

# **SUSCEPTIBILITY GENES AND PHENOTYPE MODIFIERS IN PRION DISEASES**

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## ABBREVIATIONS

ADAS-cog	Alzheimer's Disease Assessment Scale
ADGC	Alzheimer's Disease Genetic Consortium
APC	Antigen-presenting cell
APP	Amyloid precursor protein
BAF	B allele frequency
BPRS	Brief psychiatric rating scale
BSE	Bovine spongiform encephalopathy
CIBIC-P-plus	Component of the clinician's global impression of change
CDR-SB	Clinical Dementia Rating Scale Sum of Boxes
CNV	Copy number variants
CJD	Creutzfeldt-Jakob disease
CSF	Cerebrospinal fluid
DC	Dendritic cell
DWI	Diffusion-weighted imaging
EEG	Electroencephalography
ETOH	Ethanol
FAD	Familial Alzheimer's disease
FISH	Fluorescence <i>in situ</i> hybridization
FFI	Fatal familial insomnia
FLAIR	Fluid attenuated inversion recovery
GCS	Glasgow Coma Scale
GPI	Glycophosphatidylinositol
GSS	Gerstmann-Straussler-Scheinker

GWAS	Genome-wide association
HapMap samples	Set of reference samples
hGH	Human growth hormone
HMM	Hidden Markov model
IBD	Identity by descent
iCJD	Iatrogenic CJD
IL	Interleukin
IPD	Inherited prion disease
KORA	Collaborative Health Research in the Region of Augsburg
LD	Linkage disequilibrium
LOH	Copy neutral loss of heterozygosity
LRR	Log R ratio
MAF	Minor allele frequency
MGB	Minor Groove Binder
MELAS	Mitochondrial, encephalopathy, lactic acidosis, and stroke-like episodes
MMSE	Mini-mental state examination
MNP	Mononuclear phagocytes
MRI	Magnetic resonance imaging
NCJDRSU	National CJD Research and Surveillance Unit
NPC	National Prion Clinic
NPMC	National Prion Monitoring Cohort
OPD	Open reading frame
OPRI	Octapeptide repeats insertion
PCA	Principal components analysis
PCR	Polymerase chain reaction



PK	Proteinase K
PNG	Papua New Guinea
PSWC	Periodic sharp wave complexes
PSEN	Presenilin gene
PRNP	Prion protein gene
PrP <sup>C</sup>	Prion protein (normal cellular isoform)
PrP <sup>Sc</sup>	Prion protein (scrapie isoform)
sCJD	Sporadic CJD
SNP	Single nucleotide polymorphism
TSE	Transmissible spongiform encephalopathies
vCJD	Variant CJD
WTCCC	Welcome Trust Case Control Consortium

## ABSTRACT

Prion diseases show remarkable clinical and neuropathological heterogeneity. All reported cases with definite variant Creutzfeldt-Jakob disease (vCJD) were homozygous at *PRNP* codon 129. Heterozygosity at codon 219 has been shown to be protective against sporadic CJD (sCJD). Copy number variants (CNVs) are a novel source of genetic variability associated with susceptibility to neuropsychiatric disorders.

**Aims:** Hypotheses tested:

- The clinico-pathological phenotype of prion disease is modified by investigation findings, co-deposition of amyloid beta, tau proteins and/or candidate genetic variation.
- The MRC Scale can be used for analysis of disease progression in CJD
- Copy number variation alters the risk of prion disease in the UK and Papua New Guinea (PNG)

**Methods:** Case reports illustrated genetic susceptibility and phenotypic heterogeneity. The MRC Scale was used to assess disease progression and study power in sCJD. Real-time PCR and gene sequencing were used to assess the role of candidate genes in clinico-pathological heterogeneity. GWAS were used to assess the role of CNVs as susceptibility loci to prion diseases.

**Results:** Two patients with vCJD were heterozygous at codon 219. The MRC scale could be administered daily requiring only 90 patients to provide sufficient study power. Amyloid- $\beta$  deposition was significantly influenced by APOE  $\epsilon$ 4 haplotype in definite sCJD. Prion protein and hyperphosphorylated tau deposition were influenced by MAPTH1c haplotype. CNV duplications at chromosome 10 and 14 were significantly enriched in cases when compared to controls. The finding was confirmed using real-time PCR but was not replicated in the German cohort. Analysis

using Penn CNV revealed a nominally significant association of CNV deletion at *PARK2* gene.

**Conclusion:** Heterozygosity at codon 219 is protective against sCJD but may confer susceptibility to vCJD. Patient stratification and assessments using MRC Scale allowed adequate study power to justify future therapeutic trials. MAPTH1c haplotype played a role in both prion and tau protein deposition. Chromosome 10 and 14 duplications and deletion at *PARK2* gene may play a role in prion disease susceptibility.

## I INTRODUCTION

Prion disease is a relatively recently coined term for long-recognized transmissible disorders of humans and animals. The disease was initially observed in sheep and goats although it is now recognised in several other species. The diagnostic confirmation of a prion disease is typically based on the appearance of a pathological form of the prion protein in the body. Prion protein in a misfolded or “scrapie” form (termed PrP<sup>Sc</sup>) is thought to comprise the major if not sole component of the infectious agent of prion diseases and induces a fatal, degenerative disease (Prusiner 1982).

The first prion disease described was sheep scrapie. Scrapie is now recognized as one of several transmissible spongiform encephalopathies (TSEs), which include bovine spongiform encephalopathy (BSE or "mad cow disease") and chronic wasting disease of cervids (Hunter, 2007). Scrapie has been known since the 18th century (1732) and does not appear to be highly transmissible to humans, although occasional transmissions cannot be excluded (Jeffrey and Gonzalez, 2007). It is however demonstrably transmissible among similar animals. The affected animals compulsively scrape off their fleeces against rocks, trees or fences due to the itching sensation caused by the condition. Other clinical signs include excessive lip smacking, altered gait and convulsive collapse (Foster et al., 2001). It tends to persist in flocks and can also arise apparently spontaneously in flocks that have not previously suffered with the disease. The mechanism of transmission between animals and other aspects of the biology of disease are active areas of research. Recent studies suggest that prions may be spread through urine and persist in the environment for decades which could explain spread between flocks (Detwiler and Baylis, 2003). This and other phenomena are in contrast to all other known infectious agents such as bacteria, fungus or parasite which must contain nucleic acids, either DNA, RNA, or both. All known prion diseases affect the structure and function of the nervous system and are currently untreatable and

universally fatal. Epidemiological, molecular, cellular and clinico-pathological studies aim to provide better understanding and possible treatment and prevention of these disorders.

## **1.1 Historical overview**

Prion diseases include kuru (Collinge et al. 2006), Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome (Masters et al., 1981), fatal familial insomnia (Medori et al., 1992a; Medori et al., 1992b) in humans, and similar diseases in sheep, goats (WILSON et al., 1950) and muffs (Wood et al., 1992), transmissible mink encephalopathy, chronic wasting disease of mule, deer and elk (Williams and Young, 1980; Williams and Young, 1982), bovine spongiform encephalopathy or mad cow disease (Bradley et al., 2006; Bradley and Liberski, 2004; Collee et al., 2006; Wells et al., 1987) and its analogues in several exotic species of antelopes (Cunningham et al., 1993; Fleetwood and Furley, 1990; Kirkwood et al., 1992) and feline spongiform encephalopathy in domestic cats (Wyatt et al., 1991). The clinical features of scrapie, TSE in sheep and goats, have been known under different names for over 200 years.

The first investigator who believed that scrapie was a disease caused by a 'filterable agent' was Besnoit in 1899 while the transmissible nature of scrapie was only proved in the late 1930s by the important experiments of Cuille and Chelle. In the follow up of these reports, Gordon (Gordon WS, 1946) inadvertently replicated the finding of Cuille and Chelle using a vaccine based on formalin-fixed sheep brains in a large group of animals which developed scrapie. The transmission of scrapie from sheep to mice by Morris and Gajdusek (Morris and Gajdusek, 1963) and from goats to mice by Chandler (Chandler RL, 1963; Chandler, 1961) facilitated laboratory research and development of many new hypotheses on the aetiology and pathogenesis of the disease.

The discovery of human TSEs is related to the investigation of the cause of a 'strange encephalitis-like' disease identified in 1955 in a territory of Papua New Guinea - kuru (Liberski, 2009). Gajdusek provided one of the first descriptions of the clinical presentation of kuru. Importantly, it was later observed that

the histopathology of kuru is reminiscent of scrapie. As a result, Gajdusek went on to show that kuru, and then Creutzfeldt-Jakob disease was transmissible, to chimpanzee (Gajdusek et al., 1966). Thereafter, the potential for protein-only infectious agents (Griffith, 1967) and the term “prion” and its key hypothesis was formulated by Stanley Prusiner (Prusiner, 1982) who co-purified prion protein (PrP) with infectivity. It was postulated that the scrapie agent is a proteinaceous particle, termed PrP<sup>Sc</sup>. It appears as an abnormal fibrillar structure in scrapie, CJD and kuru-infected brains (Armstrong, 2010;Merz et al., 1984;Merz et al., 1983) by negative stain electron microscopy. These fibrillar structures were named ‘scrapie associated fibrils’ or ‘fillamentous virus’ and later labeled ‘prion rods’ and classified as a form of amyloid by Cho (Cho, 1980) and Prusiner (McKinley et al., 1983). The gene encoding PrP was cloned by Charles Weissmann’s group and the ablation of this gene led to complete resistance to scrapie (Bueller et al., 1993). A number of rare but lethal neurodegenerative diseases which affect humans share a role with abnormal prion protein in their pathogenesis. Possible similarities with the pathogenesis of more common neurodegenerative disorders and potential for outbreaks of prion diseases emphasize the importance of research in this field.

## **1.2 Prion protein, prion protein gene and prions: structure and function**

All known mammalian prion diseases involve PrP in their pathogenesis. The endogenous, properly folded, form is denoted PrP<sup>C</sup> (for *Common* or *Cellular*) while the disease-linked, misfolded form is denoted PrP<sup>Sc</sup> (for *Scrapie*, after one of the diseases first linked to prions and neurodegeneration) (Prusiner 1982). The structure of the PrP<sup>C</sup> is shown in Figure 1. Proteins showing prion-type behavior are also found in some fungi, which have been useful in helping to understand mammalian prions. Fungal prions do not appear to cause disease in their hosts although this is controversial (Lindquist et al., 2001).

Prions propagate by transmitting a misfolded protein state. When PrP<sup>Sc</sup> enters a healthy organism, it induces normally folded protein (PrP<sup>C</sup>) to convert into the disease-associated multimeric isoform (PrP<sup>Sc</sup>). It is hypothesised that the abnormally folded prion protein “seed” acts as a template to guide misfolding. The growing prion seed can fragment, these newly formed prions can then go on to convert more proteins and trigger a chain reaction that produces large amounts of the misfolded prion protein and prions (Prusiner, 1998).

Propagation of prions depends on the presence of a pool of normally folded protein from which the prions can recruit. The altered PrP<sup>Sc</sup> structures are stable and accumulate in infected tissue, causing, through mechanisms not well understood, tissue damage and cell death. This structural stability is linked to a resistance to denaturation by chemical and physical agents, making disposal and containment of these particles difficult.



Prions come in different strains, with characteristic incubation times and neuropathology, consistent in transmission within and even between species. It is hypothesized that strains are encoded in the structure of the infectious agent (Collinge and Clarke, 2007).

Prion infections are associated with remarkably prolonged but highly consistent incubation periods in inbred laboratory animals followed by a rapid clinical phase. The relationship between prion propagation, generation of neurotoxic species and clinical onset has remained obscure. Sandberg et al (Sandberg et al., 2011), building on early work necessarily sparse in time points, showed that prion propagation in mouse brain proceeds via two distinct phases: a clinically silent exponential phase not determined by prion protein concentration which rapidly reaches a maximal prion titer, followed by a distinct switch to a plateau phase. The duration of the latter phase determines time to clinical onset in a manner inversely proportional to prion protein concentration. These findings are therefore consistent with an uncoupling of infectivity and toxicity. The authors suggest that prions themselves are not neurotoxic but catalyze the formation of such species from PrP<sup>C</sup>. Production of a neurotoxic species is triggered when prion propagation saturates, leading to a switch from autocatalytic production of infectivity (phase 1) to a toxic (phase 2) pathway.

Protein misfolding and aggregation appear to be common to a larger group of neurodegenerative diseases. Characteristic pathological findings include brain vacuolization, astrogliosis and neuronal apoptosis associated with accumulation in the central nervous system of extracellular protein deposits that may have the properties of amyloid fibrils (Aguzzi, 2005; Aguzzi and Heikenwalder, 2006; Chesebro et al., 2005; Westermarck, 2005). Although identical in their primary structure PrP aggregates are protease resistant (Leffers et al., 2005; McKinley et al., 1991; Sunde et al., 1998) and it appears that they are misfolded PrP species (PrP<sup>Sc</sup>).

The human PrP gene is found on chromosome 20 and encodes a proto-protein containing 253 amino acids before being processed. The first 22 residues are cleaved after translation whereas the last 23 amino acid residues are cleaved prior to addition of glycosylphosphatidylinositol anchor to serine at position 230.

The human prion protein gene comprises two exons with the entire open reading frame contained within the larger second exon (Mead, 2006; Oesch et al., 1985). The two domains of PrP are distinct in terms of the corresponding genetic variation of *PRNP*. Figure 3 illustrates the structure of the prion protein gene. Codons 51-91 of the N-terminal domain encode a 5-mer repeat region consisting of a nonapeptide followed by four identical octapeptides. Alterations in the number of repeats are known polymorphisms and pathogenic mutations, but there are very few point mutations or common single nucleotide polymorphisms (SNPs) in this region. Point mutations causing IPD and a number of SNPs are found in the C-terminal domain. A common coding polymorphism at codon 129 of *PRNP* between methionine and valine (rs1799990, c.385A>G) has a critical role in susceptibility and modification of prion disease.

There are three types of pathogenic *PRNP* mutations: point mutations leading to an amino-acid substitution or premature stop codon, and alteration of the octapeptide repeats (>1 deletion or >3 insertions) (Figure 3). Over 60 different mutations have been described: some are typically associated with particular clinical categories of prion disease, while others are associated with a spectrum of clinical phenotypes, often with striking phenotypic variability. Despite the rarity of *PRNP* mutation, the protracted clinical course of some IPDs lead to a high population prevalence relative to sporadic and acquired prion diseases.

Prion protein is normally extracellular and attached to the cellular membrane by its GPI anchor. Prion proteins of mammals are highly conserved (although not exceptionally so compared with other human proteins) as indicated by human PrP sequence identity with sheep (94%), chimpanzee (99.2%) and cows (92.8%).

There are a number of studies suggesting a probable function of prion protein (Figure 4). However, despite this a definite and consistent physiological role of PrP remains unknown (Hu et al., 2007; Steele et al., 2007b). Although the precise function of PrP is not yet known, it is possibly involved in the transport of ionic copper to cells from the surrounding environment (Brown et al., 1997). Studies have also proposed roles for PrP in cell signaling or in the formation of synapses (Kanaani et al., 2005), or in the maintenance of myelin (Bremer et al., 2010).

Ablation of the prion protein gene (*Prnp*), which encodes PrP, was instrumental in an attempt to understand the structure and function of normal PrP. However, no obvious phenotype in PrP knock-out mice has been described. Studies of PrP function have often relied on intuition and serendipity. There are a multitude of phenotypes described in PrP deficient mice however, the critical question remains about the primary phenotypes associated with PrP deletion and its implication on the function of PrP. Many years of study of the role of PrP<sup>c</sup> in normal cell biology gives it an almost unique place within protein database (Figure 4). It seems that PrP has evolved (and survived) to perform a function that does not have a precedent amongst its GPI anchored cell-surface proteins, perhaps representing a new type of plasma membrane ecosystem (Westaway et al., 2011). In a context where we await a clarifying insight into the structure and function of PrP<sup>c</sup>, the discovery of GPI-anchored N-glycosylated Doppel (Dpl) and Shadoo (Sho) proteins are exciting because they have homology to the N- and C- halves of PrP<sup>c</sup> itself (Westaway et al., 2011) and collectively these proteins comprise a prion protein gene family. Dpl

and Sho may be simpler - and more "understandable" - entities that can be pursued in parallel to PrP<sup>C</sup>, and could contribute greatly to the understanding of the biology of mammalian prion proteins from fresh directions. Dpl has a profound role in successful gametogenesis that warrants close scrutiny and a case for deeper study can be made for Sho, a recently discovered CNS-expressed protein with many parallels to established aspects of PrP biochemistry.

Transgenic mouse models have greatly contributed to our knowledge of prion pathophysiology. Shmerling et al (Shmerling et al., 1998) demonstrated that PrP<sup>C</sup> deficient mice are resistant to scrapie which is induced by mouse adapted sheep prions. Interestingly, they demonstrated that PrP lacking residues 32-121 or 32-124 caused severe ataxia and neural death limited to the granular layer of the cerebellum as early as 1-3 months after birth. However, attempt to transfer disease of these mice did not succeed. Thus, PrP lacking residues 32-121 or 32-134 induced neural dysfunction (neurotoxicity) without infectivity.

Early attempts to identify a function for PrP were hindered by the absence of phenotypic defects in PrP-null mice and of conserved structural or sequence motifs (Aguzzi et al., 2008a). Despite these initial setbacks, subsequent studies have uncovered that PrP-null mice differ from their wild-type counterparts in many more subtle activities, including circadian cycles, neuroprotection, synaptic function, stem cell renewal and proliferation (Aguzzi et al., 2008b; Steele et al., 2007a; Wilson et al., 2009) and two recent studies have shown possible new roles for PrP. During zebra fish development, knockdown of either of the duplicated PrP genes induces loss of cell adhesion and altered localisation of E-cadherin and Fyn Tyr kinase, phenotypes that are suppressed by expression of murine PrP (Malaga-Trillo and Sempou, 2009). In adult mice, regulated proteolysis and expression of PrP on the neuronal cell surface is required for maintenance of myelination through a non-cell autonomous route (Bremer et al., 2010). Together, these

studies may suggest that PrP has a pleiotropic role in vivo, perhaps mediating its broad effects through an activity in cell signalling pathways (Westergard et al., 2007).

While the absence of neurodegeneration in PrP null mice was initially considered to be incompatible with a loss-of-function model for prion diseases, subsequent studies have shown that expression of PrP fragments induces spontaneous neurodegeneration in PrP-null mice (Baumann et al., 2007; Shmerling et al., 1998). Based on these observations, PrP toxicity during TSE infection could be explained as a loss of some PrP functions but not others (Westergard et al., 2007). Consistent with this idea, PrP must be expressed on the surface of neurons to mediate TSE pathogenesis following infection (Brandner S et al., 1996; Chesebro et al., 2005; Mallucci G et al., 2003) suggesting that normal localization of the protein is required to elicit neurotoxicity.

### **1.3 Prion toxicity and infectivity**

It is accepted that PrP<sup>Sc</sup> is a refolded partially protease resistant and infectious form of the normal cellular isoform of PrP<sup>C</sup> encoded by the highly conserved single-copy gene *PRNP* (Prusiner, 1982). The mechanism by which PrP<sup>Sc</sup> induces brain dysfunction is not well defined. Importantly, neurotoxicity in prion disease cannot be explained by loss of normal functional activity of PrP<sup>C</sup> since mice lacking PrP<sup>C</sup> do not show signs of neurodegeneration and do not develop prion disease (Bueler et al., 1993; Mallucci G et al., 2002). Furthermore, in mouse models, transplant of neural tissue over-expressing PrP<sup>C</sup> into PrP<sup>C</sup> knock-out mice was easily infected with PrP<sup>Sc</sup> but infectivity was localised within grafted tissue while surrounding PrP<sup>C</sup> deficient tissue was not affected. Thus, it appears that PrP<sup>C</sup> is required for PrP<sup>Sc</sup> induced neurotoxicity (Brandner S et al., 1996). Similarly, post-natal knock down of PrP expression in a conditional knockout model caused no overt phenotype and allowed rescue of prion disease (Mallucci et al., 2003; Mallucci et al., 2002).

Sandberg et al (Sandberg et al., 2011) describe a long plateau in prion infectivity during the incubation time to prion disease, suggesting the uncoupling of neurotoxicity and infectivity in the mechanism of prion propagation. It appears therefore that PrP<sup>Sc</sup> can propagate using PrP<sup>C</sup> as a substrate and then PrP<sup>Sc</sup> may cause cell death using membrane bound PrP<sup>C</sup> although the exact mechanism of how this occurs is still unknown. Alternatively, there are multiple biochemical pathways leading from PrP<sup>C</sup> with the generation of infection and toxicity being distinct. Recent data point to the activation of the unfolded protein response in the pathways associated with neurotoxicity in prion disease (Moreno et al., 2012).

#### **1.4 Prion biology and disease pathogenesis**

Prion diseases are the prototypical and extensively characterized examples of protein conformation-based phenotypic traits and can be defined by proteinaceous infectious particles (Griffith, 1967). These factors were originally identified as infectious entities associated with a group of transmissible neurodegenerative diseases in mammals— the causative agent of which is resistant to treatments that damage nucleic acids (Prusiner, 1998). The fact that prions could act as infectious agents despite the absence of a nucleic acid genome led to the formulation of the “protein-only” or prion hypothesis as a self-perpetuating conformer of a host prion protein (Prusiner *et al.*, 1982).

The infectious conformer of PrP<sup>Sc</sup> was predicted to recruit and convert the normal conformer PrP<sup>C</sup> into the PrP<sup>Sc</sup> form through contacts between specific regions of the protein, and refolding, thereby ‘replicating’ the agent during infection. The appearance of similar conformers in mammals is typically associated with emergence of disease, although subclinical infections have been described (Hill AF and Collinge J, 2003). Despite this clearly disadvantageous phenotype for PrP, mounting evidence suggests that prion-like conversions of other proteins may be a mechanism for regulating protein function and survival advantage (Si *et al.*, 2003). Strong evidence supports a direct role of PrP in clinical disease. Depletion of PrP post-infection extends incubation times and reverses both neuropathology and behavioural defects induced by the infection (Mallucci G *et al.*, 2007; White *et al.*, 2008).

## **1.5 Prion protein strains and species barrier**

The prion hypothesis originally suggested that PrP<sup>Sc</sup> catalyzed the conversion of the PrP<sup>C</sup> in the context of a heterodimer of the two isoforms (Griffith, 1967; Prusiner, 1982). However, subsequent studies have revealed that soluble PrP<sup>C</sup> binds to an oligomer of PrP<sup>Sc</sup>, which stimulates remodelling upon incorporation into these complexes (Collinge and Clarke, 2007), see Figure 2, the “seeding” model. Such a mechanism will progressively increase the size of prion complexes and requires fragmentation to occur to generate more infectious entities. Prion proteins in both mammals and yeast can adopt a range of self-replicating conformers known as prion strains, which are thought to confer distinct clinico-pathological phenotypes by assembling into aggregates with different physical properties (Collinge and Clarke, 2007; Legname et al., 2006; Tanaka et al., 2006). These differences are believed to be specific to different rates of conversion of the soluble protein and of fragmentation of prion complexes to generate new propagons, and thereby the efficiency of conformational self-replication, providing a molecular basis for the phenotypes (Angers et al., 2010; Bruce, 1993; Ghaemmaghami et al., 2009; Li et al., 2010; Manuelidis et al., 1997). The existence of mixtures of prion strains *in vivo* raises the possibility that prion phenotypes may not simply reflect the physical properties of a single conformer but rather the collective and dynamic behaviour of the various forms present.

Prion strains have been associated with highly variable clinical phenotypes including changes in incubation periods (Bessen and Marsh, 1992; Kimberlin and Walker, 1978) the efficacy of inoculation routes and transmission rates to other species. Similarly, prion disease pathology, duration and clinical symptoms in humans are altered by the coexistence of prion strains (Cali et al., 2009; Parchi et al., 2009). Thus, the interplay between prion strains directly affects prion phenotypes. These studies suggest that



prion strains compete for a limiting host component necessary for developing a prion infection and the competition between existing strains may similarly be affected by the efficiency with which each conformer is replicated in the tissue (Shikiya et al., 2010).

The physical interaction between prion proteins is an essential event in conformational self-replication. Naturally occurring PrP polymorphisms in animals and man alter TSE characteristics and, in extreme cases, the susceptibility to prion disease (Morales et al., 2006). A well-characterized genetic modifier of prion diseases is Met/Val 129 polymorphism of the prion protein gene (Goldfarb et al., 1989;Owen et al., 1990). Although both PrP129 homozygotes (Met/Met or Val/Val) and heterozygotes (Met/ Val) are susceptible to prion disease, the genotype at this position affects the pattern of PrP<sup>Sc</sup> accumulation in the brain and the clinical symptoms, incubation period and duration of disease (Morales et al., 2006). Disease progression is typically more rapid in homozygotes than in heterozygotes, providing a potential explanation for the over-representation of homozygotes in some TSEs. Heterozygous interference appears to be a general phenomenon, as both animals and humans heterozygous for other PrP alleles show a similar overdominance (Dickinson et al., 1968;Foster and Dickinson, 1988;Goldmann et al., 1994;Jeong et al., 2005;Westaway et al., 1987).

In animal models of prion infectivity, the species barrier has been a well-recognised phenomenon (Collinge and Clarke, 2007), but increasing interest has arisen with the realization that variant CJD appeared in the human population following transfer of BSE from cattle (Bruce et al., 1997;Collinge and Clarke, 2007;Collinge et al., 1996). Studies now suggest that species barriers are the outcome of interactions between prion strains and sequence variants of PrP, which reflect the rate of conformational self-replication in the recipient (Laurent, 1998). Species barriers may manifest as a complete block of disease development (Kimberlin and Walker, 1978) or, alternatively, as a prolonged

incubation period or subclinical infection following first passage that is shortened on subsequent passage within the same species. In both cases, replication of the infectious species occurs during the asymptomatic phase (Hill AF et al., 2000; Race and Chesebro, 1998) but the toxic species apparently does not reach the threshold concentration required for clinical disease within the organism's natural lifespan (Dickinson et al., 1975). Thus, interspecies transmissibility may be operationally defined by the ability of the recipient protein to efficiently replicate the conformation imposed by the template. The concept of a conformational replication barrier to interspecies transmission also explains an early observation that such transitions are often accompanied by a change in prion strain (Kimberlin et al., 1989; Pattison and Jones, 1968).

## **1.6 Prions and the immune system**

Although prions do not induce an immune and inflammatory response typical for infection, both mechanisms of innate and acquired immunity play a role in prion pathogenesis and possible development of protection strategy. Complement has been shown to be activated early during prion pathogenesis by as yet undetermined mechanisms and may constitute the first active response to infection perhaps by binding to C1q and Factor H (Michell, 1996). This binding occurs specifically when prion protein is conformationally modified to represent the conversion to the disease-associated isoform (Blanquet-Grossard et al., 2005). The role of complement in prion pathogenesis has recently been subject to review (Mabbott, 2004). In brief, prion or TSE agents are opsonized by complement components including C1q and C3, most likely via the classical complement activation pathway, which may aid in their targeting of the agent to lymphoid follicles. Mice lacking in complement components C1qa, C2 or C3 revealed deficient peripheral prion pathogenesis (Klein et al., 2001;Mabbott et al., 2001). The production of complement components by mononuclear phagocytes (MNP) has been shown to alter their function (Hartung and Hadding, 1983).C1q enhances the receptor-mediated uptake of disease-associated PrP by classical dendritic cells (Flores-Langarica et al., 2009). The role of complement during prion pathogenesis is further complicated by the observation that the interactions of the individual complement components with PrP<sup>Sc</sup> appear to differ depending on the strain of the infectious agent (Hasebe et al., 2012). As with most innate immune functions within the CNS a clear neuroprotective/neurodegenerative dichotomy is described in the literature, indicating that complement activation may lead to both protective and degenerative effects dependent upon the context and the responsive and regulatory mechanisms involved.

A recent study (Michel et al., 2012) showed that mice overexpressing the cervid prion protein are susceptible to chronic wasting disease, but mice lacking complement acceptors C21/35 expression completely resist clinical disease upon peripheral infection. Mast cells have been implicated in prion pathogenesis due to their high expression levels of cellular prion protein (PrP<sup>C</sup>) and their ability to travel to the brain (Haddon et al., 2009). The role of mast cells in the brain is thought to be neuro-modulatory and their transport to the brain is linked to steroidal hormones and sexual activity or anxiety behaviors (Silver et al., 1996). The presence of mast cells in the brain and their ability to shed expressed PrP upon activation (Haddon et al., 2009) may have implications for prion pathogenesis within the CNS.

Mononuclear phagocytes (MNP) may also play a role in prion pathogenesis. The expression of cellular prion protein in MNP has been associated with phagocytic ability and modulation of inflammatory responses (de Almeida et al., 2005; Nitta et al., 2009; Uraki et al., 2010). Evidence suggests that macrophages (generally identifiable by the markers integrin alpha M, Macrosialin or the F4/80 antigen) degrade the prion agent (Sassa et al., 2009). The degradative and prion clearance abilities of macrophages appear to be down-regulated when macrophage activation is stimulated by danger signal molecules (Gilch et al., 2007). Again the mechanism of degradation is unknown but represents a critical therapeutic target as it constitutes a natural regulatory mechanism to prion pathogenesis. Stimulating or enhancing this degradative ability may improve peripheral resistance to prion infection.

An alternative hypothesis is to suggest that in prion infection with low or poor replicative ability (*i.e.*, due to the factors mentioned above) the balance is switched in favor of degradation of agent, thereby preventing or dramatically slowing pathogenesis. This may occur both within any given cell and not exclusively to degradative or phagocytic cell types. In fact it is well known that there are limited *in vitro* cell infection models in the prion field due to the relevant inability to infect numerous cell types with a

variety of prion strains even within the same species (Neale et al., 2010) and due to the complexity of the host innate immune response.

Castro-Seoane et al (Castro-Seoane et al., 2012;Klohn et al., 2013) studied the kinetics of prion accumulation in various splenic cell types at early phase of infection. Prions were detectable in B and T lymphocytes and macrophages but the highest infection titres were seen in plasmacytoid dendritic cells and natural killer cells. Furthermore, they showed a release of prions from infected cells via exosomes suggesting that intercellular dissemination may contribute to rapid prion colocalisation in the lymphoreticular system.

More recently Klöhn et al (Klohn et al., 2013) reviewed the current paradigms for antigen-sharing pathways which may be relevant to better understand dissemination of rogue neurotoxic proteins. Recent evidence of a remarkably fast splenic prion accumulation after peripheral infection of mice, resulting in high prion titers in dendritic cells (DCs) and a release of prions from infected DCs via exosomes, suggest that intercellular dissemination may contribute to rapid prion colonization in the lymphoreticular system. Evidence from retroviral infections shows DCs and other antigen-presenting cells (APCs) share viral antigens by intercellular transfer to warrant immunity against viruses. Evolved to adapt the immune response to evading pathogens, these pathways may constitute a portal for unimpeded prion dissemination owing to the tolerance of the immune system against host-encoded prion protein.

Antigen presenting cells have long been identified as critical to prion pathogenesis. While evidence exists for both cell-free and cell-mediated transport of prions to lymphoid tissues (Michel et al., 2012), removal of the cell-mediated trafficking severely hampers prion pathogenesis. Prion infection of 'plt' mice deficient in chemokines (CCL19/CCL21) revealed delayed pathogenesis following transcutaneous

infection attributable to impaired CCR7-mediated chemotaxis of DC (Levavasseur et al., 2007). Transient depletion of CD11c-expressing cells (a commonly used marker indicative of classical DC) revealed the ability to completely block or severely impair pathogenesis via oral and intraperitoneal routes (Cordier-Dirikoc and Chabry, 2008; Raymond et al., 2007).

Within the CNS the innate immune response is mediated by specialized MNP known as microglia. Following peripheral prion infection the microglia show signs of activation after neurons and astrocytes have responded. These findings suggest that microglia do not respond directly to presence of misfolded prion protein *per se* but may require priming by other CNS cell types. Once activated the microglial population expands and has been shown to upregulate various markers including Trem2, SiglecF, CD200R, and Fcγ receptors (Lunnon et al., 2011).

Immunity-associated candidate genes have been identified by observational techniques e.g., following gene expression profiling and numerous genes have been screened for a role in prion pathogenesis via knockout transgenic mouse models (Tamguney et al., 2008). The majority of such studies have determined that knockout of an individual component does not prevent prion pathogenesis. From these candidates all have previously been implicated in prion pathogenesis, but interleukin-4 (Il-4), interleukin-6 (Il-6) and interleukin-12 (Il-12) have been shown not to be required. Interleukin-10 (Il-10) knockout mice revealed major alterations to, but not prevention of, prion pathogenesis.

## **1.7 Genetics of prion disease**

Prion diseases are known to have powerful genetic determinants. The strongest genetic association of a common genotype with any disease has been observed in variant CJD (Mead et al., 2009a). All patients with neuropathologically proven variant CJD have been homozygous for methionine at polymorphic codon 129 of the prion protein gene (*PRNP*). Homozygous genotypes at codon 129 also strongly associate with sCJD (Palmer et al., 1991), iatrogenic CJD (Collinge et al., 1991), early age of onset of inherited prion disease (IPD) (Poulter et al., 1992) and kuru (Cervenakova et al., 1999). Although this is a powerful effect, about a third of the population exposed to BSE are homozygous for methionine at *PRNP* codon 129, whilst only around 220 individuals have developed vCJD to date, implicating the potential important role of other susceptibility loci. Another genotype, heterozygosity at codon 219, commonly found in several Asian populations, has been shown to be protective against sCJD (Lukic et al., 2010; Shibuya et al., 1998).

The prion protein gene is known to affect incubation times and disease susceptibility in humans, mice, and sheep (Carlson et al., 1986; Collinge et al., 1991; Hunter et al., 1994; Palmer et al., 1991; Westaway et al., 1994). In *PRNP*, a polymorphism occurs at codon 129 where either a methionine or valine may be encoded. Acquired and sporadic prion diseases occur mostly in homozygous individuals, and a protective effect of heterozygosity is also seen in some inherited cases (Baker et al., 1991; Collinge et al., 1991; Palmer et al., 1991). Mouse quantitative trait loci (QTL) mapping studies using simple crosses have successfully identified many loci linked to prion disease incubation time (Lloyd et al., 2001; Lloyd et al., 2002; Manolakou et al., 2001; Moreno et al., 2003; Stephenson et al., 2000). A new report has added to these data using recombinant inbred lines (Iyegbe et al., 2010). Haplotype analysis and genotyping representative SNPs identified *Cpne8* as the most promising candidate. The role of *Cpne8* in prion

disease has not been established but it may be implicated in PrP processing and targeting as Cpn8 is a member of the copine family that are  $\text{Ca}^{2+}$  dependent phospholipid binding proteins thought to be involved in membrane trafficking (Tomsig and Creutz, 2002). Furthermore, two polymorphisms in the murine PrP gene (*Prnp*) have been described where *Prnpa* (Leu-108, Thr-189) and *Prnpb* (Phe-108, Val-189) are associated with short and long incubation times, respectively (Carlson et al., 1988; Carlson et al., 1986; Moore et al., 1998; Westaway et al., 1987).

