may function as a CSF test, and justify further larger scale work with larger sample sets and using more "pure" control samples which would be needed to validate an assay robustly.

Materials and methods

CSF samples

In order to assemble a panel of CSF samples for testing I searched the MRC Prion Unit database for all archived CSF samples and reviewed the associated clinical information to identify suitable samples with appropriate consent in place to allow their use in research. Ten samples from patients with confirmed prion disease diagnoses were identified from this initial search. These were all samples of CSF obtained at autopsy, from patients with a range of different prion disease diagnoses (see Table 4).

I subsequently made arrangements (in collaboration with Professor Sebastian Brandner, Consultant Neuropathologist at University College London Hospital) for large volume CSF samples to be routinely collected at autopsies for patients with suspected prion disease carried out at University College London Hospital whenever appropriate consent was provided, in order to prospectively accumulate further samples. These included samples from patients with a range of prion disease types and also some from patients found to have an alternative, non-prion diagnosis at autopsy (see Table 4).

To obtain samples of CSF collected at lumbar puncture during life, we collaborated with the Neuroimmunology Department laboratory at the Institute of Neurology, University College London, to identify archived diagnostic CSF samples for patients with prion disease, and also a panel of CSF samples for patients that had an alternative non-degenerative diagnosis to act as controls. 19 samples were identified as an initial panel. These samples are also listed in Table 4, along with brief diagnostic/clinical details. All lumbar puncture CSF samples had been processed on receipt to the Neuroimmunology Department Laboratory with centrifuge at 2500 rpm for 9 minutes at room temperature, and then the acellular supernatant stored at -80°C (personal communication from Mr Miles Chapman, Senior Laboratory Technician, Neuroimmunology Department). Following transfer

to the MRC Prion Unit they were stored at -70°C. There was no difference in the processing or storage of samples from patients with suspected prion disease and those from other patients.

Clinical details related to the samples were obtained from existing databases (MRC Prion Unit tissue archive database, and Dementia Research Centre CSF database), with confirmatory information obtained from review of clinical notes where necessary.

Diagnosis & other clinical info		Method of collection	Number of samples
Non-neurodegenerative controls*		Lumbar puncture	12
Non-prion, neurological disease controls	AD	Post mortem – prospective	1 (Large volume)
	Vascular and AD	Post mortem – prospective	1 (Large volume)
	Anti-VGKC encephalitis	Post mortem – prospective	1 (Large volume)
	Lewy body disease	Post mortem – prospective	1 (Large volume)
Definite sCJD		Lumbar puncture	4
		Post mortem – archived	2
		Post mortem – prospective	9
Definite vCJD		Lumbar puncture	1
		Post mortem – archived	3
Inherited prion disease	E200K	Lumbar puncture	1
	P102L	Post mortem – archived	2
	6-OPRI	Post mortem – archived	2
	7-OPRI	Lumbar puncture	1
Definite iatrogenic CJD (growth hormone)		Post mortem - archived	1

Table 4. Details of CSF samples assembled. AD = Alzheimer's disease. VGKC = voltage gated potassium channel. *Diagnoses of non-neurodegenerative controls: Schizophrenia (4), epilepsy (2), headache (2), depression, conversion disorder, small fibre neuropathy, autism.

Brain homogenate samples

Brain homogenate samples were used for several experiments, to provide a source of abnormal disease-associated PrP that could be "spiked" into either buffer or CSF. These were all archived samples prepared previously by colleagues at the MRC Prion Unit. Prior to use they were stored at 10% of their original concentration in phosphate-buffered saline (PBS), at -70°C. They included: vCJD patient human brain homogenate, sCJD patient human brain homogenate, normal human brain

homogenate, wild-type FVB mouse brain homogenate; PrP-null knockout FVB mouse brain homogenate.

Standard DDA protocol for experiments with CSF and dilute brain homogenates

The starting point for the project was the standard direct Detection Assay protocol developed for its use as a blood test, and used to generate the masked panel data published in Edgeworth et al, 2011[129]. This was used as the initial standard protocol, with variations introduced as the project progressed, which are detailed in the relevant sections below. The standard protocol adapted for use with CSF or dilute brain homogenate was as follows:

Stainless steel powder (<45µm, *Goodfellow*) is washed thoroughly using 2% triton-x-100 (*Sigma*), 70% ethanol and sterile double-distilled water (ddH_2O). It is then separated into individual aliquots of 23mg of steel powder, each in 115µL ddH_2O as a suspension and allowed to settle. The supernatant liquid is removed immediately prior to use.

DDA capture buffer is prepared so that the concentrations of its constituents *after addition of the test sample*, will be as follows: 100mM tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 8.4, 2% bovine serum albumin (BSA), 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) detergent, 1 complete protease inhibitor tablet (*Roche*)/50ml, and 10µL benzonase (Grade II, *Merck*)/50ml.

For the standard protocol the test sample is diluted 1:100 into the DDA capture buffer, and the initial concentrations of the buffer constituents are identical to that used for the blood experiments described in Chapter 2: 101mM tris(hydroxymethyl)-aminomethane (Tris) buffer at pH 8.4, 2.04% bovine serum albumin (BSA), 2.04% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate

(CHAPS) detergent, 1 complete protease inhibitor tablet (*Roche*)/50ml, and 10µL benzonase (Grade II, *Merck*)/50ml.

All subsequent steps are carried out in a Containment Level III laboratory, adhering to all relevant local operating procedures.

CSF and/or dilute brain homogenate samples to be tested are thoroughly thawed from storage at -70°C. Using the dilution required for each experiment, test samples are mixed with DDA capture buffer to give a final volume of 800µL, and the mixture added to a single 23mg steel powder aliquot. This is then incubated overnight on an Eppendorf Thermomixer at a temperature of 18°C shaking at 650rpm.

Wash solution is prepared, consisting of phosphate-buffered saline and 0.05% Tween detergent. The supernatant is removed from each steel powder sample, and these are then each washed 5 times using 1mL of wash solution for each wash. After the fifth wash is removed, dry steel samples are "heat-shocked" for 5 minutes at 110°C, and then allowed to cool.

Biotinylated anti-PrP antibody ICSM18B (*D-Gen Ltd*) is prepared at a concentration of 0.6µg/ml, and 50µL added to each steel powder sample. These are then incubated for 1 hour on a Thermomixer at 37°C shaking at 750rpm.

Supernatant is removed and samples are washed a further 3 times using 1mL of wash solution for each wash. Horseradish peroxidase conjugated *NeutrAvidin-HRP* (Pierce) is prepared at 1:100 000 dilution, and 50µL added to each dry steel powder sample. These are then incubated for 45 minutes on a Thermomixer at 37°C shaking at 750rpm.

Supernatant is removed and samples are washed a further 3 times using 1mL of wash solution for each wash. *Femto* chemiluminescent SuperSignal reagent (*Pierce*) is prepared by mixing equal volumes of the 2 constituent parts as per the manufacturer's instructions. 60µL is added to each steel powder sample. Each steel powder sample is then resuspended in this liquid and separated equally between 3 wells of a black, 96-well, flat-bottomed ELISA plate (*Greiner*), so that each contains 20µL of *Femto* reagent and approximately 7.67mg of steel powder.

A dilution series of NeutrAvidin giving dilutions of $1:1x10^6$, $1:1x10^7$ and $1:1x10^8$ is also prepared. 20µL of each of these is added to 3 replicate wells.

A further 80µL of Femto reagent is added to each well, and the plate is then immediately read using a M100 plate reader (*Tecan*), using luminescence settings.

The mean luminescent signal measured from the 3 wells for each sample is taken as the DDA result for that sample.

Quality control for experiments using CSF and dilute brain homogenate

When this project was begun, the only samples known to produce a reliable positive result in the DDA were whole blood samples from patients with vCJD. It was therefore necessary to use these as positive control samples for quality control initially (as they were used in the experiments described in Chapter 2). However, these samples represent a finite and extremely precious resource, as the total number of such samples stored is small, and at present there are no opportunities for acquiring new samples as there were no patients with vCJD currently alive in the UK at the time. I therefore aimed to establish alternative positive and negative controls in the early stages of this project which did not require the use of vCJD patient blood. These consisted of brain homogenate from a vCJD

patient and from a normal individual diluted in the DDA capture buffer, and are described in the course of the experiments below.

Testing of dilute PrP-null and wild type FVB mouse brain homogenates

Homogenated samples of (1) wild type FVB mouse brain, (2) PrP-null (knockout) FVB mouse brain, and (3) normal human brain were obtained (prepared by colleagues at the MRC Prion Unit). Prior to use, these were stored at 10% of their original concentration in phosphate-buffered saline (PBS), at -70°C. 10-fold dilution series of each brain homogenate were prepared using standard DDA capture buffer as diluent, to give concentrations of 10^{-2}, 10^{-3} and 10^{-4}. These were then tested using the standard DDA protocol, including a further 1:100 dilution into DDA capture buffer, giving final brain homogenate concentrations during incubation with steel powder of 10^{-4}, 10^{-5} and 10^{-6}. The results of this experiment are shown in Figure 14.

Testing of dilute vCJD brain homogenate

Samples of human brain homogenate taken from (1) a patient with vCJD, and (2) an individual without neurological disease were obtained (prepared by colleagues at the MRC Prion Unit). Other aliquots of the same samples have previously been used in transmission bioassay and Western blotting experiments, confirming the presence of vCJD infectivity and protease-resistant PrP in the vCJD sample, and the absence of detectable infectivity or protease-resistant PrP in the normal sample. These brain homogenate samples were stored at 10% of their original concentration in PBS, at -70°C prior to use. A 10-fold dilution series of each was prepared using the standard DDA capture buffer to give concentrations ranging from 10^{-2} to 10^{-7}. These were then tested using the standard DDA protocol, including a 1:100 dilution into the DDA capture buffer, giving final brain homogenate concentrations during incubation with steel powder ranging from 10^{-4} to 10^{-9}. The results are shown in Figures 15 and 16 below.

A similar experiment was carried out using an alternative buffer in place of the standard DDA capture buffer. The buffer consisted of: *Opti-MEM* media (*Gibco*) + 10% Foetal calf serum + 1% penicillin/streptomycin + 10^{-4} PrP-null FVB mouse brain homogenate. This buffer was based on that used in previous experiments by members of the Molecular Diagnostics group involving binding of brain-derived prion infectivity to steel wires for transfer to cell culture [131], as this had convincingly shown binding of infectivity to steel in the absence of blood. The method was otherwise identical to that in the previous experiment. The results are shown in Figure 17.

Testing of vCJD brain homogenate/CSF spikes

To test whether brain-derived vCJD-associated abnormal PrP could be detected with the DDA in the presence of CSF, 10-fold dilution series of vCJD and normal brain homogenate samples were prepared using one of the large volume non-prion neurological disease control CSF samples as diluent (obtained at autopsy from a patient with cerebrovascular disease and Alzheimer's pathology), to give brain homogenate concentrations from 10^{-2} to 10^{-6}. These were then tested using the standard DDA protocol, including a further 1:100 dilution into the DDA capture buffer, giving final brain homogenate concentrations during incubation with steel powder ranging from 10^{-4} to 10^{-8}, and a final CSF concentration of 10^{-2}. The experiments were also repeated without the heat shock step of the standard DDA protocol.

For these experiments, vCJD and normal brain homogenate diluted in DDA capture buffer to a concentration of 10^{-2} were used as positive and negative controls for quality control purposes, as the primary objective was to investigate the effect of the presence of CSF compared with results obtained in the previous experiments using brain homogenate diluted in DDA capture buffer (see above).

A further set of experiments were performed testing the same dilution series of brain homogenates diluted in control CSF, but with a modification to the standard DDA protocol so that the test sample was only diluted 1:10 in DDA capture buffer. This gave final brain homogenate concentrations of 10^{-3} to 10^{-7} and a final CSF concentration of 10^{-1}. Constituents of the DDA capture buffer were adjusted to ensure that their final concentration during the incubation with steel powder was unchanged. These experiments were also repeated without the heat shock step of the DDA protocol.

The results of these experiments are shown in Figures 18 and 19.

Further experiments were performed to investigate alterations of assay conditions and components aiming to optimise its performance with this analyte, and ideally to allow differentiation at lower concentrations of abnormal PrP. All of these experiments consisted of varying one parameter from the standard DDA protocol at a time and comparing this directly with the standard protocol.

Parameters investigated were:

1) Heat shock temperatures of 70°C, 80°C, 90°C and 100°C
2) Use of an alternative, more highly biotinylated batch of ICSM-18B antibody;
3) Use of an alternative preparation of steel powder [316 vs 304].

Results of these experiments are shown in Figures 20, 21 and 22.

Testing of sCJD brain homogenate/CSF spikes

To test whether brain-derived sCJD-associated abnormal PrP could be detected with the DDA in the presence of CSF, 10-fold dilution series of sCJD and normal brain homogenate samples were prepared using one of the non-prion neurological disease control CSF samples as diluent (obtained at autopsy from a patient with cerebrovascular disease and Alzheimer's pathology), to give brain

homogenate concentrations from 10^{-2} to 10^{-6}. These were then tested using the standard DDA protocol, including a 1:100 dilution into the DDA capture buffer, giving final brain homogenate concentrations during incubation with steel powder ranging from 10^{-4} to 10^{-8}, and a final CSF concentration of 10^{-2}. The experiments were also repeated without using the heat shock step of the standard DDA protocol.

For these experiments, sCJD and normal brain homogenate diluted in DDA capture buffer to a concentration of 10^{-2} were used as positive and negative controls for quality control purposes, as the primary objective was to investigate the effect of the presence of CSF compared with results obtained in the previous experiments using brain homogenate diluted in DDA capture buffer (**iii** above).

A further set of experiments were performed testing the same dilution series of brain homogenates diluted in control CSF, but with a modification to the standard DDA protocol so that the test sample was only diluted 1:10 in DDA capture buffer. This gave final brain homogenate concentrations of 10^{-3} to 10^{-7} and a final CSF concentration of 10^{-1}. Constituents of the DDA capture buffer were adjusted to ensure that their final concentration during the incubation with steel powder was unchanged. These experiments were also repeated without the heat shock step of the DDA protocol.

The results of these experiments are shown in Figures 23 and 24.

Testing of diagnostic patient CSF samples

As there was evidence of differentiation between vCJD brain homogenate and normal brain homogenate diluted into CSF/buffer, albeit only at relatively high concentrations, I proceeded with testing real patient CSF.As none of the modifications to the standard DDA protocol had led to any consistent improvement in its performance, the unmodified standard protocol was used, with CSF in

place of blood. A panel of samples was tested using the standard DDA protocol, in batches including several non-neurodegenerative control CSF samples (see Table 4) and several LP-derived CSF samples from sCJD +/- vCJD patients. Overall, 11 control samples, 4 sCJD samples and 1 vCJD sample were each tested on 2 separate occasions.

vCJD and normal brain homogenate diluted into 2 separate samples of PM-derived control CSF to a concentration of 10^{-2} were used as positive and negative controls respectively, for quality control purposes. The 2 PM-derived CSF samples were also tested without addition of brain homogenate, for comparison.

The results of the 2 separate assay runs on the same panel of samples were adjusted by standardising to the luminescent signal from a 1:10 million dilution of the NeutrAvidin reagent tested on the same plate. Results for these experiments are shown in Figure 25.

Results

Dilute PrP-null and wild type FVB mouse brain homogenates

The results of this experiment are illustrated in Figure 14 (luminescence results shown on both standard and logarithmic scale).

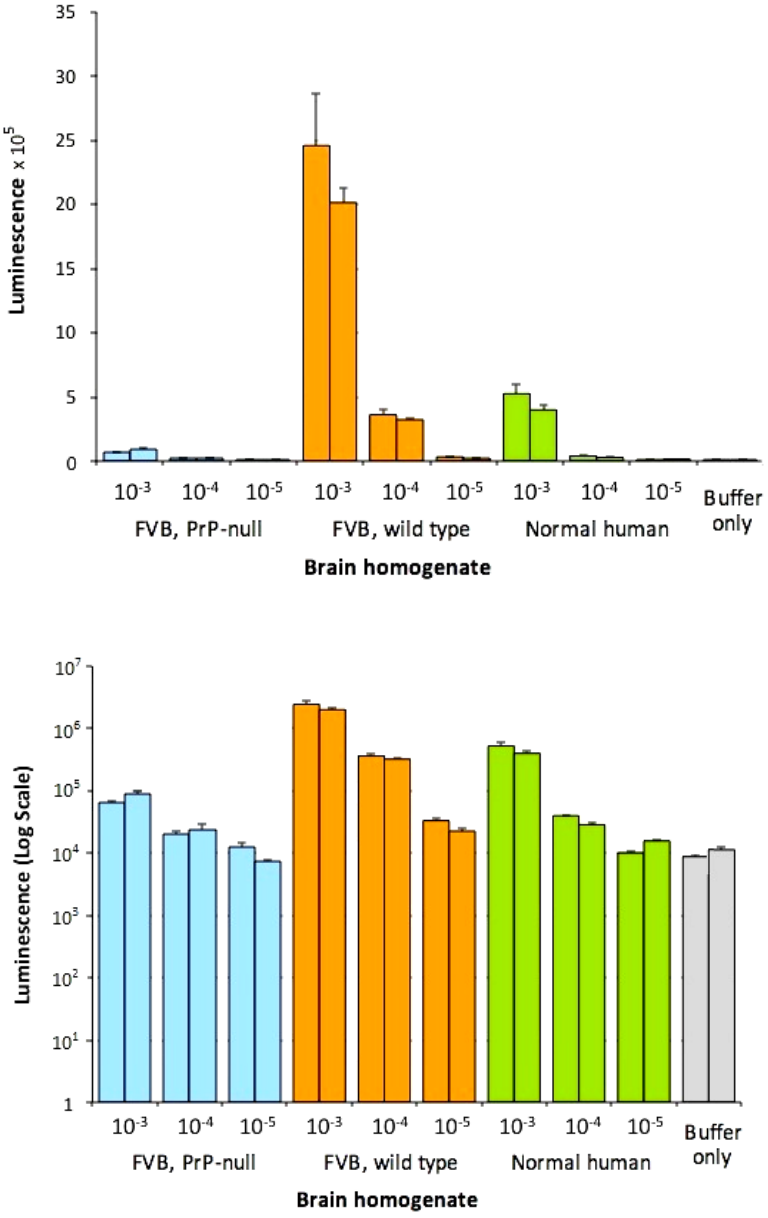


Figure 14. Direct detection assay testing 3 brain homogenates at a range of concentrations: PrP-null FVB mouse brain, wild-type FVB mouse brain, and normal human brain. Bars show mean and SD of luminescence across 3 replicate wells. Each concentration of each brain homogenate was tested in 2 separate assay runs. The same data is shown against linear and logarithmic scales in the two charts.

Wild type mouse brain homogenate produced much higher luminescent signal than equivalent dilutions of PrP-knockout mouse brain homogenate, suggesting that PrP^c was binding to the steel powder and accounting for the high signal. Although there was a dilution effect seen with PrP-knockout mouse brain, with reducing signal from reducing concentrations, the absolute levels were much lower than for wild type brain homogenate. Equivalent dilutions of normal human brain homogenate also produced higher luminescent signal than the PrP-knockout mouse brain (although not as high as the wild type FVB mouse brain), suggesting that human PrP^c is also able to bind to the steel powder and be detected by the assay. However, as it is not possible to directly compare this with a PrP-knockout human brain homogenate, confounding effects from other non-PrP constituents of the human brain homogenate cannot be entirely ruled out.

Dilute vCJD brain homogenate

As shown in Figure 15, testing a range of dilutions of vCJD and normal human brain homogenate in DDA capture buffer suggested that the assay could differentiate vCJD from normal at higher concentrations (10^{-4}). However, at lower concentrations, there was not reliable differentiation, and the luminescent signal was not significantly above that produced by steel powder without any biological sample added.

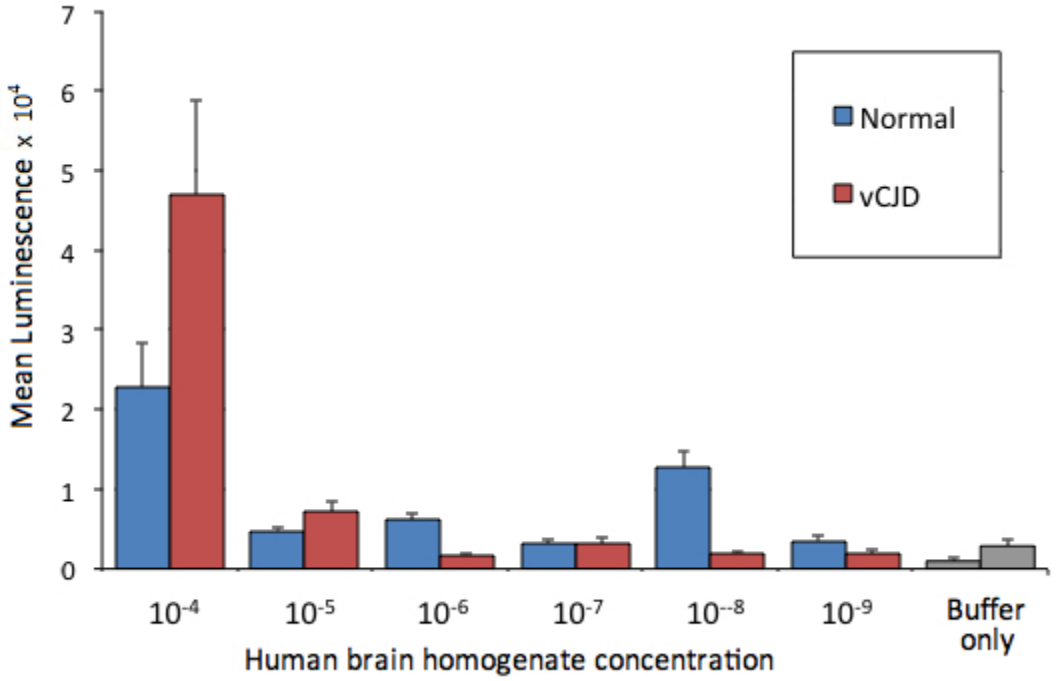


Figure 15. Direct detection assay testing vCJD and normal human brain homogenates at a range of dilutions in the standard DDA capture buffer. Bars show mean luminescence and SD across 3 replicate wells.

Following on from these initial results, vCJD and Normal brain homogenates diluted to 10^{-4} concentration were tested "head-to-head" on 5 separate occasions to ensure that there was robust and reliable differentiation. These results are shown in Figure 16. A paired 2 sample t-test comparing the adjusted means from 5 replicate assays for 10^{-4} dilution vCJD and normal brain homogenate, gives a p value of 0.0059.

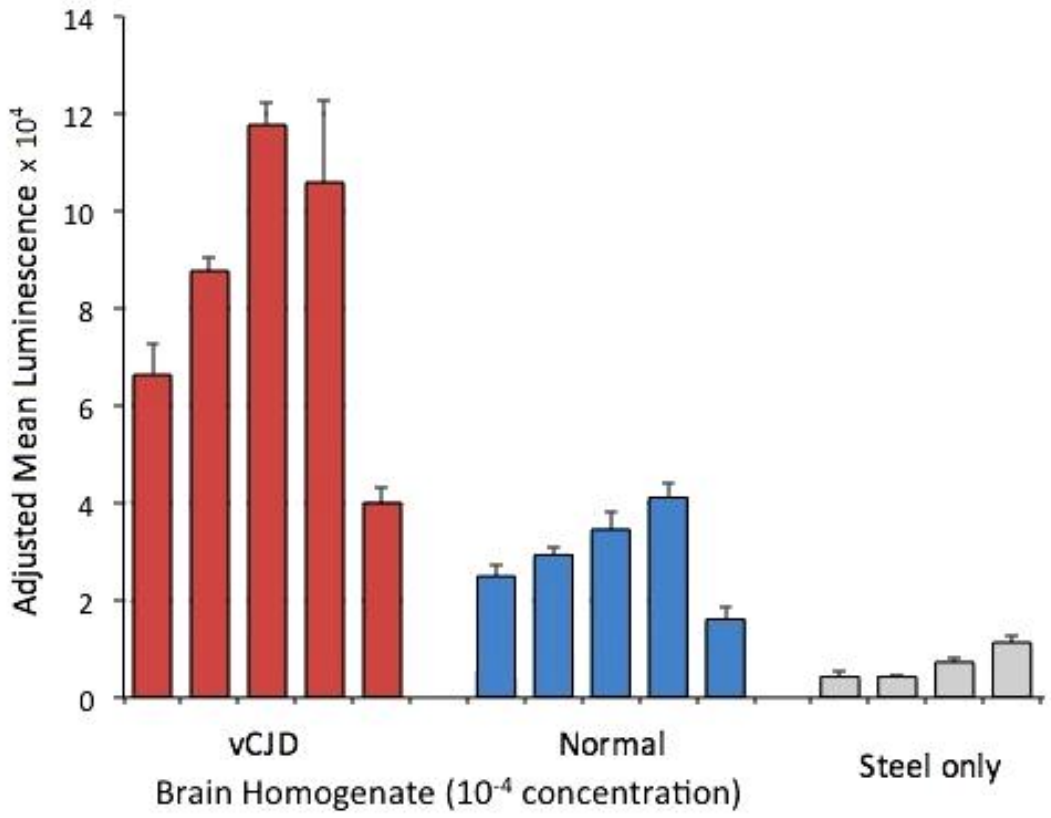


Figure 16. Repeated DDA testing of vCJD and normal human brain homogenates diluted to 10^{-4} in standard DDA capture buffer, on 5 separate occasions. Bars show mean and SD of luminescent signal across 3 replicate wells (adjusted in proportion to the mean luminescent signal from a standard 10^{-7} dilution of NeutrAvidin tested on the same plate, to allow direct comparison of results from different assay runs).

On the basis of these results, 10^{-4} dilutions of vCJD and normal brain homogenate were used as positive and negative controls for subsequent experiments.

The experiments using the alternative *Opti-MEM* based buffer did not produce evidence of differentiation of vCJD from normal brain homogenate at any of the concentrations tested, as shown in Figure 17.

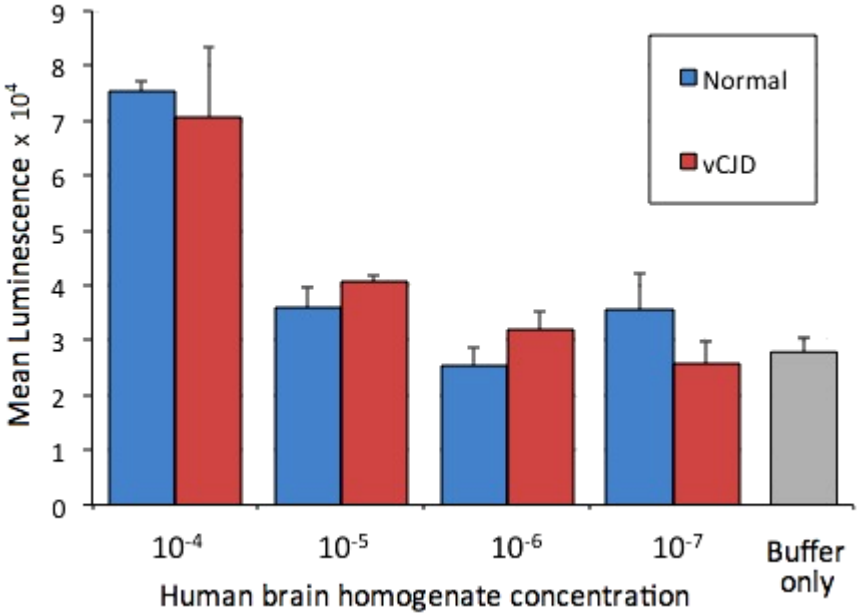


Figure 17. Direct detection assay testing vCJD and normal human brain homogenates at a range of dilutions in *Opti-MEM*-based buffer (see *Materials and methods* above for details). Bars show mean luminescence and SD across 3 replicate wells.

vCJD brain homogenate/CSF spikes

The results for testing of vCJD and normal human brain homogenates diluted into CSF at a range of dilutions are shown below. Figure 18 shows results from the experiment using a 1:100 dilution of spiked CSF into the DDA capture buffer (as used for blood in the standard assay protocol), and Figure 19 shows results from the experiment using a 1:10 dilution.

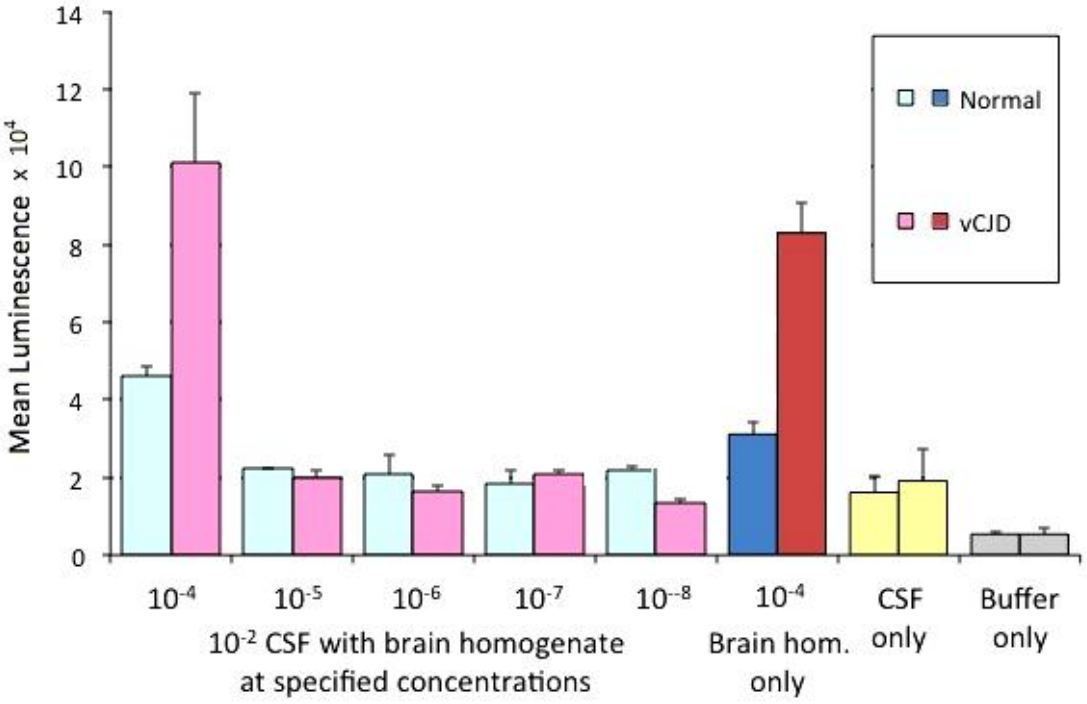


Figure 18. Direct detection assay comparing vCJD and normal brain homogenates diluted to a range of concentrations in CSF, with the "spiked" CSF diluted 1:100 into the DDA capture buffer. Brain homogenate concentrations shown are the final concentrations during incubation with steel powder (i.e. after dilution into DDA capture buffer).Brain homogenates diluted to 10^{-4} in DDA capture buffer (without CSF), unspiked CSF, and buffer-only controls included for comparison. Bars show mean and SD of luminescence across 3 replicate wells.