Although the influence of PrP gene polymorphisms on susceptibility and incubation time is well established, other lines of evidence indicate that PrP amino acid differences are not the sole genetic influence (Hill AF et al., 1999; Owen et al., 1990; Will et al., 2000). Comparison of several inbred lines of laboratory mice with the same *Prnp* genotype reveals major differences in incubation times when infected by defined prion strain (varying from 100 to 500 days), suggesting that other factors including additional genetic loci may contribute to the observed variation (Carlson et al., 1988; Dickinson, 1975; Kingsbury et al., 1983; Westaway et al., 1987). The identification of these loci in humans may allow identification of at risk individuals, allow more robust predictions of human epidemic parameters, and identify prion ligands and biochemical pathways that will allow a better understanding of prion pathogenesis and the development of rational therapeutics.

The evidence that non-*PRNP* factors exist also comes from analogy with other complex traits, specific human observations and animal studies. Familial concurrence of sCJD has been reported in the absence of a causal mutation (Webb et al., 2008a). Age at onset and death is correlated between parents and offspring in three large UK inherited prion disease kindreds suggesting a significant heritable component to phenotypic variability aside from *PRNP* (Webb et al., 2008c). Although the human observations are consistent with non-*PRNP* factors, mouse models provide the strongest evidence: these are extremely



useful as they reliably recapitulate many features of the human disease as rodents are naturally susceptible to prion disease. It is reasonable to expect that susceptibility genes and pathways identified in mice will also be relevant to human prion disease.

Lloyd et al (Lloyd et al., 2001) analysed 109 animals from an F2 intercross between 2 strains of mice CAST/Ei and NZW/OlaHsd, with significantly different incubation periods when challenged with scrapie prions. Genetic analysis showed a highly significant linkage between loci on chromosomes 2, 11 and 12 and prion disease incubation time. In fact, multiple linked quantitative trait loci identified on these chromosomes explained 82% of the total variance of incubation time. Interestingly, in another study Lloyd et al (Lloyd et al., 2002) investigated animals from the F2 intercross between the same 2 mouse strains challenged with a strain of BSE passaged twice through one of the parental mouse strains. Genetic analysis again identified linkage with 2 highly significant regions on chromosomes 2 and 11 and incubation time independently of prion strains. Two additional genes have been suggested to have an effect on incubation time: interleukin-1 receptor (IL-1) (Marcos-Carcavilla et al., 2007) and superoxide dismutase-1 (SOD-1) (Akhtar et al., 2013). It had been shown that deletion of *APP*, IL-1 gene and overexpression of human SOD-1 significantly increase survival times in infected mice (Akhtar et al., 2013). The study confirmed a highly significant association between incubation time of prion disease and SOD1 locus. Furthermore, the study showed that SOD1 deficiency significantly reduces incubation time in mice after being challenged with three different prion strains. Demonstration of mouse quantitative trait genes may be relevant for understanding genetics of human prion diseases as illustrated by the finding that *HECTD2* haplotypes are a quantitative trait gene for prion disease incubation time in mice and are associated with susceptibility to acquired human prion diseases, vCJD and kuru (Lloyd et al., 2009).

As already discussed the *PRNP* locus is highly associated with prion diseases in humans and animals irrespective of clinical presentation of disease. Mammalian prion diseases are under strong genetic influence of *PRNP* locus and this association is driven by coding variation at codon 129 of *PRNP* (rs1799990). Genetic polymorphism encoding methionine or valine amino acid is a strong genetic factor or modifier of clinico-pathological phenotype (Collinge et al., 1991; Collinge, 2005; Collinge et al., 1992; Palmer et al., 1991). As mouse study indicated the existence of several *Prnp* modifier genes (Lloyd et al., 2001; Lloyd et al., 2002; Stephenson et al., 2000) parallel studies of human genes analogous to mouse candidate genes have been done but they were underpowered because of the rarity of prion diseases. Recently Mead et al (Mead et al., 2011b) presented the first large genome wide association study (GWAS) that included 2000 samples from three populations of human prion diseases and relevant available control series. This study included samples from patients suffering from sCJD, vCJD, inherited prion disease, iatrogenic CJD and kuru collected in UK, Germany and Papua New Guinea. The analysis did not provide support for the roles of already proposed genes as potential risk factors such as *RARB-THRB* locus upstream of *STMN2* and in the *HECTD2* and *SPRN2* genes. It appears that other genetic factors may be weak in human prion disease although heritability has been suggested by sibling concurrence (Webb et al., 2008a), heritability of age of onset of inherited prion disease (Webb et al., 2008c) and documented genetic modifiers of incubation time in mice (Lloyd et al., 2001). However, in comparison with other diseases, the sample size available for analysis remains modest and weak effects would not be detected.

Sanchez-Juan et al (Sanchez-Juan et al., 2011) showed an association between a *PRNP* regulatory polymorphism G310C and sCJD. After adjusting by *PRNP* M129V genotype, the analysis showed that being a C allele carrier at *PRNP* G310C was significantly associated with a 2.4 fold increased risk of developing sCJD. In addition, haplotypes carrying *PRNP* 310C coupled with *PRNP* 129M were significantly

overrepresented in patients compared to controls. Cases of sCJD carrying a *PRNP* 310C allele presented at a younger age (on average 8.9 years younger than those without this allele), which was of statistical significance. As expected, methionine and valine homozygosity at *PRNP* M129V increased significantly the risk of sCJD, alone and adjusted by *PRNP* G310C. The findings were in keeping with the hypothesis that genetic variations in the *PRNP* promoter may have a role in the pathogenesis of sCJD.

Other explanations of the lack of associations in sCJD include the heterogeneity of this disease defined by a clinical syndrome. Several aetiologies are possible in this group including a proportion of zoonotic or iatrogenic diseases. Thus, *PRNP* genotypes were strongly associated with risk in all prion diseases and no other loci show a similarly strong or universal association in humans.

A chromosomal gene encodes PrP, a member of the *Prn* gene family that also includes *Prnd*, encoding the Doppel protein (Moore et al., 1999), and *Sprn*, encoding Shadoo (Watts and Westaway, 2007), as previously discussed. In all known PrP genes from various species, the PrP open reading frame (ORF) is encoded within a single exon although the gene itself comprises two to three exons (Basler et al., 1986; Gabriel et al., 1992; Hsiao et al., 1989; Westaway et al., 1987). The other exons contain untranslated sequences including the promoter and termination sites. The PrP promoter contains multiple copies of GC-rich repeats—a canonical binding site for the transcription factor Sp1 (Saeki et al., 1996), driving expression in many different tissues. The alignment of the translated sequences from more than 40 PrP genes shows substantial conservation between the mammalian sequences, suggesting the retention of some important function for PrP through evolution.

Variant CJD (vCJD) due to transmission of prions from cattle to humans has gained public notoriety following the BSE epidemic. Whilst the incidence of vCJD has been in decline since 2001, and the international case load remains modest at around 220, there are several outstanding issues of relevance

to public health (NCJDSU, 2009). First, it is paradoxical that an estimate of the incubation time of vCJD (based on the duration between the BSE and vCJD epidemic peaks) is so remarkably short, at around 8 years. Estimates of incubation times from acquired prion diseases that involve human-human transmission are around 12 years (for kuru, or iatrogenic CJD)(Alpers, 2008;Brown et al., 2000). Based on animal experiments one expects the incubation times involving transmission between species to be prolonged compared with intraspecies transmission. As human-human transmission incubation times can be over 50 years (Collinge et al., 2006), it would be incautious to conclude that vCJD is a completed epidemic. Secondly, there is a discrepancy between prevalence studies in the UK and the observed clinical cases suggesting, in congruence with animal studies, that subclinical infection may be more likely than clinical disease. The determinants of subclinical disease or clinical manifestation are not known. Thirdly, the propensity for and extent of human-human iatrogenic transmission remains poorly understood. Several thousand individuals in the UK have been notified that they have been exposed to blood or blood products derived from donors incubating vCJD. Much of the scientific basis for understanding these outstanding issues revolves around the susceptibility of individuals relative to their population. There are a number of other factors such as dose effect, PrP strain and environmental factors that are likely to influence the incubation time. Genetics is a tractable way to study this in order to inform the epidemiology and public health through estimation of risks to BSE prion-exposed populations.

The purpose of research into the human genetics of prion disease is not only about addressing issues derived from the vCJD epidemic. Prion diseases involve the propagation of strains with distinct clinical, pathological and biochemical features (Collinge and Clarke, 2007). This feature is a manifestation of protein-based inheritance where the transmissible agent is the product of a single host gene. Aside from human transmissibility, other aspects of the fundamental biology may not be unique to prion diseases.

Several prions in yeast have now been well characterized (Wickner et al., 2007). The autocatalytic protein misfolding that is central to prion disease is shared with other neurodegenerative conditions such as Alzheimer's and Parkinson's diseases. The extent to which these more common disorders are "prion-like" and whether they also involve strains remains an important and unresolved issue. Here, the relevance is that the identification of non-*PRNP* genetic loci that control susceptibility to prion disease or determine strains, may allow insights into fundamental molecular pathways likely to be of wider relevance in neurodegeneration such as the human proteins involved in chaperoning or degrading misfolded protein entities; or alternatively the genes involved in determining a prion strain.

In the European-led vCJD genome wide association study (Sanchez-Juan et al., 2012) two single nucleotide polymorphisms (SNPs) (rs4921542 and rs7565981) reached genome-wide significance after pooling discovery and replication populations. Rs4921542 ( $p = 1.6 \times 10^{-8}$ ) is an intronic variant in the myotubulin related protein 7 gene (*MTMR7*), which is specifically expressed in the central nervous system and dephosphorylates phosphatidylinositol 3-phosphate and inositol 1,3-bisphosphate. Rs7565981 ( $p = 4.2 \times 10^{-8}$ ) is in an intergenic region upstream of the neuronal PAS (per-ARNT-sim) domain-containing protein 2 gene (*NPAS2*), a regulatory gene belonging to a family of transcription factors. In the UK-German sCJD study, no non-*PRNP* loci achieved genome-wide significance. SNPs at the *ZBTB38-RASA2* locus were associated with CJD in the UK (rs295301,  $p = 3.13 \times 10^{-8}$ ) but these SNPs showed no replication evidence of association in German sCJD or in kuru based tests. Overall, it is likely that the *PRNP* locus contains the only strong risk factors which act universally across human prion diseases. Whilst some genome wide significant loci have been proposed in vCJD, the low incidence of this condition means that there is no way at present to generate unequivocal genetic evidence at these loci. The collective data are consistent with the findings in other diseases, of strong effects being the exception but multiple risk loci of modest effects are likely to present although more difficult to confirm.

In prion diseases large collaborative GWAS are necessary to provide statistical power for these weak effects.

## **1.8 Link with genetics of other neurodegenerative diseases**

Neurodegeneration preferentially involves the cerebral cortex in Alzheimer's disease (AD), the extrapyramidal system in Parkinson's disease and the spinal cord in amyotrophic lateral sclerosis.

Mendelian inheritance pattern has been established in inheritance of these diseases but with clinical presentations and pathology often indistinguishable from sporadic forms of the same disease suggesting the same pathophysiological mechanisms. Genome wide association studies have been used to identify susceptibility genes implying common single nucleotide polymorphisms. Although GWAS have successfully revealed numerous susceptibility genes for common disease, the odds ratio associated with these risk alleles are generally low and account for only a small proportion of estimated heritability (Hindorff et al., 2009; Manolio et al., 2009; Visscher, 2008).

### **Genetic studies**

It is postulated that early onset AD is often caused by mutation in three different genes (Tsuji, 2010). These mutations include genes encoding amyloid- $\beta$  protein precursor (*APP*), presenilin 1 (*PSEN 1*) and its homologue on chromosome 1 (*PSEN 2*). GWAS have been used to find late onset AD gene candidates related to disease risk, phenotype, biomarkers, imaging results and pathology. Apolipoprotein E (*APOE*) has been associated with late onset AD. The  $\epsilon 4$  allele (Arg 112/Arg 118) of the *APOE* gene increases AD risk fourfold in one copy and by greater than 10 fold two doses of allele. In contrast,  $\epsilon 2$  (Cys 112/Cys 112) allele of *APOE* exert protective effect while early onset familial AD mutations are sufficient but not necessary to cause AD. It is believed that in AD, *APOE* plays a role in the clearance of amyloid- $\beta$  from the brain. The first genome wide significant finding was reported for *GAB2*, proposed to influence tau phosphorylation (Reiman et al., 2007) and to modulate A- $\beta$  production by binding growth factor receptor bound protein 2 (*GRB2*) which binds amyloid precursor protein and presenilins (Nizzari et al.,

2007). In addition to *APOE*, several other genes have been shown to have a genome wide significant association in AD. A study by Harold et al (Harold et al., 2009) involved over 16,000 individuals suffering with Alzheimer's disease (AD), was the most powerful AD GWAS at the time. The established association with the apolipoprotein E (*APOE*) locus was replicated (most significant SNP, rs2075650,  $P = 1.8 \times 10^{-157}$ ) and further genome-wide significant association with SNPs at two loci were observed: at the *CLU* (also known as *APOJ*) gene (rs11136000,  $P = 1.4 \times 10^{-9}$ ) and 5' to the *PICALM* gene (rs3851179,  $P = 1.9 \times 10^{-8}$ ). These associations were replicated in the second stage of the study which involved 2,023 cases and 2,340 controls. This finding produced compelling evidence for association with Alzheimer's disease in the combined dataset (rs11136000,  $P = 8.5 \times 10^{-10}$ , odds ratio = 0.86; rs3851179,  $P = 1.3 \times 10^{-9}$ , odds ratio = 0.86).

A study by Hollingworth et al (Hollingworth et al., 2011) showed new susceptibility loci for Alzheimer's disease through a staged association study (GERAD+) and by testing suggestive loci reported by the Alzheimer's Disease Genetic Consortium (ADGC) in a companion paper. A combined analysis of four genome-wide association datasets (stage 1) was undertaken and ten newly associated variants were identified with  $P \leq 1 \times 10^{-5}$ . These variants were then tested again for association in an independent sample (stage 2). The finding of three SNPs at two loci was replicated and showed evidence for association in a further sample set (stage 3). Meta-analyses of all data provided compelling evidence that *ABCA7* (rs3764650, meta  $P = 4.5 \times 10^{-17}$ ); including ADGC data, meta  $P = 5.0 \times 10^{-21}$ ) and the *MS4A* gene cluster (rs610932, meta  $P = 1.8 \times 10^{-14}$ ); including ADGC data, meta  $P = 1.2 \times 10^{-16}$ ) are new AD susceptibility loci. The study also provided independent evidence for association for three loci reported by the ADGC, which, when combined, showed genome-wide significance: *CD2AP* (GERAD+,  $P = 8.0 \times 10^{-4}$ ); including ADGC data, meta  $P = 8.6 \times 10^{-9}$ ), *CD33* (GERAD+,  $P = 2.2 \times 10^{-4}$ ); including



ADGC data, meta  $P = 1.6 \times 10^{-9}$ ) and EPHA1 (GERAD+,  $P = 3.4 \times 10^{-4}$ ); including ADGC data, meta  $P = 6.0 \times 10^{-10}$ ).

Amyloid- $\beta$  is a proteolytic fragment generated through sequential cleavage of the amyloid precursor protein (*APP*) by  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase. Cells produce  $A\beta$  peptides of variable length. A peptide of 40 amino acids ( $A\beta_{40}$ ) is the most prevalent species secreted by cells, whereas the longer  $A\beta_{42}$  isoform appears to be the key pathogenic species and the most abundant species deposited in the brain. Major support for the amyloid hypothesis is drawn from cases of early onset familial Alzheimer's disease (FAD). The term FAD is confined to familial cases with an autosomal dominant inheritance pattern. Overall, the clinical presentation of FAD patients with *APP* and *PSEN* mutations is very similar to that of sporadic AD, which is supported by neuroimaging, biomarker and post-mortem neuropathology studies.

*APP* and *PSEN* mutations cause FAD with autosomal dominant inheritance and early disease onset. FAD is clinically and neuropathologically largely indistinguishable from the sporadic forms of AD, indicating that amyloidosis is a driving force in the aetiology of both FAD and sporadic AD. Biochemical studies have shown that *APP* mutations either shift the generation of  $A\beta$  peptides towards the highly amyloidogenic  $A\beta_{42}$  isoform or enhance the aggregation propensity of the  $A\beta$  peptides. No evidence has been found that these mutations impair the physiological function of *APP*. *PSEN* mutations also drive amyloidosis in FAD patients through changes in the  $A\beta_{42}/A\beta_{40}$  ratio. In addition, it has been proposed that *PSEN* mutations could impair other  $\gamma$ -secretase dependent and independent functions of *PSEN*. It is important, however, to note that none of these phenotypes have been comprehensively replicated in experimental models that bear relevance to the heterozygous genetic background of FAD patients with *PSEN* mutations. In the few studies that have used primary cells from FAD patients or heterozygous

knock-in mice, only single or a small number of *PSEN* mutations were investigated. It appears premature, therefore, to conclude that loss of function phenotypes like reduced NOTCH signalling that were reported in overexpression studies with FAD *PSEN* mutants are relevant to the clinical phenotype of FAD patients, or may even contribute to the pathology of sporadic AD.

### **Clinical and neuropathological features**

While Alzheimer's disease and prion disease differ in terms of incidence and to a lesser extent duration of symptoms, both processes demonstrate: (1) characteristic age of onset; (2) sporadic occurrence in the majority of cases; and (3) inherited disease based on mutations in an amyloidogenic protein in a minority of cases. Aside from kuru and rare iatrogenic cases, prion disease tends to present in middle age and in older patients (Parchi et al., 1999a). Therefore, it is not surprising that age-related pathological processes such as oxidative stress, protein cross-links, and adduct formation that are widespread early findings in Alzheimer's disease (Sayre et al., 1999), also occur in prion disease (Choi et al., 2004; Guentchev et al., 2000; Kim et al., 2001). 85% of human prion disease cases are sporadic, i.e., there is no exposure to contaminated material and no detectable mutation leading to disease. The lack of clusters of CJD throughout the world aside from kindreds with familial CJD (e.g., E200K mutation) is also consistent with the sporadic disease concept and suggests that prion disease is primarily a neurodegenerative process. In the last few years, a striking number of epidemiological, neuropathological, and biochemical similarities between prion diseases and Alzheimer's disease have been identified, in particular the fact that there are interactions between the two proteins (Jucker and Walker, 2011; Jucker and Walker, 2013) and between the signalling pathways involving both proteins. Epidemiologically and clinically, both disorders are dementing illnesses that mainly occur sporadically,

but can also be inherited. Histopathologically, both are characterised by the deposition of an extracellular amyloid that is produced by neurones. In both diseases, there is formation of amyloid oligomers and ultimately also of solid amyloid aggregates in the brain: both amyloid proteins can accumulate diffusely, or they can exhibit prominent and widespread deposition of dense amyloid plaques with a diameter of more than 200  $\mu\text{m}$ , and both elicit a considerable astrocyte and microglial reaction (Jucker and Walker, 2013).

### **Transmission studies**

A study by Eisele et al (Eisele et al., 2009) demonstrated the induction of cerebral  $\beta$ -amyloidosis in different brain areas of *APP23* transgenic mice through the injection of dilute amyloid- $\beta$  containing brain extracts. Once the amyloidogenic process has been exogenously induced, the nature of the induced amyloid- $\beta$  deposition was determined by the brain region of the host. The authors further tested the transmission of cerebral  $\beta$ -amyloidosis to *APP23* transgenic mice through the contaminated steel wires which were only prevented by plasma sterilization, but not boiling of the wires before implantation. The study suggested that very small amounts of amyloid- $\beta$  containing brain tissue in direct contact with the CNS can induce cerebral beta-amyloidosis. The observation is reminiscent of prion-like mechanisms including the propagation of pathology suggesting common pathways in the pathogenesis of the two conditions.

The concept of prion-like induction and spreading of pathogenic proteins has recently been expanded to include aggregates of tau,  $\alpha$ -synuclein, huntingtin, superoxide dismutase-1, and TDP-43, which characterize such human neurodegenerative disorders as frontotemporal lobar degeneration,

Parkinson/Lewy body disease, Huntington disease, and amyotrophic lateral sclerosis. A recent finding by Jucker et al (Jucker and Walker, 2011) showing that the most effective amyloid- $\beta$  seeds are small and soluble intensifies the search in bodily fluids for misfolded protein seeds that play a role in the proteopathic cascade, and could serve as predictive diagnostics and the targets of early, mechanism-based interventions.

## **1.9 Genome wide association studies - copy number variants**

Variation is the currency of human genetics. These differences between individuals can take many forms including: single nucleotide polymorphisms (SNPs), tandem repeats, small insertions and deletions or larger variants involving thousands-millions of nucleotides due to deletions, insertions and duplications referred to as copy number variants (CNVs). There are also other chromosomal rearrangements such as inversions and translocations (also known as copy neutral variations) and copy neutral loss of heterozygosity (LOH). Insight into human DNA variation has been facilitated by two major projects: the Human Genome Project (Collins and McKusick, 2001) which sequenced the human genome and the HapMap project (2003) which identified and annotated the sequence location of over one million SNPs and their associations with nearby variants in key populations.

The annotation of SNPs to their location within the human DNA sequence facilitated the development of arrays containing hundreds of thousands of SNPs and CNV probes used as markers for genotyping.

Utilizing these technologies, the frequency of alleles and CNVs in cases versus controls is made possible, forming the basis for the now routine genome-wide association (GWAS) case-control study. Platforms developed for high-throughput genotyping allow hundreds of thousands of DNA samples to be genotyped with high accuracy for up to around a million SNPs and CNV probes simultaneously in a single procedure.

Despite their overall success, standard genome-wide association studies have limitations, they generally test for associations of single nucleotide polymorphisms with a relatively high minor allele frequency (MAF) (typically >1%). The strategy of focusing on common SNPs in genetic association studies effectively considers the “common-disease-common-variant (CDCV)” hypothesis, i.e. risks for common diseases are conferred by multiple common variants albeit of variable strength of effect. GWAS based

on a quarter of a million to one million common SNPs rely on linkage disequilibrium (LD) mapping, the non-random association of alleles of adjacent loci on a chromosome (Li and Leal, 2008). A set of common SNPs (chosen either randomly, for technological reasons or selected to optimize the capture of other SNPs by LD) provided by the existing high-throughput genotyping platforms can cover the genome well enough to capture a large proportion of all the common variants at unmeasured loci. The “rare variants hypothesis” proposes that a significant proportion of inherited susceptibility may be due to the combination of the effects of a series of rare or low frequency variants of a number of different genes, each conferring a moderate-strong increase in relative risk. Such rare variants are more likely to be private to populations or families because of founder effects and genetic drift which makes these SNPs less well understood and harder to comprehensively incorporate in off-the-shelf genotyping technologies. The debate about the importance of common or rare variants is relevant to study design, but inevitably rather pointless as it has already been established that variants of all population frequencies may be significant risk factors in complex traits.

GWAS is based on a case–control association design to determine if one or more alleles are more frequent in cases, reflecting the genetic region of a risk variant. It is important to understand that a region identified may contain multiple genes or non-gene factors that have effects at a distance. Generalities in recent case–control association studies are that risk is usually conferred by multiple genetic factors each contributing only modest effects, and that variants are rarely associated with coding changes in proteins, rather, presumably acting by altering expression of a gene in the broadest sense. Because of the large number of hypotheses tested, there is great potential for false-positive associations. To overcome these issues a statistical correction is applied, conventionally the Bonferonni correction which requires a p value of less than around  $5 \times 10^{-8}$  for a locus to be considered to have

evidence for genome-wide association and to have a high chance of being replicated (Petretto et al., 2007).

A typical genome wide association study with about 2,000 cases and controls has the power to detect only moderate effects with an MAF of 5% at the stringent genome-wide significance level ( $5 \times 10^{-8}$ ). To overcome this constraint, a very large discovery sample size is usually required which may not always be possible, particularly in case of rare diseases such as prion disease and certainly for vCJD. Replication in an independent sample is necessary to solidify evidence for association and rule out biases such as genotyping error or population stratification. Replication should be conducted in a similar population with a sample size adequate to detect a similar magnitude of effect and significance using the same genetic model.

Genome wide association studies have allowed the analysis of structural genetic variation (McCarthy et al., 2008). CNVs have recently gained considerable interest as a source of genetic diversity with well-known examples causing neurodegenerative diseases (Blauw et al., 2010; Nuytemans et al., 2009). Several large-scale studies have reported the presence of extensive copy number variation in humans, suggesting that CNVs may account for a significant proportion of human phenotypic variability, including disease susceptibility (Feuk et al., 2006). They represent the most dynamic form of human genetic variation from one generation to the next, peppered throughout the genome and recently associated with conditions such as schizophrenia, autism and mental retardation. Several theories have been proposed to explain the widespread occurrence of CNVs in the human genome such as non-allelic homologous recombination and non-homologous end joining (Frazer et al., 2009).

A widely used whole-genome SNP genotyping platform, the Illumina Human Hap 550 BeadChip (Gunderson et al., 2005), assays more than half a million SNPs in parallel (median SNP distance  $\sim 3$  kb),

permitting kilobase-resolution detection of CNVs. The assay combines specific hybridization of genomic DNA to arrayed probes with allele-specific primer extension and signal amplification, thus achieving a high signal-to-noise ratio in genotype calling (Gunderson et al., 2005). The assay does not require PCR-based amplification, thus detected signals are less susceptible to biases caused by differential amplification of given chromosomal regions. In addition to the total fluorescent intensity signals from both sets of probes/alleles at each SNP (referred to as 'log R ratio'), the Illumina platform also allows inference of the relative ratio of the fluorescent signals between 2 probes/alleles at each SNP (referred to as 'B allele frequency'). Furthermore, data normalization at each SNP on the Infinium platform is performed by comparison of signals from a set of reference samples (eg. HapMap samples) leading to less signal variation between SNPs. Conventional methods for CNV identification on the Illumina platform involve examination of intensity signals (implemented in the LOH-plus module of the BeadStudio software), which identifies copy number changes by calculating the mode of B Allele Frequency for SNPs in a sliding window along the chromosome. While simple to implement, the sliding window approach has limited and relatively coarse boundary resolution for detected CNVs. A recently described algorithm, QuantiSNP, incorporates the log R Ratio and B Allele Frequency simultaneously in a hidden Markov model (HMM) framework (Colella et al., 2007). As demonstrated by simulations and by studies on individuals with known large aberrations, QuantiSNP significantly improves the resolution of CNV detection. The development of algorithms that both accurately model the signal measures and integrate more available data (e.g., genotype frequency, family relationship) can potentially lead to further improvement of CNV detection.

CNV Partition plug-in algorithm is supported by Genome Studio and has been optimised for speed and accuracy using log R and B allele frequency in combination to identify chromosomal aberrations, estimate copy number value and calculate per-region confidence scores. It is fast to analyse and report



(~1min per sample) and detects CNV regions and estimates CNV values with high accuracy (Winchester et al., 2009). It identifies small CNV regions (<100 kb) and provides confidence score for each locus.

CNVs account for a significant proportion of genetic diversity, with consequences in term of evolution and disease susceptibility (Conrad and Hurler, 2007). Consequently, their detection and association with quantitative traits and clinical phenotype constitute an important step towards a better understanding of disease etiology. However, accurate detection of copy number variants remains challenging. There are numerous factors in the data generation and computational analyses that can lead to spurious associations. The sheer amount of data that can be generated for a single subject imposes challenges in terms of data interpretation. Several large- scale aberrations (Dowjat and Wlodarska, 1981; Nister et al., 1987; Pepler et al., 1968) were identified before the development of higher resolution techniques. Fluorescence *in situ* hybridization (FISH) has increased this resolution, enabling the detection of *sub-microscopic* CNVs that could not be detected with karyotyping. Today, the most widely used techniques can be classified as amplification-based polymerase chain reaction (PCR), hybridization-based (FISH, comparative genome hybridization, and SNP arrays) or sequencing-based. These techniques differ in precision, throughput, and resolution.

A combination of rare and disruptive variants of large effect can contribute to different phenotypic outcomes, such as intellectual disability, epilepsy, autism and schizophrenia. Primary mutations sensitize individuals to disease from secondary mutational events, which compound at the molecular level to modify the outcome and severity. The mode of inheritance for both primary and secondary mutations, as well as the size and gene content of copy-number variants, is a critical determinant in distinguishing syndromic disorders from mutations with phenotypic variation. The overall burden of genes that are

affected by large variants may eventually be of prognostic significance, allowing clinicians to better anticipate long-term outcomes when the variants are discovered in affected persons.

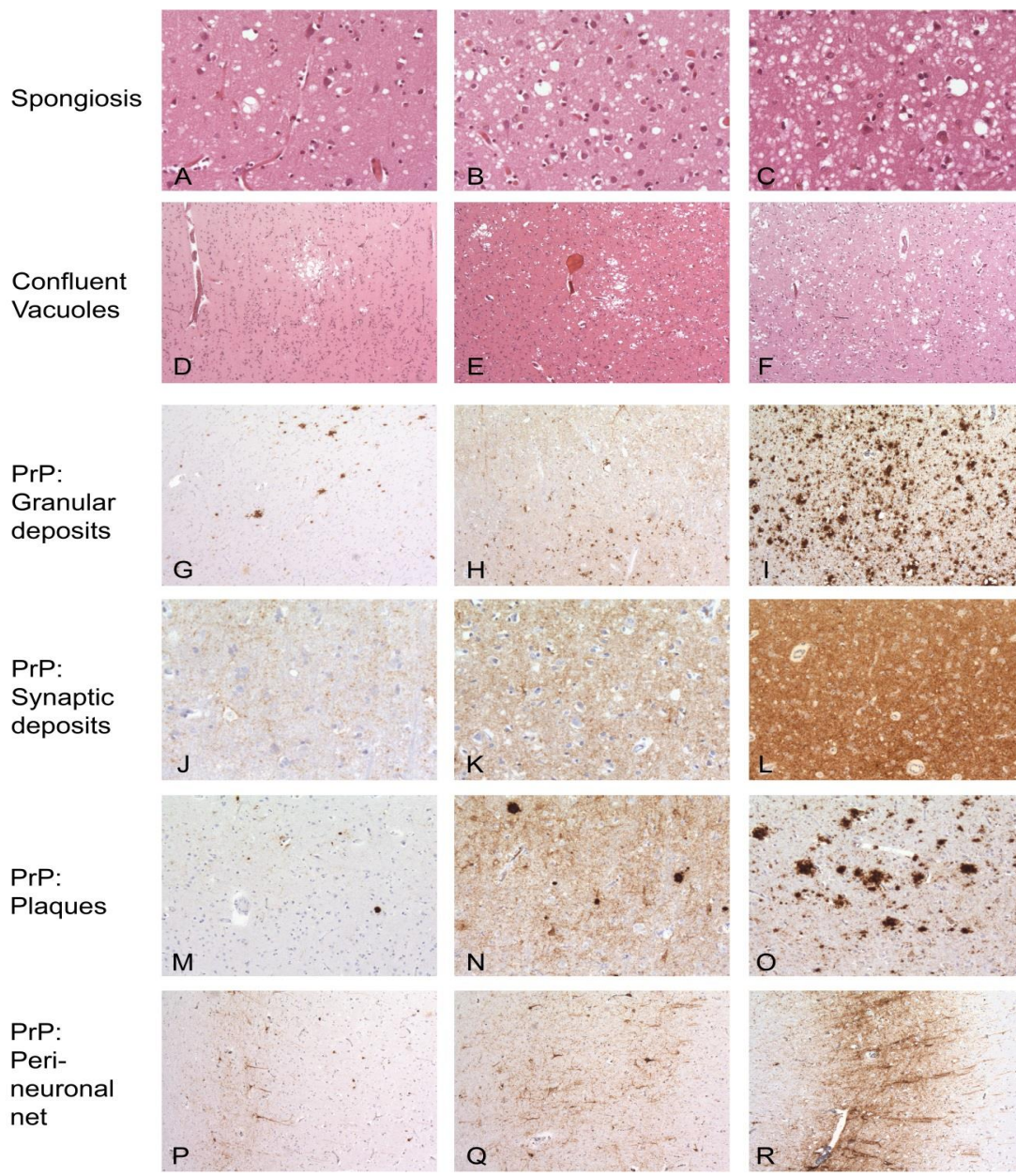
### **1.10 Neuropathology of prion diseases**

The classical triad of spongiform change, neuronal loss, and gliosis (astro- and microglia) is the neuropathological hallmark of prion diseases (Reiniger et al., 2011). Neuronal loss and gliosis may be present in many other conditions of the CNS, for example, Krabbe disease, caused by a deficiency of the enzyme galactosylceramidase. Progressive Spongiform Leukoencephalopathy (PSL) -- which is a spongiform encephalopathy—is not a prion disease, although the etiological factor which causes it among heroin smokers has not yet been identified (Kriegstein et al., 1999). Prion to PrP immunohistology, the crucial feature was *spongiform change* accompanied by neuronal loss and gliosis. This spongiform change is characterized by diffuse or focally clustered small round or oval vacuoles in the neuropil of the deep cortical layers, cerebellar cortex or subcortical grey matter, which might become confluent. Spongiform change should not be confused with non-specific spongiosis. This includes status spongiosus (“spongiform state”), comprising irregular cavities in gliotic neuropil following extensive neuronal loss (including also lesions of “burnt-out” CJD), “spongy” changes in brain oedema and metabolic encephalopathies, and artifacts such as superficial cortical, perineuronal, or perivascular vacuolation; focal changes indistinguishable from spongiform change may occur in some cases of Alzheimer's and diffuse Lewy body diseases. Very rare cases might not be diagnosed by these criteria. Then confirmation must be sought by additional techniques such as PrP immunoblotting, molecular biologic studies, or experimental transmission (Budka et al., 1995).

The spongiform change may be mild, moderate or severe (illustrated in Figure 5) and is characterised by diffuse or focally clustered, small, round or oval vacuoles in the neuropil of the deep cortical layers, cerebellar cortex or subcortical grey matter, which might become confluent. Presence and distribution of spongiform change vary greatly between cases and disease subtypes. An almost constant location in

sCJD is the head of the caudate nucleus. On the contrary, spongiform changes are rarely present in the brainstem and spinal cord, although PrP accumulation can be demonstrated at these sites. Extensive sampling from various brain areas (including frontal, temporal, and occipital lobes, basal ganglia, and cerebellum) is normally done in every suspected case. However, one block of tissue with typical histological changes and/or unambiguous PrP<sup>Sc</sup> immunoreactivity is sufficient for a definite diagnosis. Brain biopsy has been found to have a diagnostic sensitivity of 95% of sporadic CJD cases in which the disease has been confirmed at autopsy or by experimental transmission. This procedure is however, usually restricted to rare instances where a treatable alternative diagnosis is suggested by clinical or investigation findings. In sCJD, the regional distribution of spongiform change in distinct patterns was shown to depend upon PrP<sup>Sc</sup> fragment sizes and glycotypes, and codon 129 genotype of *PRNP* (Budka, 2000). However, some prion diseases have equivocal, little, or no spongiform change, such as inherited prion diseases due to D178N mutation that is specifically characterised by prominent thalamic atrophy with profound astrogliosis.

### Spongiform change and prion protein deposition in sporadic CJD



**Figure 5.** Spongiform change and PrP deposition in sCJD: spongiosis (**a–c**) and patchy confluent vacuolisation (**d–f**). Patterns and intensities of commonly observed prion protein deposits: (**g–i**) coarse granular deposits of prion protein, which may form confluent aggregates. (**j–l**) Synaptic PrP, ranging from mild to severe, and (**m–o**), formation of plaques in sCJD. (Images obtained from 'Reiniger L, Lukic A, Linehan J, Rudge P, Collinge J, Mead S, Brandner S. Tau, prions and A $\beta$ : the triad of neurodegeneration', *Acta Neuropathol.* 2011 Jan;121(1):5-20).

### **1.11 Clinico-pathological characteristics of human prion diseases**

Traditionally, the human prion diseases have been classified into clinic-pathological syndromes: CJD, Gerstmann-Sträussler-Scheinker disease, fatal familial insomnia and kuru, an epidemic prion disease of the Fore linguistic group of Papua New Guinea. Alternatively, they can be grouped by aetiology, as inherited, acquired, or sporadic (unknown) cause. sCJD occurs with a seemingly random case distribution and an annual incidence of around one per million per year. Around 10-15% of human prion disease is inherited; almost all cases of familial prion disease have been associated with coding mutations in the prion protein gene (*PRNP*), of which more than 60 mutations are described (Mead, 2006), see Figure 3. The acquired prion diseases include iatrogenic CJD, kuru and variant CJD, and arise from accidental exposure to human prions through medical and surgical procedures (especially acquired dura mater transplantation or cadaver-derived growth hormone therapy), participation in endocannibalism in the case of kuru, and exposure to BSE infected meat. Most types of prion disease are highly heterogeneous in terms of clinical presentation, incubation time (in acquired types) and age of onset.

### 1.11.1 Sporadic CJD

The annual mortality rate of sCJD is roughly 1-2 per million per year and is broadly similar worldwide and between sexes. It peaks in the seventh decade and then appears to decline with advancing age ([www.cjd.ed.ac.uk/documents/report22.pdf](http://www.cjd.ed.ac.uk/documents/report22.pdf)). There have been occasional reports in young adults aged <40 years. The core clinical feature is a rapidly progressive cognitive decline which may be preceded by a change in personality, psychiatric features or sleep disturbance. It may only be a matter of weeks between presentation and death. Definitive diagnosis is by autopsy and is characterized by spongiform change, neuronal loss, astrocytosis and prion protein deposition.

During life, patients can be diagnosed with possible or probable sCJD according to established WHO criteria ([www.cjd.ed.ac.uk](http://www.cjd.ed.ac.uk), Table 1). A study by Tschampa proposes the introduction of brain MRI for diagnosis of CJD (Tschampa et al., 2005). The data confirm that basal ganglia and cortical hyperintensities represent the most frequent MRI findings in sCJD and are present in most cases. The authors suggested modifying the diagnostic criteria to include detection of either hyperintensity in the basal ganglia (both caudate nucleus and putamen) or in at least two cortical regions (from either the temporal, parietal or occipital cerebral cortices). Characteristic brain MRI lesion patterns are helpful in establishing a diagnosis of sCJD and may help to identify atypical sporadic disease forms. Atypical cases may have a protracted clinical course or present with unusual clinical features. Such cases are more frequently associated with a valine homozygous genotype at codon 129. The diagnosis of sCJD should be questioned if there is a history of dementia longer than 2 years, if the typical neurological signs are absent, or if there is evidence of inflammation. Periodic sharp waves are not seen in a third of EEGs in definite CJD cases and MRI shows high signal on T2 weighted scans in the caudate and putamen in about

two-thirds of definite CJD (Tschampa et al., 2005). Proposed diagnostic criteria according to Tschampa et al (Tschampa et al., 2005) for sCJD are presented in Table 1.

The differential diagnosis of sCJD includes: Alzheimer’s disease and other dementias (usually in older patients), cerebral vasculopathies, chronic encephalitis (including Hashimoto’s encephalitis), encephalopathy (due to anoxic brain damage or a paraneoplastic cause) and white matter diseases such as multiple sclerosis.

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### **I. Clinical signs:**

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1. Dementia
  2. Cerebellar or visual
  3. Pyramidal or extra - pyramidal
  4. Akinetic mutism
- 

### **II. Tests**

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1. Periodic sharp wave complexes on EEG
  2. Detection of 14-3-3 protein in the CSF (in patients with disease duration of less than 2 years)
  3. High signal abnormalities in caudate and putamen or at least 2 cortical regions (temporo-parietal-occipital) either in DWI or FLAIR
- 

### **III. Diagnosis**

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4. Probable sporadic CJD  
Two out of I and at least one out of II
  5. Possible sporadic CJD  
Two out of I and disease duration less than 2 years
- 

**Table 1** shows the proposed diagnostic criteria according to Tschampa et al (Tschampa et al., 2005) for sCJD patients to include the characteristic MRI changes associated with sCJD. For the diagnosis of probable sCJD, two out of the listed clinical signs and at least one of the listed tests is necessary. For a diagnosis of possible sCJD two out of the listed clinical signs and disease duration of less than 2 years is required.



### 1.11.2 Inherited prion disease

The incidence of inherited prion disease (IPD) is around 10-15% of the total. All cases so far have been associated with pathogenic mutations in the open reading frame (ORF) or prion protein gene and are dominantly inherited. A proportion of IPD cases have been reported without a positive family history of the disease, and many cases do not share clinical features with classical CJD, hence the term inherited or genetic prion disease is used by many in preference to 'familial CJD'. Many cases of IPD have relatively slowly progressive phenotypes, such as Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI) and inherited prion disease due to Y163X mutation in the prion protein gene (termed PrP systemic amyloidosis). There are three types of mutation, point mutation, altering an amino acid, point mutation generating premature stop codon, and alteration of the number of octapeptide repeats by insertion or >1 deletion (OPRI). There is much variation in the penetrance of different mutations as evidenced by considerable intra-familial and intra-generational phenotypic variability. For this reason in some cases the disease is not reported in the first degree relatives. Inherited prion disease can be diagnosed by *PRNP* analysis, a method which has allowed recognition of a wider phenotypic spectrum of human prion disease to include atypical dementias and fatal familial insomnia (FFI). No such pathogenic mutations are present in sporadic and acquired prion disease. Inherited prion disease is generally associated with an early age at onset and a longer duration of illness than sCJD. However, the phenotype is remarkably variable, ranging from an aggressive disease indistinguishable from sCJD to a prolonged slowly progressive dementia over more than 20 years. The frequency of mutations differs worldwide; however, geographical or ethnic clusters of IPD cases with mutations E200K, V210I, D178N, and V180I have been reported from Slovakia, Israel, Spain, Italy, Chile, and Japan (Gambetti et al., 2010; Nozaki et al., 2010). Multiple strains, although yet remain unidentified, may be involved in the pathogenesis of the disease. Clinical phenotypes related to most mutations overlap with those of sCJD

(Gambetti et al., 2010;Nitrini et al., 1997). The exception is the phenotype carrying T183A- 129M haplotype which is characterised by fronto-temporal features such as aggressive behaviour, hyperorality, verbal stereotypes and often parkinsonian signs although there are not enough cases to consider this a distinctive phenotype (Anghinah et al., 2006).

### 1.11.3 Gerstmann-Sträussler-Scheinker (GSS) disease

GSS is an autosomal dominant prion disease associated with point mutations in the prion protein gene, defined by the classical neuropathological changes. Most typically they show a slowly progressive ataxic syndrome. The most frequent mutation associated with GSS involves a proline-to-leucine substitution within the unstructured N-terminal region of the PrP at codon 102 (P102L), segregating with methionine at codon 129 on the mutant allele (Hsiao et al., 1989). Other mutations have been described either at the N-terminal or at the C-terminal PrP region. The genetic heterogeneity of GSS partly explains the variability in age of onset, disease duration, and the ample number of clinical variants. However, phenotypic heterogeneity is also observed both within and between pedigrees with the same mutation, especially in P102L GSS cases (Barbanti et al., 1996; Hainfellner et al., 1995; Webb et al., 2008b). Accordingly, while in some P102L patients progressive gait disturbances, truncal ataxia, lower limb hyporeflexia and dysarthria are the presenting clinical features, followed over years by dementia and pyramidal signs, in other cases a rapidly evolving dementia, indistinguishable from sCJD, is observed. As yet, the molecular basis for such clinical variability remains to be fully explained. Neuropathological examination in GSS P102L shows marked variation in the degree and brain regional distribution of spongiform changes, which largely correlates with the biochemical detection of proteinase K (PK)-resistant PrP<sup>Sc</sup> with a core fragment of 21 kDa (Ghetti et al., 1996; Parchi et al., 1998; Piccardo et al., 1998). As opposed to the variable topographical distribution of spongiform degeneration, typical multicentric plaques are widely distributed throughout cerebral and cerebellar cortical and subcortical regions, and appear to be constituted by an abnormal PrP isoform that upon PK-digestion generates an internal 8 kDa fragment (PrP8), overall spanning from residues 74–90 to 146–153; this fragment is natively present in brain extract of GSS P102L, although is enriched after PK treatment. Experimental

transmission in mice of GSS P102L brain tissues with only PrP8 induces PrP-amyloid deposition, but not spongiform encephalopathy (Piccardo et al., 2007).

#### **1.11.4 Fatal Familial Insomnia (FFI)**

FFI due to D178N mutation presents between 20 and 72 years (average, 49 years) and is characterised with insomnia and stuporous episodes with hallucinations and confusion, autonomic dysfunction, and often myoclonus, spasticity and seizures (Medori et al., 1992b). Some of the signs differ between 129MM and 129MV subjects. Insomnia, myoclonus and autonomic malfunction are often more severe in 129MM subjects, while in 129MV subjects ataxia, dysarthria and seizures often predominate. Insomnia has not been reported in C1DD178N–129VV. Routine electroencephalography (EEG) activity is generally slowed and may demonstrate periodic sharp wave complexes (PSWC) associated with myoclonus in patients with long disease duration. Polysomnography is an invaluable diagnostic tool in FFI as it shows markedly shortened sleep time and disorderly transition between sleep stages (Sforza et al., 1995). These changes are less severe in the 129MV patients. PET scanning that demonstrates hypometabolism in the thalamus and cingulate cortex, more widespread in 129MV patients than in 129MM patients. Neuropathological changes are the defining features of FFI. It is characterised by loss of neurons and astrogliosis in the thalamus in all cases and in the inferior olives in most cases (Medori et al., 1992b; Taberner et al., 2000). PrP deposits are usually absent, but may be detected in the molecular layer of the cerebellum with a stripe-like pattern and in the subiculum-entorhinal region (Almer et al., 1999).

### 1.11.5 Inherited prion disease associated with octapeptide repeat insertional mutations

Prion disease is associated with 4–12 extra 24-bp repeats in the *PRNP* gene (Mead, 2006). As in other inherited prion diseases, the amino acid at codon 129 may influence the phenotypic effects of the repeat-expansion mutations. The clinico-pathological phenotype is highly variable (Capellari et al., 1997; Collinge et al., 1996) and tends to become less pronounced when cases are grouped according to the number of repeats.

A study by Kaski et al. (Kaski et al., 2011) on patients with 4-OPRI mutations showed that the risk of clinical disease is increased by a combination of the mutation and a susceptibility haplotype on the wild-type chromosome. The predominant clinical syndrome is a progressive cortical dementia with pyramidal signs, myoclonus and cerebellar abnormalities that closely resemble sCJD. Autopsy showed perpendicular deposits of prion protein in the molecular layer of the cerebellum. *PRNP* genotyping showed that all patients were homozygous for methionine at polymorphic codon 129. In addition, at a single nucleotide polymorphism upstream of *PRNP* thought to confer susceptibility to sCJD (rs1029273), all patients were homozygous for the risk allele (combined  $p = 5.9 \times 10^{-5}$ ). These findings may provide a precedent for understanding apparently sporadic neurodegenerative diseases caused by rare high-risk mutations.

In contrast, the mean age at onset in patients with 5 or more extra repeats is 32 years (range, 21–61 years). The vast majority of these patients present with a slowly progressive syndrome characterised by mental deterioration, cerebellar and extra-pyramidal signs often lacking the PSW complexes on EEG examination.

Histopathologically, the cases with 4 or fewer extra repeats are indistinguishable from classical CJD, whereas the cases with 5–7 extra repeats are heterogeneous and may show either CJD-like change, GSS-like features, or may be difficult to classify either as GSS or CJD changes.

### 1.11.6 E200K mutation

E200K is particularly prevalent in Jews of Libyan and Tunisian origin and Slovaks with an incidence of CJD about 100 times higher than the world-wide average (Lee et al., 1999). Patients with CJD due to E200K–129M haplotype usually have a mean age at onset of 58 years (range, 33–84 years) and a mean illness duration of 6 months (range, 2–41 months)(Kahana and Zilber, 1991;Simon et al., 2000). The presentation includes cognitive abnormalities, cerebellar signs, visual signs and myoclonus (Kahana and Zilber, 1991). Patients also present with signs of motor and sensory peripheral neuropathy found rarely in sCJD patients. The EEG shows slowing in all patients while about 75% display PSW complexes (Brown et al., 1991). Brain atrophy is detected in about one-third of patients (Chapman et al., 1993). The CSF 14-3-3 protein is elevated in almost all CJD cases with E200K mutation (Rosenmann et al., 1997).

The E200K–129V haplotype has been reported in five subjects from apparently unrelated families, and all have type 2 PrP<sup>Sc</sup>. In contrast to those of E200K–129M, the phenotypes of the E200K–129V haplotype are similar to those of sCJDV2 (Parchi et al., 1999b), presenting as ataxia at onset followed by myoclonus and PSW complexes on EEG examination. PrP<sup>Sc</sup> accumulates predominantly in the cerebellum where it forms plaque-like structures. Spongiform degeneration, astrogliosis and neuronal loss in various combinations of severity are invariably present. They are generally severe and widespread in the cerebral cortex and milder in the striatum, diencephalon, and cerebellum. The extent of astrogliosis and neuronal loss appears to be related to disease duration. PrP immunohistochemistry is consistently positive throughout the brain with a punctate or 'synaptic' pattern apparently correlated to the histological lesions.



### 1.11.7 Variant CJD (vCJD)

The first 10 patients of new variant or simply of variant CJD (vCJD) were reported in April 1996 in UK (Will et al., 1996). Over 220 vCJD cases have been reported hitherto from 8 European and 4 non-European countries (USA, Canada, Saudi Arabia, and Japan). Figure 6 shows the incidence of BSE and vCJD in the period from 1987 to 2012. The incidence of vCJD peaked following the BSE epidemic and continued to decline from 2000 (Figure 6). Reported rate of sCJD has been increasing possibly because the condition is now better recognised. The age range of disease onset in vCJD is between 16 and 74 years and they manifest a predominance of psychiatric symptoms later advancing to cerebellar ataxia and/or progressive dementia. This is in contrast to sCJD where the average age of disease onset is 65 years (Brown, 1996) and disease duration is usually much shorter than in vCJD.

The majority of vCJD cases have been reported from the UK alone (Beekes, 2010). Psychiatric and behavioural symptoms of vCJD may include agitation, aggression, depression, anxiety, apathy, emotional lability, insomnia, poor concentration, paranoid delusions, recklessness, or withdrawal; a combination of two or more of these symptoms appear in most of the patients (Spencer et al., 2002). Some patients may also show signs of sensory disturbance such as pain, paresthesia and dysesthesia. The neurologic symptoms occur at least 6 months after the onset of psychiatric symptoms and include cerebellar ataxia, cognitive impairment, involuntary movements which may be dystonic, choreiform or myoclonic. Incontinence of urine, progressive immobility, and akinetic mutism are the late onset signs. Death often occurs because of intercurrent infections. The mean age at onset of symptoms is 29 years, and the progression or total duration of the disease spans 18 months in average, which is similar to that reported for kuru and iCJD linked to treatment with hGH (Belay, 1999). The observation of posterior thalamic high signals characterized by 'pulvinar' or 'hockey stick' signs on T2 diffusion or FLAIR MR

imaging and the detection of PrP<sup>Sc</sup> in tonsillar biopsy are important for the specific diagnosis of vCJD. The neuropathological hallmark of vCJD is the presence of kuru-type amyloid plaques surrounded by spongiform lesions “the florid plaques” which also have been seen in scrapie but never in other human prion diseases (Will, 2003). Spongiform changes occur most evidently in the basal ganglia and thalamus with sparse distribution throughout the cerebral cortex. The florid plaques and misfolded PrP immunohistochemical staining have occurred predominantly in the cerebellum and cerebrum. However, sufficient amounts of abnormal PrP are detected in the lymphoreticular system of vCJD patients (Will, 2003). The detection of misfolded PrP in the lymphoreticular system has confidently been used for the diagnosis of vCJD (Ironsides et al., 2006). To date, all definite vCJD patients have been 129 MM homozygotes (Bishop et al., 2009), with one possible case of MV vCJD reported in 2009 (Kaski et al., 2009). Very soon after the report on 10 vCJD cases, epidemiological studies, experimental transmission of the disease to cynomolgous macaques and mice (wild as well as transgenic) and biochemical strain typing linked the etiology of vCJD to infection from BSE prions. The same or diverse phenotypes may develop on primary and secondary transmission of vCJD and BSE to mice expressing human PrP, depending on the source of inoculum and the PrP sequence of the recipient, and the 129 MV heterozygotes may be less resistant to vCJD transmission as compared to BSE transmission (Wadsworth et al., 2004). Moreover, at least 4 pathologically confirmed cases including 1 with subclinical or potentially preclinical infection have been associated with the secondary vCJD transmission via blood transfusion (Wroe et al., 2006). These reports of secondary iatrogenic transmission and the possibility of vCJD occurrence with long incubation periods in individuals with 129 MV and 129 VV PrP genotypes have raised serious public health concerns (Ironsides et al., 2006). Some cases of kuru and iatrogenic CJD (iCJD) have occurred after incubating the disease for almost 50 and 30 years, respectively (Collinge et al., 2006).

Edgeworth et al (Edgeworth et al., 2011) developed a solid-state binding matrix to capture and concentrate disease-associated prion proteins and coupled this method to direct immunodetection of surface-bound material. Quantitative assay sensitivity was assessed with a serial dilution series of  $10^{-7}$  to  $10^{-10}$  of vCJD prion-infected brain homogenate into whole human blood, with a baseline control of normal human brain homogenate in whole blood ( $10^{-6}$ ). The test allowed detection of a  $10^{-10}$  dilution of exogenous vCJD prion-infected brain from a  $10^{-6}$  dilution of normal brain, an assay sensitivity that was orders of magnitude higher than any previously reported. All 15 samples which scored positive in this study were from patients with vCJD, showing an assay sensitivity for vCJD of 71.4% and a specificity of 100%. These initial studies provide a prototype blood test for diagnosis of vCJD in symptomatic individuals, which could allow development of large-scale screening tests for asymptomatic vCJD prion infection although the validation of such a test may prove to be challenging.

### 1.11.8 Iatrogenic CJD (iCJD)

Duffy et al described a case of iatrogenic CJD in a person who received cadaveric corneal transplant from a patient of CJD (Duffy et al., 1974). Several cases of human prion disease, since then, have been associated with iatrogenic transmission of CJD by the use of stereotactic intracerebral EEG needles or neurosurgical instruments, cadaveric dura mater grafts and intramuscular injections of contaminated cadaveric pituitary-derived human growth hormone (hGH) and gonadotrophin hormone. The highest proportion of iCJD cases is attributed to treatment with hGH and dura mater grafts. Most of CJD cases linked to treatment with hGH have occurred in France, while those linked to treatment with dura mater grafts have mainly occurred in Japan (Nozaki et al., 2010). Between the late 1950s and 1985, about 30,000 children were treated with hGH worldwide, and the overall proportion of CJD cases from the treated population has been estimated to be about 1/100. It is believed that the likely PrP<sup>Sc</sup> contamination of pituitary-derived hGH and dura mater grafts resulted from the practice of batch processing. Before the production of recombinant hGH, it was a pharmaceutical practice to process 5,000-20,000 cadaveric pituitary glands in a single batch for the extraction of the hormone. The presence in the batch of a few pituitary glands from unknown CJD cases may be responsible for contamination. With the availability of recombinant hGH and the initiation of separated processing of individual dura mater grafts since 1987, the probability of occurring future iCJD cases appears to be very low. A peak in the incidence of iCJD cases seems to have passed, however those with long incubation periods may still occur (Collinge et al., 1991;Rudge et al., 2015). The clinicopathological features of CJD linked to treatment with hGH resemble those of kuru. The majority of UK cases were 129 VV, while codon 129 MM was thought to be a risk factor in other countries. The incubation periods may be in the range of 4.5 to over 25 years with a mean of 12 years (Collinge et al., 1991). In contrast, the clinicopathological features of CJD linked to neurografting of dura mater or the use of neurosurgical

instruments resemble those of sCJD. The mean duration of illness is 18 months and the incubation periods may be in the range of 1.5-18 years with a mean of about 6 years. In Japan, the minimum risk of developing iCJD has been estimated to be approximately 1/3000 graft recipients (Will, 2003).

### 1.11.9 Kuru

Kuru is the first human prion disease that was shown to be transmissible to chimpanzees by intracerebral introduction of brain homogenates from kuru patients (Gajdusek et al., 1966). Kuru has occurred exclusively in the Fore linguistic group of Papua New Guinea Eastern Highlands and the neighbouring peoples with whom they intermarried. There was a practice among these groups to consume the dead bodies of their relatives as a mark of respect and mourning (ritualistic cannibalism). Women and young children of both sexes were more exposed to the risk material such as brain and viscera than adult men who usually had preferentially to consume muscles. The kuru epidemic killed 1-2% of the population per annum at its peak. Some villages became even devoid of adult women. With a ban on ritualistic cannibalism in the mid-1950s imposed by Australian authorities, the incidence of the disease started to decline steadily. Kuru was introduced to Western medicine in late 1950s, although the first case of kuru was observed around 1920. The Western scientists were very soon able to prove ritualistic cannibalism as the etiology of the disease. The older kuru patients who experienced an exposure to the infection before the ban can still be seen. The incubation periods of kuru in such patients would be more than 50 years (Collinge et al., 2006; Liberski and Brown, 2008). Kuru has imposed strong selection pressure in the affected Fore groups on *PRNP* especially at codons 127 and 129. Heterozygosity at these *PRNP* codons is a resistance factor for kuru and can be seen with marked prevalence among survivors of the kuru epidemic (Mead et al., 2009b). Kuru has 3 clinical stages namely ambulant (still can walk), sedentary (only can sit up), and terminal (unable to sit up independently). An ill-defined prodromal period characterized by headache and pain usually in the joints of legs may precede these stages. Cerebellar ataxia, tremors, choreiform and athetoid movements are distinctive and prominent clinical signs. Shivering amplifiable by cold was the symptom on the basis of which the disease was named "kuru". The most prominent clinical feature of sCJD, the dementing illness can also

occur in some cases but it happens during the final disease stages only (Collinge et al., 2008).

Neuropathological features such as spongiosis, neuronal loss, and astrocytic microgliosis can variably be observed in CNS usually in the grey matter.

The PrP<sup>Sc</sup> deposition is observed in CNS only (Brandner et al., 2008). The neuropathological property which distinguishes kuru from sCJD is the presence of numerous kuru plaques, spherical bodies with a rim of radiating filaments. Kuru is believed to be caused by the consumption of prion infected brain tissue and experimental transmission studies have shown similarity between the molecular and pathobiological properties of prions causing kuru, sCJD and iCJD (Wadsworth et al., 2008a). Similar studies have also revealed differences in transmission dynamics, peripheral pathogenesis and the neuropathology properties of kuru and vCJD prions (Wadsworth et al., 2008a). As both kuru and vCJD are caused by infection via oral route, these differences can be attributed to the strain type or dose effect rather than the route of infection (Brandner et al., 2008).

### **1.12 Phenotypic variation in prion disease**

Prion diseases show a remarkable extent of phenotypic heterogeneity (Gao et al., 2011). These variations, observed as differences in age at presentation, disease duration, clinical features, investigation findings and pathological phenotype may be genetically determined, associated with a particular prion protein strain or with specific environmental factors. A better understanding of these mechanisms may lead to classification of prion diseases based on the causes rather than the effects of this heterogeneity. Such classification is likely to be more accurate and may result in a wider detection and more precise diagnosis of prion diseases.

A recent study by Gao et al (Gao et al., 2011) investigated clinical heterogeneity in 259 Han Chinese patients with sCJD. 163 patients with probable sCJD and 96 patients with possible sCJD were included in the study. Progressive dementia was the most common presenting symptom. Psychiatric features were present in a large number of patients, followed by pyramidal or extrapyramidal disturbance, cerebellar syndrome, slowly progressive dementia and the least common was presence of visual disturbance. The proportion of patients with slowly progressive dementia (persistent longer than one year) in the group of possible sCJD were slightly higher than that of probable sCJD, but the difference was not statistically significant. Almost all probable and possible sCJD patients had progressive dementia in their clinical courses. Except for dementia, the presence of four other clinical manifestations were included in the diagnostic criteria; visual or cerebellar disturbance, pyramidal or extrapyramidal dysfunction and akinetic mutism, were compared between two groups. Pyramidal or extrapyramidal dysfunction was the most commonly observed sign in both probable and possible patients, which accounted for 77.9% and 79.2%, respectively. Visual or cerebellar disturbances were noted comparably in probable (57.1%) and possible (58.3%) sCJD patients. Akinetic mutism appeared in 38.0% of probable and 30.2% of possible



sCJD patients. Myoclonus was more frequently observed in the group of probable sCJD, showing statistically significant differences (Gao et al., 2011). As an important diagnostic marker for sCJD, the data for CSF 14-3-3 protein detected using Western blot and EEG recordings were collected through the NCJDSU surveillance network. Two patients with relatively long clinical course (more than 500 days) were CSF 14-3-3 positive, but had negative EEG findings. Although probable sCJD patients show high ratios of both positive CSF 14-3-3 protein and presence of PSWC on EEG, only approximately 45% of patients who have undergone those two examinations were positive for both. It emphasizes again the important role of CSF 14-3-3 protein and EEG findings in the diagnosis of sCJD. In addition, the data show that the presence of myoclonus is associated with the presence of PSWC on EEG.

Phenotypic heterogeneity is also observed in patients with inherited prion disease. Studies of heterogeneity in patients with IPD provides a unique opportunity to characterize this diversity and examine possible modifying factors amongst individuals in whom prion disease has been initiated by the same defined genetic mutation. As the inherited prion diseases comprise a significant proportion of early-onset dementia, an appreciation of their wide range of clinical presentation is important for differential diagnosis.

A study by Mead et al (Mead S et al., 2006) was done on patients from southeast England with inherited prion disease caused by a prion protein octapeptide repeat insertion mutation. Genealogical and clinical record review, together with the characterization of the mutation-linked single nucleotide polymorphism and microsatellite haplotype, suggested a single founder for both this large kindred and a smaller family. The study investigated the phenotype of 86 affected individuals and another 84 individuals known to be at risk of inheriting the disease. Clinical onset, typically with cognitive impairment, can be strikingly early when compared with other inherited or sporadic prion diseases. The

study investigated the effect of PrP genotype, candidate genes and prion strain type on clinical, neuroradiological and neuropathological phenotype. The transmission characteristics of prions from affected individuals resembled those of classical sporadic Creutzfeldt-Jakob disease. One surprising finding was a strong inverse correlation between age of onset and disease duration. The PrP gene polymorphic codon 129 was found to confer 41% of the variance in age of onset but interestingly this polymorphism had no effect on disease duration suggesting different molecular mechanisms involved in determining disease onset and rate of clinical progression.

Zarranz et al (Zarranz et al., 2005) investigated the phenotypic heterogeneity in patients with fatal familial insomnia (FFI). FFI is associated with D178N mutation when the mutant allele encodes methionine in the polymorphism of codon 129, whereas the encoding of valine by the mutant allele would produce the CJD phenotype (Goldfarb et al., 1992). Around 30 FFI families have been reported world-wide so far (Kovacs et al., 2002; Montagna et al., 2003; Spacey et al., 2004). Although insomnia is the most characteristic early symptom of the D178N-129M mutation, only three patients complained of insomnia as the first symptom. Seven patients presented with gait ataxia. Ill-defined psychological changes or cognitive decline were the first symptoms in another seven patients, a finding already pointed out in the literature (Almer et al., 1999). However, it may happen that in the absence of a spontaneous subjective complaint, abnormal nocturnal sleep pattern is under recognised.

Homozygosity at codon 129 of the prion protein gene increases susceptibility to all forms of prion diseases. This polymorphism plays an important role in modulating the clinico-pathological phenotype. Padovani et al (Padovani et al., 1998) compared several variables such as age of onset, disease duration, histopathological findings, and abnormal PrP deposits between patients homozygous and heterozygous at codon 129. They concluded that despite certain significant differences, there was considerable

overlap between both groups with regards to age of onset and disease duration. However, in some studies, duration of illness is significantly shorter in patients homozygous for methionine than in either codon 129 MV or VV patients.

### **1.13 Clinical trials**

The possibility to induce prion diseases in experimental animals that accurately reproduce the main clinical and histopathological features of human disease provided an opportunity to evaluate a range of experimental therapeutics including small molecules and monoclonal antibodies. Prion diseases are particularly tractable for drug development, as animals are naturally susceptible, leading to the discovery of prion immunotherapeutics (White et al., 2003), and small molecule therapeutics (Silber et al., 2013;Wagner et al., 2013), which suppress prion replication and delay or prevent disease progression in mice. There is however no effective therapeutic that modifies the course of the human disease.

There is a need to evaluate the effects of compounds showing effectiveness in laboratory rodents in human disease given the pressing unmet need and the fact that prion diseases comprise a clinically heterogeneous group of syndromes with genetic, acquired or sporadic aetiologies. Development of clinical trial methodology is a major challenge. The main obstacles include the fact that the disease is rare, clinical phenotype is highly variable and there is difficulty to allocate placebo therapy to patients. Collinge et al (Collinge J et al., 2009) provided PRION-1 study design based on patient preference in order to evaluate the effect of quinacrine in prion disease. Patients were offered a choice between taking the drug, not taking the drug or being randomized to immediate quinacrine or delayed therapy (24 weeks). Patients were assessed at baseline (enrollment) with follow-up at 1, 2, 4 and 6 months and then 3-monthly including a standardized clinical examination, the Glasgow Coma Scale (GCS)(Teasdale and Jennett, 1974), mini-mental state examination (MMSE) (Folstein et al., 1975), cognitive component of the Alzheimer's Disease Assessment Scale (ADAS-cog)(Rosen et al., 1984), Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962), Clinical Dementia Rating Scale sum of Boxes (CDR-SB)(Morris,

1993), Global Impression of Change (GIC)(Schneider et al., 1997), Rankin scale (Rankin J., 1957) and the Barthel Activities of Daily Living Index(Mahoney F and Barthel D., 1965). Other prion disease clinical trials have also used survival as the primary endpoint (Geschwind et al., 2013;Haik et al., 2014;Otto et al., 2004) .

Quinacrine had no beneficial effect on any of the 8 parameters. However, the Barthel and Clinician Dementia Rating Scales were the most valued and powerful scales in a simulated clinical trial of an effective therapeutic (Collinge J et al., 2009). A combination of selected subcomponents from these 2 scales may help to more accurately detect clinically relevant effects in future trials. Based on these experiments the Prion Clinic proposed the Medical Research Council (MRC) Prion Disease Rating Scale (Thompson et al., 2013). This Scale was applied in our National Prion Monitoring Cohort (NPMC) study. NPMC study is a longitudinal, observational study based on detailed clinical assessment of patients, EEG recordings, neuropathological analysis, comprehensive neuropsychological assessments and quantitative brain imaging at regular intervals to help with the diagnosis and assess disease progression. In addition, a number of rating scales were used to assess cognitive function, mobility, self-care, continence, feeding, speech and other functions affected by the disease including the new MRC Prion Unit rating scale that can be administered over the telephone. The rating scales allow accurate monitoring of disease progression that can also serve as a baseline for patient assessments in future therapeutic trials. The aim of the cohort study was to allow a unique analysis of the natural history of prion disease in ~400 patients and provide a standard for future prion disease trials.

The information collected during NPMC study has been combined with the PRION-1 trial, an open-label patient-preference study carried out to assess the efficacy of quinacrine treatment. In PRION-1 trial patients were assessed at baseline and regular follow-up intervals. Neurological assessments and

functional scales were used to objectively assess disease progression. These included standardized neurological examination, cognitive assessment - MMSE, ADAS-cog, GCS, CDR, the clinician-interview-based impression of change plus carer input (CIBIC-P-plus), BPRS, Rankin scale and Barthel activities of daily living index.

Approximately 90-120 people are diagnosed with prion disease annually in the UK (NCJDSU, 2009) with sporadic Creutzfeldt-Jakob disease (sCJD) being the most common form of human prion disease (~85%) and the most obvious target for clinical trials. As already discussed sCJD typically manifests as a rapidly progressive dementia with myoclonus and other neurological signs. Median clinical duration is ~4 months although forms with short (few weeks) and long durations (>2 years) are well recognised (Pocchiari et al., 2004).

Chen et al (Chen et al., 2013) analysed the survival time and possible influencing factors in patients with prion diseases in China. The analysis was based on the surveillance data from Chinese Creutzfeldt-Jakob disease (CJD) surveillance network from January 2008 to December 2011. A retrospective follow-up survey was performed. Median survival time of 121 deceased patients was 7.1 months, while those for sporadic CJD, familial CJD and fatal familial insomnia cases were 6.1, 3.1 and 8.2 months, respectively. 74.0% of sCJD patients, 100% of fCJD cases and 91.7% of FFI died within one year. The general socio-demographic factors and clinical findings did not significantly influence the survival times of Chinese prion patients. The survival time was comparable with that of many Western countries, but was shorter than that of Japan. Patients with acute onset and rapid progression had significantly shorter survival times.

Surveillance data for Creutzfeldt-Jakob disease in China showed that Chinese patients had similar epidemiological and clinical characteristics as prion disease patients worldwide. A study by Shi et al (Shi

et al., 2008) included 12 Chinese provinces in CJD surveillance system. A total of 192 suspected CJD and 5 IPD cases were reported; 51 probable and 30 possible sCJD cases were diagnosed. The collected sCJD cases distributed sporadically without geographical clustering or seasonal relativity and the highest incidences in both probable and possible sCJD cases appeared in the age group of 60-69. The most common symptoms were progressive dementia, cerebellar and psychiatric features. The probable sCJD patients with both typical EEG abnormalities and positive CSF 14-3-3 protein had more characteristic clinical syndromes than those with only one positive test. The polymorphisms at codon 129 of all tested reported cases showed typical patterns of Han Chinese as previously reported, with MM homozygosity being predominant and MV heterozygosity relatively rare. These data confirmed that Chinese CJD patients have similar epidemiological and clinical characteristics compared to CJD patients worldwide.

In response to the challenges faced during MRC PRION-1(Collinge J et al., 2009) and other human trials(Haik et al., 2014) (Geschwind et al., 2013), including the lack of a validated outcome measure, we studied UK patients with prion disease to optimise clinical trial methodology and develop a resource for open-label studies. In preparation for clinical trials in sCJD, we used item-response modelling to develop a functionally-orientated rating scale (MRC Prion Disease Rating Scale, or MRC Scale) with favourable statistical properties and weighted the items to represent the most impactful domains reported by patients and carers (Thompson et al., 2013). A combination of disease aetiology, severity, and genetic stratification with codon 129, proved to be an extremely powerful approach in our trial model, resulting in estimated sample sizes for adequately powered and meaningful clinical trials which are substantially smaller than those required in other more common neurodegenerative diseases.