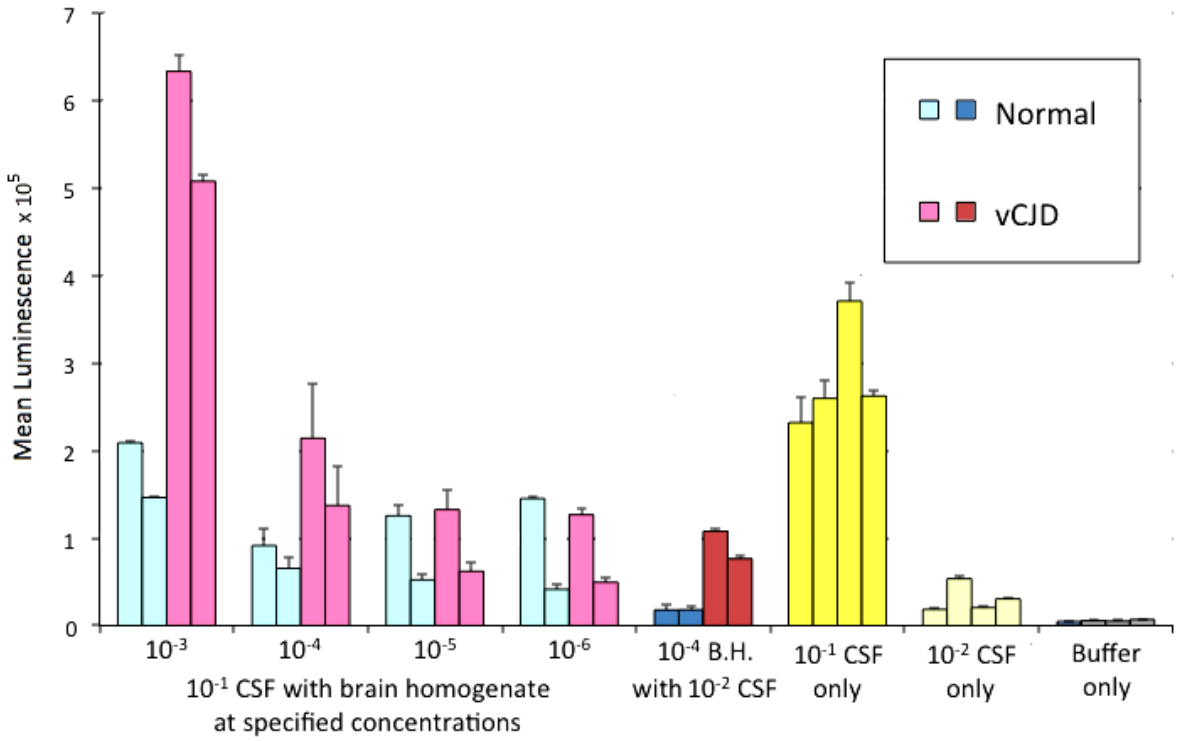


Figure 19. Direct detection assay comparing vCJD and normal brain homogenates diluted to a range of concentrations in CSF, with the "spiked" CSF diluted 1:10 into the DDA capture buffer. Brain homogenate concentrations shown are the final concentrations during incubation with steel powder (i.e. after dilution into DDA capture buffer). Brain homogenates diluted to 10^{-2} in CSF and then diluted 1:100 into DDA capture buffer, unspiked CSF at 1:10 and 1:100 dilutions, and buffer-only controls were included for comparison. Bars show mean and SD of luminescence across 3 replicate wells.

Comparing these results with those obtained from testing vCJD and normal brain homogenates diluted in DDA capture buffer in the absence of CSF (as shown in Figures 15 and 16 above), suggests that the presence of CSF, at concentrations up to 1:10, does not prevent the assay from differentiating vCJD from normal brain homogenate, when this is present at a concentration of 10^{-4} during the incubation with the steel powder.

Some secondary observations can also be made. There does not appear to be any consistent differentiation of vCJD from normal brain at lower than 10^{-4} concentrations of brain homogenate.

The protocol involving 1:10 dilution of the spiked CSF is able to differentiate initial (pre-dilution) samples with a brain homogenate concentration of 10^{-3}, while the protocol involving 1:100 dilution can only reliably differentiate samples at a concentration of 10^{-2}.

Another observation illustrated by Figure 19 is that unspiked CSF diluted 1:10 into DDA capture buffer consistently produced higher luminescent signals than samples that contained the same amount and concentration of the same CSF sample, but also contained brain homogenate, even when this was at very low concentrations. Possible reasons for this are discussed below.

Optimising assay parameters for vCJD brain homogenate spiked CSF

Figures 20, 21 and 22 show three examples of optimisation experiments in which assay parameters were varied to look for any improvement in the assay's performance (when used to differentiate between CSF spiked with vCJD brain homogenate, and CSF spiked with normal human brain homogenate). In each experiment one parameter was varied while all other aspects of the standard DDA protocol as described above were maintained. Parameters investigated were primary antibody batch and concentration (Figure 20), heat shock temperature (Figure 21) and steel powder batch (Figure 22). "304" and "316" steel are alternative stainless steel alloys with slightly different nickel and carbon contents (both manufactured by *Goodfellow*).

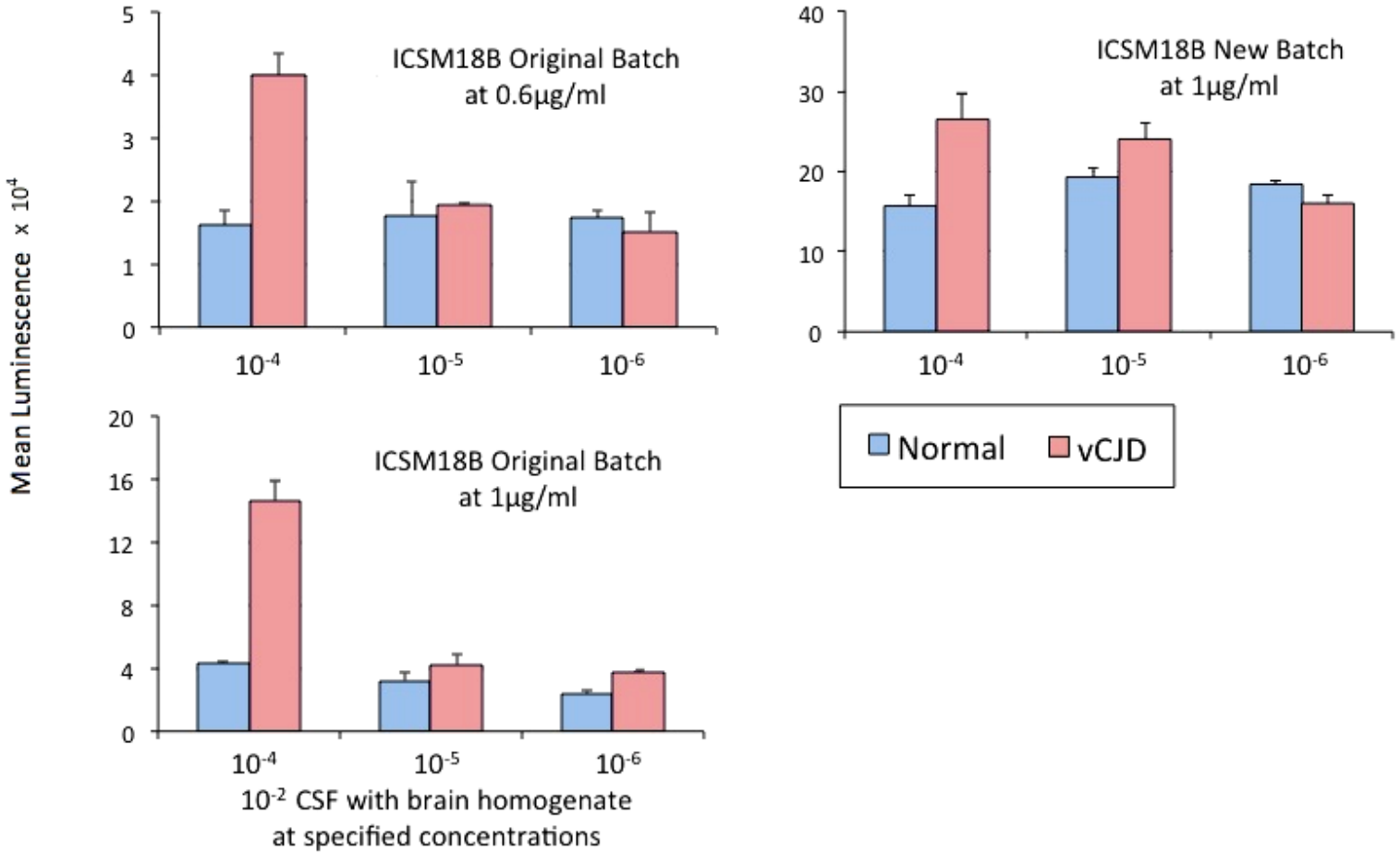


Figure 20. Direct detection assay comparing vCJD and normal brain homogenates diluted to a range of concentrations in CSF and spiked CSF diluted 1:100 into DDA capture buffer, with variation in primary antibody batch and concentration as indicated. Bars show mean and SD of luminescence across 3 replicate wells.

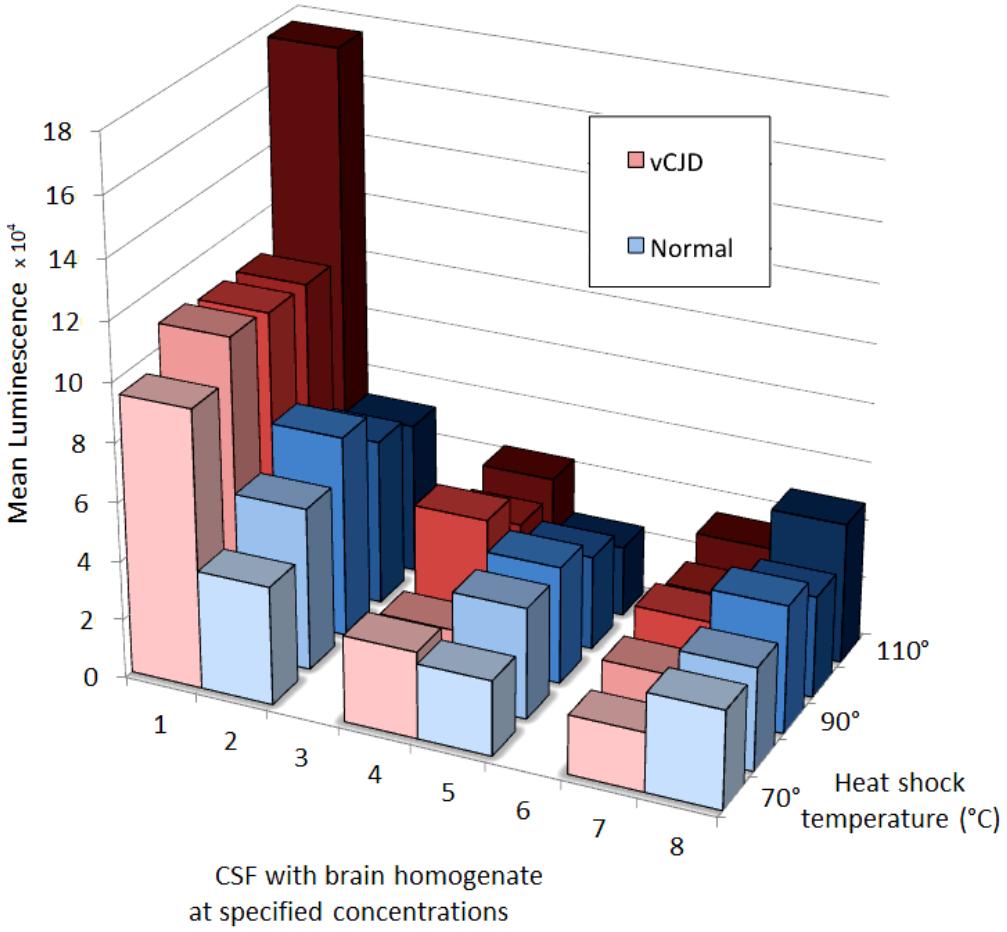


Figure 21. Direct detection assay comparing vCJD and normal brain homogenates diluted to a range of concentrations in CSF and spiked CSF diluted 1:100 into DDA capture buffer, with variation in heat shock temperature used.

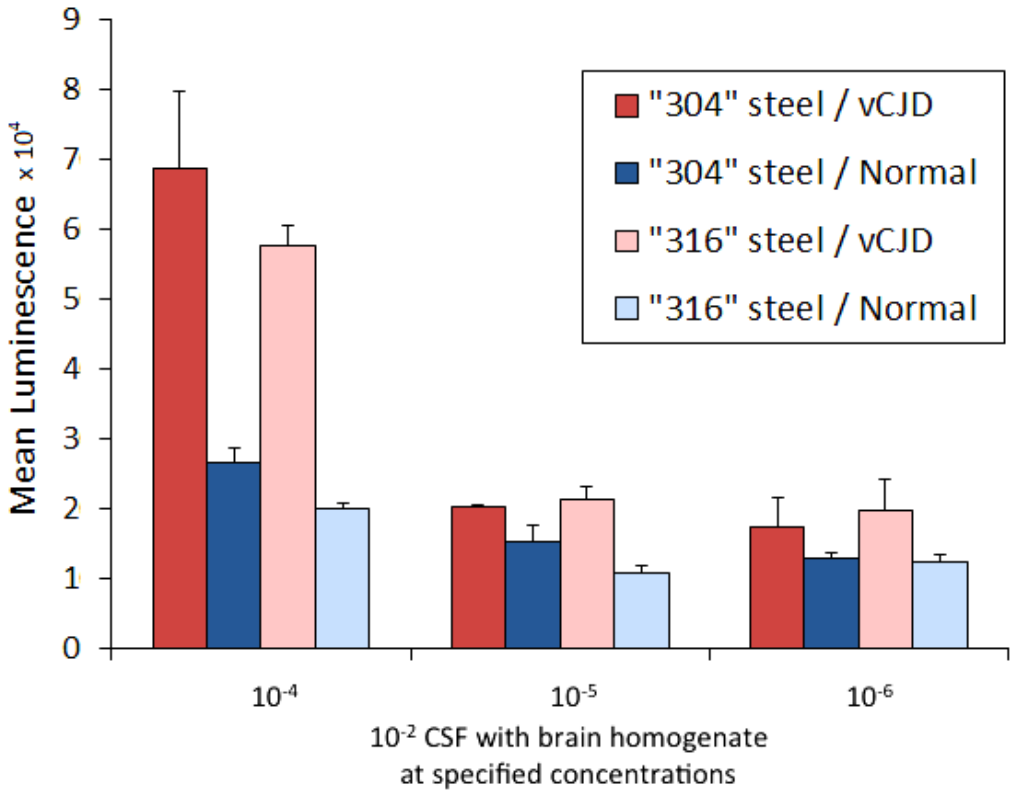


Figure 22. Direct detection assay comparing vCJD and normal brain homogenates diluted to a range of concentrations in CSF and spiked CSF diluted 1:100 into DDA capture buffer, with variation in steel powder batch used. Bars show mean and SD of luminescence across 3 replicate wells.

sCJD brain homogenate/CSF spikes

The results for testing of sCJD and normal human brain homogenates diluted into CSF at a range of dilutions are shown below. Figure 23 shows results from the experiment using a 1:100 dilution of spiked CSF into the DDA capture buffer (as used for blood in the standard assay protocol), and Figure 24 shows results from the experiment using a 1:10 dilution.

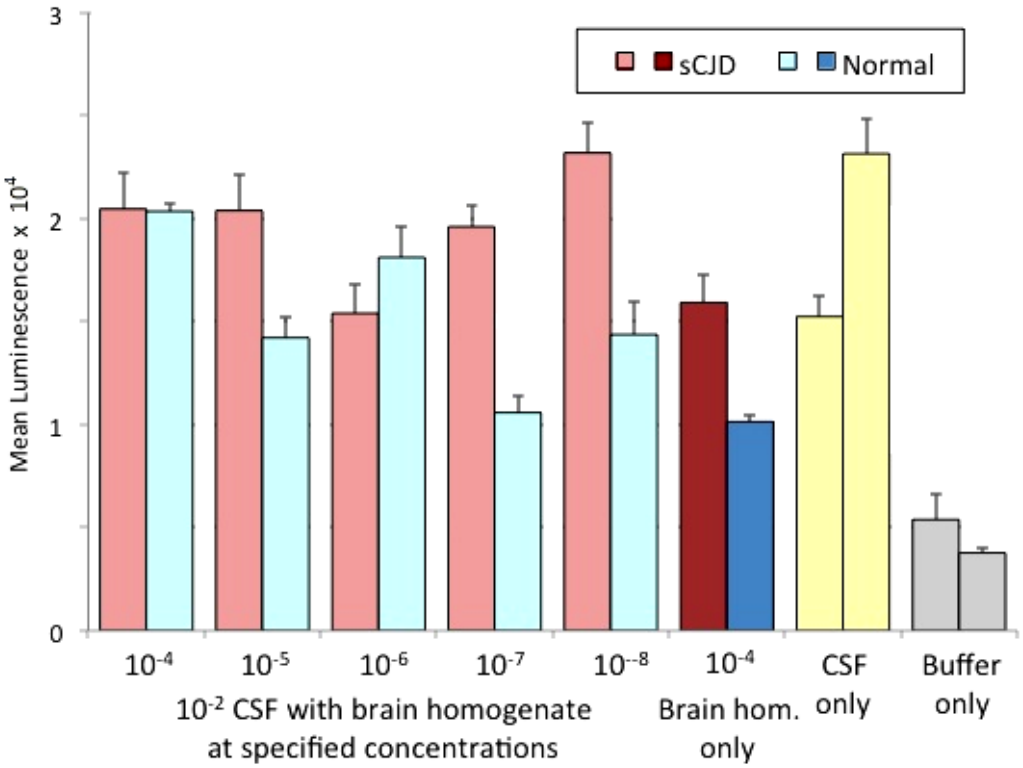


Figure 23. Direct detection assay comparing sCJD and normal brain homogenates diluted to a range of concentrations in CSF, with the "spiked" CSF diluted 1:100 into the DDA capture buffer. Brain homogenate concentrations shown are the final concentrations during incubation with steel powder (i.e. after dilution into DDA capture buffer). Brain homogenates diluted to 10^{-4} in DDA capture buffer (without CSF), unspiked CSF, and buffer-only controls included for comparison. Bars show mean and SD of luminescence across 3 replicate wells.

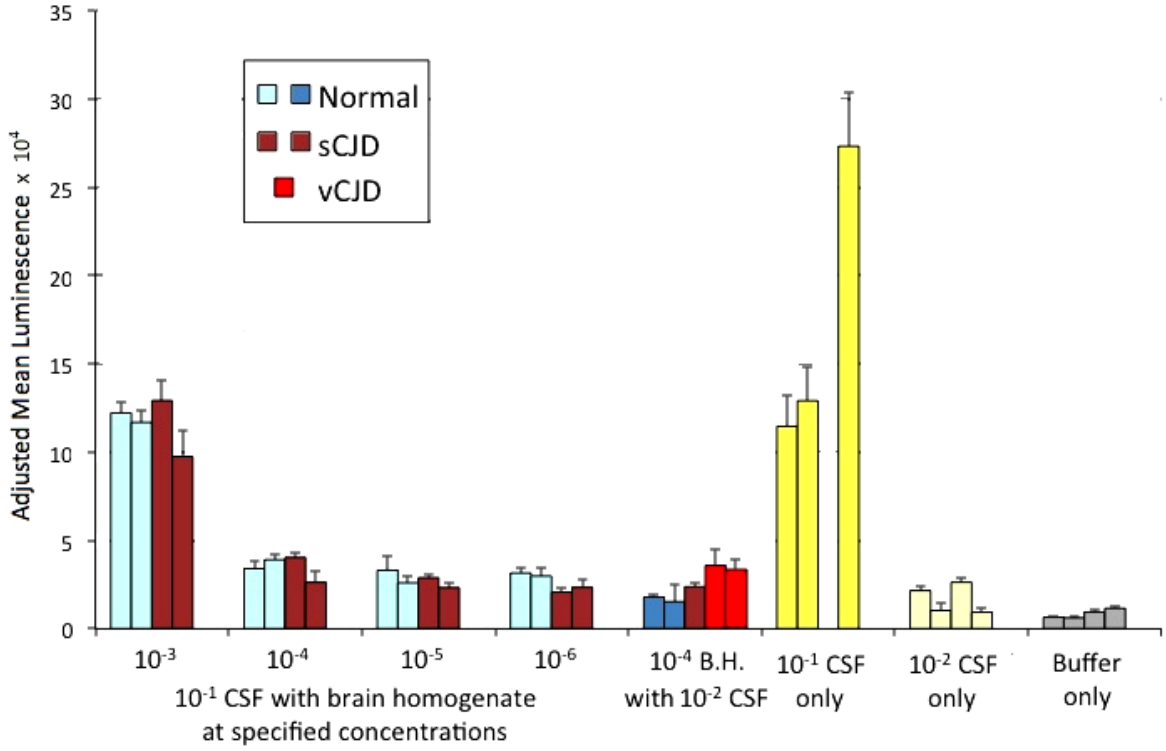


Figure 24. Direct detection assay comparing sCJD and normal brain homogenates diluted to a range of concentrations in CSF, with the "spiked" CSF diluted 1:10 into the DDA capture buffer. Brain homogenate concentrations shown are the final concentrations during incubation with steel powder (i.e. after dilution into DDA capture buffer). sCJD, vCJD and normal brain homogenates diluted to 10^{-2} in CSF and then diluted 1:100 into DDA capture buffer, unspiked CSF at 1:10 and 1:100 dilutions, and buffer-only controls were included for quality control and comparison. Bars show mean and SD of luminescence across 3 replicate wells.

Again there did not appear to be any successful differentiation of sCJD from normal brain homogenate, at any of the dilutions tested. The same phenomenon whereby unspiked CSF samples diluted 1:10 produce higher luminescent signal than the same concentration of the same CSF in the presence of dilute brain homogenate was observed, as in the equivalent experiment with vCJD.

Testing of diagnostic CSF samples

The results from this experiment are shown in Figure 25.

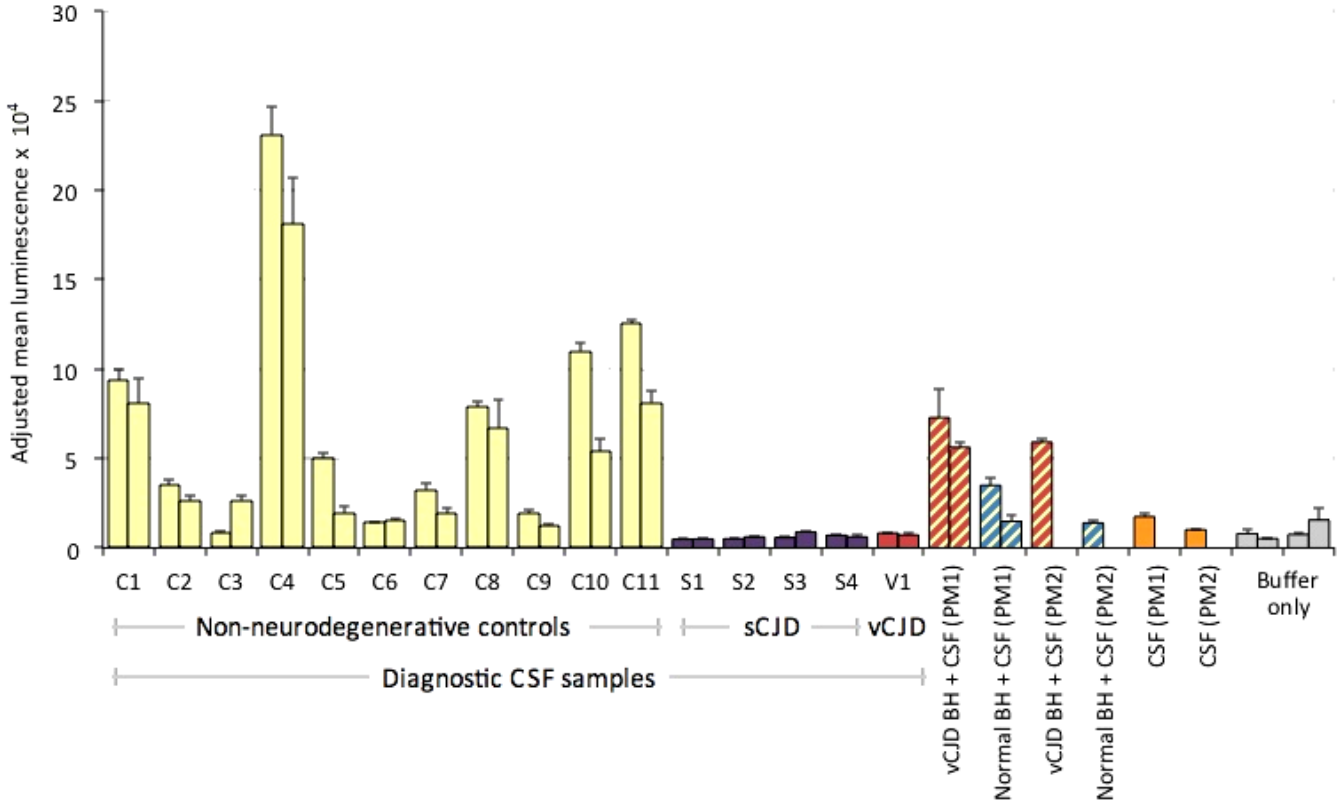


Figure 25. Testing of diagnostic, lumbar puncture CSF samples from 11 non-neurodegenerative control patients, 4 sCJD patients and 1 vCJD patient using standard Direct Detection Assay protocol. Each sample was tested on 2 separate assay runs, and the results are combined by standardising to the luminescent signal produced by a standard 1:10m dilution of NeutrAvidin tested on the same plate. vCJD and normal brain homogenates diluted to 10^{-2} in 2 different PM-derived non-prion control CSF samples (giving final brain homogenate concentration of 10^{-4} during incubation with steel powder), the 2 PM-derived control CSF samples unspiked, and buffer-only controls were included for quality control and comparison.

Unexpectedly, the samples from patients with prion disease produced consistently *lower* luminescent signal than those from non-degenerative controls. Comparing the adjusted mean luminescent signal results from these 2 groups (controls *vs* prion disease, including sCJD and vCJD) with a 2 tailed t-test gives a *p* value of 0.0087.

Discussion

The series of experiments described above represent early attempts to apply the direct detection assay, which had been developed and validated specifically as a blood test for vCJD, to cerebrospinal fluid.

As has been discussed in Chapter 2 above, the assay itself remains quite poorly understood, and its development and validation as a blood test relied heavily on empirical testing under a very large number of different conditions until a successful combination was found. From previous experience in applying the assay to blood taken from mice or hamsters, and from experiments involving blood spiked with brain homogenate (see Introduction to Chapter 2), it was clear that the assay was very sensitive to changes in conditions, in ways that could not easily be predicted or explained. As a result, it was difficult to make predictions about what effect changing any constituent element or condition of the assay would have, and this made the process of planning its application to CSF very challenging.

Unfortunately CSF does not lend itself well to a process of empirical, exploratory assay development, where it would often be useful to test a very large number of different combinations of conditions, as it is typically sampled in very small volumes.

One way in which this issue was dealt with was to make use of larger volume CSF samples obtained post mortem for some elements of the experiments described, but this is not ideal, and introduces a number of potentially problematic issues. The non-prion control CSF samples obtained at post mortem (see Table 4) were taken from patients that had died with an alternative neurological illness: namely Alzheimer's disease, or Alzheimer's disease with coexisting cerebrovascular disease. The constituents of these patients' CSF are likely to have been affected by the disease from which they were suffering, and it is possible these changes might have had an impact on the assay. For

example, other non-PrP aggregated amyloid proteins might interact with disease-associated PrP in the spiked CSF, or might compete with it for binding to the steel. The principal aim of the experiments using these CSF samples was to demonstrate that the presence of CSF did not interfere with the ability of the assay to detect disease-associated PrP, even at higher concentrations. It was felt that it was reasonable to prioritise availability of larger samples over having very strict and "clean" control CSF, but this does represent a compromise.

In other parts of the project where diagnostic CSF samples obtained at lumbar puncture were being tested, it was necessary to have more closely matched control samples for the results to be meaningful. Comparing lumbar puncture samples from prion disease patients with post mortem control samples would be unsatisfactory. I used CSF samples obtained at lumbar puncture from patients with symptoms or diagnoses not typically associated with any known changes in the CSF. It is important to remember that these are not "normal" CSF samples, but for the purposes of these experiments they provide reasonable control samples for comparison.