## **II AIMS**

- 1. To describe extreme examples of phenotypic heterogeneity using case studies and reports**
- 2. To investigate/describe phenotypic heterogeneity using an heuristic clinical scoring scheme and the MRC Rating Scale**
- 3. To establish a semi-quantitative histopathological rating scheme in prion diseases**
- 4. To test several hypotheses, based on candidate genes, that gene variants may determine the clinico-pathological phenotype of definite sporadic CJD**
- 5. To test a hypothesis that large copy number variants confer susceptibility to prion diseases using genome wide association studies**



### III MATERIAL AND METHODS

#### **2.1 Subjects**

##### **2.1.1 Patient Selection**

Prion disease samples were obtained from patients seen at the NHS National Prion Clinic, St Mary's Hospital and the National Hospital for Neurology and Neurosurgery, University College London NHS Foundation Trust, London and collaborating Institutions in Scotland, Germany and Papua New Guinea (PNG). The clinical and laboratory studies were approved by the local research ethics committee of University College London Institute of Neurology and National Hospital for Neurology and Neurosurgery and by Scotland A Research Ethics Committee (Cohort study) or the Eastern Research Ethics Committee (PRION-1 study), and relevant bodies in Germany and acting for the Government of PNG. Full participation of the PNG communities was established and maintained through discussions with village leaders, communities, families and individuals. For the Cohort study and PRION-1 trial informed consent was obtained directly from study participants or from relatives, carers or Independent Mental Capacity Advocates as appropriate. The samples and patient details were obtained by the NPC team led by Professors John Collinge and Simon Mead. Patients were diagnosed according to the WHO criteria as outlined below (<http://www.cjd.ed.ac.uk/>)(Zerr et al., 2009).

***a. Sporadic CJD***

**Definite:**

A diagnosis of definite sCJD was made by standard neuropathological techniques; and/or immunocytochemical analysis; and/or Western blot confirming protease-resistant PrP; and/or presence of scrapie-associated fibrils.

**Probable:**

Probable CJD is characterised by a rapidly progressive dementia; and at least two out of the following four clinical features:

- i. Myoclonus
- ii. Visual or cerebellar signs
- iii. Pyramidal/extrapyramidal signs
- iv. Akinetic mutism

AND a positive result on at least one of the following laboratory tests:

- a. a typical EEG (periodic sharp wave complexes) during an illness of any duration; and/or
- b. a positive 14-3-3 cerebrospinal fluid (CSF) assay in patients with a disease duration of less than 2 years
- c. Magnetic resonance imaging (MRI) high signal abnormalities in caudate nucleus and/or putamen on diffusion-weighted imaging (DWI) or fluid attenuated inversion recovery (FLAIR) and/or high signal change (“cortical ribbon”) in two cortical areas not known to be liable to false positive results

AND without routine investigations to indicate an alternative diagnosis.

**Possible:**

Possible CJD is characterised by progressive dementia; and at least two out of the following four clinical features:

- i. Myoclonus
- ii. Visual or cerebellar signs
- iii. Pyramidal/extrapyramidal signs
- iv. Akinetic mutism

AND the absence of a positive result for any of the three laboratory tests that would classify a case as “probable” (see tests a-c above)

AND duration of illness less than two years

AND without routine investigations indicating an alternative diagnosis

***b. Variant CJD (vCJD)***

Variant CJD was first described in the United Kingdom in 1996 (Will et al., 1996) and has been linked with exposure to a transmissible spongiform encephalopathy of cattle called Bovine Spongiform Encephalopathy (BSE). All patients with vCJD were thought to have acquired the disease in the UK.

Clinically, vCJD is characterised by early psychiatric or sensory symptoms, which often take the form of depression, apathy or anxiety, and occasionally unusual persistent and painful sensory symptoms.

Neurological signs, including unsteadiness, difficulty walking and involuntary movements, develop as the

illness progresses and, by the time of death, patients are completely immobile and mute. The current WHO criteria for variant CJD are summarised below:

**Diagnostic Criteria for vCJD**

IA Progressive neuropsychiatric disorder

B Duration of illness >6 months

C Routine investigations do not suggest an alternative diagnosis

D No history of potential iatrogenic exposure

E No evidence of a familial form of TSE

IIA Early psychiatric features - depression, anxiety, apathy, withdrawal and delusions

B Persistent painful sensory symptoms

C Ataxia

D Myoclonus or chorea or dystonia

E Dementia

IIIA EEG does not show the typical appearance of sCJD in the early stages of illness

B Bilateral pulvinar high signal on MRI scan

IVA Positive tonsil biopsy

A diagnosis of definite vCJD is made by inclusion of IA and neuropathological confirmation of vCJD (spongiform change and extensive prion protein deposition with florid plaques throughout the cerebrum and cerebellum). Diagnosis of probable vCJD is made following inclusion of I and 4/5 of II and IIIA and IIIB, or I and IVA. Possible diagnosis of vCJD requires I and 4/5 of II and IIIA.

***c. Iatrogenic CJD***

Iatrogenic CJD is characterised by a progressive cerebellar syndrome in a recipient of human cadaveric-derived pituitary hormone; or clinical features typical of sCJD with a recognized exposure risk such as antecedent neurosurgery with dura mater implantation.

***d. Inherited prion disease***

Inherited prion disease is characterised by definite or probable CJD plus definite or probable CJD in a first degree relative; and/or neuropsychiatric disorder plus disease-specific PrP gene mutation.

***e. Kuru***

The samples from this group were collected by the Kuru Surveillance Team led by Professor Michael Alpers and Dr Jerome Whitfield in Papua New Guinea, at the Institute for Medical Research and in the villages of Waisa and South Fore. This group also included elderly recent kuru cases with long incubation time. Kuru surveillance was conducted by many different investigators prior to 1987 (Gajdusek, Zigas, Alpers, Hornabrook and others) and in the period from 1987 to 1995 it was solely led by the Kuru Surveillance Team of the PNG Institute of Medical Research. From 1996, the surveillance was reinforced with a basic laboratory for sample processing and storage which was established in the village of Waisa in the South Fore. The kuru collection comprised young children, adolescents and adults from around the peak of the epidemic and elderly recent kuru cases with long incubation times. Samples obtained in the 1950s were stored at the NIH, c/o Joe Gibbs, Storey Landis, Carleton Gajdusek and colleagues.

***f. Elderly women resistant to kuru***

This group specifically comprised elderly women aged over 50 years at sampling from a region known to be exposed to the kuru epidemic. These women were asymptomatic at the time of sampling but were thought to have been exposed to multiple cannibalistic feasts by their own recollection. If these women have not been resistant to kuru their incubation time would have exceeded 40 years.

### 2.1.2 Healthy Controls

Publically available data from the Wellcome Trust Case Control Consortium, (WTCCC) and Collaborative Health Research in the Region of Augsburg (KORA) were used for the analysis. WTCCC is a collaboration of over 50 research groups from the UK investigating the genetics of common human diseases, such as bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type 1 diabetes mellitus, and type 2 diabetes mellitus. The WTCCC data were provided following use of quality control criteria similar to those often applied as standard in genome wide association studies (GWAS). KORA (Collaborative Health Research in the Region of Augsburg) is a research platform based on a population-based cohort study of 18,000 adults from Southern Germany. Recruitment started in 1984/85 and was performed in 4 surveys, followed by repeated investigations at regular intervals. Phenotyping was broad, with a focus on cardiovascular and metabolic diseases. KORA biobank has been used in more than hundred national and international collaborations.

As control samples for kuru and elderly women resistant to kuru, the young Fore and neighbouring linguistic groups healthy population unexposed to kuru were used. When identified either by genealogical data or microsatellite analysis first degree relatives were excluded from the analysis.

## **2.2 Phenotypic heterogeneity – National Prion Monitoring Cohort (NPMC)**

From 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 (NCJDRSU) and to the NHS National Prion Clinic (NPC). Communication between both Units several times per week ensured exchange of patient details referred to one of the Units. 85% of patients were visited by the NPC within five days of referral or NCJDRSU visit. Details of enrolment into the PRION-1 trial and Cohort Study have been published (Collinge J et al., 2009;Thompson et al., 2013).

The National Prion Monitoring Cohort (NPMC) involves comprehensive assessment of all suspected and diagnosed prion disease patients in the UK. These include all patients with probable or definite sporadic Creutzfeldt-Jakob disease, variant CJD, iatrogenic CJD, and inherited prion disease, according to updated diagnostic criteria as discussed above (Zerr et al., 2009). In addition patients thought to have prion disease but not meeting formal criteria could be enrolled following review by an expert panel. Patients were enrolled at home, hospitals and other healthcare settings around the UK from 2008 onwards (Figure 9).

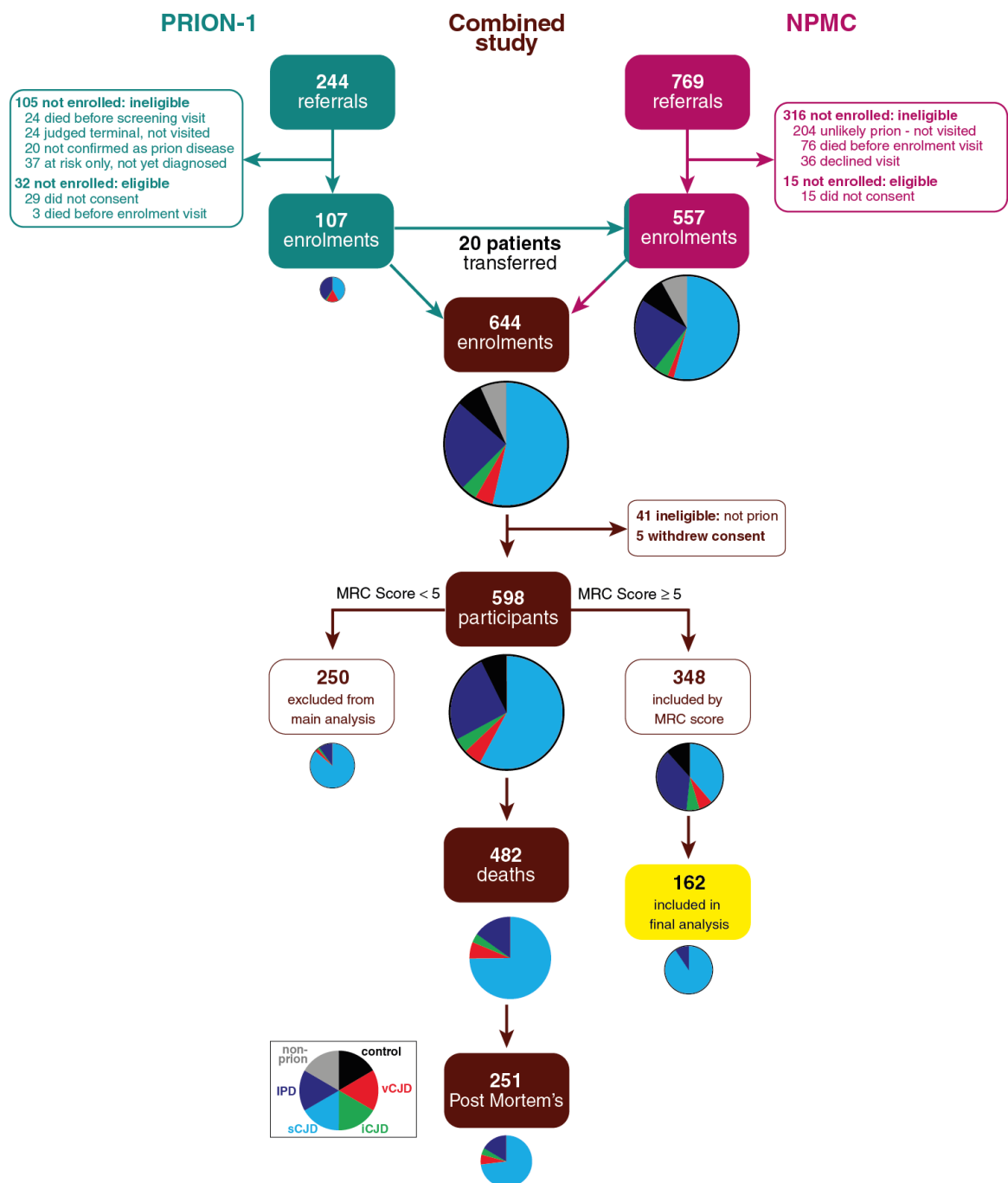
Together with a specialist nurse I travelled within the UK to assess patients suspected to have prion disease following a telephone discussion with the local hospital teams. I met with patients and their families, reviewed the clinical notes, took clinical history and performed full neurological examination. Where this was possible, I performed a detailed cognitive assessment using short cognitive examination, clinical dementia rating, brief psychiatric rating scale and Barthel index of daily living. These data were later used to design MRC Prion Disease Rating Scale.



I also discussed the possibility of familial CJD and counselled the patient and their relatives for genetic studies. I took blood samples for genetic testing and research with the agreement of the patient and their family. I reviewed the MRI scan and collected a copy to be reviewed by our neuroradiologist, Dr Hyare at the National Prion Clinic. I discussed the neuropathological studies as the only way of obtaining definitive diagnosis and obtained written consent if the family wished to proceed with this. I discussed the patients with the rest of the team during our weekly clinical meetings.

Where this was possible, I did a follow-up visit to assess disease progression and advice on the symptomatic management. Between the visits I was in regular contact with the local teams and patient relatives to advice on symptomatic management and disease progression.

I enrolled over 100 patients to the National Prion Monitoring Cohort Study and performed follow up visits where this was possible. A total number of referrals and eligible patients enrolled is summarized in Figure 9.



**Figure 9** Flowchart of enrolment and selection of patient subset for the main study to develop a clinical trial model in sporadic CJD. Figure shows the number of patients recruited in PRION-1 trial and the NPMC study until June 2014. About a third of patients also had post-mortem studies which allowed a definitive diagnosis to be made.

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

(I) symptomatic patients with sporadic CJD, variant CJD, iatrogenic CJD and forms of inherited prion disease likely to have rapid clinical progression (in the Cohort study, due to 4-octapeptide repeat insertion, E200K, D178N, E196Q, E211Q or V210I mutations)

(II) symptomatic inherited prion disease patients likely to have slow progression (in the Cohort study, those with 5 and 6-octapeptide repeat insertion, P102L, P105L, Q212P, A117V or Y163X mutations), and

(III) at-risk and healthy control individuals.

Patients in stratum (I) had face-to-face follow-up assessments initially every 6 to 8 weeks. Several outcome measures and rating scales were recorded along with a systematic history and examination. The analysis was focussed on the MRC Scale (Thompson et al., 2013). If clinical progression measured by the MRC Scale proved to be slower than the expected significant deterioration over this interval (ie there was minimal or no change as determined by an overall clinical impression over 6-8 weeks), study physicians could decide to reduce follow-up frequency for subsequent assessments (up to a maximum interval of 24 weeks). Patients in strata (II) and (III) had follow-up assessments every 6 to 12 months. From May 2010 patients in stratum (I) also had telephone assessments of the MRC Scale up to every 2 weeks between follow-up assessments. These were discontinued if there was minimal change between consecutive assessments (at worst possible score) (Figure 9). During the visits patients were assessed by one clinician and one specialist nurse from the National Prion Clinic team. The initial assessment included cognitive analysis, clinical examination and a blood test to look for a possible genetic cause of prion disease and take samples for research. Further follow up visits were made to repeat these assessments and document accurately disease progression as already discussed. The initial

investigations were usually performed at the local hospitals where the patient first presented. Results of the blood tests, CSF analysis, brain imaging and EEG recordings were then forwarded to the NPC team. A subset of patients was investigated at the National Hospital for Neurology and Neurosurgery (NHNN) where they were looked after by the NPC team. They had a clinical assessment by one of the doctors, full neuropsychological analysis, MRI using the 3T scanner which allows more detailed imaging and EEG/neurophysiology testing.

The clinical parameters investigated were demographics, presenting clinical features, cognitive deficit on presentation, presence of visual symptoms, severity of cerebellar signs, pyramidal features, involuntary limb movements and sensory disturbance. The consensus opinion of two study physicians was used to allocate each patient to one of seven empirically-recognised clinical subtypes of CJD, based on the earliest clinical features which impaired function (not simply the first symptoms as these are often non-specific and irrelevant to subsequent course). Patients with sCJD were sub-classified into Heidenhain variant if visual disturbances such as cortical blindness, oculomotor impairment, and/or visual hallucinations were documented as the first presenting symptom (Appleby et al., 2009). The Oppenheimer-Brownell variant of sCJD included patients who presented with ataxia in the absence of cognitive and visual impairment. Cases presenting with dementia, memory impairment, executive dysfunction, language impairment, and/or confusion in the absence of visual, cerebellar, and mood symptoms within the first week of illness were classified as the pure cognitive variant. Patients with cognitive symptoms and ataxia, without visual impairments and affective symptoms during the initial illness phase, were classified as having classic CJD. Patients who were reported to have symptoms of depression, mood lability, mania, hypomania, and/or anxiety within the first week of illness were classified as having the affective variant if they did not meet inclusion criteria for the Heidenhain group.

Thus categories defined were:

1. visual or Heidenhain subtype associated with cortical blindness, double vision, hemianopia
2. ataxic subtype
3. pure cognitive subtype eg. isolated frontal, parietal, speech output or amnesic syndrome or combination of these
4. psychiatric subtype associated with mood disorder, delusional state, marked agitation in the absence of ataxia, motor or movement disorder
5. thalamic subtype in which the key feature was insomnia
6. stroke-like subtype with abrupt or subacute onset hemiplegia and
7. classical CJD subtype which does not fit into the above categories, typically a combination of rapidly progressive multi-focal dementia, with ataxia and myoclonus.

Data were recorded on paper forms and returned to a central database at the NPC.

Demographic features and the presenting clinical symptoms coding scheme are summarised in Table 2.

Components assessed		
Demographic data	Disease duration	days
	Age	years
	Gender	M, F
	Codon129	MM, VV, MV
	Diagnosis type	sCJD, IPD, iCJD, vCJD
Components assessed		Score
Presenting symptoms	Visual	0-1
	Ataxia	0-1
	Pure cognitive problems	0-1
	Psychiatric problems	0-1
	Thalamic	0-1
	Stroke-like symptoms	0-1
	Classical CJD	0-1

**Table 2** shows demographic data such as disease duration, age of onset, gender and disease category to include sCJD, inherited prion disease, iatrogenic CJD and variant CJD. Disease duration and age of onset were expressed as quantitative trait while gender was labelled as categorical value, male or female. Also included were molecular genetic data, for example, *PRNP* codon 129 polymorphism included homozygosity for methionine or valine and heterozygosity for methionine/valine labelled as MM, VV or MV. Presenting symptoms at disease onset included visual symptoms, ataxia, pure cognitive problems, psychiatric, thalamic, stroke-like symptoms and classical CJD. These were categorical values depending on whether they were absent (0) or present (1) at disease onset.

Different clinical features characteristically associated with prion disease were noted during the first visit by the National Prion Clinic team. The first visit was prioritised for classification as this was thought to be the most informative, particularly for patients suffering with sCJD who deteriorate rapidly and often become mute and bed-bound by the second clinical assessment, thus detailed clinical examination was difficult to perform. If it was unclear from the history obtained by the family or relative we often

referred back to the clinical notes or contacted GP surgeries to check what the patient initially complained of.

Cognitive function was assessed by performing a MMSE and GCS in more severely affected patients. The scores were documented as 0-30 and 0-15 respectively. Presence and absence of visual symptoms to include cortical blindness, oculomotor impairment and visual distortions were documented as binary values. Involuntary limb movements categorised as tremor, myoclonus or chorea were also documented as absent or present. Presence or absence of sensory abnormalities particularly important in patients with variant CJD was documented as present or absent. Frontal release signs were generated as a single figure by adding different components such as glabellar tap, pout reflex, grasp reflex and rooting to create a single number from 0-4. Severity of cerebellar signs assessed by examining different systems such as gait, limb coordination and eye movements were expressed as a single value 0-17. Severity of pyramidal features was assessed by grading the severity of increased tone and reflex abnormalities. A value generated by adding these components was thought to be a more accurate representation of the severity of each system involvement in prion disease.

With informed consent, Sanger sequencing of the prion protein gene (*PRNP*) was done to identify mutations that cause inherited prion disease and polymorphic genetic variants (Figure 3) (Wadsworth et al., 2008b). With informed consent, autopsy was done in 60% at The National Hospital for Neurology and Neurosurgery, Queen Square or other hospitals in the UK; all were reviewed by Professor Sebastian Brandner. For the purposes of this study, autopsy results were used to confirm the diagnosis of human prion disease and provide tissue for molecular studies. PrP<sup>Sc</sup> type was determined by Western blotting of frontal lobe tissue as previously described and according to the London Classification system (Wadsworth et al., 2008b).

Magnetic resonance brain imaging (MRI) was performed at The National Hospital for Neurology and Neurosurgery, Queen Square, or obtained from local hospitals where the patient was initially investigated. The basal ganglia, cerebral cortex and thalamus were reviewed by a consultant neuroradiologist and each region was independently documented as clinically normal/abnormal for high signal change on T2, FLAIR or diffusion weighted imaging. The MRI study was not blinded to diagnosis. CSF analysis was done at NCJDRSU or The National Hospital for Neurology and Neurosurgery, Queen Square. The 14-3-3 protein was documented as normal/abnormal; S100b protein was recorded in ng/ml (normal range <0.55 or <0.61). Electroencephalogram investigation reports from hospitals were obtained and recorded as normal/abnormal for background, and whether periodic sharp wave complexes were observed. The difficulty with this was that the reports were subjective as reported by a Neurophysiologist at the local hospital.

MRC Scale measurements (see below) were transformed to the underlying Rasch scale used in their development using RUMM2030 (Humphry and Andrich, 2008) all subsequent analyses were done on this scale which runs from 100 (best possible score) to 0 (worst possible score).



### **2.3 DNA extraction from blood**

DNA extraction from blood was performed using Nucleon™ (BACC3 Kit, Nucleon BACC Genomic DNA Extraction Kit, GE healthcare, Life Sciences, Amersham, UK). The following protocol was used: 10 ml of Reagent A (as part of the 'Illustra' DNA extraction kit) was added to a 50 ml polypropylene tube followed by 30ml of 18.2MΩ deionised water and then 3-10ml of whole blood. The mix was centrifuged at 1300G for 5 minutes and the supernatant was removed. 2 ml of reagent B was added and the pellet was re-suspended. Re-suspended pellet and solution were transferred to a 15 ml polypropylene tube. 500µl of sodium perchlorate was added and mixed and the tube was incubated at 65°C for 25mins. 2 ml of chloroform stored at -20°C was added to the tubes and mixed thoroughly. 300µl of Nucleon resin was added and centrifuged at 1300G for 3 min. The upper phase was carefully transferred to a fresh 15ml tube. 1-2X volumes of 100% ethanol (EtOH) stored at -20°C was added and the tubes inverted several times to allow DNA precipitation. DNA was spooled out using the glass pipette and allowed to air dry for approximately 2 minutes. DNA was subsequently transferred to TE in a labelled tube and placed at 37°C for one hour to assist with re-dissolving. DNA was quantified using a Nanodrop spectrophotometer and the concentration was noted.

## **2.4 Allelic discrimination using real time Polymerase Chain Reaction**

All genotyping assays were undertaken on the 7500 Fast Real Time PCR (Applied Biosystems, UK) using pre-prepared 96 well plates. DNA plates containing 20ng/ $\mu$ l were used as reservoirs for genotyping assays. All reactions contained a total volume of 5  $\mu$ l mix. 1  $\mu$ l (20ng) of the reaction volume was added as the final addition by multi-channel pipette from the DNA plate.

Custom Primers and Minor Groove Binder (MGB) (Applied Biosystems, UK) probes were diluted to 90 $\mu$ M and 20 $\mu$ M respectively, to represent 100X concentrations, prior to storage. Each well contained the equivalent of: 2.5  $\mu$ l of ABI 2X Genotyping Mix, 0.05  $\mu$ l forward primer 1 (900nM), 0.05  $\mu$ l reverse primer 1 (900nM), 0.05  $\mu$ l Probe 1 (Probe labelled Vic was assigned to allele 1) (200nM), 0.05  $\mu$ l Probe 2 (Probe labelled Fam was assigned to allele 2), (200nM) and 1.3 $\mu$ l of deionised water and 1 $\mu$ l of DNA.

Pre-designed probes for the allelic discrimination assays were supplied as a mix at either 20X or 40X concentrations (40X mixes were diluted by half following the addition of deionised water). Each well contained the equivalent of: 2.5  $\mu$ l ABI 2X Genotyping Mix, 0.25  $\mu$ l of allelic discrimination assay at 20X concentration, 1.25  $\mu$ l of deionised water and 1 $\mu$ l (20ng/ $\mu$ l) DNA. All additions other than DNA were made as a cocktail for the number of wells plus 15%.

Plates were transferred onto SDS7500 Fast Machine for allelic discrimination using standard cycling conditions to include pre-read for baseline estimation, amplification run and a post-read. Fam probe was assigned to SNP 1 and Vic probe was assigned to SNP 2. Figure 11 shows variation in clustering due to the genotype of the target allele. For example in case of codon 129 polymorphism, there are three clearly distinguishable clusters representing the three possible genotypes at that locus (the MM, VV homozygote and the MV heterozygote polymorphism).

## **2.5 Gene sequencing**

### ***SPRN***

#### **2.5.1 PCR reaction**

MegaMix Blue (containing Taq DNA polymerase, dNTP's, MgCl<sub>2</sub>, reaction buffer and an enzyme stabiliser, Microzone, UK) was prepared containing primers at 0.5µM sufficient for 25µl reactions on a 96 well plate. The primers used are detailed below:

<b>Gene</b>	<b>Primer Name</b>	<b>Sequence (5' - 3')</b>
<i>SPRN</i>	P644	<b>GCG ATG GAG CGT GGC</b>
	P645	<b>CTT CCT CTG CGA CAG</b>
	P650	<b>CCT CTG CTC TCC AGC</b>
	P651	<b>AGC CCG CCG CCA GGC</b>

24µl of premix (containing primers at 0.5µM) was pipetted into each relevant well of a 96-well plate and 1µl DNA was added to each well from a 96 well DNA storage plate using a multi-channel pipette. The plate was sealed and spun down. It was then transferred to a PCR machine (Bio-Rad DNA Engine Tetrad 2) for DNA amplification using the following cycling conditions: 1) 94°C for 5mins, b) 94°C for 30secs, c) 63°C for 40secs for 650/1 primers and 65°C for 644/5 primers, d) 72°C for 45secs, e) steps b)-d) were then repeated 34 times and e) 72°C for 5mins. PCR products were assessed using electrophoresis of 5µl product on a 2% agarose gel stained with ethidium bromide. 5µl of HyperLadder (Bioline Reagents, Ltd, UK) was loaded and the gel viewed using the Bioradtransilluminator (Biorad, UK) and Quantity One software (BioRad Corporation, USA).

### **2.5.2 PCR product clean-up**

An equal volume of Microclean (Microzone) was added to the PCR product, mixed by vortexing and left at room temperature for 5 minutes. Plate was spun at 3000G at room temperature for 40 minutes, inverted onto tissue paper and spun again at 40G for 30 seconds to remove fluid. Pellets were resuspended by adding 50 $\mu$ l 18M $\Omega$  H<sub>2</sub>O, vortexed and left to stand for 5 min. The plate was vortexed again and spun down.

### **2.5.3 Sequencing Reactions**

For each sequencing reaction a premix was prepared to include 1 $\mu$ l of BigDye (Applied Biosystems), 5 $\mu$ l Better Buffer (Microzone), 3 $\mu$ l betaine (5M, Sigma) and 4.25 $\mu$ l 18M $\Omega$  ddH<sub>2</sub>O. 13.25 $\mu$ l of the mix was pipetted into each well of a 96 well plate. 0.75 $\mu$ l of the sequencing primer (as detailed above) was pipetted into their respective wells and 1 $\mu$ l of PCR product was added to each well. Plate was covered, spun down and placed on a PCR machine with the following cycling conditions: 1) 96°C for 1 minute, 2) 96°C for 10 seconds, 3) 50°C for 5 seconds, 4) 60°C for 4 minutes, steps 2)-4) were then repeated 24 times.

### **2.5.4 Sequencing Product Clean-up**

3.75 $\mu$ l of 0.125M EDTA (Sigma-Aldrich) was added to each sequencing reaction. 45 $\mu$ l 100% ethanol was added to each reaction, mixed by pipetting and left at room temperature for 15 minutes. Plate was spun at 3000G for 30 minutes at 4°C, inverted and spun again up to 185G. 60 $\mu$ l 70% ethanol was added and

the plate was spun at 1650G for 15 minutes at 4°C. Plate was inverted and spun at 185g for 1 minute. It was then placed on a PCR block, uncovered, at 37°C for 5 minutes to remove final traces of ethanol.

#### **2.5.5 Capillary electrophoresis of Sequencing Products**

10µl Hi-Di (Applied Biosystems) formamide loading solution was added to each well. Plate was covered, spun down and samples denatured by placing on PCR block held at 95°C for 2 minutes, and transferred to ice/4°C PCR block immediately for 2 minutes. Plates were transferred onto the sequencing machine (Applied Biosystems 3730XL) and run using standard protocol (Big Dye 1.1). Both raw and analyzed data were viewed using the Sequence Analysis Software Seqscape (Life Technologies, UK). Signal strength of over 8000 relative fluorescence units (RFU) did not produce adequate data and these samples were re-injected with a shorter injection time. Where the signal strength was too low, the base-line was too noisy and the samples were re-injected with a longer injection time.

## **2.6 Genome-wide association analysis (GWAS)**

### **2.6.1. DNA sample selection**

DNA for genome wide association analysis was obtained from patients with prion disease diagnosed according to the above criteria (see section 2.1.1) (Lukic et al., 2011; Zerr et al., 2009). Most patients were of white British ethnic origin and all patients of known non-white ethnic origin were excluded. This information was based on names and geographical location for some samples. The rest of the samples were checked using principal component analysis (as discussed below, Quality control 2.5.3) and all the ethnic outliers were excluded. Study also included patients with kuru and women resistant to kuru. A cohort of German patients who all had neuropathologically confirmed sCJD was used in our replication study. These patients all had a classical presentation of sCJD. Genomic DNA was extracted from peripheral blood and brain tissue was used as a source of genomic DNA for patients where blood was not available using the methods discussed. Amplified DNA was used for a small number of samples. Samples were checked for degradation and amplified as discussed.

### 2.6.2 Genotyping

Cases included 680 German definite sCJD samples, 579 UK sCJD, 133 UK vCJD (and 5 non-UK vCJD), 165 kuru samples, 125 kuru resistant women and 286 geographically matched control individuals. All cases were genotyped on an Illumina 660K platform. Publicly available KORA controls were genotyped on 550K Illumina arrays and 5667 WTCCC2 controls on the Illumina1.2M Custom Duo array. There was no discrepancy between reported and genetic gender in the cases. Prior to analysis, samples were removed from the WTCCC2 data set by their prescribed criteria. The WTCCC2 chose criteria to be similar to those often applied as standard in GWA studies. Individuals were excluded if they displayed a disproportionate number of heterozygous or missing calls. Related individuals were excluded according to identity by descent (IBD) (as discussed below). Individuals were excluded on the basis of ancestry if they differed from the majority of the collection according to a principle component analysis of HapMap individuals. Following quality control, a total of 467 WTCCC2 individuals were removed leaving 5200 samples which comprised of 2630 males and 2570 females.

### 2.6.3 Quality control

Confounding can be a major source of bias in population-based case-control studies and is caused by underlying differences between the case and control groups other than those directly under investigation. In the case of genotype distribution, the main source of confounding is **population stratification**, in which genotypic differences between cases and controls are generated because of different population origins rather than any effect on disease risk (Cardon and Palmer, 2003). Efforts were made to remove or reduce the effect of population stratification through the removal of individuals of divergent ancestry. **Principal components analysis** (PCA) was used to identify and subsequently remove individuals with large-scale differences in ancestry (Patterson et al., 2006; Price et al., 2006). PCA is a multivariate statistical method used to produce a number of uncorrelated variables or principal components from a data matrix containing observations across a number of potentially correlated variables. The principal components were calculated so that the first principal component accounts for as much variation in the data as possible in a single component, followed then by the second component and so on. When using PCA to detect ancestry the observations were the individuals and the potentially correlated variables were the markers.



## **2.7 Copy Number Variants (CNV) analysis**

### **2.7.1 CNV analysis in Genome Studio**

The CNV Partition (Bead Studio, Illumina Infinium) plug-in algorithm is supported by Genome Studio. It uses a recursive partitioning approach to provide copy number estimates, as well as copy number bookmarks. It identifies regions of the genome that are aberrant in copy number using two Infinium Assay outputs: the log R ratio (LRR) and B allele frequency (BAF). Since LRR is the logged ratio of observed probe intensity to expected intensity, any deviations from zero in this metric are evidence for copy number change. BAF is the proportion of hybridized sample that carries the B allele as designated by the Infinium Assay. In a normal sample, discrete BAFs of 0.0, 0.5, and 1.0 are expected for each locus representing AA, AB, and BB. It is fast to analyse and report (~1min per sample) and detects CNV regions and estimates CNV values with high accuracy. It identifies small CNV regions (<100 kb) and provides confidence score for each locus.

There are 2 further softwares that allow CNV detection, Quanti SNP and Penn CNV. Each algorithm has different advantages and disadvantages such as speed, accuracy of CNV detection and ability to detect small versus large deletions and duplications. In order to select the most appropriate algorithm we analysed a 10 sample project using all three softwares. The project was created in Genome Studio using a unique sample sheet. The performance and accuracy of 3 different CNV softwares was initially assessed. Following CNV analysis, PLINK, a genome wide analysis tool was used to estimate type, genomic location and frequency of CNV deletions and duplications. It allowed estimation of genome-wide CNV burden as well as specific association analyses. The primary analysis was a  $\chi^2$  test with the use of empirical p values. Three different algorithms were used to detect copy number value, confidence score, chromosomal location and copy number size. A preliminary CNV analysis was

performed with a 10 sample project using 3 different algorithms: CNV Partition, Penn CNV and Quanti SNP. It showed the total number of CNV deletions/duplications detected as well as the lowest and highest confidence value. CNV Partition detected the highest number of CNVs of even small CNV deletions and duplication with a high CNV confidence value suggesting good dependability and was therefore used for further analysis of the complete sample set.

## 2.7.2 Quality Control

Prior to analysis, samples were removed from the data sets by their prescribed criteria:

1. If there were fewer than 20 SNPs/CNV markers and if they had a low SNP density (>15kb per marker).
2. CNVs were excluded if > 50% of their length overlapped regions of segmental duplications, as defined by the 'Segmental Dups' track in UCSC.
3. We joined any CNVs that appeared to be artificially split by the algorithm if the length of the sequence between two CNVs was <50% of the length of the larger CNV. Consistent with previous CNV studies in a closely related disorder (Chapman et al., 2013), we analyzed only rare CNVs (<1%) due to the problems of reliably calling more common CNVs with standard SNP arrays. We filtered out the common CNVs present in >1% of each sample using PLINK and performed association tests.
4. All of these CNVs were found at <1% frequency in the control population and included more than 20 markers
5. The CNVs were separated according to size as: >100kb, >500kb and >1MB and quality score of >50 was used.
6. Of the total 27 >1000kb duplications seen in European case samples (from Germany, France and UK), 3 overlap in a region of chromosome 16 (NCBI build 36, 15,387,380-16,197,033) comprising 7 genes, although this same region was involved in 6 large duplications in control samples. Due to differences related to platform and filtering we have not directly compared these frequencies with other studies.
7. In PNG sample analysis a 1% filter was applied as the control population was small, and the population genetics of the Eastern Highland region has not been studied in detail.