If work on the DDA in CSF reached a stage where a potential diagnostic assay for clinical use was being validated, the choice of control samples would need to be carefully considered. While comparison with entirely normal control samples from healthy volunteers is scientifically appealing, it must be remembered that the role of a clinical diagnostic test is usually to distinguish between a specific condition and other conditions that might produce similar clinical features. The results will therefore be more useful in practice if a comparison group of samples from patients with other diagnoses are included – this approach was taken in the masked panel experiment validating the DDA as a vCJD blood test[129].

The results of these experiments suggest that the standard Direct Detection Assay is able to detect brain-derived vCJD-associated abnormal PrP in the presence of CSF, but that the same is not true for sCJD-associated abnormal PrP.

vCJD brain homogenate could only be differentiated from normal brain homogenate at a concentration of 10^{-4} or above, whether in the presence of CSF or not. In contrast, previous experiments involving testing of vCJD brain homogenate spiked into normal control blood published in Edgeworth et al 2011[129] showed that concentrations from 10^{-7} to 10^{-10} of vCJD brain homogenate, in the presence of blood, produced significantly higher luminescent signal in the assay than a 10^{-6} concentration of normal brain homogenate in blood, with the impression of a dilution effect in the luminescent signal across the dilution series of vCJD brain homogenate: this is shown in Figure 5 in the Introduction of Chapter 2 above, which is reproduced from Edgeworth et al 2011[129].

The reasons for this striking discrepancy must either relate to the effect of the presence of blood in the assay, or on other conditions or constituents that varied between these previous experiments and the current experiments. This should be investigated further, including seeking to determine whether one or more components in normal blood act as cofactors for the assay improving its sensitivity. If these could be identified, they could be added to the buffers used for non-blood analytes.

Conclusions on current performance in diagnostic (lumbar puncture) CSF samples

The results of the testing of diagnostic CSF samples using the standard DDA protocol shown in Figure 25 are striking, but are not easy to explain. The luminescent signal produced by the sCJD and vCJD samples was consistently lower than that from the non-neurodegenerative controls, some of which produced very high signals.

Addressing the control samples first, it was striking that compared with normal control blood samples; the DDA results from these control CSF samples were much more variable, with some individual samples consistently producing very high signals across multiple occasions of testing. Given that none of these samples had been in any contact with tissue from patients with prion disease, it is likely to be PrP^c being detected in these samples; we know from experiments described above that PrP^c is able to produce high signal in the DDA in the right circumstances(see Figure 14). Might it be that the control samples producing very high DDA signal contain relatively high concentrations of PrP^c? This would be worth exploring: methods of quantifying PrP^c such as ELISA could be used, and the results correlated with the DDA results.

Possible explanations for unexpected results in testing of patient CSF

The finding that the sCJD and vCJD patient CSF samples produced consistently lower luminescent signal than controls was unexpected, but consideration of how this might be explained may raise important questions about how the DDA works and suggest valuable avenues for further work. Previously published work has shown that the total levels of PrP in CSF are reduced in patients with sCJD[161]. If the output of the DDA when used in CSF is dominated by the concentration of PrP^c in the test sample rather than the presence or absence of abnormal disease-associated PrP, then the lower signal from the CJD samples might be simply accounted for by the lower levels of total PrP. Why should this be the case in testing patient CSF, but not in testing dilute brain homogenate, brain-homogenate spiked CSF or patient blood? The overall protein content of CSF is very low in comparison with that of blood, or of brain homogenate. It is possible that other, non-PrP proteins present in blood and brain homogenate play an important role in the functioning of the assay. A model of competitive binding might explain this: as the detection antibody in the DDA does not distinguish normal from disease-associated PrP, and I have shown that normal PrP *can* produce high luminescent signals in the DDA in the right context, it seems likely that "blocking" of binding of PrP^c to the steel would play an important part in the assay. If this blocking is achieved by other protein

species present in the blood or brain-homogenate, but absent from CSF, this could explain the observed results. This might also account for the surprising observation of unspiked CSF producing higher luminescent signal than the same CSF spiked with normal control brain homogenate.

CHAPTER 4. FURTHER WORK ON THE DDA AS A VCJD BLOOD TEST

Introduction

Following on from the work on the Direct Detection Assay as a blood test for vCJD (as published in Edgeworth et al, 2011[129]), and further work exploring other applications for the test (including that described in the previous two chapters), a number of important unanswered questions remain regarding the assay.

One striking observation from the previous work on the DDA as a blood test for vCJD is that the optimised assay involves testing only 8µl of blood, diluted 100-fold into the DDA capture buffer. When one of the fundamental challenges of designing a vCJD blood test was to find a way to detect the very small amounts of abnormal disease-associated PrP thought to be present in blood, it seems counter-intuitive to use such a small amount of the analyte. I planned exploratory experiments to investigate ways of scaling up the assay, aiming to increase the signal to noise ratio (i.e. the ratio of luminescent signal produced by vCJD samples to that produced by normal control samples) and thereby improve the sensitivity of the assay.

Materials and methods

These experiments used the standard DDA protocol for blood (as documented in detail in the *Materials and methods* section of Chapter 2 above) as a starting point. Modifications to this standard protocol introduced in the course of the experiments are described below.

A small panel of vCJD and normal control whole blood samples that had previously been used in the published masked panel experiment and were known to produce positive and negative DDA results respectively was used.

Pooling multiple replicate steel powder samples

Six replicate samples sets, each consisting of 1 vCJD and 3 normal control whole blood samples, were tested using the standard DDA protocol (24 samples in total). Immediately prior to transferring the steel powder to the 96-well plate, 5 replicates of each sample were combined, to give 1 single (23mg) sample and 1 pooled, quintuple sample (115mg) for each blood sample. These were than each split across 3 wells of the plate as per the standard protocol, except that in order to allow equal separation of the 115mg steel samples these were suspended in 180µl of *Femto* reagent which was then split into 3 x 60µlacross the 3 wells (rather than 60µl split into 3 x 20µl). All wells were then topped up to give a total of 100µl *Femto* reagent in each well, as in the standard protocol.

Use of 24 well plates with wider wells

Adding larger steel samples to the small wells of a 96-well plate led to piling of steel in the wells. As the luminescent signal was detected from above each well, only the luminescence produced by steel particles on the top surface of the pile would be able to reach the detector and increase the overall luminescent signal. This could account for the failure of simply increasing the amount of all the constituents being added to the plate (by pooling 5 samples immediately prior to transferring to the plate as in the above experiment) to proportionately increase the luminescent signal produced. I therefore investigated the use of alternative plates with wider wells. 24-well plates were obtained, with each well having an area of1.9cm^2 (compared with approximately 34mm^2 for a 96-well plate).

Initially the above experiment using single and pooled, quintuple steel samples was repeated using the 24 well plates. Again the pooled 115mg samples were resuspended in 180µl of *Femto* reagent as in the previous experiment. All wells on the 24-well plates were topped up to a total volume of 600µl of F*emto* reagent (increased from the 100µl used for the 96 well plates), to ensure even coverage of the steel within the larger wells.

Increasing the blood input of the assay using 24 well plates

Next, 3 replicate panels of 1 vCJD and 3 normal control samples were tested using:

1) the standard DDA protocol except for use of 24 well plates (*Greiner*) (with adjustments to the *Femto* reagent volumes as described in the previous experiment), with 8µl of blood and 23mg of steel powder.
2) as above, but volume of blood increased to 16µl.
3) as above, but volume of blood increased to 16µl and amount of steel increased to 46mg.

Results

Pooling multiple replicate steel powder samples

The results of this experiment are shown in Figure 26 below. While the luminescent signal produced by the pooled quintuple vCJD sample was higher than that from the single sample, it was increased only by a factor of 1.69, and there was a relatively greater increase in the signal from the normal control samples, so that the positive to negative ratio was reduced. As each of the five samples combined for the pooled sample were identical to the single sample, we can assume that the pooled sample contained 5 times the quantity of bound NeutrAvidin, and would therefore expect it to produce 5 times the luminescent signal when mixed with an excess of the chemiluminescent reagent.

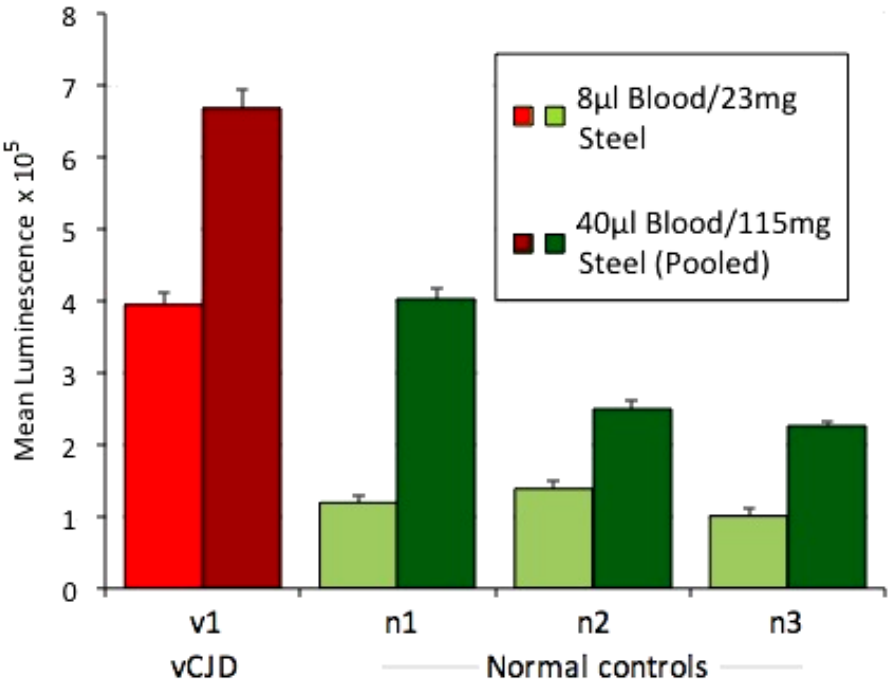


Figure 26. Direct detection assay testing of vCJD and normal control whole blood samples, as standard single samples and as quintuple, pooled samples combined immediately prior to aliquotting onto the plate, using standard 96 well plate. Bars show mean and SD of luminescence across 3 replicate wells.

An observation made during this experiment provides a possible explanation for the fact that this was not the case. When 38.3mg of steel was added to a well of the 96-well plate (each 115mg combined sample split across 3 wells), there was piling of the steel in the small well. As the luminescent signal is detected from above each well while the plate is stationary, the luminescent signal that is able to reach the detector will come from those steel particles on top of the pile, and as such is likely to be more closely related to the surface area of the top of the pile rather than the total amount of steel. Adding more steel to a small well may produce a taller pile without greatly increasing the surface area visible to the detector. This is illustrated in Figure 27. This issue could explain a "ceiling effect" in experiments aiming to increase the signal to noise ratio of the assay.

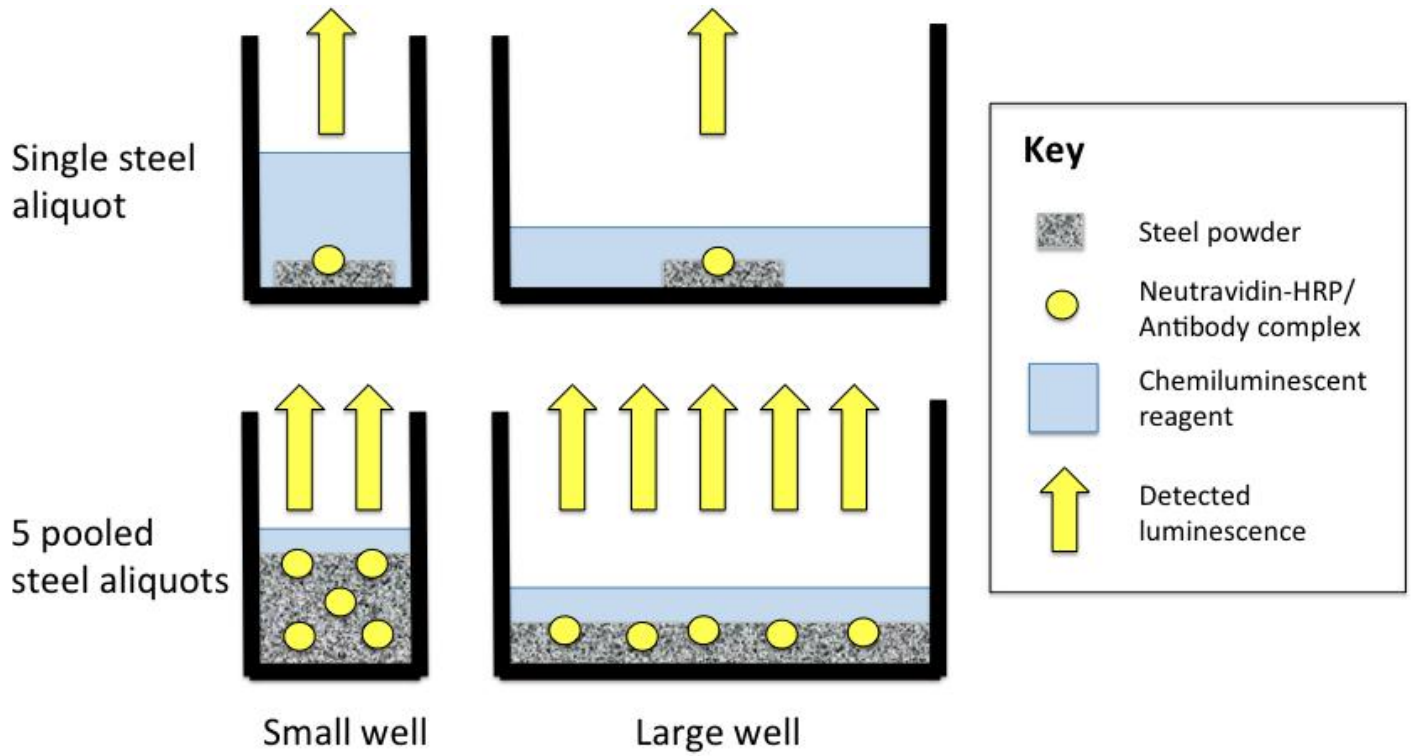


Figure 27. Schematic diagram showing suggested explanation for apparent ceiling effect when scaling up the assay using small welled plates.

Use of 24 well plates with wider wells

To investigate the above hypothesis directly the same experiment was repeated with 24-well plates used in place of the 96-well plates. The results of this experiment are shown in Figure 28.

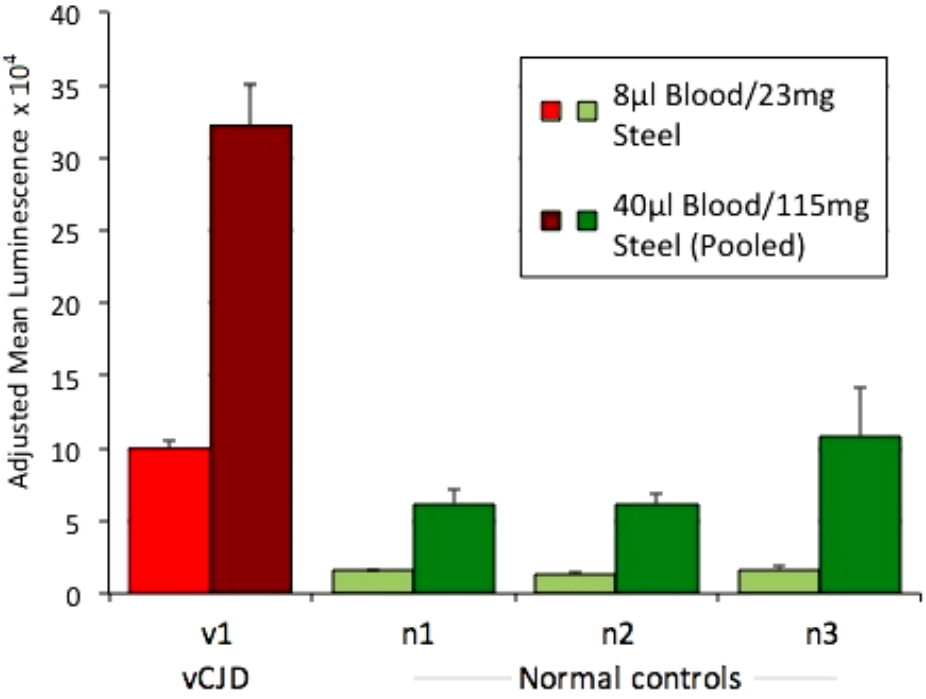


Figure 28. Direct detection assay testing of vCJD and normal control whole blood samples, as standard single samples and as quintuple, pooled samples combined immediately prior to aliquotting onto the plate, using 24 well plates. Results from 2 separate plates are combined by standardising to the luminescent signal produced by a standard 1:10 million dilution of NeutrAvidin tested on the same plate. Bars show mean and SD of luminescence across 3 replicate wells.

The pooled, quintuple vCJD sample now produced a luminescent signal 3.13 times that of the corresponding single sample. However the signal from the pooled normal samples was also increased, and the ratio of the vCJD sample to the mean of the normal control samples was 4.15 for the pooled samples compared with 6.65 for the single samples; i.e. there had been no benefit to the signal to noise ratio of the assay.

Increasing the blood input of the assay using 24 well plates

The results of this experiment are shown in Figure 29 below, and the key findings shown below:

Blood volume (µL)	Steel amount (mg)	Ratio of luminescent signal vCJD : mean of normals
8	23	2.50
16	23	3.96
16	46	5.52

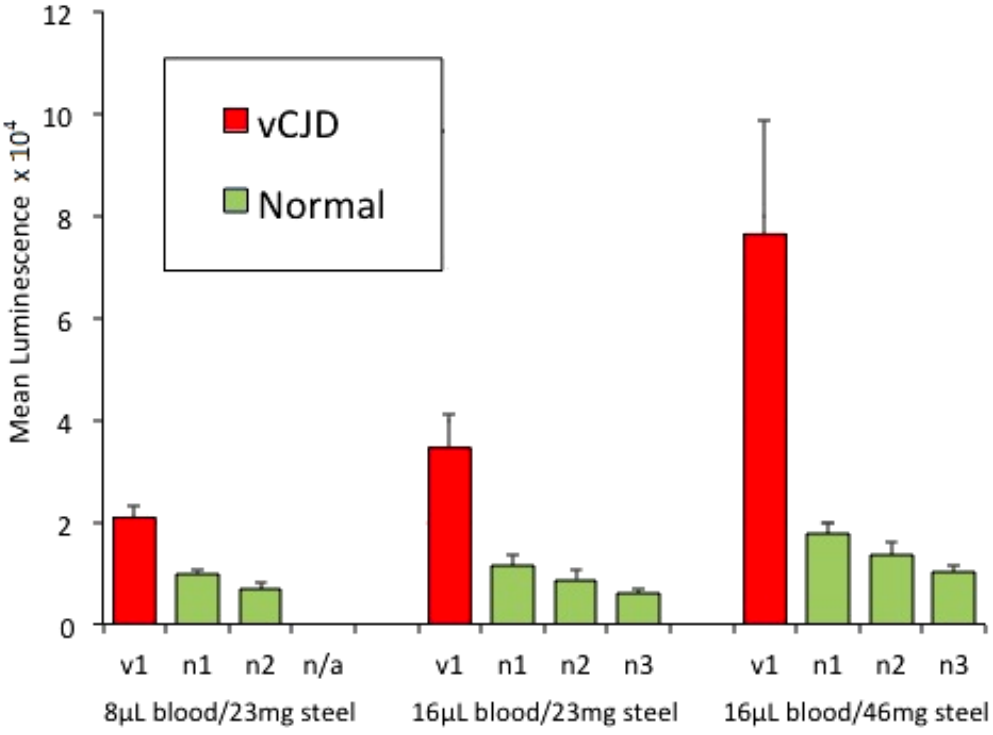


Figure 29. Direct detection assay using 24 well plates and increasing amounts of blood and steel, using panel of vCJD and normal control whole blood samples. Bars show mean and SD of luminescent signal across 3 replicate wells. The assay could not be completed for sample n3 in the 8µl blood/23mg steel sample set for technical reasons (steel lost during wash steps).

Discussion

This chapter describes a small set of exploratory experiments aiming to better understand and improve the functioning of the Direct Detection Assay as a blood test for vCJD. These experiments formed part of a wider project of the Molecular Diagnostics group aiming to improve on the standard assay, by improving our understanding of how the assay works, and by exploring interesting or unexpected observations from previous work, such as the need to dilute blood for the assay to be successful in detecting vCJD patient blood.

The results suggest that it may be possible to achieve some improvements in the signal to noise ratio of the assay by increasing the amount of blood and steel used, but that this requires some technical modifications to the assay (including use of larger-welled plates to allow dispersal of the larger amounts of steel in the well).

These experiments were very small and their conclusions should be interpreted with caution: they would require replication in larger sample sets to confirm their conclusions more robustly. Nevertheless, they are included here partly to provide examples of this empirical method of assay development, and the challenges this presents.

CHAPTER 5. PSYCHIATRIC AND BEHAVIOURAL FEATURES OF HUMAN PRION DISEASE

Introduction

Behavioural disturbance and psychiatric symptoms (BPS) have been recognized since the earliest descriptions of prion disease[172], but there has been relatively little research into their prevalence, phenomenology and treatment, or their molecular pathophysiological basis. When vCJD first appeared in the UK in the 1990s, psychiatric features were noted to be relatively prominent, particularly in the early phase of the illness, and this has been well studied in the context of the UK CJD surveillance system [102,173]. As a result, the presence of "early psychiatric symptoms" (specifically "depression, anxiety, apathy, withdrawal, delusions") is included in the WHO diagnostic criteria for vCJD[107]. A recent review of 106 pathologically confirmed cases of vCJD concluded that these early psychiatric symptoms were present in 92%[105].

In 2005, Wall et al reported a retrospective study of psychiatric symptoms in all sCJD cases evaluated at the Mayo clinic over 25 years, based on detailed review of clinical case notes[174]. This included 126 patients and found that psychiatric symptoms (excluding sleep disturbance) were present in 26% of cases at presentation, in 80% within the first 100 days after onset, and in 89% at any stage of their illness.

Appleby and colleagues have used retrospective casenote review[175] and meta-analysis of published cases[176] to investigate patterns of symptoms in sporadic CJD, and suggest the existence of a distinct "affective sCJD variant".

Published experience of pharmacological treatments in prion disease has focused on putative disease-modifying agents. Interestingly, chlorpromazine and the tricyclic antidepressants are among

a number of agents that have been found to inhibit prion replication *in vitro*[119,177] and their use in prion disease patients has been published in a handful of case reports[178-181], but these reported their failure to halt the progression of the disease rather than any usefulness to treat psychiatric symptoms. Beyond this there is no previously published evidence on which to base the symptomatic treatment of BPS in patients with prion disease.

In contrast, BPS in Alzheimer's disease (AD) and other common dementias have received increasing clinical and scientific attention in recent years. They are recognised as a major cause of morbidity in themselves, and are associated with poorer outcomes for patients and more stress for carers[182,183]. Large scale research into their treatment has produced unexpected and important results in terms of both efficacy and safety. For example, meta-analysis of clinical trials using atypical antipsychotics for agitation and aggression in AD has shown only very modest efficacy compared with placebo [184] and substantial safety concerns, with a significant increase in mortality (odds ratio 1.54) [185]. This highlights the importance of systematic study of these symptoms to allow safe and evidence-based care of patients. Several studies have used factor analysis to show that BPS in AD tend to occur in a small number of clusters or "subsyndromes"[186,187]. All studies have reported psychosis, mood and hyperactivity/agitation clusters (although specific names used vary).

The project presented in this chapter uses systematically collected clinical data from 357 patients (317 patient-years of follow-up) that have been studied in the context of the PRION-1 trial and/or the National Prion Monitoring Cohort, to carry out a comprehensive study of the behavioural and psychiatric features of the human prion diseases.

Patients and Methods

Patients

All patients were referred to the NHS National Prion Clinic, and were enrolled in the PRION-1 trial and/or the National Prion Monitoring Cohort. Uptake of enrolment in these clinical research studies is extremely high (>95% in the Cohort), so they provide a highly representative sample of patients seen in this clinical setting.

Asymptomatic individuals known to be at risk of prion disease were also enrolled in the Cohort. Individuals were eligible if they were known to have received a transfusion of whole or leucodepleted blood donated by a person who later developed vCJD, or if they were an asymptomatic first degree relative of a patient with IPD (and had not had a negative genetic test). These at risk individuals are included in selected parts of the analysis below where specifically noted.

Clinical data from PRION-1 and the Cohort study

Theses clinical studies are described in detail in Chapter 1 above. PRION-1 was an open-label, patient preference trial of quinacrine for all types of human prion disease, which recruited patients from 2001 to 2007. The trial showed no effect of quinacrine on survival or any of the rating scales used as secondary outcome measures[120,121]. For the purposes of this study the PRION-1 clinical data is therefore regarded as natural history data, irrespective of treatment with quinacrine. The National Prion Monitoring Cohort is an ongoing natural history study of all types of prion disease, which has been enrolling patients since October 2008. In both studies, patients were enrolled and followed up throughout their disease course whenever possible, with clinical data recorded by a neurologist at each assessment. For the analysis presented here, 3 particular sets of data have been used to identify patients with BPS:

1) *First symptoms*. These were recorded for all patients, specifically including the presence of behavioural and psychiatric symptoms at onset.
2) *Symptoms at time of assessment*. The presence and severity/frequency of a range of specific symptoms were recorded at each assessment. These included "Hallucinations", and "Depressive symptoms".*
3) *Indications for drug prescription*. Details of all drugs prescribed during the period of study were recorded, including indication for treatment.

Investigation results including *PRNP* genotype and MR brain imaging were also collected whenever possible. MR imaging was reviewed by a specialist neuroradiologist (Dr Harpreet Hyare), and the presence/absence of pathological signal change in 3 specific areas noted (cortex, striatum, and thalamus). Neuropathological data was collected for those patients that had tonsil or brain biopsy and/or autopsy.

Brief Psychiatric Rating Scale (BPRS)

The BPRS was administered at all PRION-1 assessments when the patient was able to co-operate. The scale used was a slightly modified version of that developed by Ventura et al[188] from the original scale developed by Overall and Gorham[189], operationalized for rating from video records. The scale consisted of 24 items, each scored on a 1-7 scale. 14 items were rated from the subject's verbal response to structured, scripted questions, and 10 from the behaviour observed throughout the 20-minute interview. The majority of the assessments were video-recorded, and then reviewed and scored by a consultant psychiatrist (Professor Angus Mackay), blinded to the score given by the interviewer and to any treatment.

**In PRION-1 symptoms were classified as "Absent", "Slight" or "Significant", whereas in the Cohort they were classified as occurring "Never", "Rarely", "Often" or "Always". For the purposes of this analysis these have been reclassified as "Absent", "Slight/Rarely", or "Significant/Often/Always".*

MRC Prion Disease Rating Scale (MRC-PDRS)

Rates of clinical decline are highly variable both within and between prion disease types. As a result, time-from-onset cannot be used to separate patients into early-, mid- and late-stages of disease, and it is more meaningful to use a direct measure of functional impairment as an indicator of progression.

Published analysis of rating scales data from the PRION-1 trial showed that a novel combination scale (combining elements of the Barthel Index[190], the Clinician's Dementia Rating Sum of Boxes[191] and the Glasgow Coma Score[192]) has advantages over any individual scale as a global measure of progression for all types of prion disease[121]. Chapter 6 of this thesis describes work developing and validating a novel rating scale based on combined elements of these three existing scales, as a bespoke scale for use in prion disease clinical trials. This work has also now been published[193]. The new scale, the Medical Research Council Prion Disease Rating Scale (MRC-PDRS), has been completed at Cohort assessments since May 2010. As its 3 constituent scales were completed at all assessments throughout both PRION-1 and the Cohort, it can be imputed for all other assessments. It gives a score from 0 (maximal impairment, worst score) to 20 (no impairment, best score).

Clinical Casenote Review

The clinical casenotes of selected patients identified from the research data were reviewed in detail, including all of those prescribed medication for BPS. The review aimed to obtain more clinical detail of the BPS, including their timecourse, phenomenology, and any reported effects of treatment (beneficial or adverse).

Genome-wide Association and Candidate SNP Analysis

Genome–wide association analysis was performed on a subset of 170 cases (113 sCJD, 33 IPD, 22

vCJD and 1 iCJD) and 5200 UK controls provided by the Wellcome Trust Case Control Consortium (WTCCC). Cases were classified according to the presence or absence of 3 phenotypes (psychotic features at any stage, mood disorder at any stage, and psychiatric symptoms at onset) as described above. Analyses were carried out comparing (1) cases with each phenotype (Prion+) with cases without the phenotype (Prion–), and (2) Prion+ cases with controls (i.e. 2 analyses for each of the 3 phenotypes, giving a total of 6 analyses). Individuals were included if they had been genotyped previously for inclusion in the genome-wide association study of susceptibility to prion disease published by Mead et al, 2012[194]. Genotyping and quality control had been carried out previously for the earlier study, and this current study used the archived genotype data.
For detail of patient and control samples, genotyping, quality control and analysis see Mead et al. In the current study, Fisher's exact test was used for the association analysis because of the relatively small number of patients. 518 938 SNPs were included in the analysis after quality control. Ethnic outliers detected using a multidimensional scaling plot were excluded.

A small selection of candidate SNPs were also specifically examined in a hypothesis driven manner, in light of the limited statistical power that could be achieved in the genome-wide analysis given the relatively small number of cases. By reviewing previously published genome-wide association studies in psychiatric conditions characterised by psychosis and/or mood disorder (schizophrenia [195], bipolar affective disorder [196-198] and major depressive disorder [199]a list of candidate SNPs were identified that have shown association with these conditions at genome-level significance, on the basis that these might also show association with the behavioural/psychiatric phenotypes in prion disease. These SNPs are listed in Table 20 below. The codon 129 polymorphism of *PRNP* (SNP rs1799990), was also included as this is known to modify other aspects of the phenotype of prion disease (as reviewed above) and also to confer susceptibility to prion disease (as shown in the GWAS for prion disease mentioned above [194]). There was no evidence of population structure in the UK and no corrections were made.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel and IBM SPSS Statistics. Logistic regression analysis was used to model:

(1) The effect of MRC-PDRS score on the odds of specific BPS being present at the time of assessment, with disease type included as a covariate to account for the uneven distribution of disease types across the range of MRC-PDRS scores;

(2) Factors predicting the presence of specific BPS in all symptomatic patients, with age at onset, gender, disease type and PRNP codon 129 genotype as covariates (279/ 317 patients had all data available and were included);

(3) Factors predicting the presence of specific BPS in sCJD patients, with age at onset, gender, PRNP codon 129 genotype and distribution of pathological signal change on MR brain imaging as covariates (103/204 patients had all data available and were included).

Results

Patients

PRION-1 and the Cohort (up to 28/08/2011) provide clinical data on a total of 374 patients, including 204 with sporadic CJD, 25 with variant CJD, 80 with inherited prion disease, 8 with iatrogenic prion disease due to exposure to contaminated human growth hormone, 30 at risk of inherited prion disease, and 10 at risk of variant CJD (Table 5). The remaining 17 patients were found to have an alternative, non-prion diagnosis after enrolment: either at autopsy or as result of additional investigation results (e.g. elevated serum voltage-gated potassium channel antibody) or clinical progression that was not in keeping with prion disease (e.g. spontaneous recovery). These non-prion patients are excluded from the analysis.

Diagnosis		Number of Patients	
		Symptomatic	At Risk
Sporadic CJD		**204**	**-**
	Definite	102	-
	Probable	82	-
	Possible	20	-
	Codon 129 MM	89	-
	Codon 129 MV	48	-
	Codon 129 VV	32	-
	Not genotyped	35	-
Variant CJD		**25**	**10**
	Definite	18	-
	Probable	7	-
	Codon 129 MM	24	4
	Codon 129 MV	1	2
	Not genotyped	0	4
Iatrogenic CJD (hGH)		**8**	**-**
	Definite	4	-
	Probable	4	-
	Codon 129 MM	1	-
	Codon 129 MV	5	-
	Codon 129 VV	1	-
	Not genotyped	1	-
Inherited Prion Disease		**80**	**30**
	Insertion mutations: "IPD-OPRI"		
	4-OPRI	3	0
	5-OPRI	8	1
	6-OPRI	19	2
	Point mutations: "IPD-Point"		
	P102L	24	11
	A117V	7	2
	E200K	8	8
	D178N	4	6
	Y163X	3	0
	Others (P105L, Q212P, V210I, E211Q)	4	0

Table 5. Summary of patient diagnoses. hGH = Human growth hormone. OPRI = Octapeptide repeat insertion.

All patients included here had a working clinical diagnosis of prion disease at the time of enrolment, and patients have only been excluded from the analysis if there is clear clinical or neuropathological evidence that they did not have prion disease. This may lead to a small number of non-prion cases being included, but this is in keeping with the population seen in clinical practice. Neuropathological diagnosis of neurodegenerative disease during life is rare, and so a degree of diagnostic uncertainty must be acknowledged and clinical decisions made in this context.

Grouping of behavioural and psychiatric symptoms (BPS)

Symptoms occurring together in individual patients seemed to be divisible into the 3 main groups that have been established from factor analysis in Alzheimer's disease: psychotic symptoms; agitated symptoms; and mood disorder. Commonly described symptoms and behaviours, and how these were grouped, are shown below.

Psychotic symptoms	Agitated symptoms	Mood disorder
"Hallucinations"	"Agitation"	"Low mood"
"Paranoia"	"Aggression"	"Depression"
"Delusions"	"Behavioural disturbance"	"Emotional lability"
+/- "Agitation"	"Resistiveness"	"Apathy"
+/- "Aggression"	"Wandering"	"Suicidality"
+/- "Behavioural disturbance"	"Panic attacks"	"Withdrawal"
	"Shouting"	"Anxiety"

Prevalence of BPS

The prevalence of BPS (overall and by symptom group) occurring at any stage of disease is shown in Table 6. 253/317 (80%) of symptomatic patients had BPS noted at any stage of disease. There was variability between disease types, but even the lowest prevalence (in IPD-point) was over 70%. Psychotic symptoms were most common in vCJD and sCJD, while depressive symptoms were more

common in vCJD and IPD. Agitated symptoms were most common in IPD-OPRI (but may be underestimated overall for reasons discussed below).

	n	At disease onset				At any stage							
		Any BPS		Only BPS		Any BPS		Any Agitated		Any Psychotic		Any Mood	
All patients	317	141	**44.5%**	28	**8.8%**	253	**79.8%**	57	**18.0%**	117	**36.9%**	132	**41.6%**
sCJD	204	88	**43.1%**	15	**7.4%**	161	**78.9%**	33	**16.2%**	87	**42.6%**	68	**33.3%**
vCJD	25	23	**92.0%**	6	**24.0%**	24	**96.0%**	7	**28.0%**	12	**48.0%**	16	**64.0%**
iCJD	8	2	**25.0%**	0	**0.0%**	6	**75.0%**	0	**0.0%**	2	**25.0%**	4	**50.0%**
IPD All	80	28	**35.0%**	7	**8.8%**	62	**77.5%**	17	**21.3%**	16	**20.0%**	44	**55.0%**
IPD OPRI	30	16	**53.3%**	5	**16.7%**	26	**86.7%**	10	**33.3%**	7	**23.3%**	15	**50.0%**
IPD Point	50	12	**24.0%**	2	**4.0%**	36	**72.0%**	7	**14.0%**	9	**18.0%**	29	**58.0%**

Table 6. Prevalence of behavioural disturbance and psychiatric symptoms in all symptomatic patients and in individual prion disease types, at disease onset and at any stage of disease.

Some patients developed BPS at an early stage, before the onset of dementia and/or progressive neurological symptoms, while others only developed symptoms in the context of established neurocognitive decline.

The prevalence of BPS at onset for each disease type is also shown in Table 6. BPS were particularly common at onset in vCJD, but also common in IPD-OPRI and sCJD. BPS as the *only* symptoms at onset occurred in a smaller proportion of patients, in a similar pattern across disease types. The presence of behavioural disturbance at onset was separately recorded and was more common than any individual BPS-group.

Description of BPS

Synthesising information from the various sources described above, the characteristics of the psychiatric and behavioural symptoms seen in these patients can be described. Unless specifically noted, patients from all prion disease types are considered together.

Psychotic symptoms

Psychotic symptoms occurred with and without associated agitation or behavioural disturbance. By far the most common was visual hallucinosis. Visual hallucinations occurred both in the context of other progressive visual symptoms (such as visual distortion and agnosia), and as an isolated phenomenon. Distressing visual distortions and illusions were also common. Several patients described unpleasant splitting and twisting of faces and other objects: “like a Picasso”. In terms of hallucination content, there was a striking abundance of animals, and also human figures. In a few instances, patients described multi-modal hallucinations (e.g. seeing, hearing, feeling and smelling rats crawling over the body) but this was rare and was always associated with a particularly florid visual hallucinosis.

In more advanced patients episodes characterized by a wide-eyed, staring and sometimes fearful expression were frequently observed. Some of these patients had previously reported visual hallucinations at an earlier stage.

A smaller number of patients were noted to have delusions, occurring both with and without hallucinations. Delusional content tended to be relatively simple (e.g. delusions of theft or infidelity). A number of patients were noted to have fixed ideas based on a failure to distinguish events on television from reality. Several patients reported vivid sensory experiences outside the limits of their real sensory field, such as 'seeing' someone in another part of the house or a distant location ('extracampine' hallucinations), often with associated delusional thought content.

Possible Schneiderian first-rank symptoms were noted in 2 patients with vCJD. A 54 year old man with irritability, agitation, visual hallucinations and myoclonus reported feeling “as if someone is taking over my mind”, and a 30 year old man was noted to have “thought interference, delusional thinking and possible hallucinations” prior to the onset of any frank neurological symptoms.

Agitated symptoms

Agitation and behavioural disturbance (including aggression) also frequently occurred in the absence of psychotic features. Typical descriptions included “irritability”, “resistiveness” and “hostility”. Other over-active behaviours such as wandering and repetitive vocalizations were also included in this group.

In contrast with psychosis, these symptoms were commonly noted to occur in response to environmental or physical stimuli (most commonly administration of personal care).

In some patients, particularly those in the early stages of IPD-OPRI, non-psychotic behavioural

disturbance occurred in the context of disinhibition, impulsivity and other signs of frontal lobe dysfunction.

Patients with advanced disease often showed evidence of agitation without any behavioural evidence of hallucinations. This was often reactive to stimulus and often co-existed with stimulus-sensitive myoclonus and an exaggerated startle response, which are common features in the late stages of all prion diseases. This was observed in some patients who had had agitated symptoms noted earlier in their disease course, but also in many who had not.

Mood disorder

Some patients who were studied in the early stages of disease, and also during the presymptomatic/at-risk phase had symptoms of low mood combined with typical biological symptoms consistent with a "classical" depressive illness.

Patients in the early stages of vCJD were often noted to have predominant symptoms of social withdrawal, irritability, anxiety and low mood, in keeping with previous descriptions of this patient group.

Patients in the early to mid-stages of disease were more commonly noted to have emotional lability, characterized by sudden, unprovoked episodes of tearfulness (or more rarely laughing or elation). Patients at this stage were also often noted to have social withdrawal and apathy, albeit in the context of deteriorating expressive language and frontal executive function.

Affective symptoms in patients in the late stages of disease could rarely be characterized. Whereas the presence of agitated or psychotic symptoms may sometimes be inferred from the behaviour of a hypokinetic and mute patient, this is rarely true of mood disorder.

Suicidal ideation was noted in a few cases, all in the early to mid-stages of IPD. This varied from a patient with minimal cognitive impairment making enquiries regarding physician-assisted suicide, to a patient with marked frontal executive deficits voicing suicidal intent impulsively and with incongruous affect. Only one suicide attempt was identified from the clinical case notes reviewed (a patient in the early stages of IPD-OPRI took an overdose on the anniversary of his mother's death from the same condition), and there were no completed suicides.

Other symptoms

Almost all symptoms noted could be classified into the three groups above, but a few others did occur. Obsessive thoughts were reported in a handful of patients. Compulsive or repetitive behaviours were included in the agitated symptom group. One patient with IPD due to D178N mutation had a diagnosis of obsessive-compulsive disorder pre-dating onset of cognitive and neurological symptoms by many years. A recurrent sensation of "déjà vu" and a strong sensation of derealization were reported by two patients in the very early stages of sCJD.

BPS and Disease Progression

Figure 30 shows the proportion of assessments at which (A) hallucinations and (B) depressive symptoms were noted to be present when patients were at different stages of disease progression, as assessed by the MRC-PDRS.It also shows the proportion of assessments where the presence of each symptom could not be assessed, predominantly because of aphasia and severe global impairment in late stage disease.

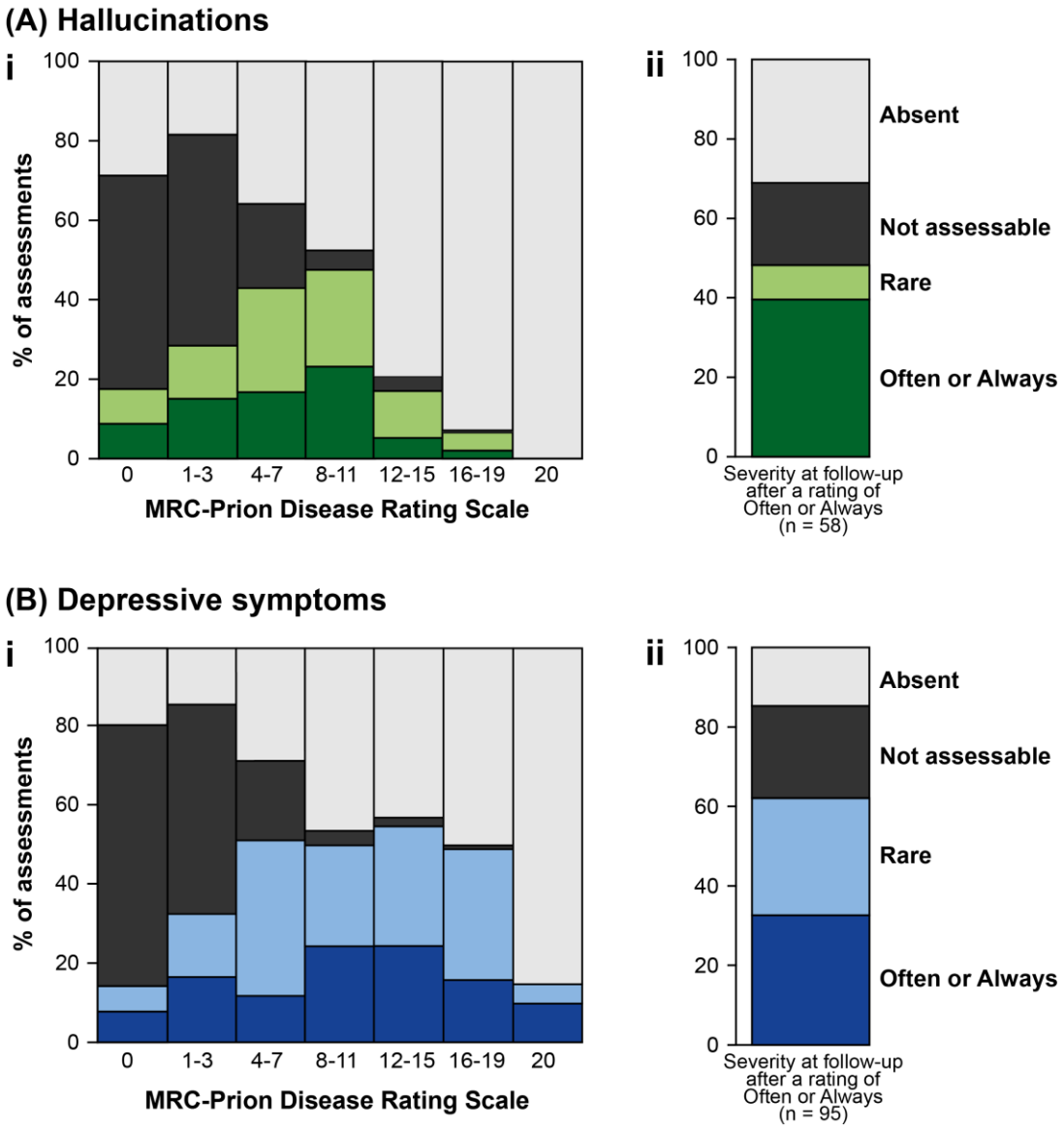


Figure 30. Natural history of symptoms. Prevalence of Hallucinations and Depressive Symptoms at all assessments grouped by stage of disease progression ((A)i and (B)i), and severity rating of Hallucinations and Depressive Symptoms at the follow-up assessment after a rating of "Often" or "Always" ((A)ii and (B)ii). NB. Disease progression assessed by MRC-PDRS score (see text): 0 = maximal impairment, akinetic and mute; 20 = no functional impairment.

It is important to note that the distribution of MRC-PDRS scores varies between different disease types: the majority of sCJD patients already had low scores at enrolment, while many IPD patients were enrolled with much higher scores.

Depressive symptoms were observed at a similar rate across the range of functional impairment until the very severely impaired range where they often could not be assessed. In contrast, hallucinations were not observed in the most mildly impaired, and became more prevalent as impairment increased. In keeping with this, logistic regression showed a highly significant effect of MRC-PDRS score on the odds of hallucinations being present ($p < 0.001$), adjusted for disease type. There was no significant effect of MRC-PDRS on the odds of depressive symptoms being present ($p = 0.078$), although there was some trend towards increasing depressive symptoms at greater levels of functional impairment (Tables 7 and 8).

Independent variables		Logistic Regression		
		Odds Ratio	95% CI	p value
MRC Score	Per point	**0.935**	**0.909 - 0.963**	**<0.001**
Diagnosis	sCJD	**1**	**(ref)**	**<0.001**
	vCJD	**0.327**	**0.122 - 0.874**	**0.026**
	iCJD	1.334	0.461 - 3.864	0.595
	IPD OPRI	**0.083**	**0.025 - 0.279**	**<0.001**
	IPD Point	**0.254**	**0.121 - 0.531**	**<0.001**
	Constant	**0.783**		0.171

Table 7. Logistic regression of MRC-PDRS score and disease type vs. Hallucinations

Independent variables		Logistic Regression		
		Odds Ratio	95% CI	p value
MRC Score	Per point	0.981	0.961 - 1.002	0.078
Diagnosis	sCJD	1	(ref)	0.328
	vCJD	1.31	0.661 - 2.597	0.439
	iCJD	0.601	0.167 - 2.16	0.435
	IPD OPRI	0.644	0.37 - 1.12	0.119
	IPD Point	0.858	0.497 - 1.482	0.583
	Constant	**0.412**		**<0.001**

Table 8. Logistic regression of MRC-PDRS and disease type vs. Depressive Symptoms.

To examine the natural history of BPS in individual patients, all assessments at which Hallucinations or Depressive symptoms were noted to be occurring “Often” or “Always” where the patient went on

to have another assessment were identified, and the severity of the symptom at this second assessment was noted (Figure 30). Both symptoms improved at the second assessment in about 40% of cases, and could no longer be assessed due to progression of disease in about 20% of cases. It was checked whether any of these patients had relevant symptomatic treatments (e.g. antidepressants, antipsychotics) started between these two assessments. Only 8 of 58 serial assessment pairs identified for hallucinations, and 8 of 95 pairs identified for depressive symptoms captured initiation of a relevant symptomatic treatment, so most of those patients in whom the symptoms improved had not been treated, or continued on established treatment. The small number of cases that were newly treated are analysed further under *Pharmacological Treatment* below.

Brief Psychiatric Rating Scale (BPRS) in Prion-1

The BPRS was completed in 39 of the 101 symptomatic patients in PRION-1. The remainder were already too impaired at enrolment to complete the lengthy patient interview required. As a result most of the BPRS data collected is from patients at an early stage of disease, and the more slowly progressive disease types are over-represented. In total, 175 assessments were completed in these 39 patients.

126 of the BPRS assessments were video-taped and then reviewed by a consultant psychiatrist (Professor Angus MacKay), who was blinded to the original assessor's score. Figure 31 shows a Bland-Altman Plot for the total BPRS scores in these assessments, illustrating the level of agreement between the original assessor and the consultant psychiatrist. There was a strong bias towards the original assessor giving a higher total score.

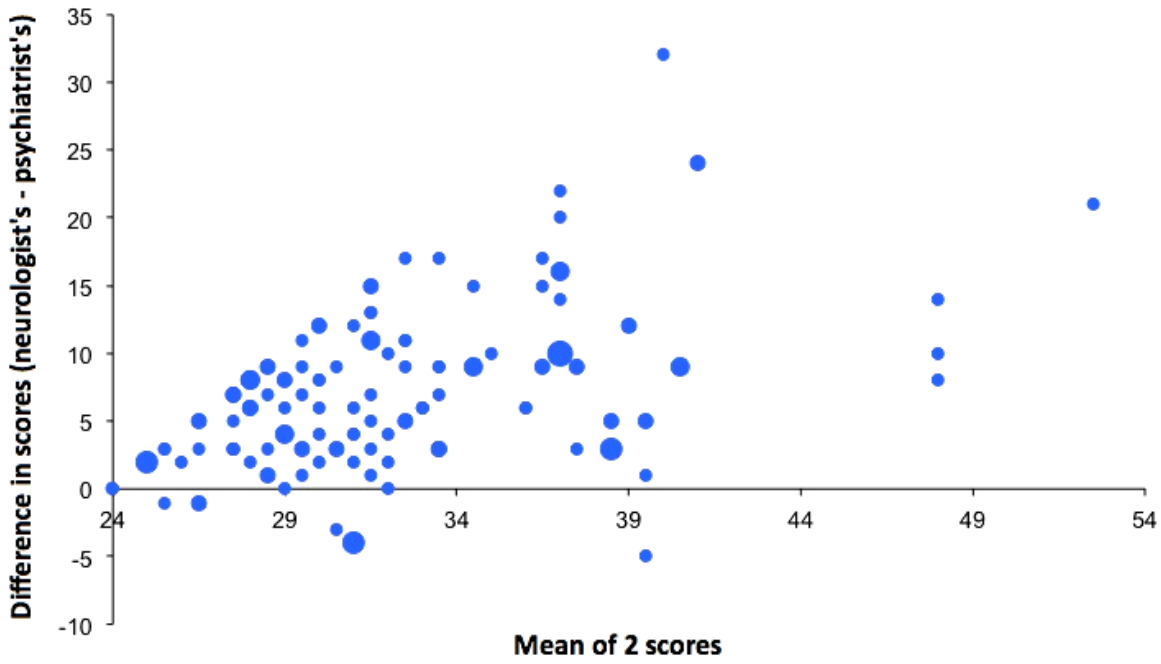


Figure 31. Bland-Altman plot comparing total Brief Psychiatric Rating Scale (BPRS) scores given at the initial, face-to-face assessment (neurologist) and at review of the video-taped assessment (psychiatrist), from 126 assessments. The psychiatrist reviewing the video was blinded to the original assessor's score.

To assess the reliability of individual sub-components of the BPRS, Cohen's Kappa scores were calculated for the agreement of individual symptom scores. By convention Cohen's Kappa values are interpreted using the descriptors proposed by Landis and Koch[200], whereby <0 = "no agreement"; 0–0.20 = "slight agreement"; >0.20–0.40 = "fair agreement"; >0.40–0.60 = "moderate agreement"; >0.60–0.80 = "substantial agreement", and >0.80-1.0 = "almost perfect agreement". These results are shown in Table 9. They ranged from -0.03 (no agreement) to 0.47 (moderate agreement). If scores were collapsed so that each symptom was classified either absent or present (regardless of severity) and Kappas recalculated, they ranged from -0.04 (no agreement) to 0.66 (substantial agreement).

BPRS Subcomponent	1 - 7 Score κ	Present/Absent κ
Somatic concern	0.22	0.41
Anxiety	0.23	0.46
Depression	0.26	0.40
Suicidality	0.47	0.66
Guilt	0.15	0.33
Hostility	0.14	0.30
Elated mood	0.10	0.18
Grandiosity	0.00	0.00
Suspiciousness	0.32	0.36
Hallucinations	0.38	0.51
Unusual thought	0.24	0.24
Bizarre behaviour	-0.01	-0.02
Self-neglect	-0.03	-0.04
Disorientation / inappropriate affect	0.02	0.06
Conceptual disorganisation	0.07	0.07
Blunted affect	-0.01	-0.02
Emotional withdrawal	-0.02	-0.03
Motor retardation	-0.02	-0.02
Tension	-0.01	-0.01
Uncooperativeness	0.18	0.18
Excitement	0.00	0.00
Distractability	-0.02	-0.03
Motor hyperactivity	0.21	0.44
Mannerisms and posturing	0.08	0.17

Table 9. Cohen's Kappa (κ) values for agreement on individual BPRS subcomponents between the initial, face-to-face assessment (neurologist) and at review of the video-taped assessment (psychiatrist), from 126 assessments. Kappa values have been calculated both for the 1 to 7 score used in the BPRS, and by collapsing these down to a dichotomous, absent/present score.

In light of the generally poor levels of agreement, only the data from the consultant psychiatrist's rescoring of the videoed assessments were included in further analysis.In total 126 BPRS assessments in 38 of the 101 symptomatic patients in PRION-1 were scored from video by the consultant psychiatrist (Professor Angus Mackay), and this data is presented here.

Figure 32 shows the proportion of assessments at which each symptom sub-component was scored

as being present and the level of severity. Most symptoms were very rarely scored as being present. Six of the seven most frequently recorded symptoms fall within the Mood Disorder grouping used above: Depression, Somatic Concern, Anxiety, Suicidality, Guilt and Self-neglect. In contrast, the psychotic symptoms were very rarely recorded.

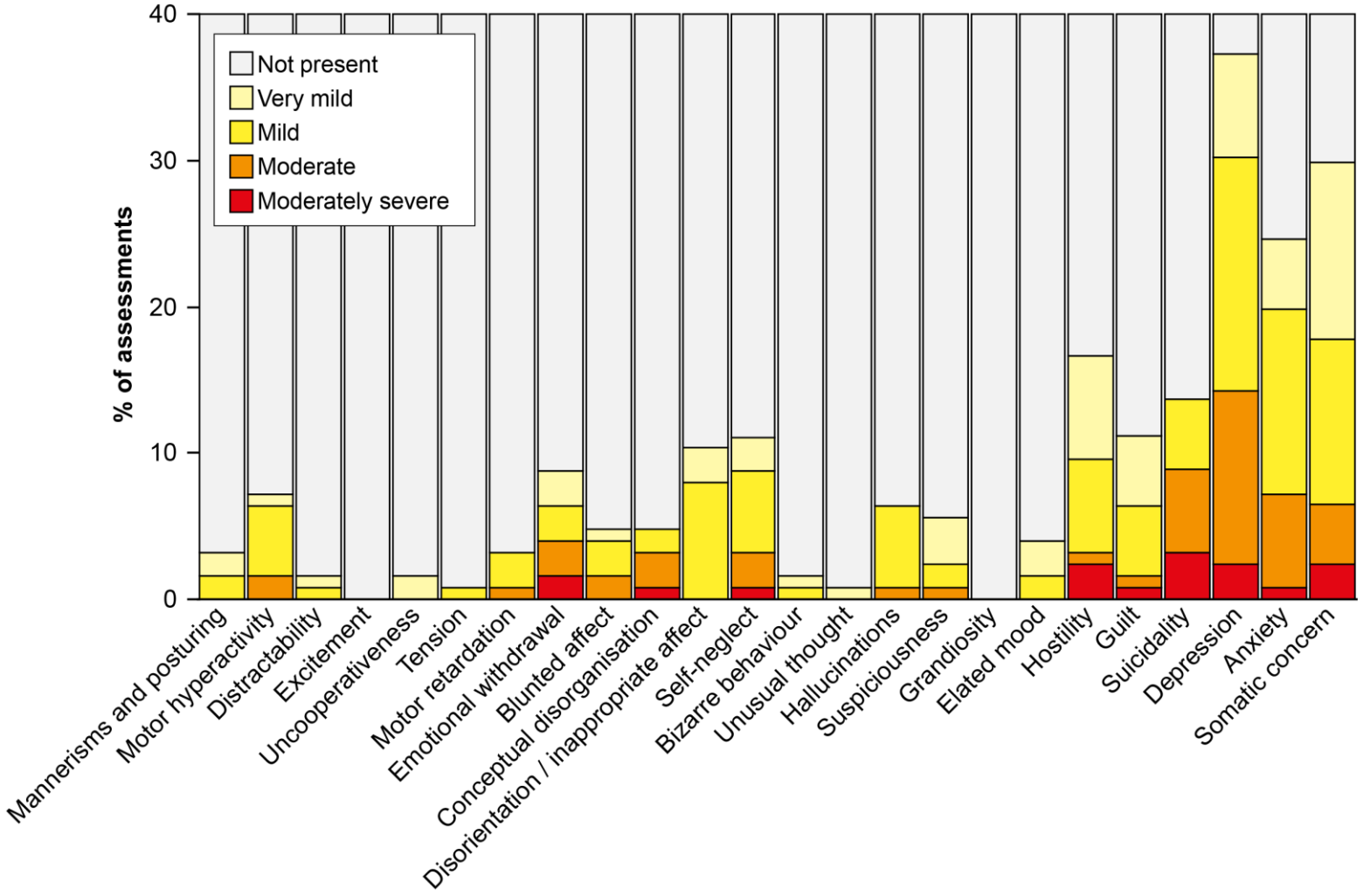


Figure 32. BPRS. Breakdown of BPRS by symptom category, showing proportion of assessments scored at each severity level (out of total of 139 assessments in 38 patients).

This provides a more detailed profile of the symptomatology in a sub-group of less severely impaired patients (those that were able to co-operate with the lengthy BPRS interview). These results are in keeping with those presented above: in the earlier stages of prion disease symptoms of mood disorder predominate, while psychotic symptoms are rare (but may emerge as disease progresses).

Pharmacological Treatment

Commonly prescribed classes of medication for BPS were antidepressants (for mood disorder and agitated symptoms), benzodiazepines (for agitated symptoms and psychotic symptoms), antipsychotics (for agitated symptoms and psychotic symptoms) and acetylcholinesterase inhibitors (for psychotic symptoms).

For each drug class/symptom group combination, Table 10 shows the number of prescriptions and the duration of treatment that were studied, as well as details of reported efficacy, adverse events and reasons for discontinuation as established from review of the clinical case notes. For all drug class/symptom group combinations most prescriptions were continued until death or advanced disease, or were ongoing at the end of the period of study. None of the adverse events attributed to the treatments were severe, and they very rarely led to discontinuation.

Medication class	Number of prescrip-tions studied	Duration of treatment (days)		Clinical notes record…				Stopped due to…						Ongoing at last follow-up		Side effects noted
		Total	Median course length	Efficacy		Side effects		Side effects		Lack of benefit		Death or advanced disease				
Agitated symptoms																
Benzodiazepines	**39**	5683	23	6	**15.4%**	4	**10.3%**	0	**0.0%**	0	**0.0%**	24	**61.5%**	8	**20.5%**	Sedation (4)
Antipsychotics	**23**	7253	149	10	**43.5%**	3	**13.0%**	0	**0.0%**	0	**0.0%**	14	**60.9%**	7	**30.4%**	Extrapyramidal (2), Sedation (1)
Antidepressants	**6**	3995	750.5	1	**16.7%**	2	**33.3%**	1	**16.7%**	1	**16.7%**	0	**0.0%**	4	**66.7%**	Weight gain (1), Headache (1)
Psychotic symptoms																
Benzodiazepines	**32**	3280	42	7	**21.9%**	4	**12.5%**	0	**0.0%**	0	**0.0%**	26	**81.3%**	1	**3.1%**	Sedation (4)
Antipsychotics	**31**	6266	61	9	**29.0%**	6	**19.4%**	0	**0.0%**	1	**3.2%**	22	**71.0%**	4	**12.9%**	Sedation (6)
AChE Inhibitors	**5**	2027	236	3	**60.0%**	0	**0.0%**	0	**0.0%**	0	**0.0%**	2	**40.0%**	3	**60.0%**	None
Mood disorder																
Antidepressants	**45**	18315	301.5	16	**35.6%**	4	**8.9%**	1	**2.2%**	2	**4.4%**	22	**48.9%**	13	**28.9%**	Sedation (1), Agitation (1), Weight gain (1), Unpleasant taste (1)

Table 10. Drug treatment of BPS. Observational data for all drug classes prescribed 5 or more times for each symptom group. NB: Some patients received more than one agent (most commonly antipsychotic and benzodiazepine) but in most these were not started simultaneously. As far as possible the relative clinical benefit and/or adverse effects of each were determined from careful review of the clinical notes. If this was not clear, the benefit or adverse effect was attributed to both. Specific medications prescribed from each class are listed in the text.