Individuals were excluded if they displayed a disproportionate number of heterozygous or missing calls. Related individuals were excluded according to identity by descent (IBD). Individuals were excluded on the basis of ancestry if they differed from the majority of the collection according to a principle component analysis of HapMap individuals. Gender discrepancy between the supplier and the inferred gender also led to sample removal. Individuals were excluded if the mean of their A and B allele intensities were outliers when compared with the sample at large. Samples were excluded from the Genome Studio project if they had a low average SNP genotyping call rate (<98%). For CNV Partition project cases (670K array) and controls (1.2M Custom Duo) were merged using a unique sample sheet. Only probes common to both projects were used and chromosome X was excluded from the analysis to avoid false positive associations created by the software due to the absence of 2<sup>nd</sup> chromosome X in males.

### **2.7.3 CNV analysis in UK patients**

A total of 708 UK samples (509 sCJD, 123 vCJD and 76 inherited prion disease samples) were analysed and compared to 902 Wellcome Trust controls. Patient samples were analysed on Illumina Human-Hap 670 array and the publicly available WTCCC2 controls on 1.2M Custom Duo array. The data was imported into Genome Studio and the 2 projects were analysed using CNV Partition. When more case and control samples became available the analysis was repeated using Penn CNV.

### **2.7.4 Real time PCR in UK sporadic CJD**

In order to validate the findings following genome wide CNV analysis, real time PCR was used for validation. All genotyping assays were undertaken on the 7500 Fast Real Time PCR machine (Applied Biosystems) using pre-prepared 96 well plates containing DNA at 20ng/ml. 1 µl of DNA was added to reaction premix as the final addition. Each well contained: Applied Biosystems 2.5 µl 2X Genotyping Mix, 0.25 µl of 20X allelic discrimination assay mix, deionised water 1.25 µl and DNA 1.0 µl (20ng/µl). Plates were transferred onto the SDS7500 Fast Machine for allelic discrimination using standard cycling conditions to include pre-read for baseline estimation, amplification run and a post-read.

### **2.7.5 CNV analysis in German sporadic CJD**

In order to replicate findings detected in UK patients CNV analysis was performed in German definite sCJD patients. A total number of 635 German sCJD cases and 822 control samples were analysed. The cases were analysed on 670K Illumina Genome Studio platform and controls on an older 550K Illumina platform. The 2 projects were merged using a unique sample sheet. CNV analysis was performed in Genome Studio using CNV Partition and PLINK for case/control association analysis.

### **2.7.6 Validation using real time PCR**

In order to confirm the findings following genome wide CNV analysis, real time PCR was used for validation. All genotyping assays were undertaken on the 7500 Fast Real Time PCR machine (Applied Biosystems) using pre-prepared 96 well plates containing DNA at 20ng/ml. 1 µl of DNA was added to reaction premix as the final addition. Each well contained: Applied Biosystems 2.5 µl 2X Genotyping Mix, 0.25 µl of 20X allelic discrimination assay mix, deionised water 1.25 µl and DNA 1.0 µl (20ng/µl). Plates were transferred onto the SDS7500 Fast Machine for allelic discrimination using standard cycling conditions to include pre-read for baseline estimation, amplification run and a post-read.

### 2.7.7 Statistical analysis

Data management and statistical analysis were performed using PLINK (Renteria et al., 2013). PLINK is an open-source whole genome association analysis toolset, designed to perform a range of basic, large-scale analyses in a computationally efficient manner. In our study PLINK was used for genotype/phenotype data analysis that has already been called from raw intensity data.

Cohorts of CJD patients were compared with relevant controls using allelic and genotypic models. Age at death (or sampling) was used as a quantitative trait in analysis of kuru patients. Age at clinical onset expressed as deviation from the mean expected onset for each IPD mutation was used as a quantitative trait in the IPD cohort. Meta-analyses were done using PLINK. Imputation was done using IMPUTE2 using data provided by the WTCCC and 1000 genomes project. IMPUTE2 uses a fine-scale recombination map and a densely genotyped reference panel to "fill in" missing genotypes in a study dataset, which might consist of cases and controls typed on a commercial SNP chip. By estimating the genotypes of SNPs that were not in the original study data, imputation allows a much larger set of SNPs to be tested for association. This can increase both the power to detect association signals and the signal resolution near a causal or associated variant.

Conditional analyses were done at the PRNP locus using rs1799990 in additive and genotypic models. Stratified analyses were done using all three genotypes at rs1799990. Age of clinical onset in UK sCJD was used either as a quantitative trait or as a threshold for stratification (include only sCJD age at onset, 60 years).

Case-control labels were permuted within each of the four CJD cohorts, and a genome-wide meta-analysis was performed on the permuted data set using the same analysis as for the observed data set.

This was repeated 500 times generating crude estimates for the significance of this statistic.

The statistical case/control analysis for a given quantitative trait was analysed using PLINK commands based on Wald parametric statistical test. The quantitative phenotypes analysed were:

1. Cognitive function
2. Visual symptoms
3. Involuntary limb movements
4. Sensory abnormalities
5. Frontal release signs
6. Cerebellar features
7. Pyramidal features



## **2.8 Neuropathological analysis in prion disease**

Ethics approval for these studies was obtained from the Local Research Ethics Committee of UCL Institute of Neurology, National Hospital for Neurology and Neurosurgery. Informed consent was obtained from relatives to use tissue for research. Clinical data, disease history, *PRNP* mutation and polymorphism at codon 129 were available for majority of cases analysed.

Autopsies were carried out by Professor Brandner and Dr Reiniger laboratory in a post-mortem room designed for high risk autopsies. Brain tissue was stored in buffered formalin. The frontal, temporal, parietal, occipital lobes and cerebellum were sampled and immersed in a 10% buffered formalin for up to one week, immersed into 98% formic acid for one hour and post fixed for 24 hr in 10% buffered formalin. Tissues were then processed through graded alcohols and embedded in paraffin wax.

### **2.8.1 Antibodies, immunohistochemistry and electron microscopy**

Antibody staining and immunohistochemistry was performed by the neuropathology team using anti-PrP ICSM35 D-Gen Ltd, London, UK raised in Prnp<sup>0/0</sup> mice against recombinant human PrP (Isaacs et al., 2008) and anti-PrP KG9 TSE resource Centre, Roslin Institute University of Edinburgh (Bell et al., 1997). ICSM35 recognises residues 93-102 and KG9 binds to residues 140-180 of human PrP.

Immunofluorescence and preparation for transmission electron microscopy was performed by the neuropathology team using thin tissue blocks. Resin sections were stained with Toluidine blue and suitable regions selected for tissue EM. Images were recorded on a 4Megapixel SIS Megaview digital camera.

### 2.8.2 Examination and quantification

Gross inspection of the whole brain in sporadic Creutzfeldt-Jakob disease often did not reveal obvious abnormalities. More commonly, there was some degree of cerebral atrophy, which was diffuse or focal. Based on the preferential involvement of specific regions the analysis involved assessment of frontal, parietal, temporal, occipital and cerebellar lobes. Each of the lobes was investigated for the presence and distribution of spongiform degeneration and prion protein deposition. In order to investigate a possible multiple protein pathology and neuropathological similarities with other neurodegenerative conditions we looked at the presence of amyloid- $\beta$  and phosphorylated tau in brains from prion disease patients. Similarly, we investigated the association of neuropathological abnormalities in sCJD, inherited prion disease, iatrogenic and variant CJD with polymorphism at codon 129 of the prion protein gene, age at disease onset and disease duration.

I was initially taught by Professor Brandner and Dr Reiniger on how to recognise characteristic pathological CJD changes and grade them according to the devised criteria. Together with Dr Reiniger I performed of spongiform change, prion protein, amyloid- $\beta$  and tau protein distribution and deposition in prion disease patients. I performed scoring of a subgroup of slides. The aim of our study was to ascertain the extent to which misfolded proteins associated with other neurodegenerative diseases are found at autopsy examination of human prion diseases.

Table 5 summarises key features we observed in the neuropathological analysis. Prion protein accumulates to form granular, synaptic, plaque deposits and peri-neuronal net. The deposits vary in severity and were graded from mild, moderate to severe. In our study the grading was based on the appearance according to an example image (Figure 5, introduction). A more detailed description of spongiform change, PrP, amyloid- $\beta$  and tau deposits is summarised in Table 5.

Feature	Pattern	Score1	Score2	Score3
Spongiform degeneration		Fine, diffuse	Intermediate	Severe – large partly confluent vacuoles
Prion protein	Granular	Occasional disseminated granules, or infrequent patches. Can be associated with synaptic PrP	Frequent disseminated and partly confluent granules. Often associated with synaptic deposits	Very dense, often confluent granules. Can dominate the entire grey matter
	Synaptic	Low density or patchy	Intermediate, diffuse	Strong, diffuse
	Plaques	Low frequency	Intermediate frequency	High density/frequency
	Perineuronal net	Occasional patches or very fine, delicate decoration of neurones. Typically deep cortical layers.	Contiguous network of perineuronal labelling. Mini plaques often present	Dense network of perineuronal labelling. Often small or medium sized plaques. Also combined with synaptic deposits
Amyloid-β	Diffuse	Rare diffuse amyloid-β deposition	Frequent diffuse deposits	Very dense, often widespread diffuse deposits
	Plaques	Equivalent to CERAD low	Equivalent to CERAD intermediate	Equivalent to CERAD high
Tau phosphorylation	Prion protein associated	Very occasional stubs or rod-like inclusions\75 per 10 HPF	Moderately frequent density of rod-like inclusions (75–500/10 HPF	Frequent deposition of rod or stub like inclusions ([600/10 HPF), often forming coarse granular aggregates
	Amyloid-β associated	Rare threads	Occasional tangles, threads if intermediate density	Frequent tangles and dense meshwork of threads

**Table 5.** describes typical changes associated with prion protein, amyloid-β and phosphorylated tau deposition. The changes are scored from 1-3 to indicate mild, moderate and severe deposition. In some cases where tau deposition was very high a score of 4 was used. This was rare and was only used to score occasional very heavy tau deposits (The table shows only grading from 1-3 for each of the proteins).

Neuropathological changes in prion disease such as neuronal loss and gliosis accompany many other conditions of the central nervous system, whereas spongiform change is moderately specific to prion diseases. Spongiform change is characterised by diffuse or focally clustered, small, round or oval

vacuoles in the neuropil of the deep cortical layers, cerebellar cortex or subcortical grey matter, which might become confluent (Figure 5, Introduction). The semi-quantitative analysis was performed based on the typical pathological appearance. The deposits were graded from mild to severe using scores 0-3. In some cases where tau deposition was very high a score of 4 was used. This was rare and was only used to score occasional very heavy tau deposits. (The table shows only grading from 1-3 for each of the proteins). The severity of the abnormal appearance was subjective and established by two different observers performing analysis together (AL was one of the two). One slide for each of the areas frontal, parietal, temporal, occipital lobe and cerebellum were analysed and the final score taken as an average of the above areas. The examination was performed on a LEICA DM2500 with a 40X Pan Apochromat objective. The typical pathological features that were looked for when the scoring was performed are illustrated in Figure 5 in Introduction.

In addition to prion protein deposits,  $\beta$ -amyloid and hyperphosphorylated tau were investigated in the brains of patients with prion disease. These were scored according to intensity but also the typical distribution of deposits. Table 6 shows how a single score was obtained to illustrate both distribution and intensity of each of the three proteins. For prion protein a single score was generated depending on whether the deposits were plaques or synaptic. They were associated with a score from 1-3 to indicate severity of the deposition (for example score S2 would indicate moderate synaptic prion protein deposition). Amyloid- $\beta$  deposits were labelled as P or A depending on whether they formed diffuse or dense deposits and were associated with a score from 1-3 depending on the severity of deposition (eg. score A2 would indicate moderate diffuse amyloid- $\beta$  deposition). Tau deposits usually occurred as threads or stubs and were graded from 1-4 depending on the severity of deposition.

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## Scoring for prion protein, amyloid- $\beta$ and hyperphosphorylated tau

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<b>Prion protein</b>		
<i>Quality</i>		
S	Synaptic	
P	Plaques	
<i>Intensity</i>		
0	Negative – no prion protein deposition	
S1	Weak synaptic deposition	
S2	Moderately dense deposition	
S3	Dense, generalised synaptic PrP	
P1 (D1,2,3)	Small plaques	D1 low density: occasional plaques
P2s or (D1,2,3)	Predominantly medium size plaques	D2 intermediate density with relatively frequent plaques
P3s or (D1,2,3)	Predominantly large plaques	D3 High density of plaques with frequent, densely packed plaques
<b>Tau</b>		
0	No tau at all	
1	Very occasionally small threads or stubs in the grey matter	
2	Occasional threads and stubs	
3	Frequent stubs and threads in the grey matter. Accentuation around plaques.	
T (1,2,3)	Occurrence of tangles (frequency)	
<b>Amyloid-<math>\beta</math></b>		
0	No A beta deposition	
P1	Sparse	
P2	Moderate	
P3	Dense / frequent	
A1	Diffuse amyloid beta, occasional patches	
A2	Diffuse amyloid beta, frequent patches	
A3	Diffuse amyloid beta, very widespread, occasionally confluent to non-core plaques	

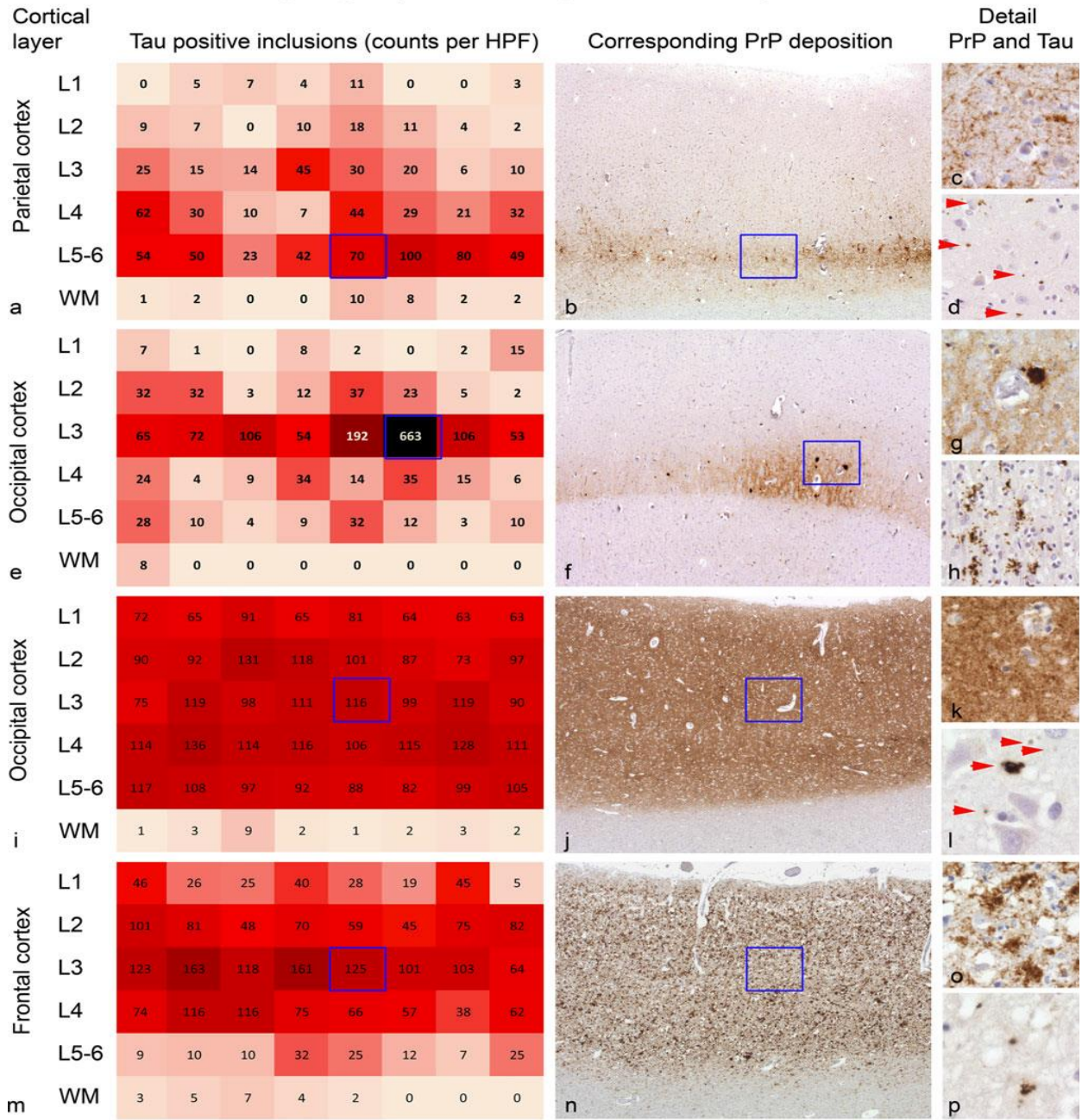
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**Table 6** shows combined score generated to demonstrate both the quality and intensity of prion protein hyperphosphorylated tau and amyloid- $\beta$  deposition. Where the 2 scores were different the highest score was used to generate the combined score that best describes the protein deposits.

Amyloid- $\beta$  forms diffuse and plaque deposits, whereas tau phosphorylation usually accumulates in the vicinity of amyloid- $\beta$  and prion protein. Tau protein can however, be present independently in which case it has a slightly different appearance.

Due to its varied aetiology and appearance quantitative assessment of hyperphosphorylated tau deposits was done using a heat map (Figure 12) to enable counting of the number of tau rods and granules in a high power field. The heat map displays the counts of hyperphosphorylated tau inclusions in a data matrix. Larger values are represented by dark red or black squares (pixels) and smaller values by lighter squares. A parallel tissue section was also produced and stained for prion protein to assess the PrP deposition in the same area that corresponds to the heat map. Figure 12 shows the count and colour coded representation (heat map). The tau inclusions were counted in different areas for each of the lobes as illustrated in the figure. The corresponding prion protein deposition is also demonstrated.

## Tau phosphorylation corresponds to PrP deposits



**Figure 12.** The heat map illustrates quantitative assessment of hyperphosphorylated tau deposits. Figure shows the count and colour coded representation. Middle column shows a parallel tissue section, stained for abnormal PrP showing deposits corresponding to the tau heat map. Right column shows high power magnification of the areas indicated by a blue square in the heat map and in the overview of the PrP labelled section (corresponding to roughly 25% of the blue square). (Figure copied and adapted from *Tau, prions and Aβ: the triad of neurodegeneration*. Reiniger L, Lukic A, Linehan J, Rudge P, Collinge J, Mead S, Brandner S. *Acta Neuropathol*. 2011 Jan;121 (1):5-20.)

### III RESULTS

#### **3.1 Phenotypical heterogeneity and susceptibility locus in variant CJD- clinical case reports**

Prion diseases show marked phenotypic heterogeneity in terms of disease duration, age at onset, clinical, investigation and neuropathological features. These differences may be genetically determined, associated with a particular prion protein strain or specific environmental factors. In order to illustrate this heterogeneity we provide clinical and investigation findings of 2 patients with clinical presentation typical of sCJD who were subsequently diagnosed with vCJD following neuropathological analysis, confounding expectations.

We also present 2 cases with an unexpected polymorphism at codon 219. All neuropathologically proven vCJD patients were homozygous for methionine at codon 129. In this study we describe 2 patients with diagnosis of vCJD and a rare polymorphism at codon 219. Heterozygosity at codon 219 (E219K) is known to have a protective effect in Japanese patients with sCJD (Shibuya et al., 1998). This finding is intriguing as the polymorphism appears to be neutral or may even confer increased susceptibility to vCJD. We also wished to test the hypothesis that the prion protein coding sequence is a crucial determinant of clinical phenotype.



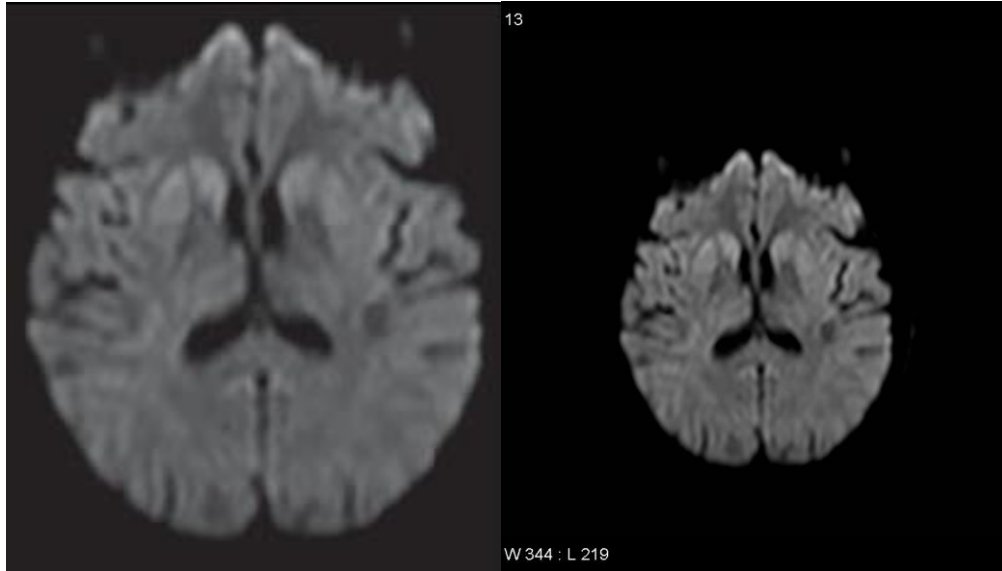
**3.1.1 Atypical clinical presentation in two patients with variant CJD, correctly diagnosed following neuropathological analysis.**

As already discussed variant CJD (vCJD) has been causally linked to BSE and typically affects young individuals in contrast to sCJD (Will et al., 1996). It is associated with a rapid cognitive decline, psychiatric and sensory disturbance. In patients with vCJD, the PrP<sup>Sc</sup> is deposited in the lymphoreticular system and has a characteristic appearance following partial protease digestion and Western blotting (Wadsworth et al., 2001). MRI typically shows high signal intensity in the pulvinar nucleus on T2 weighted images (Will et al., 2000). Pre-mortem diagnosis can be assisted by tonsillar biopsy (Hill AF et al., 1999). Accurate pre-mortem diagnosis is important for patients and their families, in terms of a differential diagnosis including treatable causes, prognosis and choices about experimental therapeutics. Phenotypic variability due to atypical clinical presentation in vCJD can lead to misdiagnosis and have implications regarding potential experimental therapeutics (Allroggen et al., 2000). We describe clinical and investigation features in 2 patients prompting a diagnosis of sCJD by the updated WHO criteria (<http://www.cjd.ed.ac.uk/documents/criteria.pdf>). The correct diagnosis of vCJD was made at autopsy in both cases. Both patients had MRI findings typical of sCJD with greater signal hyperintensity in the caudate and putamen than the pulvinar nuclei which was further confirmed with quantitative analysis. The age at presentation in the first patient was unusually late at 54.

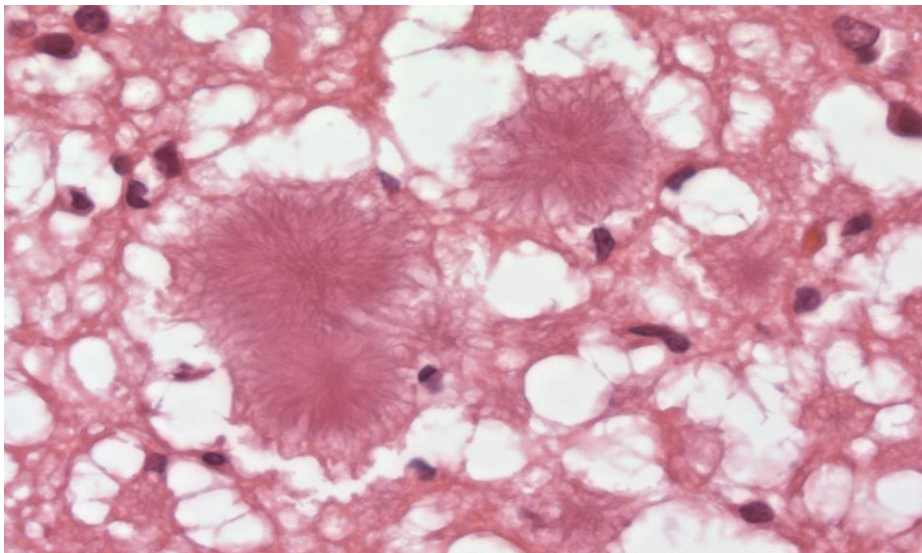
**CASE 1**

A 54 years old lady presented with a 7 month history of episodic memory abnormalities, depression, occasional paranoid delusions, somnolence and vivid dreams. She subsequently developed in-coordination and intermittent upper limb twitching and her daughter noticed a breathing abnormality characterized by heavy breathing and 'humming'. Her gait became unsteady and she required assistance

for walking. She developed an obvious short-term and topographic memory deficit. On admission to hospital her MMSE was 11/30 and she had a broad-based, ataxic gait. During a six week admission she continued to deteriorate in terms of mobility, speech and swallow. When examined by the NPC she was drowsy, disoriented in time and place and responded verbally with a few appropriate words. On cranial nerve examination there was vertical gaze palsy, improved by doll's head movements, implicating a supranuclear component and a prominent pout reflex. In the upper limbs there was an alien limb phenomenon and cogwheel rigidity which was more pronounced on the left. In the lower limbs there was bilateral spasticity. The tendon reflexes were brisk throughout with extensor plantar responses. She had great difficulty in standing due to a combination of ataxia and apraxia and was doubly incontinent. She continued to deteriorate becoming bed-bound and mute and died 11 months into the illness. The MRI showed abnormal high signal in the caudate and basal ganglia which was greater than in the thalamus as shown by quantitative studies (Figure 13). The CSF analysis was unremarkable apart from being positive for 14-3-3 protein. The EEG revealed generalized slow wave activity. Extensive investigations to exclude an autoimmune, inflammatory and a para-neoplastic disorder were negative. *PRNP* gene analysis revealed no mutations and the codon 129 genotype was methionine homozygous. The post-mortem examination was diagnostic of vCJD. A detailed neuropathological examination of the brain tissue revealed multiple 'florid' plaques in the cerebral and cerebellar cortex, severe spongiform change, severe thalamic gliosis and marked accumulation of the disease-associated prion protein in diffuse and pericellular deposits in the cerebrum and cerebellum (Figure 14).



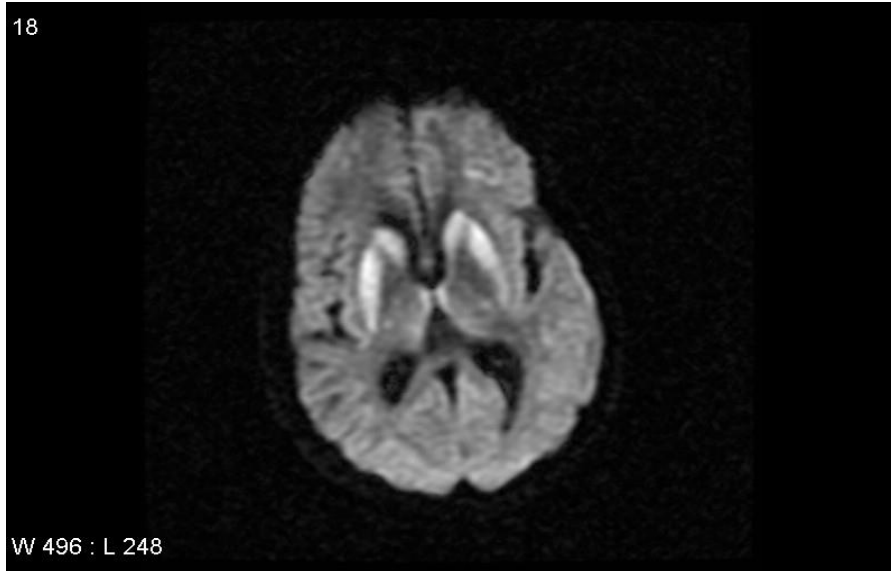
**Figure 13.** Diffusion Weighted Imaging (DWI) at two levels demonstrate hyperintensity in the basal ganglia bilaterally. The signal intensity ratios of the deep grey nuclei compared to white matter performed are: caudate 234.7/48.6; putamen 220.6/48.6 and pulvinar 195.1/48.6. High signal in the basal ganglia was greater than in the thalamus, thus the pulvinar sign was not present as expected in vCJD. The FLAIR images were degraded by movement artifact and were therefore undiagnostic although the high signal in the basal ganglia appeared greater than that in the thalamus (*Images kindly provided by Dr Hyare*).



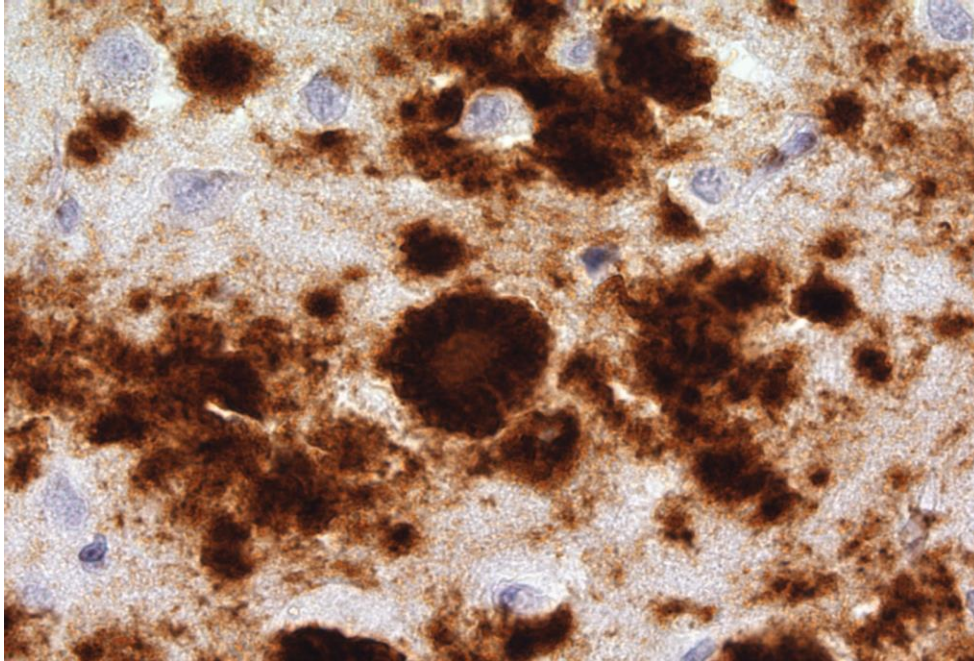
**Figure 14.** There is a severe spongiform change accompanied by gliosis in the cortex with striking depositions of florid plaques which often appear in clusters and are typically seen in vCJD (H&E stain, 40x) (*Images kindly provided by Prof Brandner and Dr Reiniger*).

## CASE 2

A 24 years old patient complained of pain in the right leg attributed to heavy manual work which spontaneously resolved within 2 weeks. Two months later he became unsteady, speech was slurred and his personality changed in that he became socially withdrawn and apathetic. He had several falls and intermittent episodes of incontinence which did not appear to cause him any concern. He was admitted to the hospital and his behaviour continued to deteriorate with aggressive outbursts and increasing withdrawal. There was a suggestion of pre-morbid personality change as during the previous 2 years at school his academic achievement had declined and he had subsequently dropped out of a course in graphic design. On admission the examination revealed moderately severe cognitive impairment with a MMSE of 12/30. He performed poorly on frontal executive tasks, had some language impairment and dyscalculia with intact visuo-perceptual functions. There was a moderate dysarthria. Muscle tone was increased in both upper and lower limbs and reflexes were symmetrically brisk with extensor plantar responses. He had a markedly ataxic gait requiring assistance of two to walk. He continued to deteriorate and when re-examined by NPC he could only utter occasional words, had moderate dysphagia, bilateral grasp reflexes and occasional myoclonic jerks. Myoclonus became more severe with intermittent choreiform movements in the arms. He subsequently became essentially mute, deteriorated in terms of mobility and swallow and died 9 months into the illness. The MRI of the brain showed abnormal signal return from the basal ganglia and dorso-medial thalamus and the frontal cortical grey matter especially on the left on T2, FLAIR and DWI sequences (Figure 15). The EEG showed diffuse slow wave abnormality. The CSF examination was unremarkable apart from a greatly elevated tau protein (14-3-3 protein was negative). Extensive investigation failed to reveal an alternative autoimmune, paraneoplastic or inflammatory cause for the illness. *PRNP* gene analysis showed no mutations and the codon 129 genotype was methionine homozygous.



**Figure 15.** MRI image demonstrates hyperintensity in the caudate and putamen which is greater than in the thalamus on DWI. The signal intensity ratios for the deep grey nuclei compared to white matter performed on image are: caudate 201.2/137.4; putamen 181.7/137.4 and pulvinal 165.7/137.4. Therefore, the pulvinal sign was not present as expected in vCJD. The appearance on FLAIR images also supported these findings (*Images kindly provided by Dr Hyare*).



**Figure 16.** Immunohistochemistry for abnormal prion protein demonstrates frequent formation of small to medium sized plaques which frequently form confluent aggregates. In addition, there is intracellular deposition of prion protein forming stellate deposition (ICSM35 antibody, 40x). The findings are consistent with vCJD. (*Images kindly provided by Prof Brandner and Dr Reiniger*).

Phenotypic variability as demonstrated by an unusually old age at disease onset and sporadic-like MRI appearance is not commonly reported in vCJD. One of the challenges faced by clinicians is dealing with the variability of clinical symptoms, signs and investigation findings in prion disease, which may pose a serious diagnostic challenge as evidenced by the cases described. It may result in a delayed diagnosis and delayed inclusion in therapeutic trials when relatives/patients desire to do so. In our experience of 60 biopsies, by far the largest series worldwide, tonsillar biopsy has 100% sensitivity and specificity, at any stage of the disease (Lukic et al., 2011). Prion protein deposition in the tonsil can be patchy, and at least 20 germinal centers need to be examined (Ironsides et al., 2000). A recent study shows that detection of disease-associated prion proteins in blood is possible and may allow a rapid, non-invasive

screening of individuals with suspected vCJD (Edgeworth et al., 2011; Jackson et al., 2014) although the test has been positive in sCJD samples. This may be of particular importance for the diagnosis of atypical cases in the evolution of vCJD outbreak associated with an altered clinical phenotype, investigation findings and genetic analysis. The challenge remains in ascertainment of genetic loci that may be associated with the phenotypic variation by altering the pathway of protein misfolding or by their involvement in the mechanism of chaperoning or degrading misfolded proteins or alternatively the genes involved in determining a prion strain. A better understanding of these genes may allow an insight into the pathogenesis of prion protein misfolding and allow stratification to help clinical trials or improve recognition of diagnostic markers in prion disease.

### **3.1.2 PRNP codon 219 may represent a susceptibility polymorphism to variant CJD**

As already discussed, variant Creutzfeldt-Jakob disease (vCJD), an acquired prion disease transmitted by exposure to bovine spongiform encephalopathy prions, was first recognized in the mid-1990s (Knight, 2006; Will et al., 1996). More than 200 cases have been identified worldwide, most in the United Kingdom. In neuropathologically confirmed vCJD (or probable vCJD according to WHO criteria), every patient who has undergone genotyping to date has been homozygous for methionine at a common polymorphism of the prion protein gene (*PRNP*) at codon 129. This genetic variant has profound susceptibility effects across all categories of prion disease (Collinge, 2005). However, two thirds of the population carries this genotype whereas only around 220 patients developed clinical vCJD in a population widely exposed to BSE implicating the potential importance of other genetic loci in susceptibility to disease. A further genetic polymorphism has been reported at codon 219 in which guanine (G) is replaced by adenine (A) in the first position of codon 219, resulting in substitution from glutamic acid (E) to lysine (K). This polymorphism is common in east Asians, with an allele frequency of 0.06 in the healthy population. It has not been reported in patients with sCJD in the Korean and Japanese populations and is, therefore, considered strongly (perhaps completely) protective against this disease (Jeong et al., 2005; Shibuya et al., 1998). Eleven patients and controls with E219K all had Asian ethnicity or ancestry based on a heterogeneous diagnostic referral series totaling more than 1800 samples at the MRC Prion Unit (Beck et al., 2010) We detected this polymorphism in 2 patients with clinical and investigation findings consistent with vCJD suggesting its possible role in susceptibility to this form of prion disease. We investigated the possibility that *PRNP* variants are determinants of clinical phenotype in variant CJD.