Antidepressants for mood disorder

There were 45 prescriptions of antidepressants for mood disorder, in 42 patients. Drugs prescribed were Citalopram (21); Fluoxetine (7); Mirtazapine (6); Sertraline (3); Amitriptyline, Escitalopram (2 each); Clomipramine, Paroxetine, Trazodone and Venlafaxine (1 each).

In total 18,315 days of treatment were studied. Most of this experience was in either sCJD cases, where treatment courses tended to be shorter (median 111 days), or in IPD cases, where courses were longer (median 565 days). 16 out of 45 (36%) prescriptions of antidepressants for mood disorder were noted to have apparent clinical benefit. 12/16 of these were in patients with IPD. Apparent adverse effects were noted in 4 cases (9%): agitation, sedation, weight gain and a persistent unpleasant taste. None of these were severe, and in only one case did they lead to discontinuation of the prescription (unpleasant taste). 21 patients (50%) died while being treated.

Antipsychotics for psychotic symptoms

There were 31 prescriptions of antipsychotics for psychotic symptoms, in 29 patients. These were Quetiapine (14), Olanzapine (8), Risperidone (6), Haloperidol (2) and Levomepromazine (1). Quetiapine appears to have been favoured because of a wish to avoid worsening or precipitating extrapyramidal symptoms.

In total 6,266 days of treatment were studied. Most of this experience was in sCJD and vCJD cases, and most treatment courses were short (median 61 days). However, a few patients with IPD and atypical sCJD had prescriptions continuing for several years. 9 out of 31 (29%) prescriptions of antipsychotics for psychotic symptoms were noted to have apparent clinical benefit. An apparent adverse effect was noted in 6 cases (19%); this was sedation in all cases. This often occurred in the context of severe neurological disability, and was not recorded as a reason for discontinuation of the prescription in any cases. 19 patients (66%) died while being treated.

Benzodiazepines for psychotic symptoms

There were 32 prescriptions of benzodiazepines for psychotic symptoms, in 26 patients. These were Lorazepam (12); Diazepam, Midazolam (6 each); Clonazepam (4); Temazepam (3) and Alprazolam (1).

In total 3,280 days of treatment were studied. Most of this experience was in late-stage disease. In a number of cases myoclonus was noted as a joint indication. Most treatment courses were short (median 42 days). 7 out of 32 (22%) prescriptions were noted to have apparent clinical benefit. An apparent adverse effect was noted in 6 cases (19%); this was sedation in all cases. This often occurred in the context of severe neurological disability, and was not recorded as a reason for discontinuation of the prescription in any cases. 21 patients (81%) died while being treated.

Acetylcholinesterase inhibitors for psychotic symptoms

There were 5 prescriptions of acetyl cholinesterase inhibitors for psychotic symptoms, in 5 patients. These were Donepezil (4) and Rivastigmine (1).

In total 2,027 days of treatment were studied. Prescriptions were started in moderately advanced patients, all of whom had hallucinations, and courses were relatively long (median 236 days). 3 out of 5 (60%) prescriptions were noted to have apparent clinical benefit. No adverse effects were recorded. 2 patients (40%) died while being treated.

Antipsychotics for agitated (non-psychotic) symptoms

There were 23 prescriptions of antipsychotics for agitated symptoms, in 23 patients. These were Quetiapine (11), Haloperidol (6), Risperidone (4), and Olanzapine (2). Again, Quetiapine seems to have been favoured because of a wish to avoid worsening or precipitating extrapyramidal

symptoms.

In total 7,253 days of treatment were studied. This included very short treatment courses at the end stage of sCJD (minimum 7 days) and much longer courses in slowly progressive IPD-OPRI (maximum 1506 days, median 149 days). 10 out of 23 (43%) prescriptions were noted to have apparent clinical benefit. Apparent adverse effects were noted in 3 cases (13%): extra-pyramidal symptoms (2 cases) and sedation. None of these were severe, and they were not recorded as a reason for discontinuation of the prescription in any cases. 11 patients (48%) died while being treated.

Benzodiazepines for agitated (non-psychotic) symptoms

There were 39 prescriptions of benzodiazepines for agitated symptoms, in 37 patients. These were Midazolam (16); Diazepam (12); Lorazepam (7); and Clonazepam (4).

In total 5,683 days of treatment were studied. Most of this experience was in late-stage disease. In a number of cases myoclonus was noted as a joint indication. Most treatment courses were short (median 23 days). A few courses were much longer (maximum 2472 days), particularly in slowly-progressive IPD with chronic agitation and behavioural disturbance. 6 out of 39 (15.4%) prescriptions were noted to have apparent clinical benefit. An apparent adverse effect was noted in 4 cases (10.3%); this was sedation in all cases. This often occurred in the context of severe neurological disability, and was not recorded as a reason for discontinuation of the prescription in any cases. 24 patients (65%) died while being treated.

Antidepressants for agitated (non-psychotic) symptoms

There were 6 prescriptions of antidepressants for agitated symptoms, in 5 patients. These were Amitriptyline (2), Mirtazapine (2), Fluoxetine (1) and Sertraline (1).

In total 3,995 days of treatment were studied. All 5 patients had IPD-OPRI, were treated at relatively early stages of disease, and treatment courses were long (median 750.5 days). 1 out of 6 (16.7%) prescriptions was noted to have apparent clinical benefit. Apparent adverse effects were noted in 2 cases (33%): weight gain and headache, and were recorded as a reason for discontinuation of the prescription in one case (headache). No patients died while being treated.

Effect of pharmacological treatments on natural history of BPS

To obtain a more objective assessment of efficacy, patients who were assessed as having Hallucinations or Depressive symptoms "Often" or "Always" at a study assessment and then went on to have another assessment were identified, and those in whom any appropriate treatment was started in the intervening time were compared with those in whom it was not. Only 8 of 58 serial assessment pairs identified for hallucinations, and 8 of 95 pairs identified for depressive symptoms captured initiation of treatment. There was no significant difference in the number of patients improving with treatment or without for either symptom (Fisher's exact tests).

Factors Predicting Presence of BPS

The full results for the logistic regression analyses for factors predicting presence of BPS are shown in Tables 11 - 18. Nominal (uncorrected) p values are quoted below and in the tables.

Independent variables		Total	With psychotic symptoms		Logistic Regression		
		n	n	%	Odds Ratio	95% CI	p value
Diagnosis	sCJD	170	70	41.18%	***1***	**(ref)**	**0.001**
	vCJD	25	12	48.00%	0.654	0.175 - 2.443	0.528
	iCJD	7	1	14.29%	**0.1**	**0.01 - 0.955**	**0.045**
	IPD OPRI	29	7	24.14%	**0.239**	**0.075 - 0.767**	**0.016**
	IPD Point	48	9	18.75%	**0.189**	**0.075 - 0.476**	**<0.001**
Age at Onset	Per year				0.979	0.953 - 1.006	0.125
PRNP Codon 129	MM	159	54	33.96%	***1***	**(ref)**	**0.008**
	MV	83	37	44.58%	1.959	1.064 - 3.607	**0.031**
	VV	37	8	21.62%	0.488	0.201 - 1.181	0.112
Gender	Male	134	50	37.31%	***1***	**(ref)**	
	Female	145	49	33.79%	0.944	0.556 - 1.601	0.831
Constant					2.646		0.303

Table 11. Logistic regression for variables affecting presence of psychotic symptoms in all types of prion disease (in 279 patients)

Independent variables		Total	With mood disorder		Logistic Regression		
		n	n	%	Odds Ratio	95% CI	p value
Diagnosis	sCJD	170	57	33.53%	*1*	ref	0.372
	vCJD	25	17	68.00%	3.044	0.838 - 11.054	0.091
	iCJD	7	4	57.14%	1.689	0.326 - 8.741	0.532
	IPD OPRI	29	15	51.72%	1.555	0.559 - 4.327	0.397
	IPD Point	48	28	58.33%	1.96	0.917 - 4.192	0.083
Age at Onset	Per year				0.984	0.96 - 1.009	0.204
PRNP Codon 129	MM	159	67	42.14%	*1*	ref	0.11
	MV	83	43	51.81%	1.684	0.933 - 3.037	0.083
	VV	37	11	29.73%	0.76	0.331 - 1.745	0.517
Gender	Male	134	53	39.55%	*1*	(ref)	
	Female	145	68	46.90%	**1.68**	**1.002 - 2.818**	**0.049**
Constant					0.955		0.958

Table 12. Logistic regression for variables affecting presence of mood disorder in all types of prion disease (in 279 patients)

Independent variables		Total	With agitated symptoms		Logistic Regression		
		n	n	%	Odds Ratio	95% CI	p value
Diagnosis	sCJD	170	28	16.47%	*1*	ref	0.657
	vCJD	25	8	32.00%	0.615	0.135 - 2.81	0.531
	iCJD	7	0	0.00%	<0.001	0 - ???	0.999
	IPD OPRI	29	10	34.48%	1.067	0.322 - 3.531	0.916
	IPD Point	48	7	14.58%	0.511	0.18 - 1.45	0.207
Age at Onset	Per year				**0.966**	**0.936 - 0.997**	**0.03**
PRNP Codon 129	MM	159	35	22.01%	*1*	ref	0.699
	MV	83	13	15.66%	0.763	0.359 - 1.618	0.48
	VV	37	5	13.51%	0.711	0.245 - 2.068	0.531
Gender	Male	134	27	20.15%			
	Female	145	26	17.93%	0.961	0.511 - 1.809	0.902
Constant					2.172		0.476

Table 13. Logistic regression for variables affecting presence of agitated symptoms in all types of prion disease (in 279 patients)

Independent variables		Total	With BPS at onset		Logistic Regression		
		n	n	%	Odds Ratio	95% CI	p value
Diagnosis	sCJD	170	74	43.53%	**1**	**(ref)**	**<0.001**
	vCJD	25	23	92.00%	**9.682**	**1.687 - 55.584**	**0.011**
	iCJD	7	2	28.57%	0.359	0.06 - 2.159	0.263
	IPD OPRI	29	16	55.17%	1.105	0.396 - 3.081	0.848
	IPD Point	48	11	22.92%	**0.297**	**0.128 - 0.692**	**0.005**
Age at Onset	Per year				0.991	0.966 - 1.017	0.493
PRNP Codon 129	MM	159	80	50.31%	1	(ref)	0.071
	MV	83	37	44.58%	1.205	0.667 - 2.176	0.537
	VV	37	9	24.32%	**0.425**	**0.182 - 0.990**	**0.047**
Gender	Male	134	66	49.25%	1	(ref)	
	Female	145	60	41.38%	0.907	0.54 - 1.525	0.714
Constant					1.614		0.601

Table 14. Logistic regression for variables affecting presence of BPS at onset in all types of prion disease (in 279 patients)

Independent variables			Total	With BPS at onset		Logistic Regression		
			n	n	%	Odds Ratio	95% CI	p value
Age at Onset		Per year				***0.935***	**0.889 - 0.984**	**0.01**
PRNP Codon 129		MM	46	19	41.30%	*1*	(ref)	0.333
		MV	33	17	51.52%	1.197	0.447 - 3.210	0.72
		VV	24	6	25.00%	0.464	0.135 - 1.594	0.223
Gender		Male	46	20	43.48%	*1*	(ref)	
		Female	57	22	38.60%	0.945	0.387 - 2.311	0.902
MRI	Striatum	Abnormal	79	32	40.51%	*0.826*	0.204 - 3.352	0.789
		Normal	24	10	41.67%			
	Thalamus	Abnormal	60	24	40.00%	0.813	0.266 - 2.484	0.717
		Normal	43	18	41.86%			
	Cortex	Abnormal	83	36	43.37%	1.205	0.342 - 4.242	0.771
		Normal	20	6	30.00%			
Constant						65.816		0.033

Table 15. Logistic regression for variables affecting presence of BPS at onset in sCJD (in 103 patients)

Independent variables			Total	With psychotic symptoms		Logistic Regression		
			n	n	%	Odds Ratio	95% CI	p value
Age at Onset		Per year				*0.962*	0.917 - 1.009	0.113
PRNP Codon 129		MM	46	19	41.30%	***1***	**(ref)**	**0.048**
		MV	33	20	60.61%	2.031	0.759 - 5.436	0.158
		VV	24	6	25.00%	0.411	0.121 - 1.387	0.152
Gender		Male	46	19	41.30%	*1*	(ref)	
		Female	57	26	45.61%	1.632	0.665 - 4.003	0.285
MRI	Striatum	Abnormal	79	35	44.30%	*1.257*	0.318 - 4.974	0.744
		Normal	24	10	41.67%			
	Thalamus	Abnormal	60	27	45.00%	0.816	0.271 - 2.456	0.717
		Normal	43	18	41.86%			
	Cortex	Abnormal	83	38	45.78%	0.961	0.281 - 3.284	0.95
		Normal	20	7	35.00%			
Constant								

Table 16. Logistic regression for variables affecting presence of psychotic symptoms in sCJD (in 103 patients)

Independent variables			Total	With mood disorder		Logistic Regression		
			n	n	%	Odds Ratio	95% CI	p value
Age at Onset		Per year				*0.961*	0.914 - 1.01	0.116
PRNP Codon 129		MM	46	14	30.43%	*1*	(ref)	0.599
		MV	33	12	36.36%	1.522	0.538 - 4.304	0.428
		VV	24	7	29.17%	0.824	0.239 - 2.845	0.76
Gender		Male	46	12	26.09%	*1*	(ref)	
		Female	57	21	36.84%	2.024	0.784 - 5.226	0.145
MRI	Striatum	Abnormal	79	23	29.11%	*1.169*	0.292 - 4.674	0.826
		Normal	24	10	41.67%			
	Thalamus	Abnormal	60	16	26.67%	0.41	0.128 - 1.309	0.132
		Normal	43	17	39.53%			
	Cortex	Abnormal	83	25	30.12%	0.547	0.159 - 1.874	0.337
		Normal	20	8	40.00%			
Constant						8.89		0.257

Table 17. Logistic regression for variables affecting presence of Mood Disorder in sCJD (in 103 patients)

Independent variables			Total	With agitated symptoms		Logistic Regression		
			n	n	%	Odds Ratio	95% CI	p value
Age at Onset		Per year				*0.969*	0.913 - 1.029	0.304
PRNP Codon 129		MM	46	5	10.87%	*1*	(ref)	0.179
		MV	33	9	27.27%	3.327	0.92 - 12.038	0.067
		VV	24	4	16.67%	1.585	0.34 - 7.386	0.558
Gender		Male	46	7	15.22%	*1*	(ref)	
		Female	57	11	19.30%	1.812	0.575 - 5.709	0.31
MRI	Striatum	Abnormal	79	14	17.72%	*1.452*	0.259 - 8.14	0.671
		Normal	24	4	16.67%			
	Thalamus	Abnormal	60	10	16.67%	0.5	0.124 - 2.023	0.331
		Normal	43	8	18.60%			
	Cortex	Abnormal	83	15	18.07%	1.049	0.224 - 4.921	0.951
		Normal	20	3	15.00%			
Constant						0.675		0.863

Table 18. Logistic regression for variables affecting presence of Agitated Symptoms in sCJD (in 103 patients)

Presence of psychotic symptoms at any stage was independently predicted by specific disease types (sCJD > IPD) and codon 129 genotypes (MV > MM) with a high level of significance. Presence of BPS at onset was independently predicted by specific disease types (vCJD > sCJD > IPD-Point). Female gender reached borderline significance as an independent predictor of mood disorder at any stage (OR 1.68, p = 0.049), as did younger age at onset as an independent predictor of agitated symptoms (OR = 0.966 per year, p = 0.03), although in light of the degree of multiple testing these results should be treated with caution.

In the sCJD-only analysis, the presence of BPS at onset was independently predicted by younger age at onset (OR = 0.935 per year, p = 0.01), and no other strongly significant predictors were found.

Genome-wide association and candidate SNP Analysis

Table 19 shows details of the SNPs with the most significant associations in each analysis. No SNPs reached the standard threshold for genome wide significance in association studies ($P<5 \times 10^{-8}$).

Phenotype	SNP	Chr	BP	MAF	Closest gene (RefSeq)	Distance (kb)	Prion+ vs Controls		Prion+ vs Prion-	
							GWAS p-value	OR	GWAS p-value	OR
Psychotic Features	rs1055569	6	31548061	0.291	HCG26	Intragenic	**2.66E-06**	**2.36**	1.08E-04	2.49
	rs4413654	6	31549328	0.219	HCG26	1.164	**3.69E-06**	**2.43**	1.02E-04	2.63
	rs2516440	6	31548476	0.294	HCG26	0.312	**4.48E-06**	**2.33**	1.08E-04	2.49
	rs4077732	11	11493063	0.330	GALNTL4	Intragenic	2.96E-03	0.53	**1.65E-06**	**0.30**
	rs7231996	18	69417499	0.131	LOC100505817	249.395	0.01	1.81	**3.14E-06**	**5.56**
	rs6440974	3	156553159	0.099	LOC100507537	58.976	6.56E-05	2.59	**4.47E-06**	**5.27**
Mood disorder	rs7789850	7	140947080	0.026	AGK	Intragenic	**2.16E-06**	**4.60**	2.46E-04	7.64
	rs12789145	11	94047384	0.084	PIWIL4	53.149	**3.58E-06**	**0.00**	7.75E-06	0
	rs6867820	5	121882424	0.224	SNCAIP	54.731	**6.03E-06**	**2.24**	9.74E-05	2.61
	rs1219407	11	121249388	0.073	SORL1	215.633	8.75E-05	2.60	**3.94E-06**	**7.60**
	rs478903	11	121190975	0.089	SORL1	181.294	1.35E-04	2.39	**6.65E-06**	**6.17**
	rs761998	20	14257952	0.352	FLRT3	Intragenic	0.02	0.64	**6.69E-06**	**0.35**
BPS at onset	rs10509125	10	61596872	0.402	ANK3	Intragenic	**1.43E-06**	**2.09**	3.50E-05	2.52
	rs561437	13	109734228	0.499	COL4A1	Intragenic	**4.77E-06**	**0.49**	1.15E-04	0.42
	rs7151968	14	47671186	0.205	LOC100506433	337.219	**5.83E-06**	**2.12**	1.60E-03	2.23
	rs4743805	9	106909655	0.165	SLC44A1	137.095	5.892E-03	1.66	**6.35E-06**	**4.51**
	rs9472202	6	44129264	0.208	C6orf223	47.592	3.778E-05	1.98	**8.41E-06**	**3.32**
	rs7040444	9	15049821	0.213	LOC389705	40.099	2.933E-03	0.52	**1.17E-05**	**0.30**

Table 19. Most strongly associated single-nucleotide polymorphisms in each of the genome-wide association analyses: Prion+ vs Controls and Prion+ vs Prion- for each of 3 psychiatric/behavioural phenotypes. SNP = single-nucleotide polymorphism; Chr = chromosome; BP = base pair position; MAF = minor allele frequency; GWAS = genome-wide association; study; OR = odds ratio for minor allele; Prion+ = prion disease cases with phenotype; Prion- = prion disease cases without phenotype.

The single most significant association was in the Prion+ vs Controls analysis for psychiatric symptoms at onset, for a SNP (rs10509125) lying within the Ankyrin 3 (*ANK3*) gene ($p = 1.43 \times 10^{-6}$, OR 2.09). In the Prion+ vs Prion- analysis association for this SNP was less significant but effect size was greater ($p=3.5 \times 10^{-5}$, OR=2.52).

A cluster of 3 SNPs on chromosome 6p were most strongly associated with the presence of psychotic features in the Prion+ vs Controls analysis (rs1055569; $p = 2.66 \times 10^{-6}$, OR 2.36), and also showed association in the Prion+ vs Prion– analysis ($p = 1.08 \times 10^{-4}$, OR 2.49). These SNPs span the 5' end of the non-protein-coding HLA complex group 26 (*HCG26*) gene.

The most significant association in the mood disorder analyses was in the Prion+ vs Controls analysis for a SNP lying within the acylglycerol kinase (*AGK*) gene (rs7789850; $p = 2.16 \times 10^{-6}$, OR 4.6). *AGK* encodes a mitochondrial membrane protein involved in lipid and glycerolipid metabolism.

Closely associated SNPs rs1219407 and rs478903 which showed the strongest association with Mood Disorder in the Prion+ vs Prion- analysis lie about 200 kilobases from the *SORL1* gene. rs6867820, another SNP that showed association with Mood Disorder, lies 55 kilobases from the SNCAIP gene, which encodes Synphilin-1.

Table 20 summarises the association results for the candidate SNPs identified from previously published GWAS in psychiatric conditions (schizophrenia[195], bipolar affective disorder [196-198] and major depressive disorder [199] and in prion disease[194]. The SNP implicated by the prion disease GWAS is *PRNP* codon 129. As expected, this showed some association in all of the analyses comparing prion disease patients with controls, and this was particularly strong in the analysis of patients with BPS at onset vs controls: patients with variant CJD are over-represented in this group and codon 129 is known to be very strongly associated with this disease sub-group. None of the candidate SNPs identified from GWAS in psychiatric conditions showed evidence of association with the

behavioural/psychiatric phenotypes in prion disease in our analysis, in light of the number of tests being performed.

Candidate SNPs identified from other GWAS							Psychotic symptoms				Mood disorder				BPS at onset			
							Prion+ vs Controls		Prion+ vs Prion-		Prion+ vs Controls		Prion+ vs Prion-		Prion+ vs Controls		Prion+ vs Prion-	
SNP	Chr	BP	MAF	Closest gene (RefSeq)	Identified in GWAS for…	Reference	GWAS p-value	OR	GWAS p-value	OR	GWAS p-value	OR	GWAS p-value	OR	GWAS p-value	OR	GWAS p-value	OR
SNPs implicated in GWAS for psychiatric conditions																		
rs6932590	6	27356910	0.27	*PRSS16*	Schizophrenia	Stefansson et al	0.35	0.82	0.86	0.96	0.23	0.79	0.64	0.89	0.67	0.93	0.42	1.23
rs3131296	6	32280971	0.15	*NOTCH4*	Schizophrenia	Stefansson et al	0.01	0.43	0.4	0.71	0.02	0.49	0.68	0.85	3.42E-03	0.44	0.3	0.67
rs9960767	18	51306000	0.05	*TCF4*	Schizophrenia	Stefansson et al	0.54	0.75	0.32	0.59	0.8	0.91	0.55	0.74	0.93	0.97	0.65	0.81
rs12807809	11	124111495	0.17	*NRGN*	Schizophrenia	Stefansson et al	0.24	1.3	0.59	1.17	0.01	1.66	0.02	1.95	0.88	0.97	0.16	0.68
rs13211507	6	28365356	0.12	*PGBD1*	Schizophrenia	Stefansson et al	0.09	0.56	0.4	0.71	0.14	0.65	0.68	0.85	0.58	0.87	0.21	1.64
rs6913660	6	27199404	0.19	*HIST1H2BJ*	Schizophrenia	Stefansson et al	0.17	0.7	0.18	0.66	0.63	0.9	0.87	0.96	0.69	0.92	0.99	1.01
rs1938526	10	61970389	0.06	*ANK3*	BPAD	Ferreira et al	0.95	1.02	0.49	0.74	0.79	1.09	0.59	0.8	0.37	1.29	0.87	1.07
rs1064395	19	19222735	0.16	*NCAN*	BPAD	Cichon et al	0.31	1.26	0.45	1.25	0.29	1.25	0.42	1.26	0.16	1.31	0.18	1.48
rs2251219	3	52559827	0.39	*PBRM1*	BPAD/MDD	McMahon et al	0.2	1.26	0.27	1.29	0.28	1.2	0.38	1.22	0.1	1.29	0.09	1.46
rs2289247	3	52702297	0.41	*GNL3*	BPAD/MDD	McMahon et al	0.04	1.44	0.04	1.58	0.17	1.26	0.23	1.3	0.07	1.32	0.06	1.51
rs4238010	12	3988578	0.13	*CCND2*	MDD	Muglia et al	0.18	1.37	0.88	1.05	0.86	0.96	0.07	0.58	0.48	1.16	0.33	0.76
SNP implicated in GWAS for prion disease (*PRNP* codon 129)																		
rs1799990	20	5E+06	0.35	*PRNP*	Prion disease	Mead et al	0.02	0.51	0.58	0.83	3.40E-03	0.46	0.25	0.68	2.70E-04	0.46	0.07	0.56

Table 20. Summary of association results from our analysis for candidate single nucleotide polymorphisms identified from previously published genome-wide association studies of psychiatric conditions (schizophrenia, bipolar affective disorder and major depressive disorder) and prion disease. BPS = behavioural disturbance or psychiatric symptoms; SNP = single-nucleotide polymorphism; Chr = chromosome; BP = base pair position; MAF = minor allele frequency; GWAS = genome-wide association study; OR = odds ratio for minor allele; Prion+ = prion disease cases with phenotype; Prion- = prion disease cases without phenotype; BPAD = bipolar affective disorder; MDD = major depressive disorder.

Discussion

This work represents a comprehensive study of the behavioural and psychiatric manifestations of the human prion diseases, using clinical data from a large prospective study carried out by a single team. The findings expand on those of previous smaller studies, such as that from the Mayo clinic based on retrospective casenote review of 126 sCJD cases seen over 25 years[174], and studies of vCJD by the UK National CJD Research and Surveillance Unit[102,173].

Behavioural and psychiatric features were common in all types of prion disease studied, and there is significant heterogeneity both within and between disease types. This should inform clinical care as these symptoms represent a significant burden of morbidity for patients and carers, but may easily go unreported and unrecognized unless clinicians are vigilant for them.

Knowledge of the natural history of these symptoms is also valuable in guiding discussions with patients and families, and in making decisions about care. For example, it is clear from these results that psychotic symptoms tend to emerge as the diseases progress, and this should be borne in mind by clinicians seeing patients in the early stages of disease.

Most clinical features of prion disease, which result from the irreversible loss of brain functions, do not recover once they have appeared. In contrast, this study shows that BPS fluctuate and improve in a substantial proportion of patients. This suggests that improving these symptoms with pharmacological or other interventions is feasible, but also that improvement may occur without any specific intervention. This is particularly important to bear in mind when interpreting apparent benefit from treatment, both in clinical practice and in therapeutic research.

The differential diagnosis of neurodegenerative diseases relies largely on the recognition of characteristic patterns of clinical features (although biochemical and imaging techniques are making

an increasing contribution). Awareness of the prevalence and phenomenology of BPS in prion disease should thereby assist with diagnosis. For example, visual hallucinations are well-recognized as features of Dementia with Lewy Bodies (and are included in the consensus diagnostic criteria for that disease[201]), but this study shows that they are also common in prion disease.

Poor agreement of BPRS

In addition to the BPRS, a wide range of different clinical assessments of neurological and cognitive function carried out in the PRION-1 trial were video-taped. An analysis of the inter-rater concordance between the neurologist carrying out these assessments and a senior neurologist reviewing the video tape has been published by Carswell et al [202]. The BPRS was not included. This showed that, apart from assessments relying largely on non-visual, non-auditory information (such as assessment of muscle power, or deep tendon reflexes) agreement was generally excellent, and concluded that video-taped assessments could be usefully incorporated into the design of clinical trials for prion diseases and other similar neurocognitive conditions.

This is in strong contrast to the findings presented above for BPRS, where agreement was generally very poor and the neurologist making the assessment face-to-face tended to give substantially higher scores (implying more severe symptomatology) than a senior psychiatrist scoring from video. Several factors may be contributing to this. The clinicians carrying out the PRION-1 assessments were neurologists and will have had less experience and training in the assessment of psychiatric symptoms, compared with the assessment of neurological and cognitive functions. Even using a structured rating scale such as the BPRS, the assessment of these psychiatric symptoms may be challenging. This is particularly true in a population of patients with co-existing physical and cognitive deficits, which is not the population for whom the BPRS was designed. For example, the BPRS includes subcomponents assessing “somatic concern” and “disorientation/inappropriate affect”. Deciding how to score these in patients with multiple disabling physical symptoms and

significant cognitive impairment is difficult, and open to variable interpretation on the part of the individual assessor. The results suggest that while the assessing neurologists often erred on the side of scoring these as present, the psychiatrist scored them as absent unless they were clearly present *and* were not accounted for by physical or cognitive deficits. It is also possible that the assessing neurologists' scores were influenced by observations made during other parts of their clinical assessment, rather than purely from the responses given to the structured BPRS questions which were available to the psychiatrist. For example, the same investigator will have taken a general clinical history of the patient's recent progress and symptoms from the patient and their relatives and carers during their visit, and if this included reports of psychiatric symptoms this may have influenced their scoring of the BPRS even if these were not reported by the patient themselves during the structured interview.

Overall, these results raise substantial concerns about the validity of the BPRS as a tool for assessing behavioural and psychiatric symptoms in this patient population, particularly when administered by non-psychiatrist clinicians. The most significant problem identified is that the Scale could not be completed at all in the large majority of patients, as it relies on the patient themselves co-operating with a lengthy structured interview. Several rating scales have been developed and validated specifically for assessing BPS in patients with dementia, such as the Neuropsychiatric Inventory developed by Cummings[203], although there has been no published validation of any of these in a prion disease population. These are usually administered by interview of a closely-involved carer, and are designed to be more robust to interference from cognitive or physical symptoms.

BPS are key clinical features of the prion diseases and their assessment should be part of any future clinical trial, although they are not likely to form part of a primary outcome measure assessing disease progression as this study has shown that they fluctuate through the disease course (this is discussed in detail in chapter 6). Validation of a suitable clinical rating scale for inclusion in trial

protocols and amenable to statistical analysis should therefore follow on from the work presented here. To this end, the Neuropsychiatric Inventory has now been adopted as part of the routine assessment battery of the Cohort study, based on the results of the study presented here.

Challenges in studying BPS in prion disease

In comparison with the more common dementias, there are a number of particular challenges to studying BPS in prion disease. Prion diseases are rare, with only around 1 or 2 new cases per million population each year. To study large numbers of patients it is therefore necessary to enrol patients from a wide geographical area over a long period of time. Patients deteriorate very rapidly in the majority of cases (particularly those with sCJD). Patients are often at an advanced stage of disease when the diagnosis is made, making it impossible to study a large part of the disease course prospectively. Prospective study of rapidly progressing cases is also challenging, as even very frequent follow-up assessments may fail to capture substantial clinical change. Any study of the phenomenology of psychiatric symptoms relies on patients describing their internal experiences. Expressive language dysfunction is recognized as a hallmark of prion disease and often occurs as one of the earliest cognitive changes. Patients with prion disease thereby lose the ability to describe their internal experience of any psychiatric symptoms, leaving carers, clinicians and researchers to interpret behaviours as evidence of these. For example, a wide-eyed, fearful expression without obvious cause in a mute patient may be the result of visual hallucination, but it is not possible to confirm this or to establish the phenomenology of the symptom.

In this study systematically, prospectively collected data was prioritized over retrospective review of clinical casenotes whenever possible, in order to minimize bias and allow direct and fair comparison of patients. However, as a consequence, the prevalence of BPS may be somewhat underestimated, particularly in more rapidly progressing cases where prospective study is particularly challenging.As no symptoms from the agitated (non-psychotic) symptom group were rated routinely at all

assessments (while hallucinations and depressive symptoms were), the agitated group is likely to be relatively underestimated.

To allow the above analysis to be performed it was necessary to classify the diverse symptoms described by patients, carers and health professionals into a manageable number of symptom groups. Three were used: psychotic symptoms, agitated (non-psychotic) symptoms, and mood disorder. This division performed well in terms of providing a simple system that encompassed almost all symptoms noted in the study, and allowing the data to be presented and analyzed in a relatively straightforward way. It also fits well with our overall clinical experience of this patient group, and with previously published work using factor analysis in other neurodegenerative diseases. However, it is important to note that any attempt to group complex disease manifestations in this way will inevitably be an over-simplification, and will lead to symptoms that are biologically distinct being grouped together. For example, the pathophysiology underlying a depressive illness in a person with very early disease may have little in common with that underlying emotional lability in a more advanced patient with frontal lobe dysfunction. This will make it more difficult to demonstrate biological associations with these symptom groups.

In the logistic regression analyses strong associations were identified between several biological/clinical variables and the psychotic symptom group, but not mood disorder or agitated (non-psychotic) symptoms. This might be explained by these latter groups being more biologically heterogeneous, while the psychotic symptom group, which was dominated by a single well-defined symptom (visual hallucinosis), is likely to be more homogeneous.

Although this does represent a limitation of this study, more sophisticated sub-grouping of symptoms would not be possible to achieve from the available data, and would ideally be based on detailed phenomenological descriptions of symptoms that are often impossible to obtain in these

patients.

Pharmacological treatment of BPS in prion disease

The drug treatments commonly used for BPS in this patient population (antipsychotics and benzodiazepines for agitated and psychotic symptoms, antidepressants for mood disorder) are reported to have a clinical benefit in a proportion of patients. Limitations apply to any observational treatment study and must certainly be taken into account when interpreting these results, particularly in light of the observation from this study of spontaneous improvement in these symptoms in a substantial proportion of patients. The impact of non-pharmacological measures introduced alongside drug treatment, or spontaneous improvement in symptoms may easily be misattributed to a recently prescribed drug. The study of treatment was unblinded and uncontrolled, and relied on retrospective review of case notes for evidence of benefit and adverse events. Ideally this study should be followed up with more robust clinical trials of the promising symptomatic treatments that it has identified.

The use of antipsychotic medication in patients with dementia has been shown to be associated with a significant increase in mortality, partly explained by an excess of cerebrovascular events[204,205]. It is important to consider whether the same risks exist in patients with prion disease. Most antipsychotic treatment courses in the patients studied were relatively short, principally because of the very rapid progression of disease. However there are an important minority (mostly with IPD) where progression is slower, and treatment courses longer. Patients with prion disease are also younger than the dementia population in whom the risk has been demonstrated (mean age of patients treated with antipsychotics in this study was 51.2 years). Baseline risk of cerebrovascular disease is therefore likely to be lower, and it seems likely that this would reduce the risk of this type of adverse event. However, this study is certainly not powered to demonstrate a small change against the very high background mortality of a prion disease population, so a similar risk cannot be

ruled out.

Overall, it seems likely that any risk associated with antipsychotic treatment will have less clinical impact in the context of rapidly progressive prion disease than in other slowly progressive dementias for the reasons described. Nevertheless, clinicians should keep these theoretical risks in mind when making decisions with individual patients, particularly those with more slowly progressive disease types.

This study did not allow any systematic assessment of non-pharmacological interventions for BPS in prion disease. A major challenge to the study of non-pharmacological interventions in dementia has been a lack of standardization making direct comparison difficult[206,207], and this is particularly true in a group of patients studied in a wide variety of clinical contexts over a long period of time.

Nevertheless, from the casenotes reviewed and the clinical experience of the National Prion Clinic team more generally, it is clear that non-pharmacological interventions can play a vital role in caring for patients with these symptoms. The involvement of suitably trained and experienced carers, and the care of patients in appropriate, calm and containing settings, seem to be particularly valuable.

Biological mechanisms underlying BPS in prion disease

Studying the remarkable heterogeneity of prion disease can provide valuable insights into the underlying disease process: the strong disease-modifying effect of the codon 129 polymorphism of PRNP is perhaps the defining example of this.

There were a number of significant associations in the logistic regression models for factors predicting the presence of BPS. The association of psychotic symptoms with particular disease types

and codon 129 genotypes, and of BPS at onset with diagnosis of vCJD suggests that the propagating prion strain is a dominant factor in causing these symptoms.

The presence of hallucinations was strongly associated with MRC-PDRS, the measure of functional decline, whereas the presence of depressive symptoms was not. This may suggest that hallucinations are more closely and directly related to the underlying disease process than depressive symptoms. In the absence of organic brain disease, depressive symptoms are much more common than hallucinations, and a proportion of those recorded in this study may represent a secondary, "normal" reaction (i.e. mediated by brain areas unaffected by the organic pathology).

Interestingly, in the logistic regression models restricted to sCJD, age at onset was an independent predictor of the presence of BPS at onset. This raises the possibility that the presence of psychiatric symptoms at onset may *to some extent* be a feature of prion disease in younger people, as opposed to being primarily a feature of the vCJD prion strain. In a study using pooled data from a large number of published clinical cases, Appleby and colleagues also concluded that younger sCJD patients were more likely to present with particular symptoms, including affective symptoms and behavioural disturbance[176].

As this clinical feature is currently used as a criterion for diagnosing vCJD[105] this is an interesting possibility: if older vCJD patients were less likely to exhibit this feature, it would be more difficult to distinguish them from the more numerous sCJD cases. This is of particular interest in the context of the evolving vCJD epidemic in the UK, where future patients presenting with longer incubation periods will tend to be older.

The exploratory genome-wide association studies looked for genetic loci associated with three behavioural/psychiatric phenotypes in the context of prion disease. As the number of patients

included was relatively small these studies are only powered to detect very strongly associated SNPs, and the lack of any reaching genome-wide significance certainly does not rule out the possibility that there are genetic modifiers of these phenotypes. Hypotheses are proposed here for follow up in other cohorts of prion disease or related neurodegenerative diseases[208]. Reviewing the most strongly associated SNPs from this study identified several loci that may be of interest as they have previously been implicated in genetic studies of psychiatric or other neurodegenerative disorders.

The strongest evidence of association was for a SNP lying within the ANK3 gene, in the analysis of psychiatric features at onset. There is strong evidence for association of other SNPs within ANK3 with bipolar disorder from a large collaborative genome-wide association study[196], although the genotyped SNPs reported in that study showed no evidence of association in this analysis. Ankyrin 3 is thought to participate in the maintenance/targeting of ion channels and cell adhesion molecules at the nodes of Ranvier and initial axon segments[209]. The SNPs on chromosome 6 that were found to be trending towards association with psychotic features in prion disease lie within a chromosomal region (6p21.3-22.1) that has been implicated in genetic studies of susceptibility to both schizophrenia and bipolar disorder, without a single locus emerging as the dominant source of association[195,210]. It is possible that this apparent inconsistency is related to the unusual patterns of recombination around the Major Histocompatibility Complex which also lies in this region[211]. 4 of the top 5 candidate genes for schizophrenia risk listed on SzGene.org lie within this region (www.szgene.org). Although conclusions drawn from this study must be conservative because of the small patient numbers and the failure of any SNPs to reach genome-wide significance, this suggestion of an overlap with the genetics of “primary” psychiatric disorders is intriguing.

Large genome wide association studies using both SNPs and copy number variants (CNVs) have previously identified genetic loci with an effect across multiple psychiatric and neurodevelopmental conditions, with the overlap between risk loci for schizophrenia and bipolar disorder being

particularly well established [212-216]. This has been interpreted as evidence that there are genetic risk factors for psychosis that are not disease-specific [214]. It is conceivable that a genetic factor conferring susceptibility to schizophrenia or bipolar disorder might also increase the likelihood of an individual developing psychotic features in the context of neurodegenerative disease. Our results provide an intriguing hint that this may be the case in prion disease, and we wish to encourage further investigation of this hypothesis.

The findings of this study should help to inform the diagnosis and care of patients with prion disease, and also prompt further research seeking to replicate and expand on these findings in independent prion disease cohorts. Comparison with similar studies in other diseases may also shed a revealing light on the mechanisms underlying these important and complex manifestations of neurodegeneration.

CHAPTER 6. DEVELOPING A NEW CLINICAL RATING SCALE FOR USE IN PRION DISEASE CLINICAL TRIALS

Introduction

General introduction and aims

A principal, over-arching aim of the whole field of prion disease research (as well as neurodegenerative disease research more widely) is to find disease-modifying therapeutic agents that will be of benefit to patients affected by these conditions. Up to now, no agent has been shown conclusively to alter the progression of any prion disease in patients[217]. For this goal to be achieved, two major phases must be completed: the preclinical stages, whereby potential therapeutic agents are identified and tested in the laboratory setting using *in vitro* and/or *in vivo* experiments, until there is sufficient evidence to support a potential therapeutic effect for patients; and the clinical stages, whereby the agent goes through a series of clinical trial phases to assess its safety, and finally its efficacy.

The preclinical phase of therapeutic research in prion disease has seen considerable success, with several classes of agents showing promise in both *in vitro* and *in vivo* models[217].

The transmissibility of prion disease both within and between mammalian species, leading to fatal neurodegeneration that faithfully reproduces the clinicopathological features of the human disease, provides a uniquely robust opportunity for laboratory validation of experimental therapeutics for a human neurodegenerative disease. As a result of these animal models, unparalleled in neurodegenerative disease, and rapidly advancing understanding of the molecular pathogenesis of prion disease, development of putative therapeutic agents for use in human trials (both small molecules and monoclonal antibodies that bind to PrP^{C}) has now reached an advanced stage[22,23,132,218-221].

However, if the promise of these agents is to be realised, it is vital that the methodology used in clinical trials is optimised to maximise the chances that any real beneficial effect will be demonstrated. Even a highly effective therapeutic agent may fail to produce conclusive results without an optimal trial design.

Previous clinical trials and other treatment studies in human prion disease

In planning how to optimise the design of future trials it is useful to review therapeutic studies that have been performed in the past, and consider the relative merits and weaknesses of the methods used. A systematic review of clinical studies in prion disease carried out in 2008[222] found only one double-blind randomised controlled trial (using the analgesic agent flupertine), but also reviewed a number of small case series and case reports that included some observational account of attempted treatment. The only reports that included direct comparison with untreated patients were the flupertine trial, and case series related to use of quinacrine and amantadine. Agents used in the non-comparative observational reports included antiepileptic agents (levetiracetam, phenytoin, topiramate), an antifungal agent (amphotericin), antidepressant agents (imipramine, venlafaxine), interferon, vidarabine and a variety of antioxidants and vitamin supplements. Although in a few cases some impression of transient improvement was reported, there was no dramatic effect on the patients expected disease progression or outcome with any of these treatments, and it is not possible to draw any meaningful conclusions from these isolated case reports or small case series. The review also identified several reports related to treatment of patients with the anticoagulant pentosan polysulphate; this has been examined in more detail elsewhere and is discussed below.

Experience with pentosan polysulphate in the UK and Japan

Pentosan polysulphate is a polyanion molecule chemically related to heparin, which is used as an orally administered medication for interstitial cystitis[223]. It has been found to reduce PrP^{Sc} aggregation *in vitro*, and also to prolong incubation times in some animal models[224]. When administered orally or intravenously, it has very poor bioavailability in the central nervous system, so its use when administered directly into the cerebral ventricles via a surgically placed catheter and pump has been investigated. Most of the published experience of this treatment comes from Japan, where a lot of the relevant preclinical research was carried out; and from the UK, where the treatment has been used particularly in a number of young patients with vCJD. In the UK, a formal observational study of seven patients receiving the treatment was carried out and published by Professor Ian Bone and colleagues[224]. In Japan, one "clinical trial" of 11 treated patients (with no comparison group) has been published[225]. The conclusion from these studies has been that there is no convincing evidence that pentosan polysulphate slows the rate with which patients progress to a state of very severe disability, but that there have been a number of patients who have survived for very prolonged periods (longer than any previously published patients with the same disease type), *in* such a state of severe disability while receiving the treatment. There are potential confounders in that those patients and families willing to pursue this invasive treatment option may well be more likely to pursue other potentially life-prolonging interventions such as aggressive treatment of infections in the late stages of disease, insertion of artificial feeding tubes, or even artificial ventilation, which others might feel to be inappropriate. However, it is not clear whether the long-survival of some pentosan polysulphate treated cases *have* in fact received interventions of this type, and it remains a possibility that the treatment does indeed have a real treatment effect.

In terms of lessons for future trial design, the experience with pentosan polysulphate illustrates very clearly the limitations of survival as an outcome measure. Our objective is to find a treatment that alters the progression of the disease in order to keep patients well and able to function for as long as

possible, not one that keeps patients alive after they have reached a state of akinetic mutism. Relying on survival as an outcome measure means that we may not be able to distinguish between the two.

German trial of flupertine

A trial of flupertine for human prion disease was carried out in Germany, with patients recruited from those referred to the German National CJD Surveillance Unit between 1997 and 2001[226]. The investigators acknowledged that the usefulness of survival as an outcome measure was limited, and therefore used cognitive assessment (primarily using the change in score on the ADAS-Cog scale between the initial assessment and the best score achieved during follow-up) as their primary outcome measure. To allow this, the inclusion criteria for the trial specified that patients should be able to achieve an arbitrarily set minimum score on the cognitive tests being used to be eligible for enrolment in the trial. The trial found a statistically significant difference between the treatment and placebo groups, with less deterioration in performance on the ADAS-Cog in the patients receiving flupertine. The magnitude of this difference (12 points on the ADAS-Cog) was felt to be of clinical significance. There was no significant difference in survival between the two groups. From 682 patients assessed for eligibility for the trial, only 28 met the inclusion criteria and were randomised. These all presented as sporadic CJD, but 2 were unexpectedly found to have pathogenic *PRNP* mutations, altering the diagnosis to IPD. The reasons for exclusion of the other 654 patients were reported as "either other disease or not fulfilling dementia tests", but the relative contributions from these two criteria are not reported. Surveillance data from the German surveillance unit covering the same period have been published separately[41], and report annual rates of around 100 probable or definite sporadic CJD cases covering the same period, so it is likely that the large majority of patients were excluded based on inability to engage with or achieve the minimum score in the cognitive tests. This would be in keeping with experience from the PRION-1 trial where only 9% of enrolled sCJD patients were able to complete the ADAS-Cog at their initial

assessment[121]. As a result, the patients included in the flupertine trial were highly selected and represent a very skewed sample of CJD patients, with those having slowly progressive disease and relatively preserved language and cognitive function being over-represented. In keeping with this, the distribution of codon 129 genotypes in the study population was skewed strongly away from MM homozygotes, and PrP^{Sc} types away from those with type 1 PrP^{Sc}.

This trial represents a landmark in that it was the first randomised controlled trial to be performed in patients with prion disease, and demonstrated that this is possible. Although it achieved a positive and statistically significant result ($p = 0.02$), the small number of patients, who for the reasons described were taken from a small and skewed subgroup of the overall population of referred patients, combined with the remarkable heterogeneity of clinical progression in prion disease (as clearly demonstrated in the work presented in this chapter), do raise concerns that this may have been a chance finding. The natural history and variability of the ADAS-Cog as an outcome measure in this population had not been defined prior to the trial. The applicability of the results outside of the small minority of patients that were eligible is also a limitation of the study.

French/Italian trial of doxycycline

In January 2014, Haik et al reported a randomised, placebo-controlled trial of doxycycline as a treatment for prion disease[227]. This involved patients from both France and Italy, constituting the first international collaborative trial in prion disease. In total 121 patients were recruited from amongst 663 referrals. The proportion of referred patients being enrolled in the trial was much higher in Italy than in France, with the main reason given as failure to meet the inclusion criteria, suggesting that referral patterns are different between the two countries with fewer non-prion disease patients being referred in Italy. The primary outcome was survival, with a number of clinical, laboratory and imaging measures used as secondary measures, but not presented in the published report as only a small minority of patients were able to have these measures/investigations done.

The trial found no significant difference in survival between the treatment and placebo groups (and in fact was stopped prematurely on the basis of futility after an interim analysis showed no sign of efficacy). This was in contrast to two earlier observational reports suggesting that patients treated with doxycycline had longer disease durations when compared with historical untreated cases. The authors of the trial report acknowledge that these earlier observational reports may have been subject to selection bias, with patients that have a slower disease progression being more likely to receive treatment with doxycycline as they have not yet reached a very advanced stage of disease (when attempted treatment would often be seen as inappropriate) when they are diagnosed and offered the experimental treatment, but the historical "normal" data with which they were compared will include the majority of cases who progress very rapidly. This illustrates clearly the risks inherent in over-interpreting any non-randomised treatment study, as well as specific challenges with use of historical controls.

The trial did not involve any enrolment criteria aimed at excluding patients at advanced stages of disease, other than some trial centres (those in Italy) excluding patients that had had symptoms for more than 6 months. While increasing the numbers of patients eligible and the applicability of results, it is likely that patients at very advanced stages of disease will not be able to benefit from any treatment and that their inclusion will therefore lead to trials underestimating the efficacy of a treatment. There are also ethical considerations in treating patients with a very high burden of neurological damage with treatments that may prolong survival but are far less likely to lead to any meaningful recovery of function. The ideal solution to this problem is to achieve very early diagnosis in all cases so that they can be enrolled at a stage when they have only mild disability. Although improving early diagnosis must be a priority, the speed of progression of sCJD, combined with its rarity, make it likely that many patients will continue to be diagnosed at an advanced stage of disease. It is therefore necessary to consider this issue in trial design, and consider ruling out individuals with very advanced disease. As the doxycycline trial illustrates, time-based criteria (e.g.

months from onset) are not useful for dividing prion disease patients into stages of disease progression because of the marked variability in rate of decline, so criteria based on a functional assessment would be more useful.

American trial of quinacrine

Based on the encouraging preclinical evidence regarding quinacrine's *in vitro* inhibition of prion replication discussed above in relation to the PRION-1 trial, a clinical research group led by Michael Geschwind at the University of California San Francisco also planned and carried out a clinical trial of quinacrine[228]. This enrolled patients with sCJD from 2005 to 2009, and did include criteria to exclude very severely impaired patients: patients had to be able to swallow and follow simple one-step commands to be eligible for the trial. From 425 patients referred for consideration, 69 were evaluated by the trial team, and 54 were randomized (although 3 subsequently found to have a *PRNP* mutation were not included in the analysis). The study design involved randomization to treatment with either quinacrine or placebo for a period of two months, after which patients and/or carers could elect to receive open-label quinacrine if desired. The primary outcome was survival over the course of the two month randomized treatment period. A range of secondary outcome measures including functional and cognitive rating scales were also used (including several which were used in PRION-1 and are discussed in more detail below). This design, and the sample size/power calculation used were based on experience from the same research group's observation of sCJD patients that chose to receive open label "compassionate treatment" with quinacrine compared with those that did not choose to receive this treatment, which suggested that average survival was increased from 0.9 months to 3.2 months in those receiving quinacrine, and that randomizing 30 patients to each group in a randomized trial would provide 80% power to demonstrate a doubling in survival rate over 2 months. The primary outcome analysis showed no survival benefit in the quinacrine group (and in fact a non-significant trend towards increased survival in the placebo group). Analysis of secondary outcomes suggested that the quinacrine group

declined less in scores on two functional scales (the modified Rankin scale and the Clinician's Dementia Rating (CDR)). However, given that ten secondary outcome measures were used, the levels of significance for these differences (p values of 0.03 and 0.01 respectively) are not strong, and the effect sizes are difficult to interpret: change in mean values are quoted but these have little meaning for categorical rating scales such as these, particularly when the range of scores in the study group are extremely wide. For the statistical comparison, the authors appropriately used non-parametric methods based on rank transformation of the scores (Quade's rank analysis of covariance), but only reported the p values generated by these tests. There were also some baseline differences in the groups, including somewhat lower modified Rankin scores in the Quinacrine group (suggesting that they were more functionally impaired), and some difference in distribution of codon 129 genotypes, which did not reach statistical significance in itself but nevertheless has the potential to substantially affect results as this is such a strong modifier of disease progression.

This trial provides a number of very valuable insights into the challenges of planning prion disease trials. Most strikingly, there was a great disparity between the conclusions taken from the preliminary observational study on which the trial design was based and the results of the trial itself. As seen in the PRION-1 trial[120], the choice made by prion disease patients and/or their carers regarding whether to receive a potentially life-prolonging treatment is very strongly confounded with respect to other factors affecting rate of disease progression and survival. Patients with more slowly progressive disease, who are less severely impaired at the time of making the decision, are more likely to choose to receive treatment. Although this can be taken into account by stratifying groups (e.g. by level of functional impairment at enrolment as was done in the PRION-1 analysis), there is still a chance that other, unmeasured factors will confound the results. Geschwind's quinacrine trial clearly illustrates the value of randomization in eliminating what was an apparently strong, but in fact probably spurious, treatment effect in the observational study. As decisions about trial design, including sample size, were made based on the over-estimate of treatment effect from

the observational study, it is likely that the trial was not powered to demonstrate a more modest effect that might exist. It also illustrates again the lack of an ideal outcome measure for use in this sort of trial: the authors acknowledge that functional and/or cognitive scales may have a role, and there was very wide variability in estimated difference in survival between groups of this size.

Building on PRION-1 to plan towards future clinical trials

The PRION-1 trial demonstrated that a large prion disease clinical trial in the UK is feasible, but was limited by the lack of a validated measure of clinical progression[120]. Anticipating this issue, the trial protocol included a variety of existing rating scales designed to probe neurological, cognitive, psychiatric and general functional status. Analysis of the performance of eight of these scales in PRION-1 in terms of validity, practicality and statistical power in simulated clinical trials supported the use of functionally-orientated measures relative to global, neurological, cognitive or psychiatric scales[121]. However, a number of unresolved issues remained: no single scale captured progression across the full range of functional domains affected by any of the individual categories of prion disease; patients could not be visited frequently enough to capture the very rapid decline which is typical of Creutzfeldt-Jakob disease (CJD); floor effects (large numbers of subjects with the worst possible score) were observed in all scales except the Glasgow Coma Score (GCS); and the patient sample was too small to allow reliable analysis of important aetiological or severity-stratified subgroups[229].

Some other specific challenges to the design of prion disease trials are also relevant. The heterogeneity of the diseases increases the natural variation in any outcome measure used, which will tend to reduce statistical power, so that greater numbers of subjects are needed for a beneficial effect to be successfully proven. In combination with the rarity of the diseases, this represents a substantial challenge as it may be very difficult to recruit sufficient numbers in a feasible timescale for a trial.

The majority of patients are diagnosed (and enrolled into research studies) at an advanced stage of disease, and initiating a putative disease-modifying treatment at this stage may be futile, or may be felt to be clinically inappropriate. Improving early diagnosis and referral is therefore another key parallel goal to drug development.

The project presented in this chapter aimed to build on the experience from the PRION-1 trial with the large amount of additional clinical data now available from the Cohort study, to develop a single, functionally-orientated and validated outcome measure, tailored to the particular demands of a prion disease clinical trial. As a starting point elements of three rating scales that showed relative strengths in the PRION-1 analysis and are well established in other neurological settings were modified and combined: the Barthel Activities of Daily Living Index (Barthel)[190], the Clinical Dementia Rating Sum of Boxes (CDR)[191], and the GCS[192]. The process by which this initial scale was established and then refined is described in detail below.

In developing an optimal outcome measure, statistical and methodological concerns must be balanced with clinical and human considerations. It should measure something that is important to patients and carers, so that a statistically defined benefit in a treatment trial will translate into a meaningful, worthwhile benefit for patients. To achieve this, qualitative data was collected from patients' relatives and carers on which manifestations of prion disease were of greatest concern to them, so that these could be reflected in the domains assessed by the scale.

In the PRION-1 trial many patients with very rapidly progressive disease were only assessed on one occasion, limiting the amount that they can contribute to the statistical power of a clinical trial, particularly if its outcome is the rate of change in a measure of severity[121]. To address this, the reliability of the novel scale used over the telephone was assessed, which would allow much higher

assessment frequency. The scale's reliability when administered by different healthcare professionals (doctors and nurses) was also assessed, which again would allow more frequent assessment and more flexibility in planning trial follow-up protocols.

Introduction to Rasch Analysis [230]

To validate a novel rating scale that consists of a number of ordered categorical items, several requirements must be met if the scale is to be used to generate meaningful summed total scores, change scores, and have parametric statistics applied to it (all of which would be advantageous if not essential in a clinical trial outcome measure). Rasch analysis is a method for demonstrating that these requirements are met, as well as exploring and refining other characteristics of a rating scale. It involves testing the fit of a dataset generated using a rating scale to a mathematical measurement model: the Rasch model. If problems are identified that compromise the fit, adjustments can be made to the scale to address these, and the analysis repeated using the modified dataset. In this way the scale can be improved through an iterative process, aiming to find a version that fits the Rasch model well.

To understand the Rasch model and Rasch analysis, it is helpful to consider an *ideal* rating scale, which would satisfy all of the requirements of Rasch analysis. The scale should consist of a range of items, ranging from "easy" (where most individuals in the test population achieve the threshold for the item) to "hard" (where very few do). The "difficulty" of an item is termed its *Item Location*. If an individual reaches the threshold for a "hard" item, then it should be *likely* that they will also reach the threshold for all "easier" items in the scale. The likelihood of an individual reaching the threshold for any given item within the scale should be predictable by their overall score on the scale (or their *Person Location*), representing the single construct that the scale is measuring, or the 'latent trait' in the model: this is the assumption of unidimensionality, which must be satisfied for a fit to the Rasch model to be satisfactorily achieved. This aspect of the Rasch model can be seen as a

probabilistic form of Guttman scaling[230]: a perfect 'Guttman pattern' is achieved when there is a strict hierarchy of item difficulty, so that achievement of a certain item threshold implies that all easier items are *definitely* also achieved. Rasch analysis modifies this to allow that there should be a *high probability* that easier items are achieved. The degree to which a Guttman pattern is achieved gives an indication of the strictness of the hierarchy of item difficulty.

The spread of items across the range of "difficulty" should be appropriately targeted for the population in whom the scale will be used, ideally so that average overall score on the scale lies close to the numerical midpoint of the scale, and so that there is good coverage of the full range of "ability" seen in the population.

The way that each item within a scale behaves should be the same for all individuals, and groups of individuals, in the tested population: there should be no differential item functioning (DIF). For example, an item threshold should not be more difficult to achieve for men than for women, or for older patients than for younger patients. Rasch analysis includes systematic assessment for DIF.

Particular issues may arise for a scale including items that have multiple response categories (polytomous items): for example an item that scores 0 if a person cannot mobilise at all, 1 if they need help to mobilise and 2 if they can mobilise independently. The thresholds of the response categories should be appropriately ordered: in the example, this means that patients that can mobilise independently should generally achieve a higher overall score in the scale than those who need help to mobilise, who should achieve a higher score than those that are immobile. This is systematically assessed during Rasch analysis, and if anomalies are identified these can be dealt with (for example by combining 2 adjacent response categories into a single category, or even dropping a disordered item from the scale entirely).

Ideally, there should not be a strong dependency between items within the scale: the likelihood of a particular item threshold being reached should be determined by the overall scale score (Person Location) to a greater extent than it is determined by any other individual item within it. This may not be achieved if there is redundancy between items: for example if a patient's ability to walk with assistance, and a patient's ability to walk unaided were included in a scale as separate items, one would expect the response to one to be closely dependent on the other. If this is the case, it may be appropriate to combine items to form "super-items" and eliminate this problem of dependency: in this example this would produce the polytomous item from the previous example.