## CASE 1

A 34-year-old British man presented in 2004 with a few months' history of personality change and mild episodic memory impairment. He became obsessive, socially withdrawn, and apathetic, and he began to experience difficulty with managing finances. Several months later his gait was noted to be unsteady, and he was intermittently incontinent of urine. Subsequently, he developed clumsiness in the upper limbs and difficulty manipulating fine objects, and he could no longer use a knife and fork. His gait became increasingly unsteady, and he started to fall. His mood deteriorated significantly, and he began to demonstrate suicidal ideation. He was soon unable to perform his usual daily activities. He subsequently became doubly incontinent, started to experience choreiform movements in the upper limbs, and he spoke less. He seemed to respond to visual hallucinations and experienced a disordered sleep-wake cycle. Physical examination revealed a bilateral grasp reflex. Involuntary choreiform limb movements were present, in addition to purposeful rubbing of his chest and hair. Spontaneous myoclonus of the lower limbs was present. His speech was hypophonic, mumbling, and monosyllabic. His gait was ataxic, with reduced arm swing, and he was unsteady on turning. There was increased tone and preserved power in all 4 limbs. His reflexes were symmetrically brisk, and the plantar responses were extensor bilaterally. His Mini-Mental State Examination score was 12 of 30 at the time of investigation, 12 months after symptom onset. Investigation findings were positive for cerebrospinal fluid 14-3-3 protein and a raised S-100B protein level of 1.36 ng/mL (reference range <0.38 ng/mL), with a cerebrospinal fluid protein concentration of 0.07 g/dL (reference range, 0.02-0.05 g/dL) and otherwise normal cerebrospinal fluid constituents and no oligoclonal bands. Electroencephalography revealed nonspecific slow wave activity. Diffusion weighted magnetic resonance imaging showed increased thalamic signal bilaterally, consistent with the pulvinar sign, with an additional increase in the left occipital cortex, and was thought to be most compatible with vCJD. Findings from autoimmune,

inflammatory and para-neoplastic screening were negative. Results of treponemal and human immunodeficiency virus serologic analyses were negative. Results of screening tests for Wilson disease were also negative. Mitochondrial DNA analysis showed no evidence of the MELAS (mitochondrial, encephalopathy, lactic acidosis, and stroke-like episodes) 3243 mutation. DNA analysis showed both Huntington gene alleles to be within the reference range for CAG repeats. A diagnosis of probable vCJD was made. *PRNP* Sanger sequencing was performed and showed a methionine homozygous codon 129 genotype and an E219K polymorphism. Twelve months after the illness began, the patient deteriorated further in terms of speech, swallowing, mobility, and cognitive function. He became bed bound and mute; he died 3 months later. Neither diagnostic tonsil biopsy (Hill AF et al., 1999) nor autopsy was performed.

## **CASE 2**

A 31 year old British woman of Afro-Caribbean descent presented in 2001 with episodic memory impairment which was initially noticed by her husband. She was 34 weeks pregnant when the symptoms started. Her son was born at 36 weeks by Cesarean delivery owing to antepartum hemorrhage. She had no problems caring for him initially, but she subsequently started to experience short-term memory problems and low mood, so the husband became more involved with caring for their baby. The patient became increasingly apathetic, and when she tried to return to work 6 months later she was unable to function at her previously high level. She developed gait ataxia and an involuntary choreiform movement disorder. Her husband described her as having difficulty with speech and language. At this stage, she was noted to have myoclonic jerks of both upper and lower limbs. Physical examination revealed a Mini-Mental State Examination score of 16 out of 30. Her gait was broad-based and unsteady, and her speech was slurred. She had a positive grasp reflex bilaterally and brisk finger jerks.

The patient had abnormal movements of her upper and lower limbs and a rocking movement of the body, which were suppressible only for a short period. Tone and power were normal. Her reflexes were symmetrical, and the plantar responses were flexor bilaterally. She was disorientated in time and place. There was evidence of fronto-temporal and parietal lobe cognitive dysfunction demonstrated by dyspraxia with difficulty miming, substitution of body parts as tools, and problems with motor sequencing. Investigation findings were positive for cerebrospinal fluid 14-3-3 protein. Magnetic resonance imaging showed bilateral pulvinar high signal. Electroencephalography was unremarkable. A tonsillar biopsy demonstrated abnormal prion protein (PrP) typical of vCJD on Western blotting (type 4t PrP<sup>Sc</sup> using the London classification) (Hill AF et al., 1999;Wadsworth et al., 2001). *PRNP* sequencing revealed a methionine homozygous codon 129 genotype and an E219K polymorphism. The patient deteriorated further and became bed-bound and mute. She died 16 months after the onset of symptoms. An autopsy was not performed.

	CASES	CONTROLS
E219K DETECTED	2	9
E219K NOT DETECTED	117	1683

**Summary table of E219K polymorphism in variant CJD cases and controls.** Heterozygosity at codon 219 was detected in 2 patients with diagnosis of probable and definite variant CJD in our large referral MRC series totalling 1800 patients.

Heterozygosity at codon 219 is a common polymorphism in the healthy Japanese population and in populations in Oceania, South Asia, and the Middle East, but it has not been detected in Africans or Europeans (Beck et al., 2010;Mead et al., 2003;Soldevila et al., 2003). 11 out of 1800 patients and

controls in our heterogenous referral series at the MRC (Medical Research Council) Prion Unit had E219K heterozygosity at codon 219. All 11 individuals were of Asian ethnicity or ancestry. The potential role for codon 219 in susceptibility and phenotype modification of vCJD is discussed further in section 4.1.2.

### **3.2 Clinical heterogeneity - National Prion Monitoring Cohort**

Broad enrolment criteria were selected to ensure the Cohort study was representative of prion disease in the UK (see Methods). Patients enrolled in the PRION-1 trial and the Cohort study until June 2014, were included in these analyses. 96% of eligible patients were recruited by the NPMC team and only <1% withdrew consent prior to death. A total of 2681 assessments were done over 470 patient-years of study. 94% of recruited patients met the criteria for probable human prion disease indicating good diagnostic accuracy. Neuropathological analysis was done in 60% which confirmed prion disease in all cases. A molecular diagnosis by genetic testing or biopsy was achieved in 22%, resulting in 70% of patients with a definite diagnosis of prion disease (some had a genetic and autopsy diagnosis). Clinical deterioration was measured using the MRC Scale. Rapid decline over weeks or a few months was seen mostly in sCJD patients, but also some acquired prion disease and specific inherited prion disease mutations. Slow decline over years includes almost exclusively patients with inherited prion disease typically associated with the GSS and related phenotypes and a small proportion of patients with rapid decline followed by prolonged survival at high levels of neurodisability, essentially a comatose state (MRC Scale <3), which may be in part due to exceptional supportive care. Variant CJD patients who were heterozygous at codon 219 of the prion protein gene did not show distinct clinical phenotypes however, it is difficult to make this conclusion with confidence due to a small number of patients.

The purpose of this analysis was to investigate the determinants of heterogeneity, which could help optimise clinical trials. As sCJD is the most common category of prion disease, and given the varied timescales over which functional decline occurred in stratum I and II (see Methods and Table 4), further analysis was focused only on the rapidly progressive forms of prion disease. The other patients were excluded from the analysis. In stratum I, rates of functional decline were significantly slower for

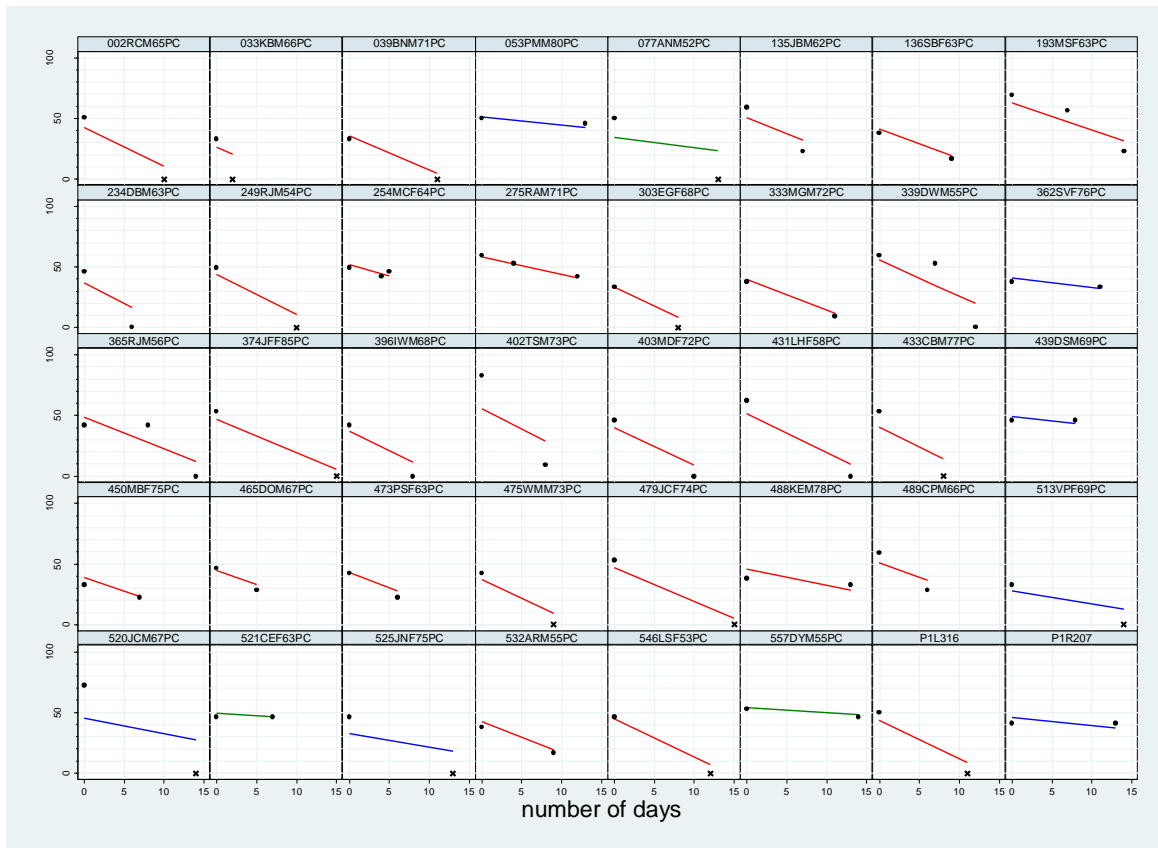
acquired prion disease versus sCJD ( $P < 0.001$ ). As incidence of acquired disease is currently very low this group of patients was also excluded from further analyses. Since rapidly progressive forms of IPD (clinically mimicking sCJD) may not be diagnosed for several weeks after prion disease itself is first identified, these patients were included, as it would not be feasible to completely exclude them from a prospective clinical trial. Finally, as many patients are diagnosed with CJD at advanced stages of neurodisability, when irreversible neuronal death has already occurred, patients with severe disease at presentation (MRC Scale  $< 5$ ) were also excluded from the analysis.

The rapidly progressive group was further subdivided according to the codon 129 polymorphism and the rate of decline measured using the MRC Scale. In the best-fitting linear mixed model in 154 rapidly progressing human prion disease patients (sCJD and fast progressing IPD), baseline MRC scale depended only on age, being 4.1 Rasch units lower for every 10 years older ( $p < 0.008$ , Table 7).

Factor	Estimate	95% CI	P (heterogeneity vs reference category)
<b>Estimates of baseline and decline</b>			
Baseline, aged 65 years, MM	53.19	(47.82,58.55)	
Baseline, aged 65 years, MV	57.88	(53.85,61.90)	0.17
Baseline, aged 65 years, VV	50.89	(45.60,56.18)	0.55
Baseline, per 10 years older at enrolment	-4.12	(-7.14,-1.10)	
Decline, per day, MM	-1.90	(-2.36,-1.45)	
Decline, per day, MV	-0.36	(-0.83,0.11)	<0.0001
Decline, per day, VV	-0.76	(-1.24, -0.28)	<0.0001
<b>Estimates of individual variability</b>			
MM: baseline	17.17	(13.31,22.17)	
MM: decline	1.36	(0.95,1.95)	
MM: correlation(baseline,decline)	0.38	(0.00,0.66)	
MM: residual error	12.80	(10.91,15.02)	
Non-MM: baseline	14.75	(12.52,17.37)	
Non-MM: decline	0.33	(0.24,0.46)	
Non-MM: correlation(baseline,decline)	0.24	(-0.02,0.47)	
Non-MM: residual error	9.20	(8.57,9.87)	

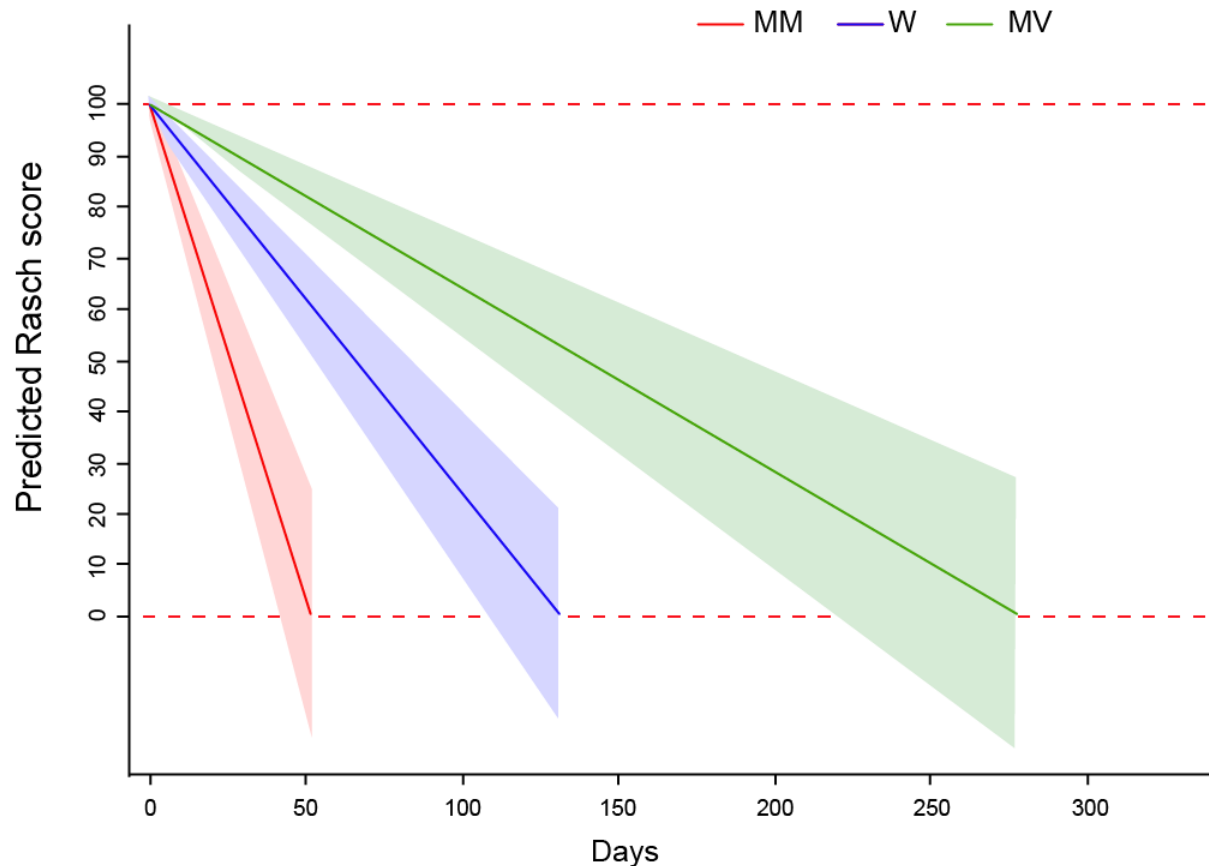
**Table 7 Model for MRC scale decline in trial-relevant population.** Model includes 824 measurements from 154 sCJD and fast IPD patients in stratum I with MRC Scale score  $\geq 5$  at enrolment. Reference category aged 65 years and was codon 129 MM. For the statistical analysis the 20-point MRC Scale was converted to the continuous Rasch score used in its development. This allowed for the more reliable imputation of rare missing questions in the MRC Scale.

Functional decline of patients with rapidly progressive CJD depended strongly on codon 129 genotype (interaction  $p < 0.0001$ ). Predicted declines from the best possible Rasch score of 100 (equivalent to 20/20 on the MRC Scale) are shown in Figures 17 and 18. Patients who were homozygous for methionine at codon 129 suffered with the fastest disease progression while the patients who were heterozygous at the polymorphic codon 129 had the slowest functional decline in this disease group.



**Figure 17.** Trellis plots illustrate model-predicted slopes of decline in function in individual patients matched to their MRC Scale observations (as converted to a Rasch score, see text). Black cross indicates proxy Rasch score of 0 at date of death. Different genotypes at polymorphic codon 129 are shown by line colour (red = MM, blue = VV, green = MV). This montage illustrates the 40 most rapidly progressive patients from a Cohort of 154. These were the most challenging from the point of view of model fitting.





**Figure 18 Predicted neurological declines from best possible score by codon 129 subtype.** The figure shows model-predicted mean declines (lines) and 95% confidence intervals (coloured bands) in Stratum 1 patients with three different codon 129 genotypes at *PRNP*. The most rapid decline was observed in patients with codon 129 MM polymorphism, followed by patient with codon 129 VV polymorphism. The slowest decline was observed in patients who were codon 129 MV heterozygous. The Rasch score approximates to 5x MRC Scale score.

Patients with sCJD/fast IPD and polymorphic codon 129 genotypes MM, VV and MV lost 10% of their function measured by the MRC Scale in 5.3 (4.2-6.9), 13.2 (10.9-16.6) and 27.8 (21.9-37.8) days. There was no evidence that codon 129 genotype significantly affected the MRC scale measurement at enrolment (in the final modelled sample,  $p=0.11$ ). However, in addition to these population-level differences in the mean declines, codon 129 genotype also significantly affected how variable individual

patients were around these means (differential random effects and residual errors,  $p=0.0001$ ). Although enrolment scores varied similarly between MM and non-MM genotype patients, subsequent declines varied significantly more in MM than non-MM, and residual errors (variability not explained by the underlying trajectories) was also significantly greater (Table 7).

Once the main model had been constructed and the potential effects of enrolment function, age, gender, disease category, and codon 129 had been considered, we further investigated whether the following additional investigation factors had important effects on baseline or decline in MRC scale:

1. clinical phenotype as assessed by the visiting physician (7 types, sCJD only; see Methods)
2. the presence or not of signal change in the basal ganglia, cortex or thalamus on MRI brain (see Methods for scoring)
3. the type of PrP<sup>Sc</sup> assessed by partial protease digestion and Western blotting (types 1-3 London classification)
4. the presence of periodic sharp wave complexes or background abnormalities on routine EEG recordings
5. the presence or not of 14-3-3 protein in routine CSF analysis, or the concentration of CSF S100b (Table 3)

Four of these factors affected decline in MRC scale when considered alone: clinical category ( $n=159$ ,  $p<0.0001$ ), strain type ( $n=53$ ,  $p=0.025$ ), presence of periodic sharp wave complexes ( $n=147$ ,  $p=0.009$ ) (as noted in the EEG reports from NHNN and local hospitals) and CSF S100b ( $n=124$ ,  $p=0.046$ ), but either these effects disappeared after adjusting for the impact of codon 129 subtype on decline (periodic sharp wave complexes, adjusted  $p=0.33$ ), or effects weakened with the effect of codon 129 subtype remaining similar to Table 7 and which were much stronger in magnitude. In the multivariate analysis, higher levels

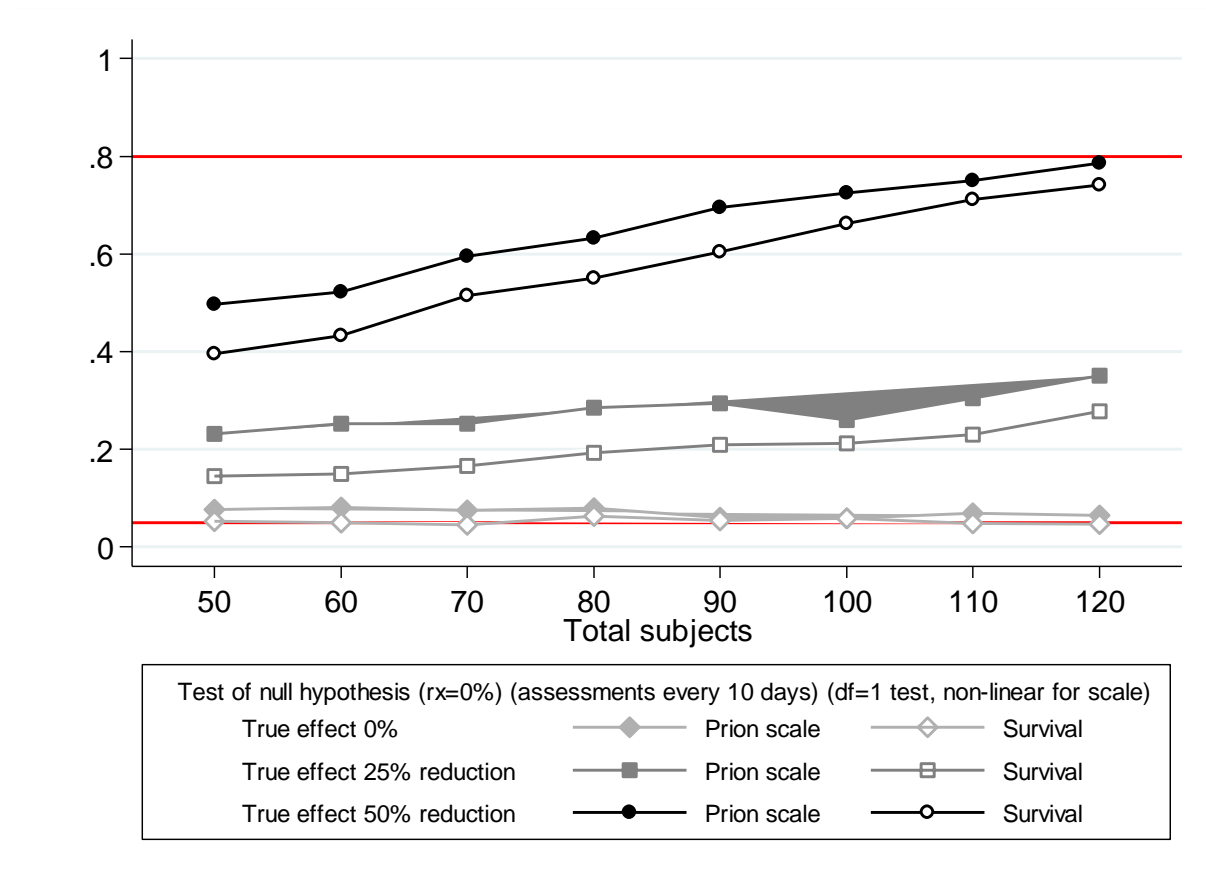
of CSF S100b, a quantitative measurement of a glial protein routinely used to assist diagnosis of CJD, were associated with more rapid decline ( $p=0.005$ ) although not at a statistical level beyond which might be expected as a result of performing tests of multiple hypotheses. Therefore the PrP gene sequence appears to be a profoundly important predictor of decline relative to other demographic or investigation factors. We note that a small proportion of rapidly progressive sCJD patients had a PrP<sup>Sc</sup> type at data freeze, which may have impacted the power to detect an effect of PrP<sup>Sc</sup> type beyond codon 129 genotype.

### ***Power calculations using the MRC Scale***

Furthermore, we used the observed enrolment frequency of codon 129 subtypes (33% MM, 43% MV, 24% VV), distribution of ages (mean 65.1 years, standard deviation 9.4), population and individual rates of decline as estimated in Table 7 to simulate cohorts of patients who might be enrolled into clinical trials of an investigational agent which reduced the rate of decline by (proportionately) 0%, 25% or 50%. We assumed a fixed percentage reduction in decline given the large absolute differences in decline observed (Table 7). We therefore estimated the impact of the intervention using a test for the percentage reduction (see supplementary materials for details). We compared the power of different sized trials to detect effects based on estimating the impact of an intervention on the MRC scale, and survival based on this.

First we considered a randomised trial which could be either placebo-controlled or open-label, although the possibility of reporting bias in an open-label trial would need to be carefully considered. Figure 19 shows the power of such a trial with total 50-120 fast-progressing patients (MM, MV and VV),

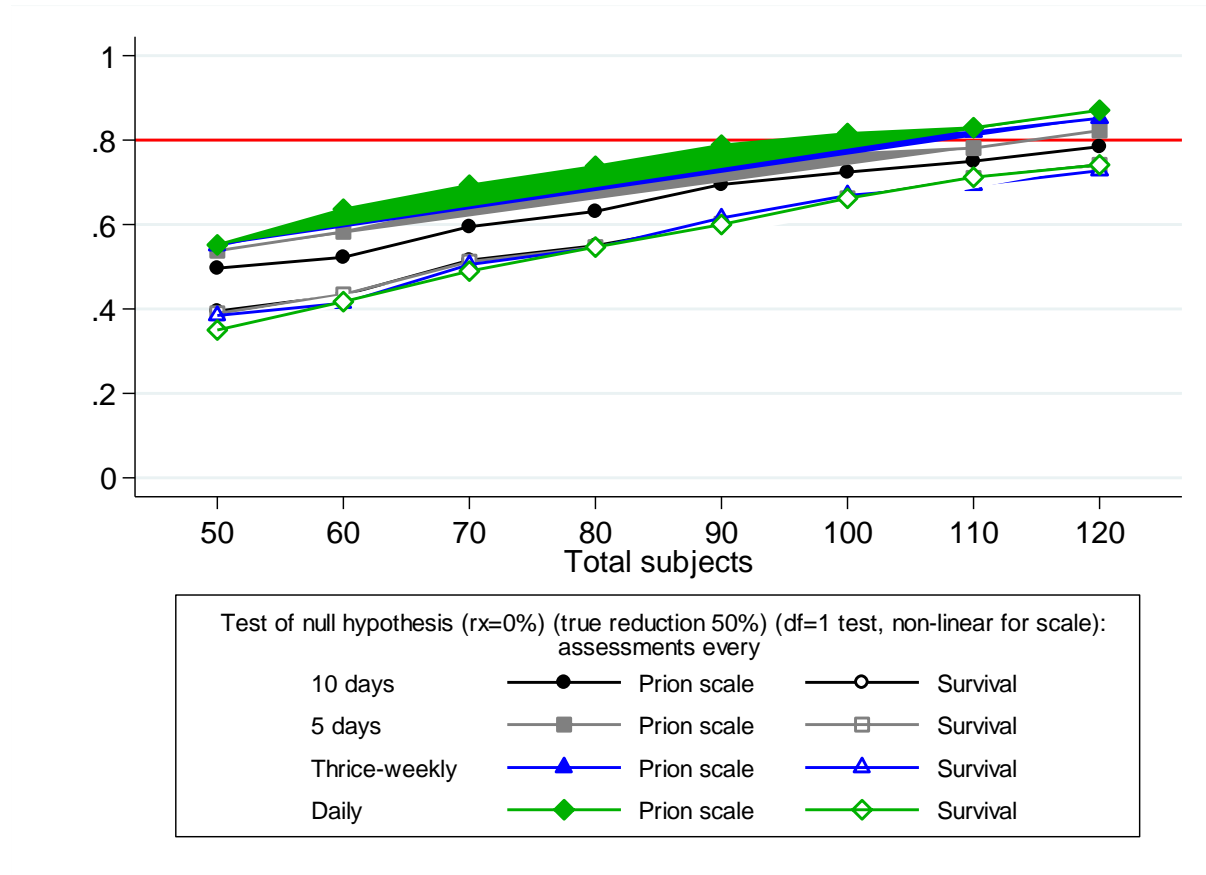
(randomised 1:1 control:intervention and assessed every 10 days, to detect a 25% or 50% reduction in decline in the MRC scale.



**Figure 19 Power for different sizes of randomised trial** Assessments every 10 days, recruiting from the fast progressing population (MM, MV and VV); varying intervention efficacy. When the true reduction is 0% (light gray line), 5% of tests would be expected to fall below a  $p < 0.05$  by chance (red line).

According to this analysis 120 patients would need to be randomised to provide 80% power to detect a 50% reduction in neurocognitive decline. The same number of patients provides 5-10% greater power from using the repeated MRC scale measurements than a survival endpoint on the same population.

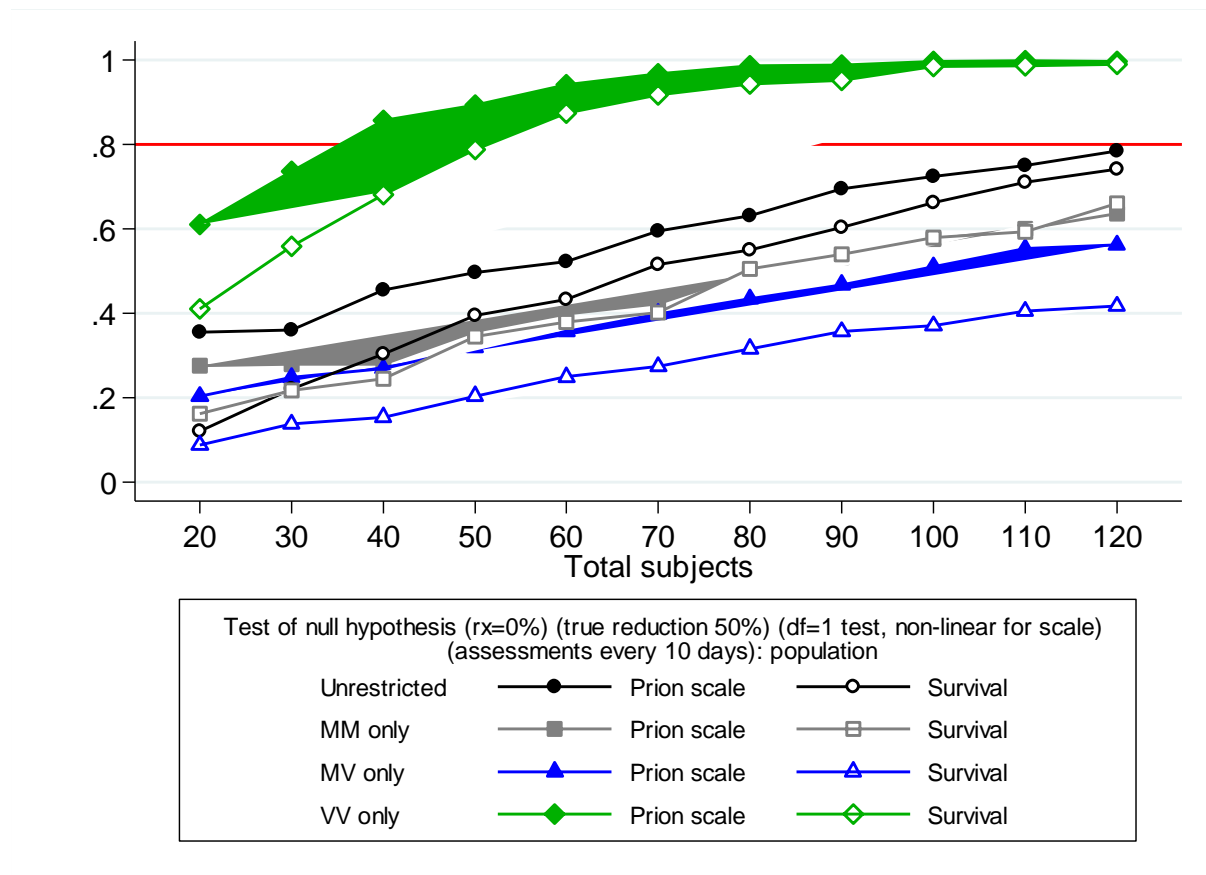
The MRC scale requires only a 2 minutes conversation to acquire information over the telephone and is highly acceptable to patients and their carers. We therefore considered the impact of more frequent measurements on power (Figure 20).



**Figure 20** Intervention has a 50% reduction in decline, recruiting from the fast progressing population (MM, MV and VV): varying number of assessments. The survival is estimated in the same manner regardless of the number of times that assessments are made; variation in the estimates of power for a survival endpoint therefore reflect only sampling variation.

The same power as achieved with 120 randomised patients measured every 10 days could be achieved by 110 patients every 5 days, 100 patients thrice weekly or 90 patients daily, as a consequence of

improved precision of estimation of rates of decline. Therefore power gains for daily measurement over a survival endpoint were 13-20% for the same number of patients. Lastly, given the substantially greater variability observed in rates of decline in the MM subgroup, we considered the impact on power of restricting a trial to different codon 129 subtypes (Figure 21).



**Figure 21** Intervention has a 50% reduction in decline, assessments every 10 days: varying recruitment from the entire fast progressing population or according to codon-129 subtype. Codon 129 VV patients showed less variation and therefore fewer patients were required to achieve the same study power compared to codon 129 MM and MV group. In this group only 40 patients were sufficient to provide 80% power to detect 50% decline in function.

Because the VV subgroup have less variability than the MM subgroup, but faster declines than the MV subgroup, only 40 randomised VV patients provided >80% power to detect 50% reductions in decline. At the observed population frequencies, however, 40 VV patients would be expected from a cohort of 167 patients in total, which would have provided greater power overall.

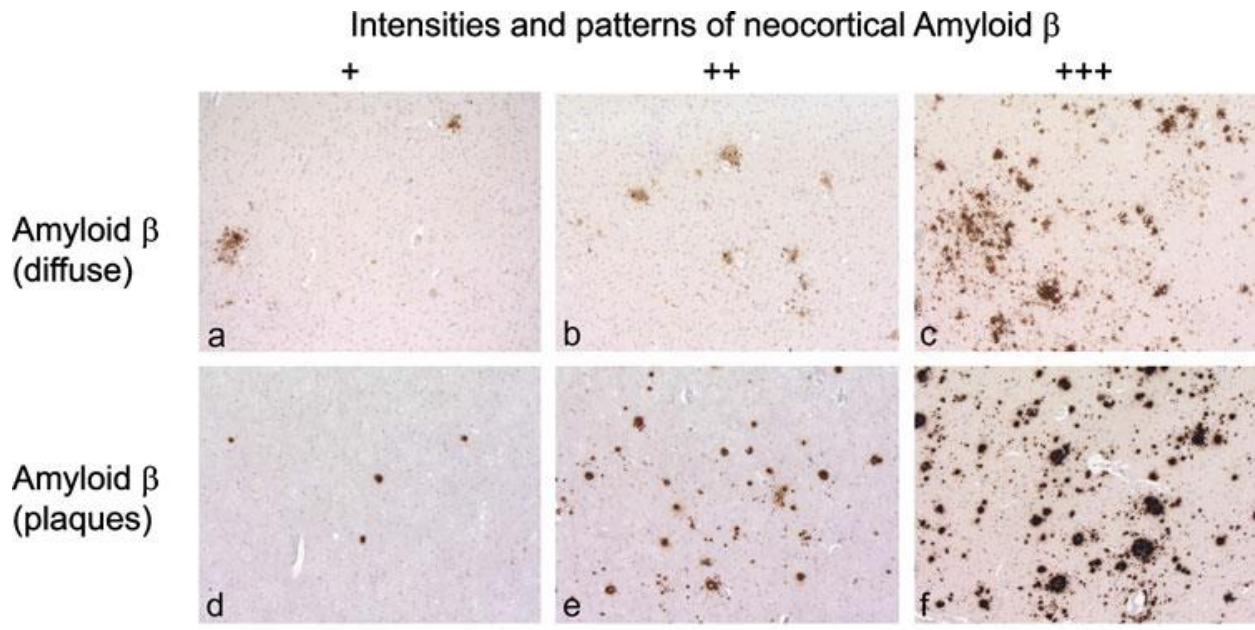
An alternative approach would be an open label study with comparisons made to the cohort natural history data presented here. Such non-randomised comparisons have well-recognised limitations, the most important being changes in external management. However, given the simulated randomised trial findings above, and the numbers already in the cohort, it is likely that similar power could be achieved with half the randomised patients.