If a novel scale can be validated with Rasch analysis using the large dataset collected from the patients enrolled in PRION-1 and the Cohort study, we can be confident that the scale will have the appropriate statistical properties to be used in a clinical trial: it will be meaningful to consider a single sum total score as an overall measure of disease severity, to compare numerical change in score or rate of change in score between groups, and to use parametric statistics to analyse these data.

Integrating different approaches to scale development

Although the statistical approach to scale development exemplified by Rasch analysis is vitally important, it must be balanced with a clinical and pragmatic perspective on the design of a rating scale. It must be ensured that the scale continues to measure factors that are meaningful to patients and carers, that it includes assessment of the full range of different domains affected by the disease, that it remains practical and easy to use both for the researcher and for the subject, and that other aspects of its reliability and validity are maintained (e.g. inter-rater reliability). To do this it is essential to bear in mind the clinical context of the patients enrolled in these studies, and also of the practical, logistical aspects of the research studies themselves, so that these can inform decisions made at all stages of the process. The scale's performance relative to existing scales can

also be examined, to assess whether it overcomes some of their limitations, particularly those identified from previous analysis of the scales data from PRION-1[121].

Establishing a natural history data set

Together, PRION-1 and the National Prion Monitoring Cohort constitute the largest single prospective clinical study of prion disease that has been carried out. Once we have established a novel rating scale specifically tailored to capture the progression of prion disease in the large dataset from these studies, we have an opportunity to illustrate the natural history of these diseases in more detail than has previously been possible. This is of considerable interest in itself, but may also play a valuable role in the planning of future clinical trials. As discussed above, the recruitment of sufficient numbers of patients to achieve statistical power in a prion disease clinical trial over a feasible timescale is challenging, and the natural history dataset established from these studies may provide a historical control group for future trials, with major benefits for statistical power. Particular issues and challenges raised by this approach are discussed in detail below.

Methods

Patient referral, clinical diagnosis and enrolment

Although these were discussed in the General Introduction above, it is useful to revisit the particulars of the system of referral and enrolment here to set the scene for this project.

A national referral system for prion diseases was set up in the UK in 2004. UK neurologists were asked by the Chief Medical Officer to refer all patients with suspected prion disease jointly to the National CJD Research and Surveillance Unit (Edinburgh, UK) and to the NHS National Prion Clinic (London, UK). This enables epidemiological surveillance, provision of specialist clinical care and also participation in clinical research, including the PRION-1 trial (2001-2007)[120] and the National Prion Monitoring Cohort study (2008-2012) (referred to henceforth as simply "Cohort study"). See [120] for details of enrolment into the PRION-1 trial, which were similar to those described for the Cohort Study below.

The Cohort study began in October 2008, and aimed to enrol all symptomatic patients with prion disease in the UK thereafter. This includes all cases of probable or definite sporadic Creutzfeldt-Jakob disease (sCJD), variant CJD (vCJD), iatrogenic CJD (iCJD), and inherited prion disease (IPD). Also eligible for enrolment are asymptomatic individuals known to be at risk of inherited prion disease (tested asymptomatic gene mutation carriers or untested first degree relatives of those with a confirmed pathogenic *PRNP* mutations), or vCJD (recipients of implicated whole or leucodepleted blood transfusion notified by the Health Protection Agency). A small group of healthy controls were also recruited (friends or relatives without pathogenic *PRNP* mutations or other known risk factors). At risk individuals and healthy controls did not contribute to the development of rating scales.

Enrolment by National Prion Clinic staff took place at hospitals, nursing homes, hospices and patients' homes around the UK. Diagnoses and eligibility were reviewed by senior National Prion

Clinic clinicians (Professor John Collinge, Dr Simon Mead and/or Dr Peter Rudge) within a week of enrolment, or prior to enrolment if there was clinical uncertainty. Probable sCJD diagnosis was made according to WHO criteria, with the addition of brain MRI as a supportive investigation following recent recommendations (pathological signal change on FLAIR or diffusion weighted sequences in the basal ganglia, thalamus and/or ≥2 cortical regions)[34]. Patients not meeting criteria for probable sCJD could be enrolled if this was thought to be the most likely clinical diagnosis at review by an expert panel. Probable vCJD diagnosis was made according to WHO criteria[231]. Inherited prion disease was diagnosed by gene test. iCJD was diagnosed using sCJD criteria with a relevant history of exposure. If patients died during the study and underwent post mortem examination, or had relevant tissue biopsy (brain or tonsil) during life, the pathological results were used to confirm diagnosis.

A further 26 patients were recruited but not included in study participant totals or any analysis because an alternative diagnosis became more likely than prion disease during follow-up, due to their clinical course (e.g. persistent improvement) and/or clinical investigation results (e.g. presence of serum voltage-gated potassium channel complex antibodies, post-mortem results).

Consent and ethics

Informed consent was obtained directly from study participants if possible, or otherwise from relatives, carers or Independent Mental Capacity Advocates as appropriate. Ethical approval was obtained from the Scotland A Research Ethics Committee (Cohort) or the Eastern Research Ethics Committee (PRION-1).

Stratification and assessment schedule

Participants were stratified at enrolment to the Cohort according to their likely rate of disease progression based on diagnosis:

(i) Symptomatic patients with sCJD, vCJD, iCJD and forms of inherited prion disease likely to have rapid clinical progression (due to 4 octapeptide repeat insertion (4-OPRI), E200K, D178N, E211Q or V210I mutations)

(ii) Symptomatic inherited prion disease patients likely to have slow progression (those with 5-OPRI, 6-OPRI, P102L, P105L, Q212P, A117V or Y163X mutations)

(iii) At-risk and healthy control individuals

Patients in stratum (i) had face-to-face follow-up assessments initially every 6 to 8 weeks. If clinical progression proved to be slower than the expected significant deterioration over this interval (i.e. minimal or no change in MRC Scale over 6-8 weeks), study physicians could decide to reduce follow-up frequency for subsequent assessments. Patients in strata (ii) and (iii) had follow-up assessments every 6 to 12 months. The following rating scales were administered at study assessments: Barthel ADL index[190], CDR[191], GCS[192], Rankin[232], Mini Mental State Examination (MMSE)[233]. In September 2009 an initial combination scale was also introduced, as described below. In addition, a systematic clinical history, neurological examination and a short neuropsychological test battery were done at all assessments, unless precluded by the level of disease severity or patient fatigue. From May 2010 patients in stratum (i) also had telephone assessments every 1 to 2 weeks between follow-up assessments. These were discontinued if there was minimal change between consecutive assessments (usually at worst possible score).

Survey of carer priorities

The Barthel ADL Caregiver Interview Summary form was used to gather semi-quantitative data from relatives/carers on the aspects of prion disease of greatest importance to them and the patients in their daily lives. Up to four symptoms or impairments of greatest concern to them at the time of assessment were ranked in order of subjective importance. This was completed at 69 assessments, by carers of patients affected by different prion disease types. These data were supplemented by

discourse analysis of 10 in-depth structured interviews with carers, which was carried out my another member of the team (Liz Ford, Research Nurse) and is not reported here.

Development pathway for novel rating scale

Building on and extending the analysis of the pre-existing scales in PRION-1[121], an iterative approach was taken to develop and refine a novel scale. An initial version took into account the relative performance and limitations of different pre-existing scales and their subcomponents in the PRION-1 analysis, the reported priorities of patients and carers, and the pooled clinical experience of the National Prion Clinic medical, nursing and neuropsychology staff. This scale version was then administered at all Cohort assessments alongside the range of scales above (from September 2009), as well as over the telephone (from May 2010).

The performance of this scale version was assessed with respect to ease of use, ability to capture clinically evident decline, inter-rater reliability (assessed using Interclass Correlation Coefficient and Bland-Altman plot), reliability of use over the telephone, floor and ceiling effects. As it consisted of a combination of slightly modified subcomponents from existing scales used in PRION-1 and throughout the Cohort, a close approximation of the scale could be calculated for all assessments carried out prior to its introduction, allowing a larger number of patients and assessments to be included in sensitivity/additional analysis.

Rasch analysis

Rasch analysis was performed with support from statistician Zoe Fox, and Research Assistant Jessica Lowe. The analysis was performed using each individual's first two initial scale version assessments after September 2009, to ensure that the analysis was not unduly affected by a small minority of patients with many assessments, while still permitting testing the function of the scale over time. Rasch analysis was performed using Rasch Univariate Measurement Model (RUMM) 2030 software,

using the partial credit variant of the polytomous model[234]. The following recommended[230] iterative steps were taken to assess and optimise the fit of the scale to the Rasch model (1) Exploring item-person interactions (to examine the degree to which the Guttman pattern was achieved) and item-trait interactions using χ^2-based fit statistics; (2) Rescoring items that demonstrated disordered thresholds (i.e. an item's scoring categories do not progress in a logical order); (3) Removing the most poorly fitting items, or combining items into super-items where appropriate; (4) Examining local dependencies between items that could confound the assumption of unidimensionality of the scale, and dropping items when misfit was still apparent; (5) Investigating differential item functioning (DIF) for gender, age, time of assessment (first vs. second assessment), assessor (doctor or nurse administering scale) and mode of assessment (telephone versus face-to-face). A random sample of later follow-up assessments were also analysed to evaluate whether there was DIF for the selection of the follow-up visit to include in the main analysis; (6) Examining local dependencies using residual correlation matrices, and exploring unidimensionality of the scale using principal components analysis to select the highest positive and highest negative items followed by post-hoc t-tests on person locations determined by these items; (7) Reviewing summary fit statistics after all modifications to the scale were made. Cronbach's Alpha statistic was used to confirm reliability of the fit statistics. The final scale, termed the MRC Prion Disease Rating Scale (or simply the MRC Scale) was selected when satisfactory fit was achieved overall, no items showed poor fit and the scale was shown to be compatible with unidimensionality. To increase numbers, a sensitivity analysis was also performed using scores derived from the individual component scales (Barthel, CDR, GCS) prior to September 2009 (the novel "Tools" item was scored as missing) for individuals who did not have two scores post-September 2009.

Results

Patients

437 patients consecutively enrolled into the Cohort and/or PRION-1 studies (up to April 2012) were included in the analysis; 240 patients with sCJD, 25 with vCJD, 12 with iCJD, and 81 with symptomatic IPD together with 34 individuals at risk of IPD, 10 at risk of vCJD, and 35 healthy controls. 311 patients died during the period of study, 192 (62%) of these underwent post mortem examination, which confirmed the diagnosis of prion disease in all cases. 81 (19%) had diagnosis confirmed in life by gene test (all IPD) or tissue biopsy (some also with post mortem examination). 165 (38%) were diagnosed using clinical and investigation findings alone.

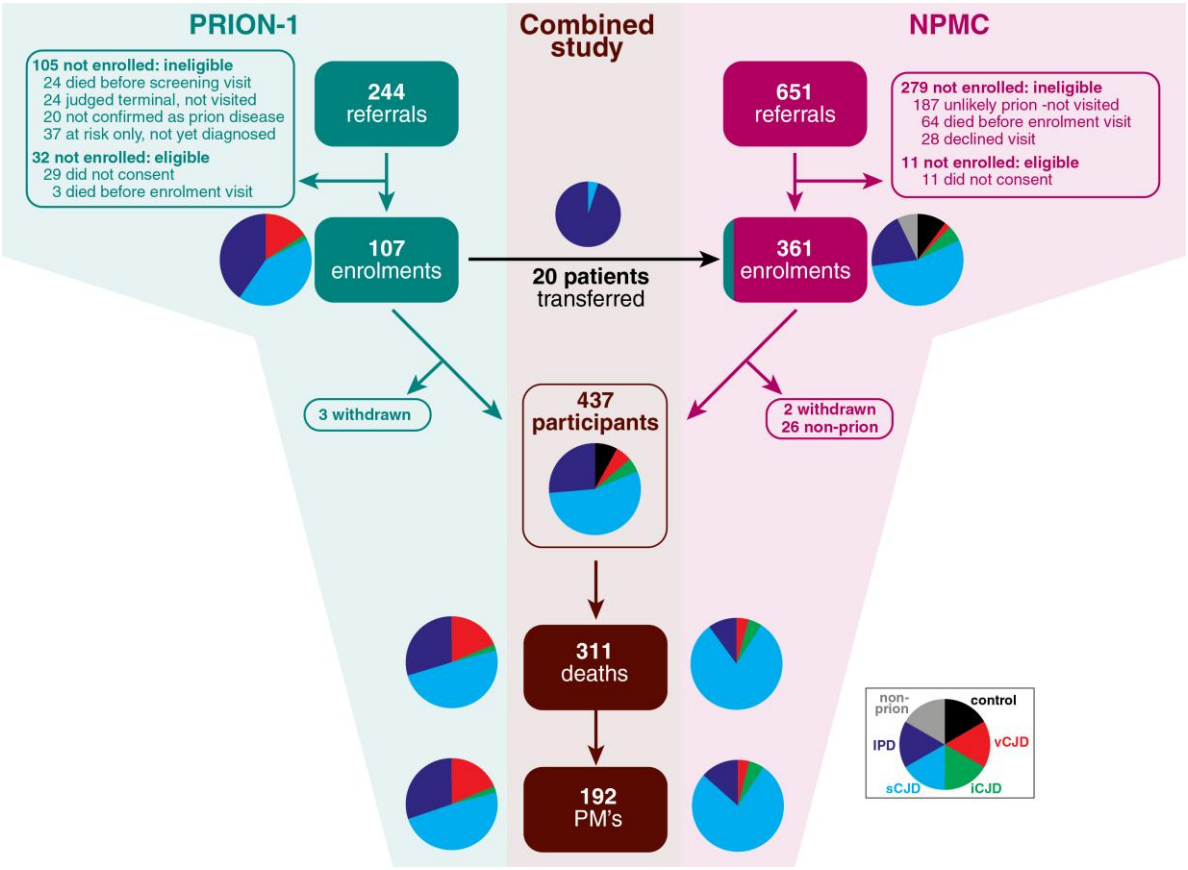


Figure 33. Study profile. iCJD = iatrogenic CJD; IPD = inherited prion disease; NPMC = National Prion Monitoring Cohort; PM = post mortem; sCJD = sporadic CJD; vCJD = variant CJD.

Figure 33 illustrates enrolment, stratification, follow-up and drop-out from the studies. 97% of symptomatic patients judged eligible for the Cohort study at the initial National Prion Clinic

assessment were enrolled, suggesting that the study is highly effective at capturing recognised prion disease in the UK. Less than 1.5% withdrew from the studies. The baseline characteristics of the patients are shown in Table 21.

	All patients	sCJD	IPD	vCJD	iCJD	At risk	Control
Enrolled	**437**	**240**	**81**	**25**	**12**	**44**	**35**
Median age (years; range)	61 (14-92)	67 (40-87)	48 (26-92)	30 (14-63)	42 (27-51)	42 (20-92)	48 (23-75)
Gender (M/F)	207/230	105/135	39/42	17/8	10/2	19/25	17/18
Median time (months) since first symptoms (IQR)	6 (3-13)	4 (2-8)	26 (7-63)	8 (5-10)	8 (5-14)	N/A	N/A
Rankin							
Number assessed	**430**	**238**	**79**	**23**	**11**	**44**	**35**
Asymptomatic (0)	75	0	0	0	0	41	34
No or slight symptoms (1/2)	29	4	18	2	3	1	1
Moderate disabilty (3)	51	18	26	6	1	0	0
Moderate to severe disability (4)	89	56	17	12	3	1	0
Severe disability (5)	186	160	18	3	4	1	0
Barthel index							
Number assessed	**424**	**238**	**76**	**20**	**12**	**43**	**35**
Median (IQR)	4 (0-19)	0.5 (0-3.5)	14.5 (2-19.5)	10 (5.6-16.1)	6.3 (2-17)	20	20
MMSE							
Number assessed	**385**	**205**	**72**	**19**	**11**	**43**	**35**
Median (IQR)	12 (0-27)	0 (0-10)	18 (5-24.8)	16 (12-21.5)	15 (1.5-27)	30 (29-30)	30
CDR							
Number assessed	**353**	**178**	**69**	**21**	**9**	**42**	**34**
Median (IQR)	11 (2-18)	18 (12-18)	8 (3-15)	11 (5.5-16)	10 (4-18)	0	0
GCS							
Number assessed	**399**	**232**	**64**	**20**	**10**	**38**	**35**
Median (IQR)	14 (10-15)	11 (9-14)	15 (11-15)	15 (13.8-15)	14 (13.3-15)	15	15
MRC Scale							
Number assessed	**388**	**212**	**67**	**18**	**12**	**44**	**35**
Median (IQR)	9 (2-19)	3 (1-8.8)	16 (5.5-19)	13 (9-17.5)	11.5 (5-17.3)	20	20

Table 21. Baseline characteristics of patients included in this study.

Scale development

Table 22 summarises the scale development process.

Initial scale		Carer priorities and clinical validity			Inter-rater reliability		Rasch Analysis		MRC Prion Disease Rating Scale		
Item	Derived from…		Problems identified	Modifications	Kappa (n = 54)	Modifications	Problems identified	Modifications	Included?	Recalculated Kappa (n = 54)	Maximum Score (Total 20)
Bowel function	Barthel	Continence	-	-	0.74	-	Fit improved by re-scoring	Adjacent levels collapsed	Yes	0.78	1
Bladder function	Barthel	Continence	-	-	0.71	-	Fit improved by re-scoring	Adjacent levels collapsed	Yes	0.89	1
Grooming	Barthel	Personal care	Personal care over-represented relative to other domains	-	0.67	-	Item dependency with Bathing	Item dropped	No	-	-
Toilet use	Barthel	Personal care	-	-	0.81	-	-		Yes	0.81	2
Feeding	Barthel	Personal care	Floor effect	Extra level added	0.70	-	Fit improved by re-scoring	Adjacent levels collapsed	Yes	0.83	2
Transfers	Barthel	Mobility	-	-	0.73	-	Item dependency with Mobility	Items merged	Yes	0.67	2
Mobility	Barthel	Mobility	"Wheelchair independent" very rarely applicable	Rewording	0.81	-	Levels disordered				
Dressing	Barthel	Personal care	-	-	0.39	Item dropped	-	-	No	-	-
Stairs	Barthel	Mobility	-	-	0.82	-	-	-	Yes	0.82	2
Bathing	Barthel	Personal care	Floor effect	Extra level added	0.66	-	Fit improved by re-scoring	Adjacent levels collapsed	Yes	0.73	1
Best verbal response	GCS	Speech	-	-	0.85	-	-	-	Yes	0.85	4
Use of tools	Novel item	Cognition	-	-	0.72	-	-	-	Yes	0.72	1
Memory	CDR	Cognition	Expressive dysphasia typically obscures verbal memory	Rewording, to account for dysphasia	0.64	-	Fit improved by re-scoring	Adjacent levels collapsed Items merged	Yes	0.70	3
Orientation	CDR	Cognition	Floor effect	Extra level added	0.80	Item merged	Item dependency with Memory/Language				
Judgement/Problem solving	CDR	Cognition	-	-	0.74	-	Fit improved by re-scoring	Adjacent levels collapsed	Yes	0.82	1

Table 22. Summary of scale development process.

Ranked symptoms and impairments reported by caregivers using the Barthel ADL Caregiver Interview Summary form were grouped into functional domains. The most frequently recorded were within domains of mobility (55/236 ranked items, 23%), personal care/continence (30/236, 13%), communication/speech (29/236, 12%), behaviour/hallucinations (29/236, 12%), eating/swallowing (27/236, 11%) and cognition/memory/navigation (21/236, 9%). Symptoms or impairments from other domains were reported in <6% of responses. These were the key domains for representation in our outcome measure.

The initial scale consisted of slightly modified versions of all subcomponents of the Barthel ADL Index, the Memory, Orientation and Judgement/Problem-solving subcomponents of the CDR, the Best Verbal Response subcomponent of the GCS and a novel subcomponent assessing ability to use tools (15 items, 35 thresholds) [121]. It was designed for completion based on a brief interview with a closely-involved relative or carer as inability of patients to participate was a major cause of poor completion rates for some scales in PRION-1. Compared with their parent scales, minor modifications were made to a number of subcomponents at this stage to make them more easily applicable to prion disease patients e.g. to account for severe expressive dysphasia/mutism, and to add additional intermediate response categories at severe levels of impairment, aiming to improve discrimination and reduce floor effects.

The initial scale was completed on a total of 977 occasions, in 266 patients. This consisted of 498 face-to-face assessments and 479 telephone assessments. The scale proved to be simple and easy to use, being completed in less than 5 minutes. In addition to this, an approximation of the initial scale could be calculated from Barthel, CDR and GCS scale data for a further 839 assessments pre-September 2009.

The initial scale was acquired over the telephone by a doctor and a specialist nurse within 24 hours of each other on 50 occasions, over the telephone by a nurse within 24 hours of a face-to-face assessment by a doctor on 2 occasions, and by both doctor and nurse attending the same visit on 2 occasions. In all cases each assessor was blinded to the other's score. Agreement across all paired assessments was good or excellent (Cohen's kappa > 0.6) for all but three items; Dressing, which was subsequently dropped from the scale; Best verbal response, which subsequently underwent item threshold re-scoring; Orientation, which was subsequently merged with Memory to form a 'super-item'.

Rasch analysis

The fit to the Rasch model of the 15 item initial scale was first assessed in all individuals across all symptomatic disease types, and demonstrated poor fit in this heterogeneous population (χ^2=166.16, df =30, p<0.0001). As Rasch analysis relies on the assumption that there is a single construct being measured by all items in a scale, the inclusion of patients with different disease types which are known to vary widely in their typical clinical progression could plausibly make it impossible to establish a fit to the Rasch model using the whole dataset. As our primary objective was to develop an outcome measure tailored to sCJD, all other disease groups were excluded and the analysis repeated using 205 records from 132 sCJD patients. Fit improved somewhat, but was still unsatisfactory (χ^2=77.01, df =30, p<0.0001) so further exploratory analyses were performed to identify the reasons for this.

The 15 individual items were examined for threshold ordering, individual item fit, differential item functioning (DIF) and local item dependencies, and changes made to address these issues as recommended with consequent improvements in fit (e.g. rescoring of disordered items, dropping of most poorly fitting item)[230]. These changes are summarised in Table 22. Item dependencies were identified between Grooming and Bathing; Transfers and Mobility; Toilet and Stairs; Bathing,

Orientation and Memory. Two "super-items" were created by combining pairs of heavily dependent items in a clinically meaningful way: Transfers and Mobility; and, Memory and Orientation. The structure and scoring of these super-items can be seen in Table 23. Once item re-scoring and local dependencies were addressed, items were dropped if fit was still not achieved.

A revised scale including all of these modifications (subsequently termed the MRC Prion Disease Rating Scale) demonstrated a good fit (χ^2=25.19, df =22 , p=0.29). The mean fit residual for all 11 items in this scale was -0.498 with an SD of 0.993, illustrating a good fit of the items to the model. Cronbach's alpha was 0.91, implying a high level of confidence in the reliability of the assessment of fit, and the Person Separation Index was 0.90. No individual item had a fit residual of magnitude >2. Examination for DIF for all items across all person factors confirmed the data to be free of DIF when Bonferroni probability adjustment for multiple testing was applied, with gender, age quartile, assessor (doctor or nurse), and assessment mode (face-to-face or telephone) included as person factors.

Principal components analysis showed that items assessing speech and cognitive functions (Speech, Memory/Orientation, Judgement and Use of tools) were positively loaded while negatively loaded items assessed some aspects of mobility, personal care and continence (Toilet, Bowels, Bladder, Stairs, Mobility/Transfers); feeding and bathing had no strong loading. Comparing the person locations from positively loaded items with a factor loading >0.4 (speech and memory/orientation) to negatively loaded items with a factor loading <-0.4 (toilet, stairs, bladder, bowels and transfers/mobility) produced significant results at the 1% level in 9/205 (4.4%) individuals and at the 5% level in 16/205 (7.8%) individuals. Overall, our analyses of multidimensionality suggest that differential progression in speech/cognitive and mobility/personal care/continence domains is the second most important dimension (after the Rasch model) but did not grossly compromise the unidimensionality of the scale and the construct of sCJD disease progression.

Including earlier data derived from the original component scales (Barthel, CDR, GCS) along with the data collected using the initial scale allowed a total of 380 scales in 239 sCJD patients to be analysed. The overall fit to the Rasch model was less good but remained acceptable (χ^2=33.75, df =22 , p=0.052), suggesting that the modified items introduced for the initial scale were performing better than pre-existing scale components. These item modifications were therefore retained in the final scale. The MRC Scale derived for rapidly progressive patients, including a rapidly progressive subset of IPD (all patients in stratum (i)) also demonstrated acceptable fit to the model (χ^2=30.14, df = 22, p = 0.11). Unsurprisingly, the MRC scale derived for IPD patients alone and for all symptomatic patients (all disease groups including stratum (ii)) continued to result in a poor fit due to the heterogeneity of clinical syndromes that comprise IPD (patients with Gerstmann-Sträussler-Scheinker syndrome typically having an early disease course dominated by ataxia and other physical impairments with relative preservation of cognitive function, while in those with 6-octapeptide repeat insertion (6-OPRI) prion disease cognitive impairments typically predominate with physical functioning relatively preserved until the later stages of the illness).

The final format of the rating scale is shown in Table 23.

Item	Category criteria	Score
Bowel function	At least one episode of incontinence in last 7 days	0
	Continent for last seven days	1
Bladder function	Always incontinent or catheterised	0
	Continent or occasional accidents	1
Toilet use	Fully dependent	0
	Needs some help	1
	Independent	2
Bathing	Fully dependent	0
	Needs some help	1
	Independent	2
Feeding	Unable or NG/PEG/RIG fed (takes nothing by mouth)	0
	Needs help but can swallow (even if unsafe)	1
	Independent	2
Transfers and mobility	Bedbound, unable to sit	0
	Can sit, but cannot mobilise OR transfer without help (from person or walking aid)	1
	Can transfer OR mobilise independently OR both	2
Stairs	Unable	0
	Needs help	1
	Indpendent	2
Best verbal response	Mute	0
	Incomprehensible sounds OR single words	1
	Sentences BUT difficulty finding words OR often confused/disorientated	2
	Normal conversation	3
Memory and orientation to surroundings	Shows no awareness of surroundings OR any evidence of memory	0
	Evidence of retaining some highly learned material (e.g. recognising familiar people) OR awareness of surroundings BUT no evidence of acquiring new material	1
	Able to retain some new information BUT memory consistently impaired	2
	Memory normal OR some impairment off and on	3
Judgement and problem solving	Unable to show any judgement or problem-solving	0
	Able to show some judgement or problem-solving, even if this is severely impaired	1
Use of tools	Unable to use any tools or objects	0
	Able to use some tools or objects, with help if necessary	1

Table 23. The final MRC Prion Disease Rating Scale.

The inter-rater reliability in administration between doctors and nurses of the final scale as a whole was excellent (Interclass Correlation Coefficient =0.96). This is illustrated in Figure 34, which shows a Bland-Altman plot for all paired assessments.

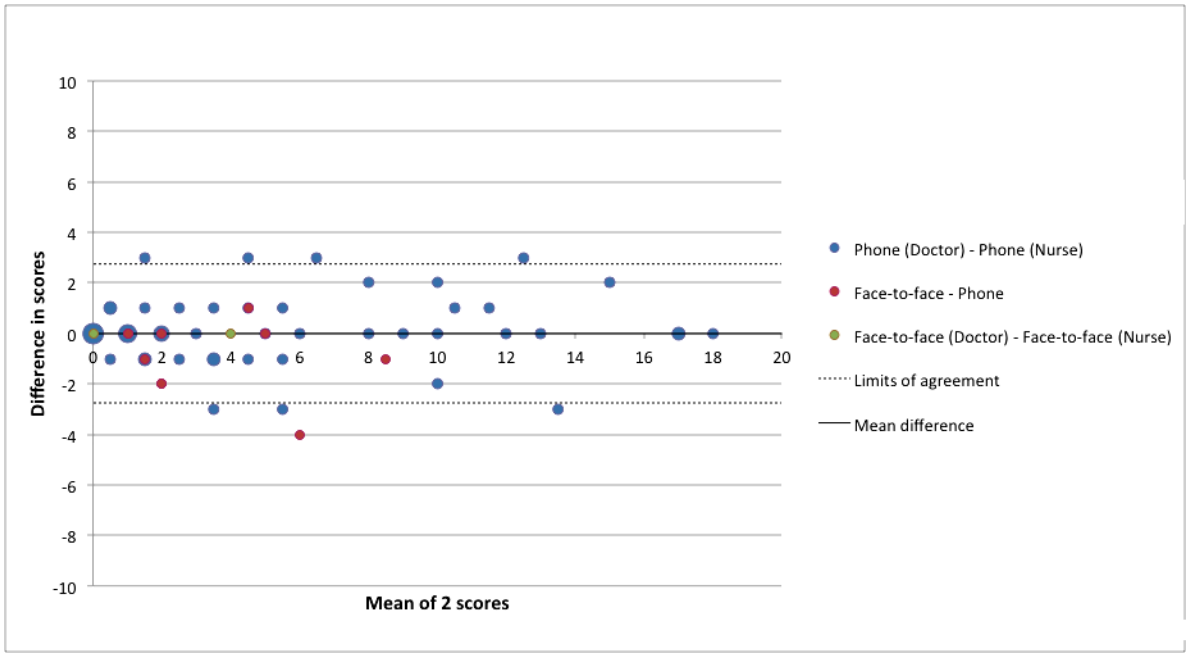


Figure 34. Bland-Altman plot illustrating agreement on paired MRC Prion Disease Rating Scale assessments. Total of 63 paired assessments included. The area of the coloured circles is proportional to the number of paired assessments from each category with that score combination: i.e. larger circles represent multiple superimposed data points.

Figure 35 shows the correlation of the MRC Scale with MMSE, Rankin and GCS across all assessments at which these scales were acquired. This illustrates the novel scale's relative resistance to floor effects, as patients at minimum (or unrecordable) score for MMSE or Rankin can still be separated by the novel scale. Whilst GCS has ability to separate patients scoring zero on the novel scale (GCS ranging 3-11), these distinctions are difficult to interpret clinically and have little or no relevance to the meaningful day-to-day functions that the scale is aiming to capture.

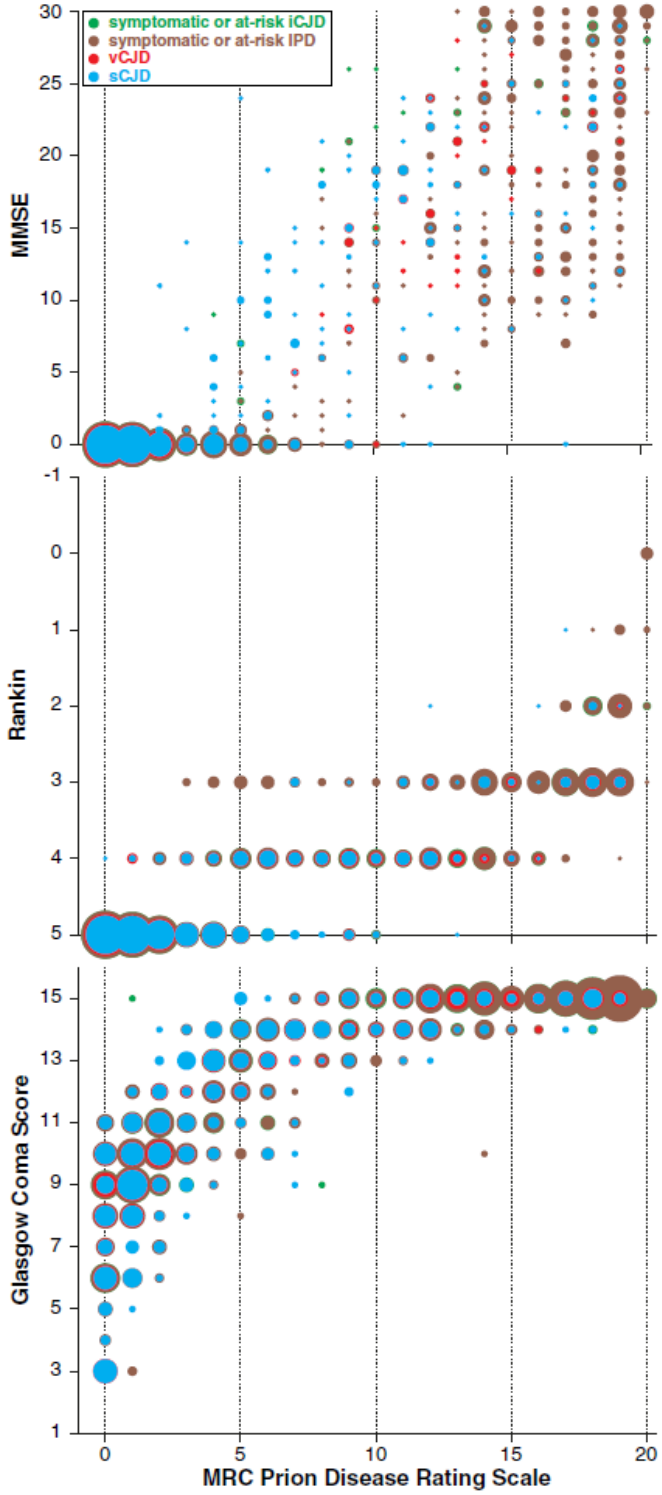


Figure 35. Correlation of the MRC Scale with other commonly used rating scales. The area coloured for each circle is proportional to the total number of patients from each aetiological group of prion disease with these scores.

The natural history of prion diseases

Figure 36 shows individual patient trajectories for the MRC Scale over time, colour-coded by disease type. Stratum (ii) patients have been included for comparison purposes with the caveat that the MRC Scale is not measuring a single progression construct in these patients, as demonstrated by the Rasch analysis reported above. Grey lines connect the last recorded score to the date of death, which is imputed as the worst-possible score (0). These plots illustrate the remarkable heterogeneity of disease progression between, and to a lesser extent within, prion disease types. Several distinct patterns of progression are apparent: rapid decline over weeks or a few months (mostly sCJD patients) and slow decline over years (almost exclusively patients with IPD). A slightly less rapid decline is observed in sCJD patients who are heterozygous at PRNP codon 129, vCJD and iCJD patients and some IPD patients (see Figures 36 and 37). In all groups, decline measured with the MRC Scale generally appears to be linear.

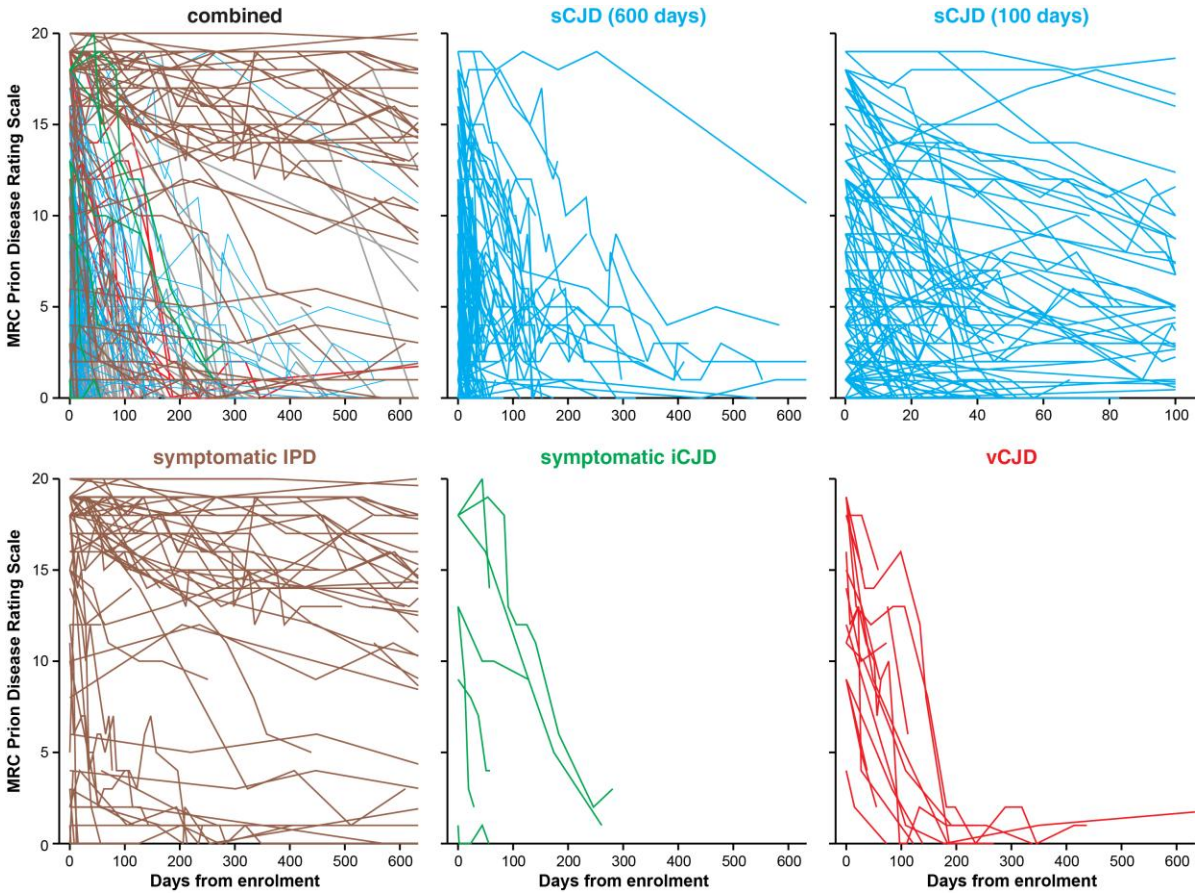


Figure 36. Trajectories of change in patients up to 600 days post enrolment for all patients (combined), sCJD, IPD, iCJD or vCJD only. Three broad patterns are seen: a slow decline in IPD patients, a rapid and somewhat variable decline in all aetiological groups, and a pattern of decline followed by a pre-terminal plateau at low levels of function in all aetiological groups. All sCJD trajectories are also shown for the first 100 days only for clarity of short duration cases. Data are also available up to 10 years in some IPD patients.

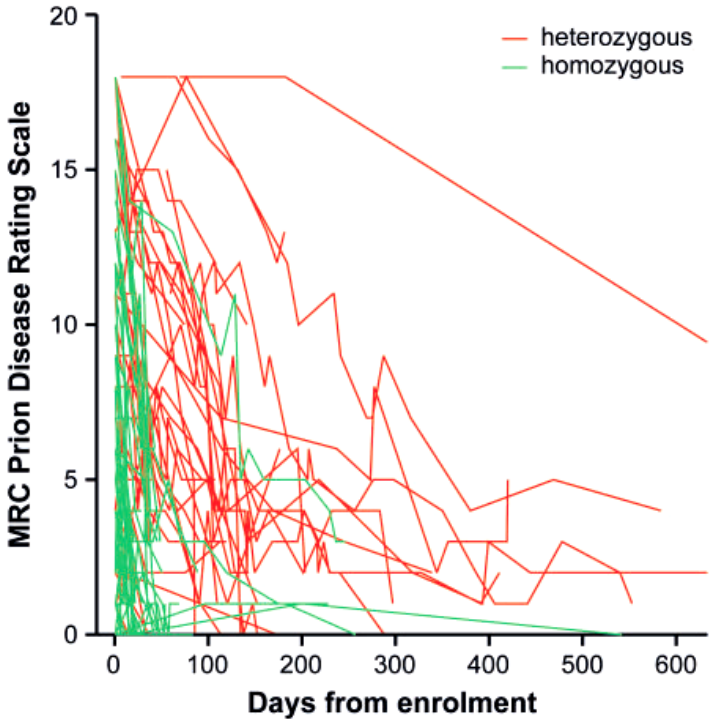


Figure 37. Trajectories of sCJD patients either homozygous (129MM or 129VV) at codon 129 of the prion protein gene or heterozygous (129MV). This genetic factor appears to be a strong determinant of rates of decline.

In most rapidly progressing patients, death occurs shortly after a very low score is reached, but in some there is an extended "pre-terminal plateau" phase at a very low score. A prion disease clinical trial design needs to consider these different patterns of decline.

Figure 38 illustrates the typical progression of a sCJD patient through the functional/cognitive milestones of the MRC Scale, and the spread of difficulty of items in the five measured domains. These observations are in keeping with clinical experience of these patient groups and are consistent with validity of the MRC Scale.

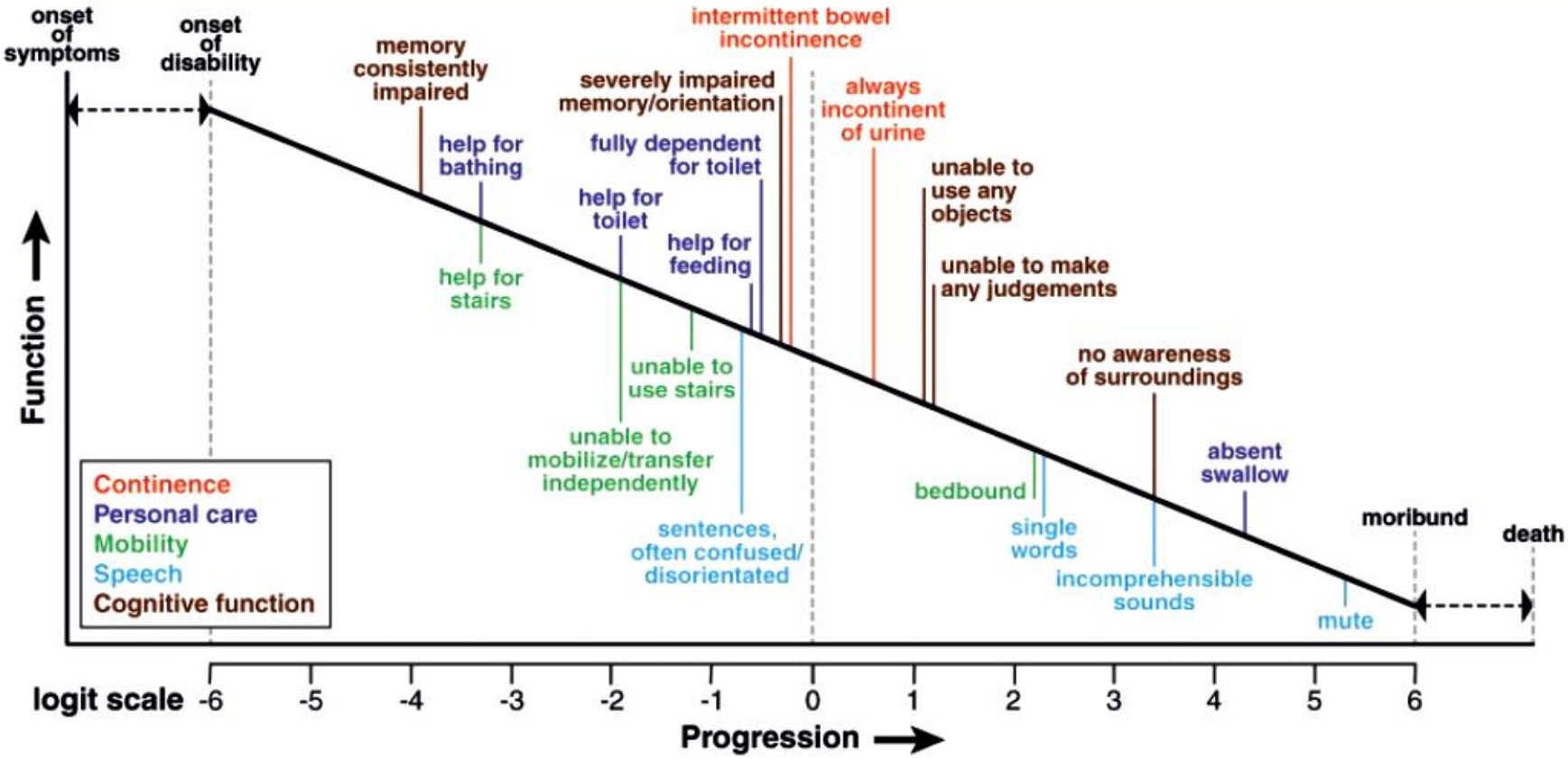


Figure 38. Representation of the pattern of decline in a sCJD patient that would be most consistent with the Rasch model. Progression is represented on the underlying logit scale used in the Rasch analysis. This diagram illustrates the validity of the progression construct, as the ordering of the items is consistent with clinical experience and there is a reasonable spread of item difficulty in different functional domains.

Discussion

This project sought to overcome fundamental obstacles on the route to developing effective treatments for prion disease: the lack of both a validated outcome measure for clinical trials and a large resource of clinical progression data. Using a range of complementary approaches, and taking into account previous analysis of rating scales data from the PRION-1 trial[121], a bespoke rating scale has been developed, refined and validated in the context of the Cohort study, the largest prospective clinical study of the natural history of prion disease. The novel outcome measure aims to maximise the likelihood of future trials giving a clear answer on therapeutic efficacy. This study also illustrates again that the advanced neurodisability at referral of sCJD patients to our clinical research team is a major outstanding problem for UK clinical trials: improved early diagnosis and referral will be key to success of clinical trials.

A new outcome measure for clinical trials

Prion disease trials have and will continue to use survival as a key outcome measure, but this has major limitations. It does not directly measure progression of disease, as patients may survive for long periods in a very advanced stage of disease or may die for reasons not directly related to disease progression (e.g. aspiration pneumonia). Existing rating scales, which are well validated in other neurological settings, are far less well suited to prion disease. For example, the MMSE and ADAS-cog, routinely used as outcome measures in Alzheimer's disease clinical trials, suffer from a marked floor effect[121] and fail to capture the profound physical impairments which are fundamental features of these diseases and may be present despite preserved cognitive function.

The need for better outcome scales is increasingly recognised in the wider field of clinical neurodegeneration research, including the role of more advanced statistical validation methods such as Rasch analysis. A 2009 paper by Black et al[235] summarises a roundtable discussion on outcome measures for Alzheimer's disease clinical trials, organised by the Alzheimer's Association, and

acknowledges the value of this approach. A pair of recently published papers presented work done using data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) study applying both traditional psychometric approaches and Rasch analysis to the ADAS-cog, and identified important limitations to the usefulness of this widely used outcome measure[236,237].

The remarkable clinical heterogeneity of prion disease, combined with its rarity, represents a major challenge to trial design. Very inclusive enrolment criteria will maximise patient numbers, but may have a paradoxically negative effect on statistical power if they greatly increase variability, or reduce the possible benefits that can be observed either due to permanent neurological damage which cannot be rescued, or due to relatively preserved functioning at enrolment. Designing a single outcome measure that can capture rapid global decline in a patient with sCJD and changes in patients with slowly progressive IPD equally well has not been possible. IPD patients are highly variable in clinical presentation with predominantly motor or predominantly cognitive progression. It is likely that the detection of subtle changes required to define the onset of disease in IPD will require analysis of neuropsychological testing which is ongoing in the Cohort study. The findings of this study suggest that, for maximum efficiency, the main group that should be targeted for future trial recruitment should be those stratum (i) patients not already at very low levels of functioning. This group of patients were also most likely to choose to take the investigational drug quinacrine in PRION-1[120].

While efforts have been made to design a scale that reflects the priorities of patients and their careers, a compromise must be struck with methodological concerns. The novel scale does not include any direct assessment of neuropsychiatric symptomatology, sleep disturbance or movement disorder (e.g. myoclonus), all of which are common features of prion disease, and are of concern to patients and carers. These features have limited value as markers of disease progression however, as they often fluctuate through the course of the disease, may improve in the later stages, and may

be significantly affected by non-disease-modifying treatments (e.g. benzodiazepines, anticonvulsants). As such they are not included in the outcome measure, but they remain clinically important features of these diseases, and other work is ongoing to improve the way in which they are assessed and treated, including that included in chapter 5 of this thesis on the behavioural and psychiatric features of prion disease.

Analyses of the baseline predictors of rates, patterns of decline, simulations of clinical trials to estimate power and fitting of linear mixed models are ongoing.

Establishing a natural history dataset and historical control group for trials

The data included in this study, combined with data that continues to be collected using the MRC Prion Disease Rating Scale through the Cohort study, represents a large and detailed natural history dataset, and it is hoped that this can act as a supplementary historical control group against which to compare treatment groups in future trials, with potentially large benefits for statistical power. To this end, the dataset has been made available to other physicians conducting clinical trials, and research groups worldwide have been encouraged to take advantage of this in planning clinical trials. To enable the direct comparison of results from different studies, we have proposed that the MRC Prion Disease Rating Scale should be adopted as a standard outcome measure for prion disease clinical research.

The use of a historical control group can be an appropriate alternative methodology to a straightforward randomised controlled trial in specific circumstances: where it is not felt that randomisation to a placebo/control arm would be acceptable, either ethically or to participants (as in recent drug industry trials of alternative monotherapy agents for treating refractory epilepsy[238,239]); or where it would be practically very difficult to enrol sufficient numbers of participants in a feasible timeframe to achieve an adequately powered study. Both of these are

relevant to a prion disease clinical trial. Experience in the PRION-1 trial showed that prion disease patients and their relatives are reluctant to be randomised, and that their decisions about whether to receive an experimental therapeutic are strongly associated with variables that have a confounding effect on outcomes, such as level of disability at enrolment[120]. The rarity of the conditions, particularly when combined with their marked clinical heterogeneity, clearly represent a challenge to enrolling sufficient numbers of patients prospectively for a well powered study.

It is therefore appropriate to include a historical control group in plans for future therapeutic clinical trials in prion disease, but there are a number of important considerations in doing so. The value of randomizing patients to concurrent treatment and control arms in a randomized controlled trials that it will tend to eliminate any confounding factors that might lead to spurious differences in outcome: including both factors that are known to be relevant and are recorded for all participants (and often presented as evidence that randomization has produced well-matched groups), but also importantly any unknown and/or unmeasured factors, as these are likely to be balanced between the two randomly assigned groups. When a historical control group is used, any factors that have changed between the collection of the control data and the treatment data have the potential to produce a confounding effect. Factors that are known to be associated with differences in the outcome may be recorded and efforts made to match the historical control group to the treatment group, but there is no way to eliminate or adjust for the effect of unknown factors.

In the case of future prion disease trials, some of the potential confounding effects may be of our own making. As discussed above, efforts are being made to improve the early diagnosis and referral of patients with prion disease, partly so that they may be offered enrolment in clinical trials at a stage before very significant neurological damage has occurred. However, any changes in the referral pattern over time will have the potential to confound the results of a historically controlled trial, and will need to be very carefully accounted for. Similarly, the increasing recognition of more

atypical clinical presentations of prion disease (particularly more slowly progressive forms of sCJD), represents another potential confounding factor.

There are particular statistical issues that must be considered in performing sample size calculations for historically controlled trials. More recent analysis of these[240] has highlighted the importance of taking into account that the outcome in the historical control group does not represent the true control population value (as had been assumed in earlier proposed methods[241]), but is itself an estimate based on a sample and is subject to random effects, and this must be incorporated into the calculation. The fact that the MRC-PDRS outcome measure has been developed and validated using a dataset which is itself to be used as a historical control group in future trials using this outcome measure, adds an additional dimension. The statistical properties of the scale when it is used to collect new prospective data in a future trial may be somewhat different from those in the dataset in which it has already been iteratively refined, due to simple natural variation.

Thoughts on wider relevance

Clinical research into rare diseases is extremely challenging for logistic, statistical and financial reasons, but it is essential that we work towards overcoming these challenges. Considered together, "rare diseases" make up a significant proportion of the burden of neurological disease, and it is essential to collect systematic data on which to base the treatment of these patients. Studying rare diseases can often provide valuable insights into more common conditions. In the wider field of neurodegeneration there is great interest in the hypothesis that templated protein misfolding mechanisms (referred to as "prion-like") may be fundamental to a wide range of other conditions including Alzheimer's and Parkinson's diseases[3]. Demonstrating a disease-modifying effect of a therapeutic agent in prion disease may therefore lead to insights with wider implications.

CHAPTER 7. GENERAL DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

This thesis presents several independent but inter-related projects from the laboratory and the clinic. The search for an effective disease-modifying therapeutic agent for prion disease is of paramount importance in this field of research, but this cannot occur in isolation. We must aim to reach a situation in which patients are diagnosed as early as possible using reliable and specific tests, and where their progression through the disease and any response to treatment can be monitored in an accurate and meaningful way both with clinical rating scales and using biomarkers. We must also continue to improve our understanding of the symptoms of these diseases and how to treat these effectively and safely; even if a disease-modifying agent is found this will remain extremely important, particularly for complex and distressing symptoms such as the behavioural and psychiatric manifestations studied in Chapter 5 above, which may themselves have an impact on patients' ability to engage with and benefit from disease-modifying treatments.

Molecular Diagnostics

Since the projects presented in Chapters 2, 3 and 4 above were performed, work on the Direct Detection Assay has continued, particularly efforts to further define its specificity with a view to a possible role as a screening test that could be applied on a large scale to improve the safety of UK blood or organ donations[135]. This study involved testing samples 5000 US blood donors (presumed to be non-BSE-exposed), 200 UK blood donors, and 352 patients with non-prion neurodegenerative diseases, and found no false positives, suggesting extremely high levels of specificity exceeding the threshold of 99.5% that have been suggested for a vCJD blood screening test (http://ec.europa.eu/health/medical-devices/files/meddev/2_14_4_ol_en.pdf). It is important to note that the sensitivity of the DDA for detecting *pre-clinical* vCJD infection is unknown as no samples taken from patients prior to the onset of symptoms are available to test, and the clinical significance of a positive test in an asymptomatic individual is unknown (particularly given the

possibility of chronic asymptomatic carrier states). Currently UK blood donations are screened for a number of infections (including syphilis, HIV, Hepatitis B and C), and if found to be positive the donors are informed and appropriately advised by a doctor regarding any need for further tests or treatment, and on their suitability to be a blood donor in future (www.blood.co.uk/resources/leaflets/tests-on-your-blood/). Clearly if the DDA is to be implemented as a screening test for blood donations, very careful consideration will need to be given to the process by which samples are tested and in what way the results are fed back to the donors.

The possible application of the DDA as a blood-based biomarker to track disease progression or response to treatment will require further investigation. Unless further cases of vCJD occur, it is not likely to have any role as a therapeutic biomarker in its current form, as it has only been found to be positive in a very small number of cases of sCJD[135] and IPD (as reported in Chapter 2), and no clear correlation with stage of disease progression has been demonstrated. If further cases of vCJD *do* appear, then re-assessing the DDA's sensitivity as a diagnostic test for vCJD prospectively will also be important because of the various methodological limitations imposed by the scarcity of samples that are discussed in Chapter 2 above.

Following on from the work using the DDA in CSF presented in Chapter 3, the surprising results of initial testing of patient CSF samples, with lower signal in prion disease patient samples than in controls, will require further investigation. In particular, looking for correlations between the DDA signal produced by any CSF sample, and its overall PrP content as measured using alternative methods such as ELISA would be useful, as one explanation of the observed results would be that the specificity for disease-related PrP is reduced when the DDA is used in CSF and it is simply acting as a quantitative test for PrP. Another question that should be explored further is whether the DDA has a differential ability to detect disease-related PrP from different prion strains (e.g. to detect

vCJD-related PrP much more readily than sCJD-related, as suggested by the brain homogenate/CSF spike experiments in Chapter 3) or disease-related PrP from different tissues: e.g. the PrP in peripheral blood may be in a very different form and environment than that in CSF or in brain homogenate.

This work on the DDA in CSF is at a relatively early stage overall, and a great deal of progress would be needed before it could be considered as a potential diagnostic marker or biomarker of disease progression. In contrast, the RT-QuIC technique (reviewed in the introduction to Chapter 3) does appear to have value as a diagnostic test, particularly for sCJD, and could also be explored as a potential biomarker that might be incorporated into clinical trials. This could be investigated most effectively by studying serial CSF samples taken from patients enrolled in prospective clinical studies such as the Cohort, so that results can be correlated with measures of disease progression, such as the MRC Scale, as well as with chronological progress through the disease and other known phenotype modifiers such as codon 129.

Although, laboratory-based (or imaging-based) biomarkers are of secondary importance to clinical outcome measures that have a direct relevance to patients, they can be extremely useful as an adjunct to these. They may help to provide proof of concept that a therapeutic agent is engaging with a disease process, and may provide an early indication that a treatment is having some effect before this becomes apparent from analysis of the primary clinical data. This is particularly true for preventative treatments (e.g. measuring cholesterol in patients taking statins to prevent cardiovascular disease), but may also be the case in situations where clinical data is likely to have a lot of inherent variability and noise (as is the case in prion disease) where it may take longer for a clinical benefit to become statistically apparent.

Clinical rating scale development

The MRC Scale has major strengths as an outcome measure for clinical studies of prion disease, and we hope that it will be adopted widely as a standard tool. This will allow pooled and/or comparative studies between different research groups, which can be of great value in the study of rare diseases such as these. The large dataset collected using the MRC Scale in the ongoing Cohort study can now be used to carry out more sophisticated statistical modelling of rates of decline in different groups of patients, and to analyse for any factors predicting this rate of decline. The ultimate aim of this process would be to stratify patients at the point of clinical trial enrolment into groups with a well-defined expected rate of decline on the MRC Scale, so that comparisons can be made within these groups with major benefits for statistical power resulting from reduction in variability.

Study of behavioural and psychiatric features

Following the work presented in Chapter 5, modifications were made to the Cohort study's protocol to include a neuropsychiatric rating scale that has been validated for use in other dementia populations, the Neuropsychiatric Inventory[203]. This should allow prospective collection of more complete and robust data than was available for this project, which identified a number of weaknesses in the way these symptoms were being assessed and recorded (e.g. under-estimation of agitated symptoms, as these were not included in the tick-box list for symptoms present at each study assessment; inability of most patients to engage with the Brief Psychiatric Rating Scale (BPRS)). As these symptoms are common and distressing, failing to adequately assess them would represent a substantial "blind spot" in any clinical research protocol, including that of future clinical trials.

In terms of plans to improve the treatment of patients with these symptoms, it is necessary to think pragmatically. The ideal would be to carry out randomised controlled trials (RCTs) of treatments that have shown promise in the observational study reported here, and these should be carried out if possible, but bearing in mind the substantial challenges to carrying out therapeutic trials in these

diseases (as discussed throughout this thesis) and the appropriate prioritisation of planning trials of putative disease-modifying agents, it is likely that we will need to base decisions about symptomatic treatment on lower quality observational data for the foreseeable future. We must remember that RCTs of treatments for similar neuropsychiatric symptoms in other neurodegenerative diseases have often not confirmed anecdotal impressions of efficacy (e.g. use of antipsychotics and antidepressants in Alzheimer's disease, as reviewed in the introduction to Chapter 5), highlighting the difficulties of interpreting less robust evidence such as that presented in this study, where there is often a tendency to over-estimate efficacy. This is particularly important in the context of the safety concerns around use of anti-psychotic medication.

Thoughts on the past, present and future of prion disease research

The strange and tragic history of prion diseases, particularly in the UK, has led to a remarkable amount of progress in this wide-ranging field of research. Despite the rarity of the diseases, they are now arguably better understood than many much more common neurodegenerative conditions. Several clinical trials have been performed showing that this is feasible in these rare and often rapidly progressive diseases, and there is very encouraging pre-clinical evidence for novel treatments, particularly mono-clonal antibodies against PrP[132,242,243].

Translating encouraging preclinical evidence into successful clinical trials and meaningful benefit for patients is a major hurdle, and one with which many different fields of neurodegeneration research are currently struggling. While prion disease research has some particular advantages that may make translation more feasible, such as the fact that the same diseases can affect both humans and laboratory animals, the challenge should not be underestimated. It is vital to appreciate that finding an effective therapeutic agent in itself will not be enough to translate this into a robustly proven therapy that will benefit patients. Approaches such as those used for the studies presented in this

thesis must occur alongside to lay the groundwork for clinical trials that will have the best possible chance of success if, or hopefully *when*, an effective therapeutic agent is found.

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