### **3.3 Histopathological heterogeneity**

Spongiform change as already discussed in the introduction is typically seen in prion disease and this is shown in Figure 5, Introduction. Prion protein deposits are detected in synaptic, granular, plaque and peri-neuronal net patterns (Figure 5, Introduction). These deposits as already mentioned vary in intensity and may, in some cases, correspond biochemically to amyloid.

Furthermore, we looked at the presence of amyloid- $\beta$  in prion disease patients. Similar to Alzheimer's disease, deposition of extracellular amyloid was observed with formation of amyloid- $\beta$  plaques and other amyloid- $\beta$  aggregates (Figures 22a–f). Amyloid- $\beta$  accumulates diffusely (Figure 22a–c), or it can exhibit prominent and widespread deposition of dense amyloid plaques with a diameter of more than 200  $\mu\text{m}$  (Figure 22d–f), and similar to prion protein deposition elicits a considerable astrocyte and microglial reaction.

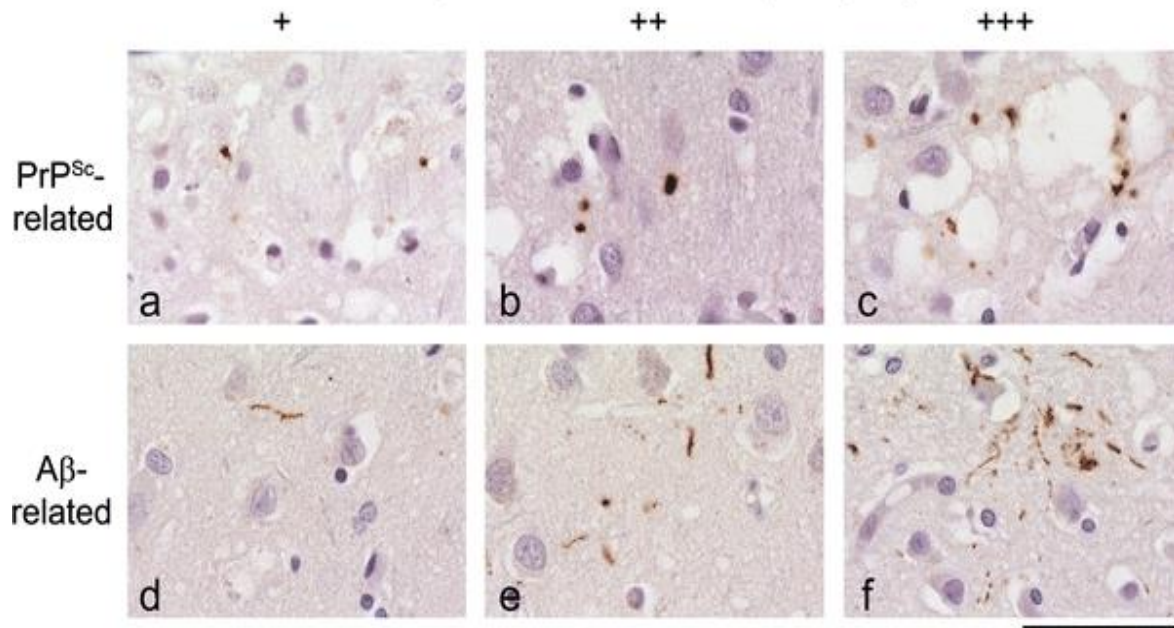




**Figure 22** Intensities of amyloid- $\beta$  deposits: a–c diffuse protein deposits without formation of plaques (ranging from mild to severe). a Score 1 (mild) describes occasional patchy deposits, occasionally seen on low power magnification. b Moderate, score 2 describes deposits that are seen in <50% of adjacent low power fields. c An example of heavier diffuse amyloid beta burden, indicating a presence in most of the low power field on a cortex section. Deposition and distribution of amyloid- $\beta$  were assessed using the CERAD (Consortium to Establish a Registry for Alzheimer’s Disease) criteria. CERAD was funded by the National Institute on Aging in 1986 to develop standardized, validated measures for the assessment of Alzheimer’s disease (AD) including clinical, neuropsychological, neuropathological and behavioral assessments of AD. Dense core amyloid beta plaques in the figure, approximately corresponding to CERAD sparse (d), intermediate (e) and high (f). Scale bar 300  $\mu$ m (a–f, g–i)

Tau accumulation accompanies most neurodegenerative processes and is often found in the vicinity of prion protein and amyloid- $\beta$  deposits. Figure 23 illustrates intensities and patterns of neocortical tau which accumulates in the vicinity of PrP<sup>Sc</sup> and amyloid- $\beta$  related deposits. Following detailed assessment of these proteins we concluded that tau pathology in areas of amyloid- $\beta$  accumulation is probably an independent (meaning distinct from the association of prion protein and tau deposition) process of tau phosphorylation that starts in the entorhinal cortex and spreads over the limbic system to extend into neocortical regions.

### Intensities and patterns of neocortical phosphorylated tau

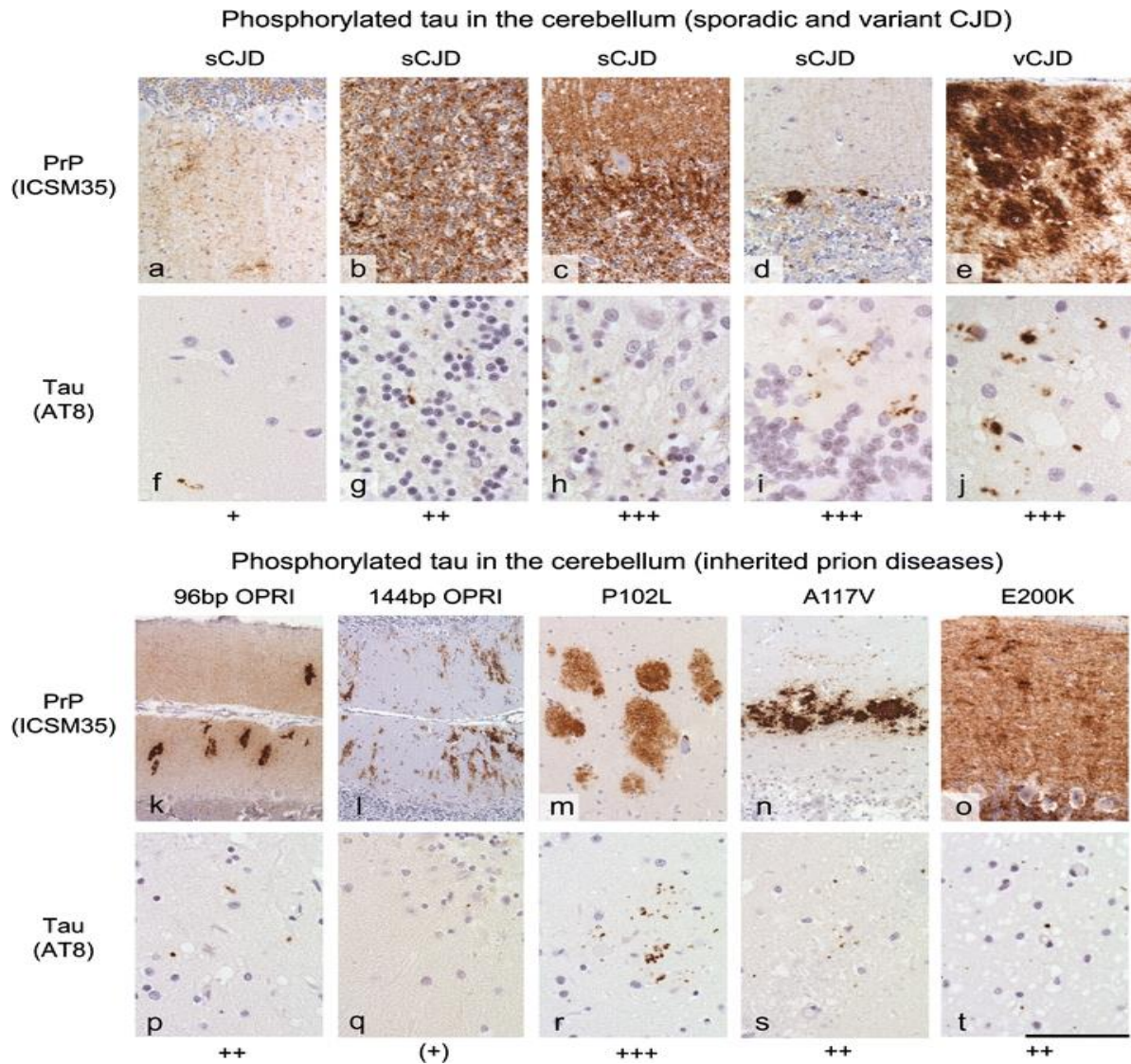


**Figure 23** Amyloid- $\beta$  or PrP induced pattern of tau phosphorylation differ: a–c shows the range of PrP induced tau deposits. The scores of tau correspond to the scores of PrP, i.e. tau score 1 is typically seen in areas with PrP score 1 and dense tau deposits (scores 2 or 3) are typically seen in areas with PrP scores 2–3 d–f. In contrast, amyloid- $\beta$  induces a threaded tau phosphorylation pattern, with elongated dystrophic neuritis but very little stub- or rod-shaped tau. All examples are from the frontal or parietal cortex. Scale bar 25  $\mu$ m (a–f).

The morphology of hyperphosphorylated tau associated with abnormal prion protein is distinct from that elicited by amyloid- $\beta$ , in particular in cases of synaptic PrP deposition (Figure 23). Prion protein related tau hyperphosphorylation appear as short stub- or rod-like structures (Figure 23a–c). The most subtle deposition forms small rod- or stub-like punctate inclusions (granules). Their shape resembles granules seen in argyrophilic grain disease (Ferrer et al., 2008). They do not extend to fibrillary or “neuritic” tau, whilst the most subtle deposition of amyloid- $\beta$  associated tau fibrils occurs in the form of thin, single neuritic threads in the cortex (Figure 23d). The next stage of amyloid- $\beta$  induced tau phosphorylation is a more frequent presence of fibrils (Figure 23e), and finally the amyloid- $\beta$  induced tau pathology amounts to a delicate network of dystrophic processes (Figure 23f), including neurofibrillary tangles. Amyloid plaques are generally surrounded by a small corona of dystrophic neurites, which are well known and have been frequently described in the literature.

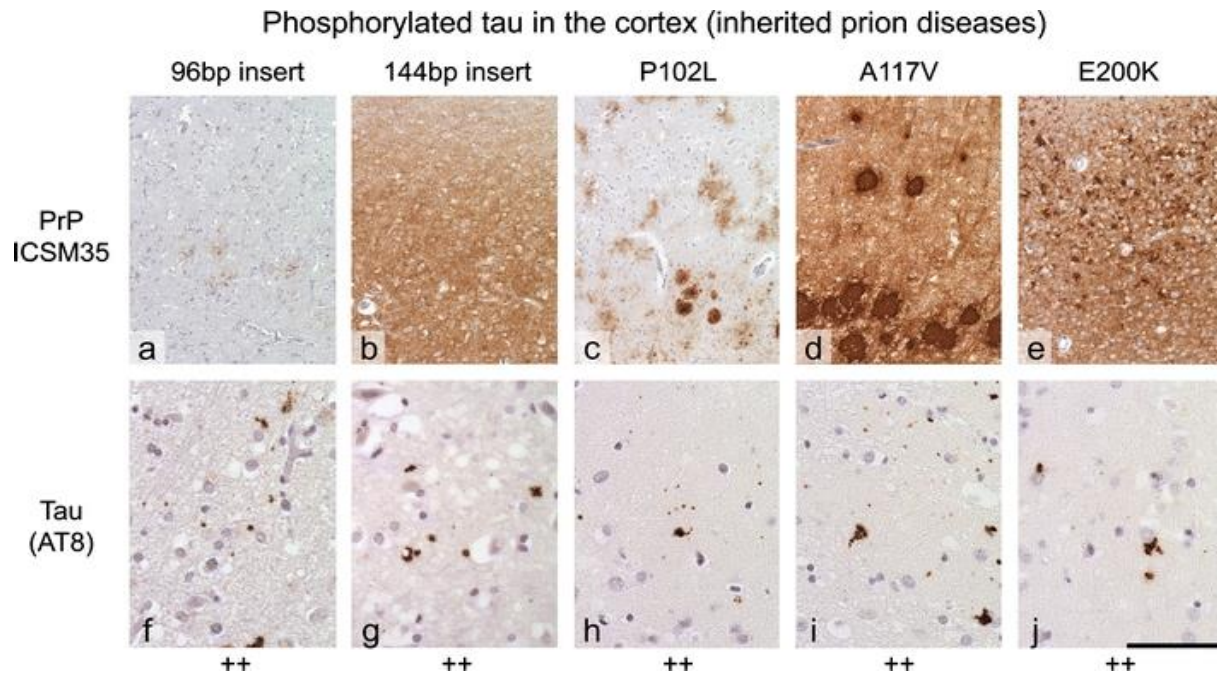
Accumulation of phosphorylated tau in the cerebellum has a characteristic appearance and usually corresponds to the severity of PrP deposits in the same regions. Figure 24 illustrates typical deposition of PrP and the corresponding phosphorylated tau in the cerebellum of sCJD, vCJD and inherited prion disease patients.

The formation of conspicuous and well-demarcated PrP plaques was typically seen in inherited forms with codon P102L (Figures 24m, 25c) and A117V mutations (Figures 24n, 25d), whilst D178N or E200K (Figures 24o, 25e) mutations showed less well-defined plaque pathology. Other mutations, such as octapeptide repeat insert (OPRI) mutations presented histologically with a unique striping pattern of the cerebellum (Figure 24k, l).



**Figure 24** Prion protein-triggered tau phosphorylation in the cerebellum: upper row shows deposition of abnormal PrP in sCJD with synaptic PrP deposition (a–c) and small plaques (d). vCJD cases showed heavy PrP burden with diffuse deposits as well as plaques (e). The corresponding tau phosphorylation is shown below (f–j). It is approximately proportional to the PrP<sup>Sc</sup> burden and is closely associated with plaques. An exceptionally strong tau deposit was consistently seen in vCJD cerebella (j). The pattern of abnormal PrP deposition is distinct for each mutation. 96 and 144 bp OPRI form a characteristic striping pattern perpendicularly to the cerebellar surface (k, l) with moderate tau labeling in 96 bp OPRI and very little tau in the 144 bp OPRI. The extent of tau phosphorylation appears to be independent of the plaque load, with P102L and A117V cases showing similar tau burden as in E200K which features synaptic PrP deposition. Scale bar 120  $\mu$ m (a–e, n, o); 250  $\mu$ m (k, l, m) and 40  $\mu$ m (f–j, p–t).

It has been observed that plaque-forming diseases are capable of generating phospho-tau deposits, but disease forms with synaptic PrP may be incapable to do so (Sikorska et al., 2009). To examine this further, we investigated patients with sCJD and compared them to cases with inherited prion disease and vCJD cases. Presence of hyperphosphorylated tau in sCJD with synaptic (Figures 24i, j) or pericellular PrP, in the absence of plaques (Figures 24a, b, e, f) has been demonstrated for the first time in this study. We detected a substantial formation of phospho-tau positive rods in vCJD (Figure 24j). In cases with concomitant amyloid- $\beta$  pathology, we found both patterns, namely granular, rod-shaped PrP-induced tau inclusions (Figure 23a–c) and thread-shaped amyloid- $\beta$  related formations (Figure 23d–f). Cases of inherited prion disease (P102L, A117V, D178N, E200K, 96 and 144 bp octarepeat insert) showed accumulation of tau directly associated with prion protein plaques (Figures 24p–t, 25f–j).



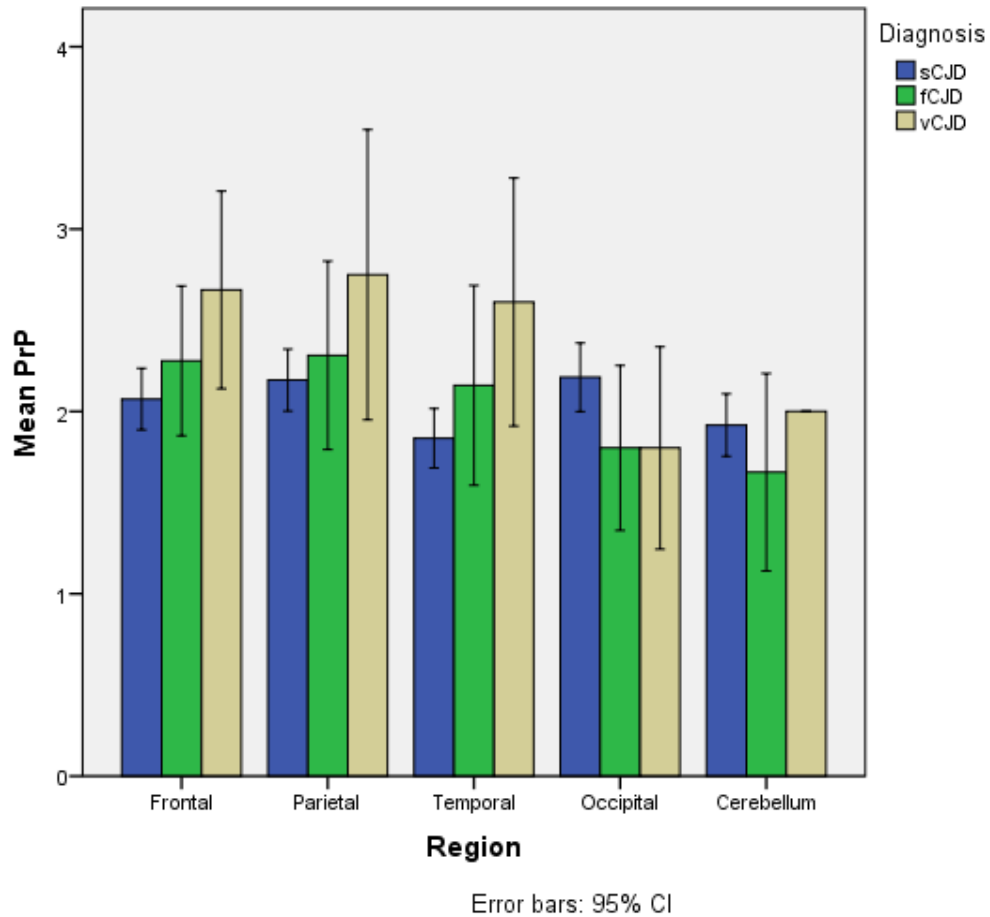
**Figure 25** PrP-induced tau phosphorylation in cortex of inherited prion diseases: upper row deposition of abnormal PrP in the frontal cortex; there is considerable variability of the intensity of PrP<sup>Sc</sup> burden and some forms are characterized by distinct patterns of plaque formation, as described before. The lower row shows tau deposits corresponding to the area depicted above. (a), 96 bp OPRI mutation with almost undetectable PrP<sup>Sc</sup> load, resulting in tau phosphorylation similar to the 144 bp OPRI (f, g), despite its significantly higher PrP<sup>Sc</sup> load (b). c This case with a P102L mutation shows predominantly diffuse deposits and only very few plaques, as compared to the very heavily plaque-forming A117V case (d), both showing similar tau hyperphosphorylation (h, i). Another case with no plaque formation, E200K (e), shows a tau load similar to all other cases (j). Scale bar a–e 240  $\mu$ m, f–j 60  $\mu$ m.

Figure 12 in Methods shows the heat map used for quantitative analysis of tau phosphorylation and PrP deposits. The data were obtained by counting tau positive inclusions in the high power field and subsequently comparing these with the corresponding PrP deposits.

Prof Brandner’s team performed post-mortem examinations and sample staining of brain tissues from 135 patients with sporadic CJD, variant CJD and acquired CJD. Prion protein, tau and amyloid- $\beta$  scores were obtained using the scoring scheme already described. We then graded the slides as no deposition, mild, moderate and severe from 0-3 (as described in Methods). In case of multiple concurrent PrP patterns, the highest individual score was used (e.g. granular score 2 and synaptic score 1 was used to obtain the final score (2)). No cases with cerebellar amyloid- $\beta$  were included in the study. In case of tau deposition the scoring ranged from 0-4 as discussed in the Methods. Genotype data at codon 129, disease duration and age at onset were compared with protein deposition and distribution.

Deposition and distribution of prion protein in different brain regions is illustrated in the bar chart below (Figure 26). There was no statistically significant difference in prion protein deposition between each of the disease forms, sCJD, fCJD and vCJD for each of the examined brain regions using the Kruskal-Wallis test as demonstrated below.

<b>Region</b>	<b>Frontal</b>	<b>Parietal</b>	<b>Temporal</b>	<b>Occipital</b>	<b>Cerebellum</b>
<b>p</b>	<b>0,104</b>	<b>0,213</b>	<b>0,056</b>	<b>0,158</b>	<b>0,646</b>

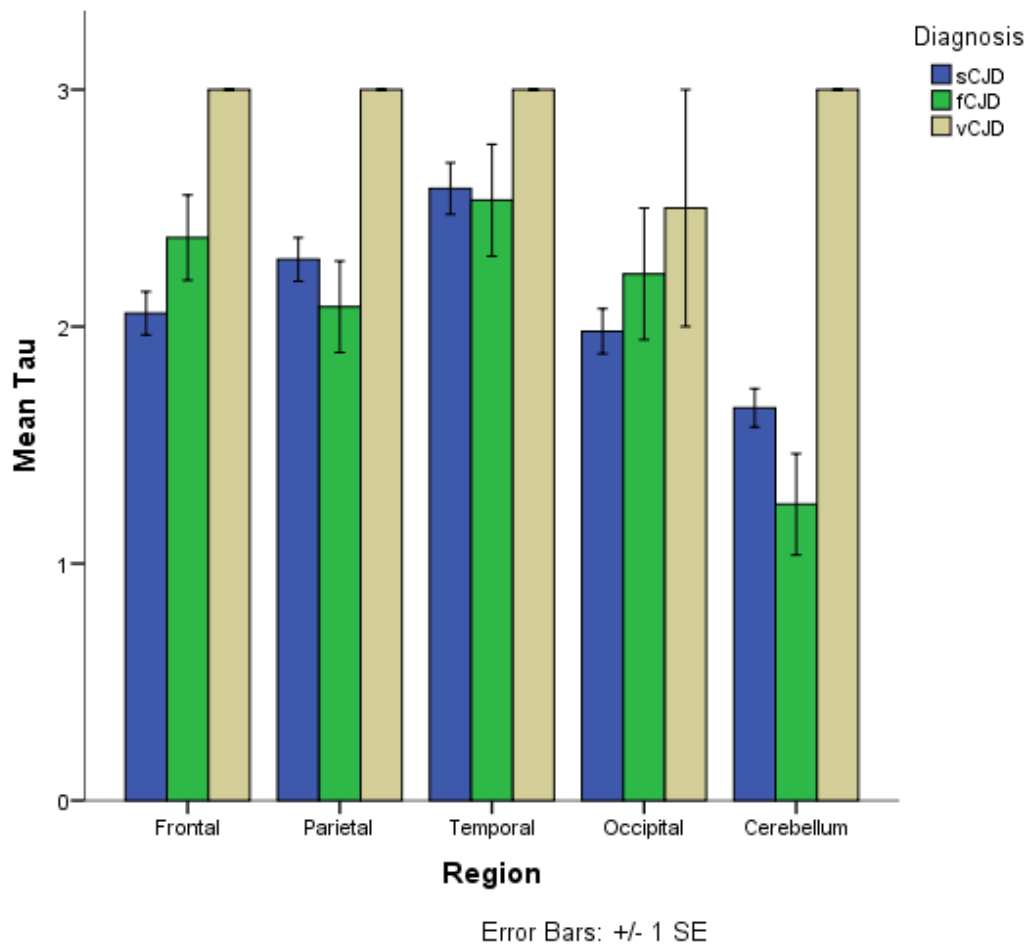


**Figure 26** shows prion protein deposition in different brain regions for each of the disease categories, sCJD, fCJD and vCJD (total of 135 cases). The colour code is used to differentiate between different disease groups. The mean PrP value was derived from the different scores ranging from 0-3 to account for no deposition, mild, moderate and severe protein accumulation. (**Mean PrP** = mean score value when all scores were added up and divided by a total number of 135 cases excluding the missing data; **Region** = brain region examined to include frontal, parietal, temporal, occipital and cerebellum).



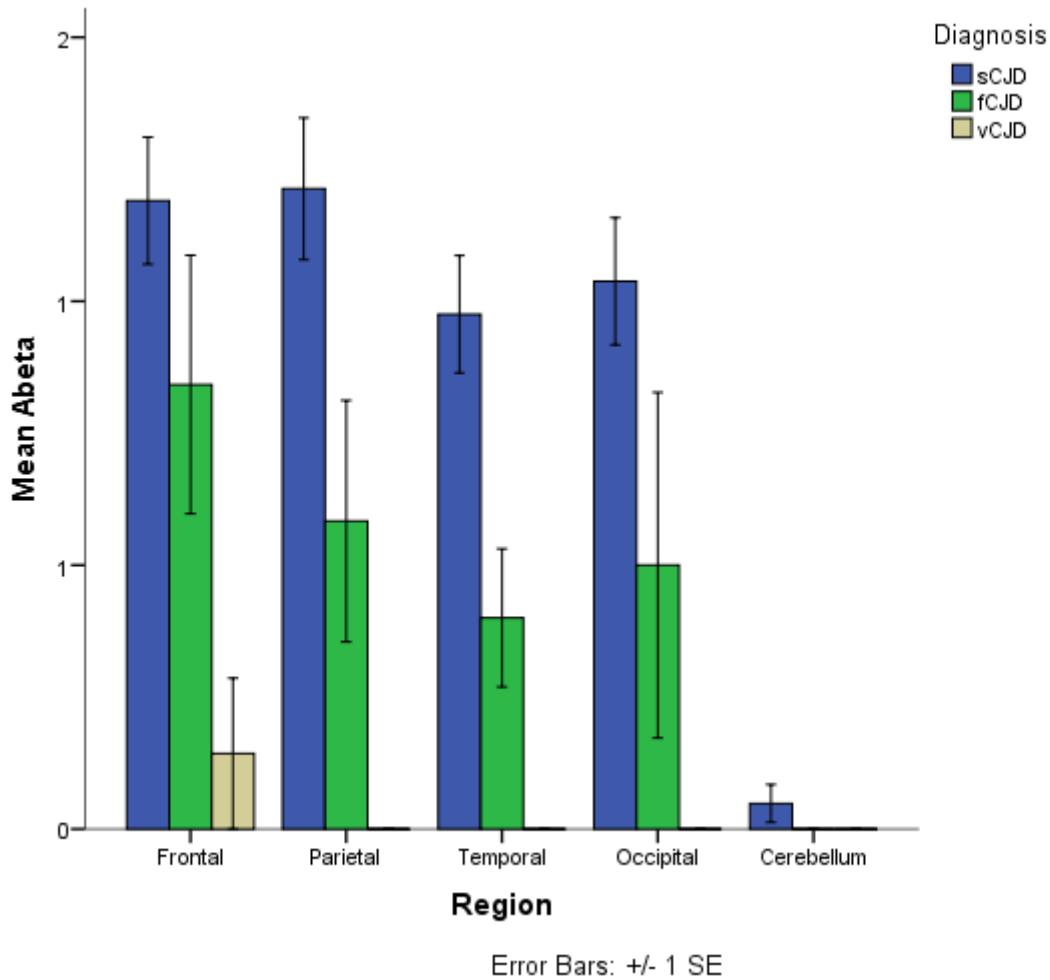
Similarly, we investigated tau protein deposition in different brain regions. This is illustrated in Figure 27.

There was no obvious difference in tau protein accumulation between different disease groups, sCJD, fCJD and vCJD using Kruskal-Wallis test.



**Figure 27** shows the mean value for tau protein value (ranging from 0-4) in each of the examined brain regions in prion disease patients. The colour code is used to differentiate between different disease groups, vCJD, fCJD and sCJD in a total of 135 CJD patients. The colour code is used to differentiate between different disease groups. The mean tau value was derived from the different scores ranging from 0-4 to account for no deposition, mild, moderate, severe and extremely high protein accumulation. (**Mean PrP** = mean score value when all scores were added up and divided by a total number of 135 cases excluding the missing data; **Region** = brain region examined to include frontal, parietal, temporal, occipital and cerebellum).

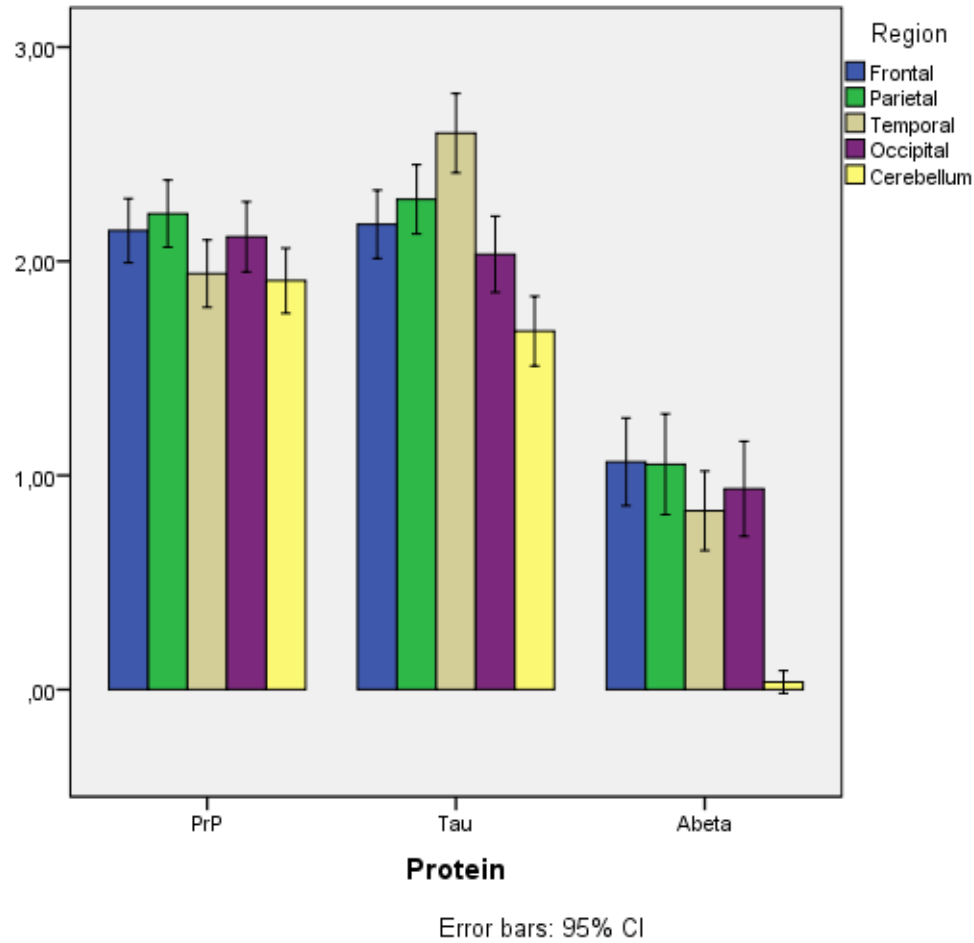
Furthermore, we investigated amyloid- $\beta$  deposition in different brain regions for each of the discussed disease categories. This is illustrated in Figure 28. Amyloid- $\beta$  deposition was lower in patients with vCJD which may be because these patients were much younger. Amyloid- $\beta$  deposition in patients with fCJD was lower than in patients with sCJD. The reason for this may be the younger age of fCJD patients but also the interaction between amyloid- $\beta$  and different prion strains. It may be that sCJD PrP<sup>Sc</sup> interaction with amyloid- $\beta$  is favored compared to the prion strain in fCJD patients. This may also account for the longer disease duration in inherited prion disease patients. There was an obviously lower deposition of amyloid- $\beta$  in cerebellum in all disease groups.



**Figure 28** shows the mean amyloid- $\beta$  value (ranging from 0-3) for each of the examined brain regions in patients with vCJD, fCJD and sCJD totalling 135 CJD cases. The mean amyloid- $\beta$  was derived when each of the scores (0-3) were added and divided by the number of samples tested. The figure shows a lower amyloid- $\beta$  deposition in patients with fCJD compared to sCJD. There was also an obviously lower deposition of amyloid- $\beta$  in the cerebellum in patients with all three disease forms. (**Mean PrP** = mean score value when all scores were added up and divided by a total number of 135 cases excluding the missing data; **Region** = brain region examined to include frontal, parietal, temporal, occipital and cerebellum).

Further analysis was performed on sCJD patients only. This subgroup included 65 patients with the diagnosis of definite sCJD. Deposition of each of the three proteins was assessed in 5 different brain regions: frontal, parietal, temporal, occipital and cerebellum.

Figure 29 shows the mean value for PrP, amyloid- $\beta$  and tau protein in the five examined regions. The Kruskal-Wallis test was used to compare the protein deposition between different brain regions. There was no statistically significant difference in the deposition of PrP and tau protein in different regions of the brain. However, there was significantly less deposition of amyloid- $\beta$  in all regions when compared with the other 2 proteins. The difference was particularly striking in the cerebellum which contains significant deposition of tau and PrP protein with complete absence of amyloid- $\beta$ .



**Figure 29** shows the mean score value for each of the three proteins: PrP, tau and amyloid- $\beta$  protein in 5 examined brain regions of 65 definite sCJD patients. The mean values on the y axis are derived from semi-quantitative analysis of protein deposition labeled from 0-3 referring to no deposition, mild, moderate and severe protein accumulation. In case of unusually high tau deposition, observed in a few cases, a score of 4 was used. The lower deposition of amyloid- $\beta$  is particularly striking in the cerebellum. This is not surprising because there is also very little PrP and hyperphosphorylated tau in this brain region. (**Mean PrP** = mean score value when all scores were added up and divided by a total number of 135 cases excluding the missing data; **Region** = brain region examined to include frontal, parietal, temporal, occipital and cerebellum).

Further analysis involved examination of each of the proteins in different brain regions and comparing them with each other. This analysis was performed on 65 sCJD patients only. Mann-Whitney test was used to assess the correlation of prion protein deposition between different brain regions. Prion protein deposition was statistically significant when parietal lobe was compared to cerebellum (Table 8).

Prion protein	Parietal	Temporal	Occipital	Cerebellum
Frontal	0.497	0.068	0.781	0.031
Parietal	-	0.014	0.352	0.005
Temporal	-	-	0.135	0.792
Occipital	-	-	-	0.070

**Table 8** shows the Mann-Whitney test used to assess the correlation of prion protein deposition between different brain regions ( $p < 0.005$  would be a suitable statistical threshold for significance that takes into consideration the multiple hypotheses tested here). Prion protein deposition was statistically significant when the parietal region was compared with the cerebellum.

Further analysis was performed tau deposition in different brain regions. Mann-Whitney test was used to assess the correlation of tau protein deposition between the examined brain areas. This showed that the lower deposition in the cerebellum is statistically significant when compared with the other brain regions. Similarly, when frontal and parietal regions were compared to the temporal and when parietal and temporal brain regions were compared to the occipital lobe the difference was statistically significant (Table 9).

Tau protein	Parietal	Temporal	Occipital	Cerebellum
Frontal	0.378	0.002	0.219	0.000
Parietal	-	0.022	0.044	0.000
Temporal	-	-	0.000	0.000
Occipital	-	-	-	0.005

**Table 9** shows the results of the Mann-Whitney test used to assess the correlation of tau protein deposition between different brain regions ( $p < 0.005$ ). The difference is statistically significant when the frontal region was compared to the temporal, when the temporal brain region was compared to the occipital and when all brain regions were compared with cerebellum.

Similar analysis was performed to assess the deposition of amyloid- $\beta$  in different areas of the brain.

Table 10 shows the Mann-Whitney test used to compare the deposition of amyloid- $\beta$  between different brain regions. The lower deposition of amyloid- $\beta$  protein in cerebellum is statistically significant when compared with the other brain regions.

A- $\beta$ protein	Parietal	Temporal	Occipital	Cerebellum
Frontal	0.958	0.150	0.658	0.000
Parietal	-	0.162	0.650	0.000
Temporal	-	-	0.362	0.000
Occipital	-	-	-	0.000

**Table 10** The Mann-Whitney test was used to assess the correlation of amyloid- $\beta$  protein deposition between different brain regions. The lower deposition of amyloid- $\beta$  in cerebellum compared to frontal, parietal, temporal and occipital region was statistically significant.



We also analyzed the correlation between different proteins and disease duration in sCJD. The correlation between disease duration and deposition of each of the three proteins, prion protein, amyloid- $\beta$  and tau protein is shown in tables 11-14. The Pearson correlation test between the mean prion protein deposition and disease duration showed that there was no statistically significant association.

		<b>PrP (mean)</b>	<b>Months</b>
	Pearson correlation	1	0.204
<b>PrP (mean)</b>	Sig (2-tailed)	-	0.058
	N	102	87
	Pearson correlation	0.204	1
<b>Months</b>	Sig (2-tailed)	0.058	-
	N	87	114

**Table 11** shows the Pearson correlation for PrP deposition and disease duration in prion disease. The association between mean prion protein deposition and disease duration was not statistically significant in patients with sCJD.

Furthermore, the Pearson correlation test showed no statistically significant association between amyloid- $\beta$  accumulation and disease duration in prion disease patients (Table 12).

		<b>Amyloid-<math>\beta</math> (mean)</b>	<b>Months</b>
	Pearson correlation	1	-0.143
<b>A-<math>\beta</math> (mean)</b>	Sig (2-tailed)	-	0.157
	N	116	99
	Pearson correlation	-0.143	1
<b>Months</b>	Sig (2-tailed)	0.157	-
	N	99	114

**Table 12** shows Pearson correlation for amyloid- $\beta$  deposition and disease duration in prion disease. The association between mean amyloid-  $\beta$  deposition and disease duration was not statistically significant in sCJD patients.

Interestingly, the Pearson correlation test showed a statistically significant association between tau protein accumulation and disease duration in sporadic CJD patients (Table 13).

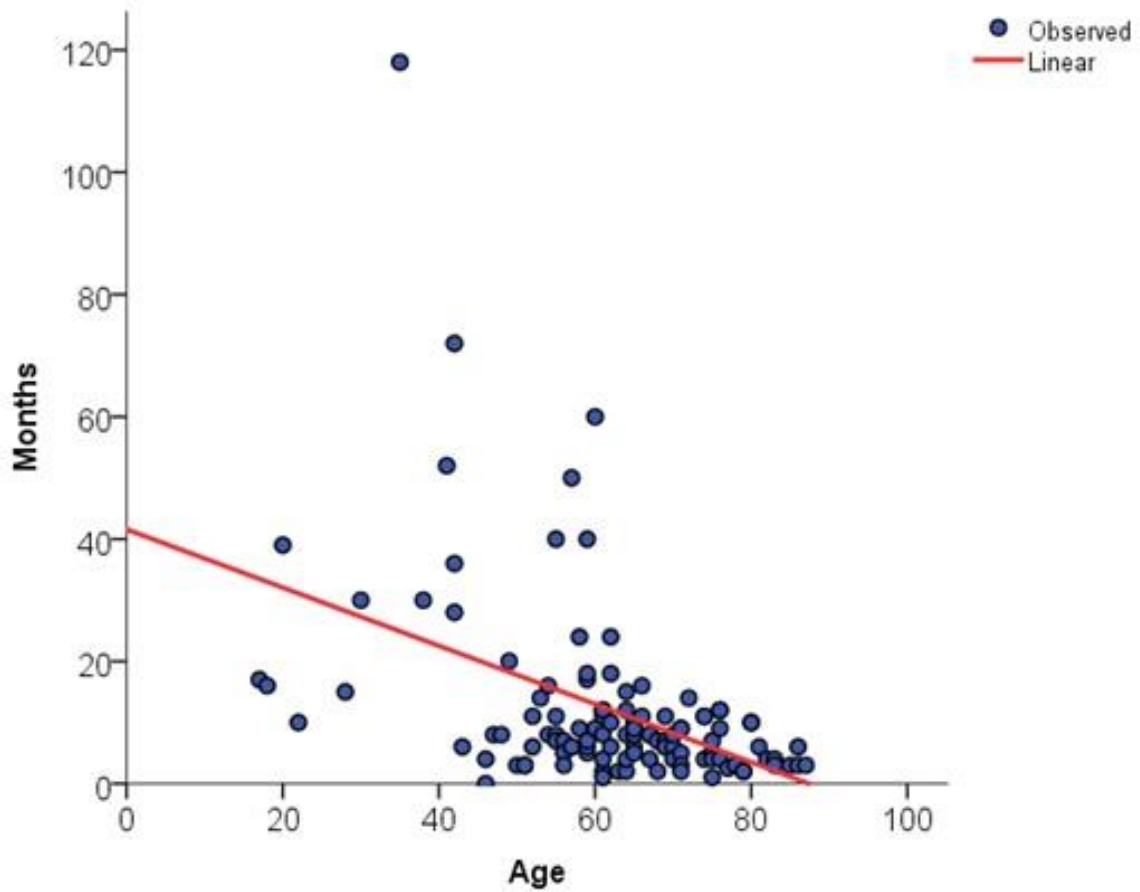
		<b>Tau (mean)</b>	<b>Months</b>
	Pearson correlation	1	0.293
<b>Tau (mean)</b>	Sig (2-tailed)	-	<b>0.007</b>
	N	98	83
	Pearson correlation	0.293	1
<b>Months</b>	Sig (2-tailed)	<b>0.007</b>	-
	N	83	114

**Table 13** Pearson’s correlation for tau protein deposition and disease duration in sCJD patients. The correlation of mean tau protein deposition and disease duration is positive and the relationship is statistically significant.

### **3.4 Candidate genes and neuropathological analysis**

Several genetic loci are known to play a role in neurodegenerative disorders likely to share common pathophysiological mechanisms. It is already known that *APOE*  $\epsilon 4$  allele (Borgaonkar et al., 1993), homozygosity at polymorphic codon 129 of the *PRNP* gene (Palmer et al., 1991) and *MAPT* haplotype (Jun et al., 2015) are genetic risk factors for Alzheimer's disease (AD), prion disease and Parkinson's disease (PD) respectively. We studied 7 candidate genes known to be associated with these diseases: *PRNP* Codon 129 (Mead et al., 2009a;Palmer et al., 1991), *PRNP* 1368-B haplotype (Mead et al., 2001), *MAPT* (Tobin et al., 2008), *APOE*(rs2075650) (Borgaonkar et al., 1993), *CLU* (rs11136000),(Harold et al., 2009;Lambert et al., 2009), *HECTD2* (rs12249854)(Lloyd et al., 2009) , *SPRN* (Beck et al., 2008).

We looked at the influence of 7 candidate genes on the age of disease onset, duration and total accumulation of prion protein, amyloid- $\beta$  and tau protein in 65 patients suffering from sCJD. The correlation of age of disease onset with duration was negative and significant at the level 0.01 which is consistent with the published literature (Appleby et al., 2009;Mead et al., 2011a;Thompson et al., 2013). This is illustrated in the graph and table below (Figure 30 and Table 13).



**Figure 30** illustrates the correlation between disease duration and age at disease onset in 65 patients with sCJD. The figure shows that the correlation of age of disease onset with duration was negative and significant at the level 0.01. This observation is consistent with the published literature.

Further analysis was performed using Kolmogorov-Smirnov, Shapiro-Wilk, Mann-Whitney and Wilcoxon tests. This analysis was performed in order to ascertain the influence of each of the candidate gene polymorphisms on clinical traits such as disease duration, age at onset, prion protein, tau and amyloid- $\beta$  accumulation. Assessment of the influence of *PRNP* polymorphisms on disease duration showed a statistically significant correlation of codon 129MM with shorter disease duration as illustrated in Table 14 and Figure 31.

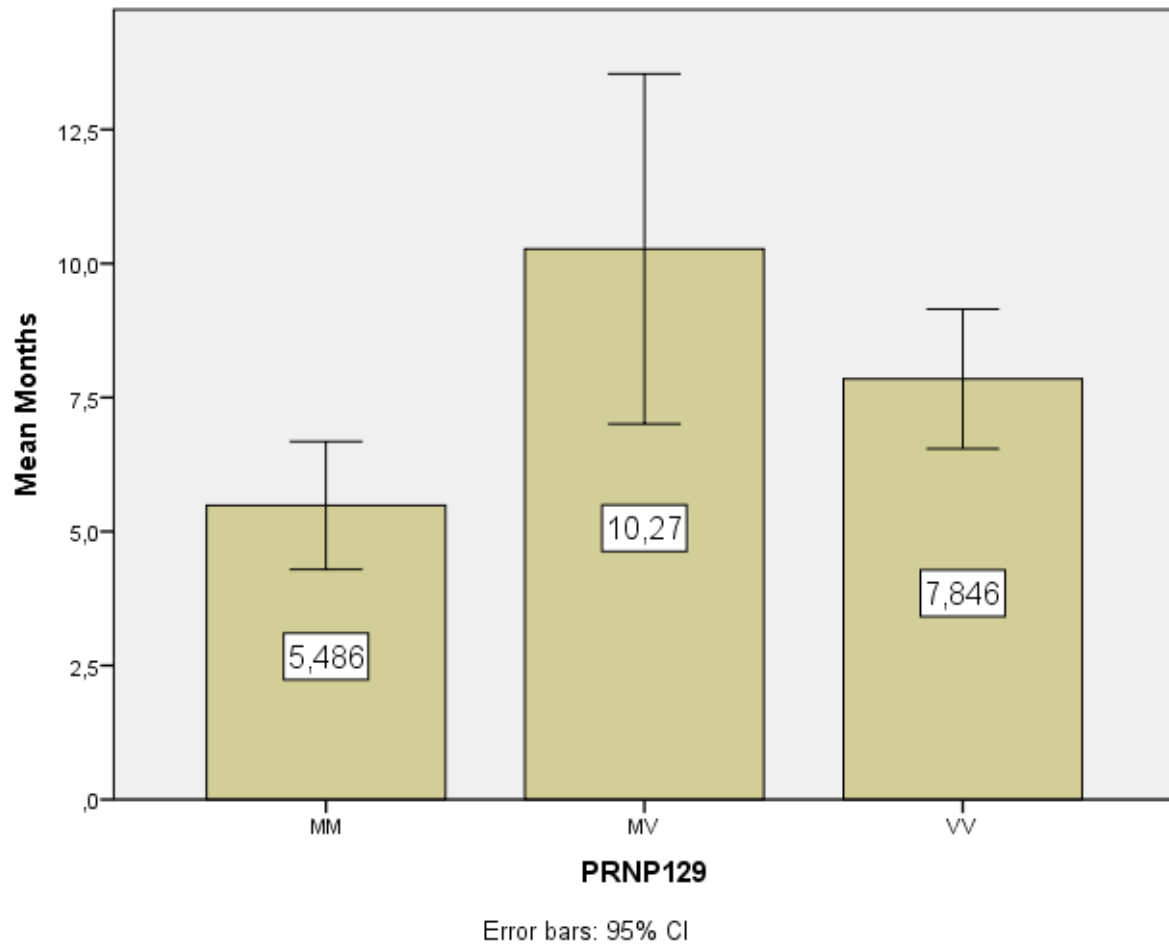
#### Tests of Normality

	PRNP 129	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	Sig	Statistic	df	Sig
<b>Months</b>	MM	0.209	35	0.000	0.870	35	0.001
	MV	0.113	11	0.200	0.967	11	0.854
	VV	0.226	13	0.068	0.908	13	0.174

**Table 14** shows the p values from a statistical analysis of polymorphisms at codon 129 versus disease duration in 65 sCJD cases. The tests of normality were performed using the Kolmogorov-Smirnov and Shapiro-Wilk statistical tests. The data do not show normal distribution.

This analysis is further illustrated in the bar chart below. The shorter disease duration is associated with methionine homozygosity (MM) at the polymorphic codon 129 (Figure 31).

### Disease duration vs codon 129 polymorphisms

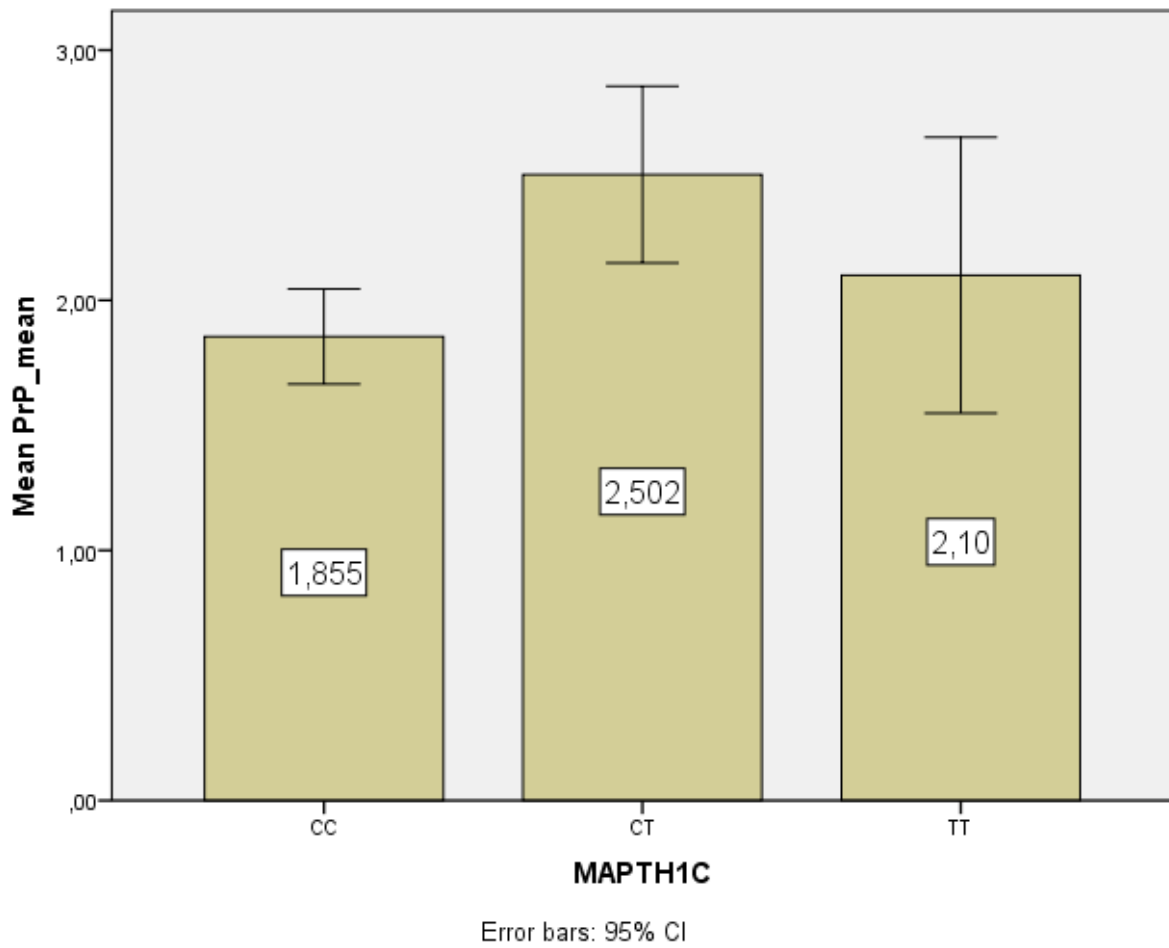


**Figure 31** shows the association of mean disease duration (months) in 65 cases of sCJD patients with different polymorphisms at codon 129 of the PRNP gene. Methionine homozygosity (MM) at the polymorphic codon 129 is associated with shorter disease duration in sCJD. Methionine valine heterozygosity is associated with the longest disease duration.

Apart from *PRNP* codon 129, the other six gene polymorphisms were not significantly associated with disease duration in CJD. None of the tested candidate genes influenced age of disease onset (data not shown). Finally, we tested the influence of the 7 candidate gene polymorphisms on the accumulation of proteins relevant to the process of neurodegeneration: prion protein, amyloid- $\beta$  and tau protein. Genotyping was performed using real time PCR and gene sequencing. The *PRNP* 129, *PRNP* 1368 B haplotype, clusterin, *HECTD2* and *SPRN* genes were not significantly associated with the total quantity of the accumulated prion protein, amyloid- $\beta$  or tau protein (data not shown). However, *MAPT* haplotype and *APOE* gene were associated with increased accumulation of some of the proteins. Prion and tau protein deposition were significantly influenced by *MAPTH1C* haplotype (Figure 32 and Table 15). The amount of accumulated amyloid- $\beta$  was influenced by *APOE* haplotype  $\epsilon$ 4 (Figure 33, Table 16) as expected but not by PrP and tau genes.



### Prion protein deposition vs MAPH1c haplotype



**Figure 32** shows the mean value of the accumulated prion protein in 65 cases of sCJD patients (y axis) vs different MAPT polymorphisms (x axis). The mean PrP value was obtained when scores 0-3 were added up and the sum divided by the number of samples scored. The genotyping for MAPT haplotype 1c was performed using real time PCR. MAPH genotype CT is associated with the highest prion protein deposition.

Multiple comparison analysis was performed using Tukey HSD test. This analysis involved multiple comparisons of each of the detected MAPT genotypes with the mean value for each of the three proteins PrP, tau and amyloid- $\beta$ . A statistically significant association was detected between MAPT polymorphisms and PrP and tau protein deposits as shown in Table 15.

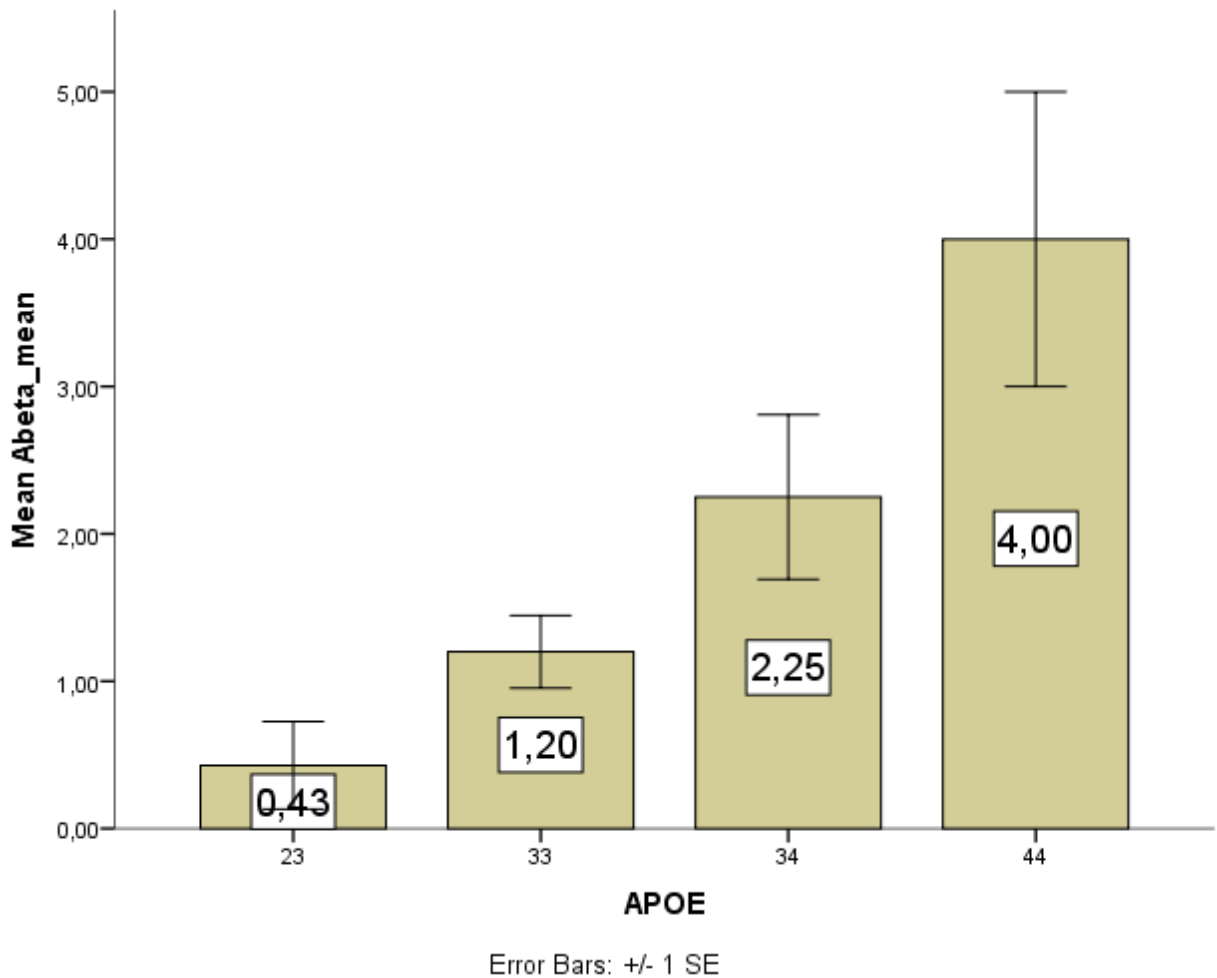
**Multiple Comparisons**  
**Tukey HSD**

Dependent Variable	(I) MAPTH1C	(J) MAPTH1C	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PrP_mean	CC	CT	-,64722*	,17375	<b>.002</b>	-1,0730	-,2215
		TT	-,24500	,24007	,569	-,8333	,3433
	CT	CC	,64722*	,17375	<b>.002</b>	,2215	1,0730
		TT	,40222	,26712	,301	-,2523	1,0568
	TT	CC	,24500	,24007	,569	-,3433	,8333
		CT	-,40222	,26712	,301	-1,0568	,2523
Tau_mean	CC	CT	-,14286	,14794	,604	-,5076	,2219
		TT	-,66667*	,21038	<b>.010</b>	-1,1853	-,1480
	CT	CC	,14286	,14794	,604	-,2219	,5076
		TT	-,52381	,23650	,085	-1,1069	,0592
	TT	CC	,66667*	,21038	<b>.010</b>	,1480	1,1853
		CT	,52381	,23650	,085	-,0592	1,1069
Abeta_mean	CC	CT	,41935	,50817	,690	-,8132	1,6519
		TT	-,83065	,79409	,552	-2,7567	1,0954
	CT	CC	-,41935	,50817	,690	-1,6519	,8132
		TT	-1,25000	,86295	,325	-3,3431	,8431
	TT	CC	,83065	,79409	,552	-1,0954	2,7567
		CT	1,25000	,86295	,325	-,8431	3,3431

\*. The mean difference is significant at the 0.05 level.

**Table 15** shows the association of scores of PrP, amyloid- $\beta$  and tau protein deposition with MAPT polymorphisms following multiple comparisons using the Tukey HSD test. The association for both accumulated PrP and tau protein with MAPT gene is statistically significant at the 0.05 level, although in consideration of multiple testing the finding needs to be replicated.

APOE ε4 is known to be associated with increased risk for development of Alzheimer’s disease. In our study increased deposition of amyloid-β protein in prion disease patients was associated with APOE ε4 genotype and this association was statistically significant as shown in Figure 33 and Table 16. This finding is interesting as it points to a possible interaction between PrP and amyloid-β (Lauren et al., 2009) although it may also be just a coincidental association.



**Figure 33** shows the mean amyloid-β deposition score in sCJD patients with different APOE alleles. APOE ε4 is known to be associated with an increased risk of AD. Increased amyloid-β deposition in prion disease patients is associated with APOE ε4 genotype and this association is statistically significant.

Multiple comparison analysis was performed using the Tukey HSD test. This analysis involved multiple comparisons of each of the detected APOE genotypes with the mean value for each of the three proteins PrP, tau and amyloid- $\beta$ . A statistically significant association was detected between APOE  $\epsilon$ 4 and amyloid- $\beta$  deposition in sCJD patients.

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) APOE	(J) APOE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
PrP_mean		44	-,61109	,37823	,384	-1,6342	,4120
	33	34	-,19442	,23519	,841	-,8306	,4418
		23	-,06442	,23519	,993	-,7006	,5718
		33	,61109	,37823	,384	-,4120	1,6342
	44	34	,41667	,41891	,753	-,7165	1,5498
		23	,54667	,41891	,566	-,5865	1,6798
		33	,19442	,23519	,841	-,4418	,8306
	34	44	-,41667	,41891	,753	-1,5498	,7165
		23	,13000	,29621	,971	-,6712	,9312
		33	,06442	,23519	,993	-,5718	,7006
	23	44	-,54667	,41891	,566	-1,6798	,5865
		34	-,13000	,29621	,971	-,9312	,6712
Tau_mean		44	-,50000	,27998	,300	-1,2628	,2628
	33	34	-,16667	,17573	,779	-,6454	,3121
		23	-,20000	,18876	,716	-,7143	,3143
		33	,50000	,27998	,300	-,2628	1,2628
	44	34	,33333	,30825	,703	-,5065	1,1732
		23	,30000	,31586	,778	-,5606	1,1606
		33	,16667	,17573	,779	-,3121	,6454
	34	44	-,33333	,30825	,703	-1,1732	,5065
		23	-,03333	,22861	,999	-,6562	,5895
		33	,20000	,18876	,716	-,3143	,7143
	23	44	-,30000	,31586	,778	-1,1606	,5606
		34	,03333	,22861	,999	-,5895	,6562
Abeta_mean		44	-2,80000*	,97099	<b>,030</b>	-5,3949	-,2051
	33	34	-1,05000	,52905	,210	-2,4639	,3639
		23	,77143	,55809	,517	-,7200	2,2629
		33	2,80000*	,97099	<b>,030</b>	,2051	5,3949
	44	34	1,75000	1,05112	,354	-1,0590	4,5590
		23	3,57143*	1,06603	<b>,009</b>	,7225	6,4203
		33	1,05000	,52905	,210	-,3639	2,4639
	34	44	-1,75000	1,05112	,354	-4,5590	1,0590
		23	1,82143	,68812	,053	-,0175	3,6604
		33	-,77143	,55809	,517	-2,2629	,7200
	23	44	-3,57143*	1,06603	<b>,009</b>	-6,4203	-,7225
		34	-1,82143	,68812	,053	-3,6604	,0175

**Table 17** shows the association of PrP, amyloid- $\beta$  and tau protein deposition with APOE polymorphisms following multiple comparisons using Tukey HSD test. The association for both accumulated amyloid- $\beta$ protein with APOE  $\epsilon 4$  is statistically significant at the 0.05 level, although in consideration of multiple testing this finding needs to be replicated.

This analysis suggests that in addition of expected associations between homozygosity at codon 129 of the *PRNP* gene and disease duration and between *APOE*  $\epsilon 4$  haplotype and amyloid- $\beta$  deposition there are further interesting associations. These include *MAPT* haplotype 1c which is significantly associated with both increased prion and tau protein accumulation suggesting a possible role of this genetic locus in the pathogenesis of prion disease. This polymorphism is known to be associated with alpha-synuclein accumulation thus suggesting possible common pathways with prion disease and other neurodegenerative conditions as previously discussed (Goedert, 2015).

### **3.5 Copy number variants as susceptibility loci to prion disease**

Copy number variants represent a potentially novel source of genetic susceptibility. In order to investigate their role in susceptibility to prion disease we analyzed CNV deletions and duplications in patients with UK sCJD, vCJD; German sCJD patients and resistance to kuru in PNG. The analysis was performed using 2 different softwares in Illumina Genome Studio. A full CNV analysis performed in this study is summarized at the end of the chapter.

#### **Pilot experiment**

Genome Studio supports several softwares for CNV data analysis to allow detection of deletions/duplications, loss of heterozygosity (LOH), their size and chromosomal location. In order to select the most appropriate software we created a 10 sample project which was analyzed using Quanti SNP, Penn CNV and CNV Partition. Each algorithm was tested for its ability to accurately detect small and large CNVs with a good confidence value. The project was created in Genome Studio using a unique sample sheet. Performance and accuracy of 3 different CNV softwares was initially assessed. PLINK a genome wide analysis tool was used to estimate type, genomic location and frequency of CNV deletions and duplications. It allowed estimation of genome-wide CNV burden as well as specific association analyses. The primary analysis was a  $\chi^2$  test with the use of empirical p values. Three different algorithms were used to detect copy number value, confidence score, chromosomal location and copy number size. The report demonstrated that CNV Partition detects the highest number of even small CNV deletions and duplications with a high confidence value suggesting good dependability. Further case/control analysis of the larger sample set was performed using CNV Partition.



### **3.5.1 Copy number variants analysis using CNV Partition**

#### **a) UK CNV detection analysis**

A total of 708 UK samples (509 sCJD, 123 vCJD and 76 inherited prion disease samples) were analyzed and compared to 902 Wellcome Trust Case Control Consortium (WTCCC) controls. Patient samples were analyzed on Illumina Human-Hap 670 array and publicly available WTCCC2 controls on 1.2M Custom Duo array. The data were imported into Genome Studio and the 2 projects were analyzed using CNV Partition. The initial analysis revealed a significant discrepancy between cases and controls. This was thought to be due to platform differences between Illumina 670K array used for case analysis and Illumina 1.2M array used for control sample analysis. In order to overcome this problem the 2 projects were merged in Genome Studio using a unique sample sheet. The merged project contained CNV probes common to both cases and controls which led to a significant reduction in the number of probes. There was also a discrepancy associated with gender. Absence of the second X chromosome in males was detected as deletion by the software creating a false positive association. We excluded the X chromosome from further analysis. In addition, the confidence value was increased to minimize false positive associations. The CNV analysis was repeated using CNV Partition and the case/control association analysis performed using PLINK.

	Total number of CNV deletions	Number of CNV deletions per person	Total number of CNV duplications	Number of CNV duplications per person
<b>Cases</b>				
<b>&gt;100kb</b>	298	0.42	563	0.795
<b>Controls</b>				
<b>&gt;100kb</b>	540	0.59	792	0.878
<b>Cases</b>				
<b>&gt;500kb</b>	11	0.0155	52	0.073
<b>Controls</b>				
<b>&gt;500kb</b>	88	0.0975	157	0.174
<b>Cases</b>				
<b>&gt;1Mb</b>	6	0.0084	12	0.017
<b>Controls</b>				
<b>&gt;1Mb</b>	28	0.031	77	0.085

**Table 18.** Summary table showing the total CNV burden in UK cases compared to controls. It shows the total CNV burden of deletions and duplications ranging from smallest (>100kb) to the largest (>1Mb). The number of CNVs per person for deletions and duplications is also shown in the table.

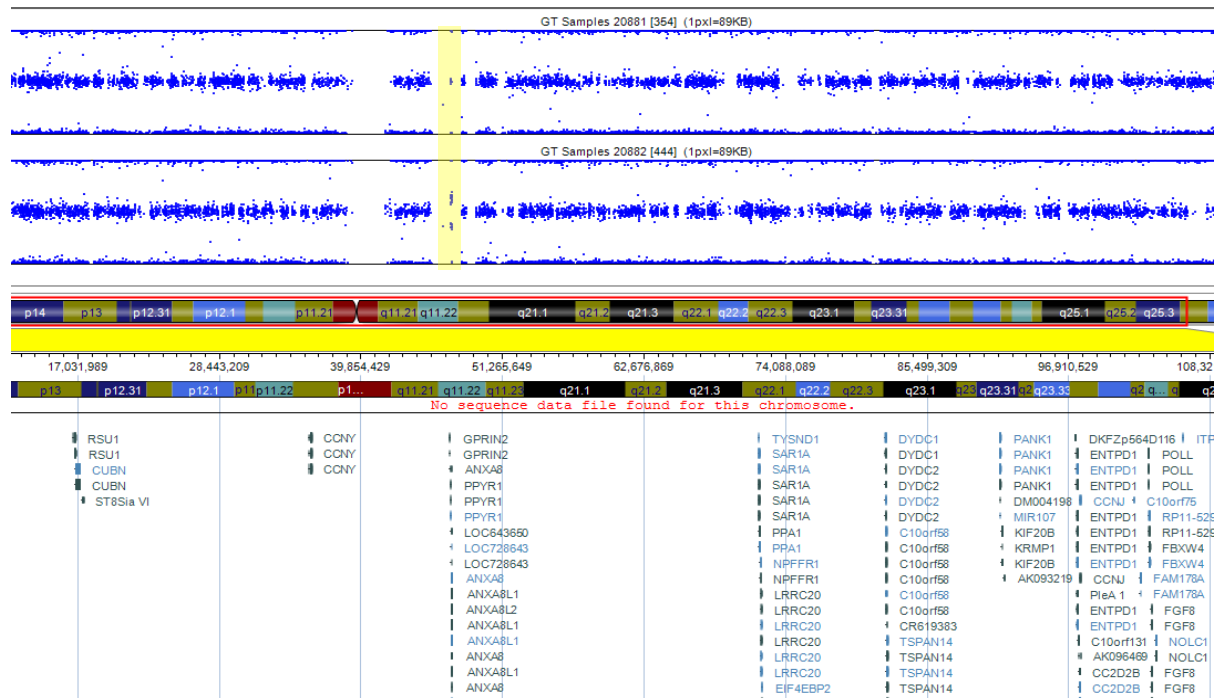
We initially looked for the presence of CNV duplications and found several loci to have a genome-wide or near genome-wide statistically significant associations in cases compared to controls. These data are summarized in the table below.

Chr location of CNV duplication	Number of duplications in vCJD cases	Number of duplication in controls	Number of duplications in sCJD cases	Number of duplications in controls	p value in vCJD	p value in sCJD
6p22.3	4/122	12/902	23/508	12/902	0.109189	0.00019998
7p12.1	5/122	1/902	19/508	1/902	1.45 x 10 <sup>-23</sup>	4.03 x 10 <sup>-8</sup>
10q11.22	6/122	23/902	46/508	23/902	0.1442	1.15 x 10 <sup>-7</sup>
12p	3/122	2/902	15/508	2/902	0.0128987	1.19 x 10 <sup>-5</sup>
12p13.31	1/122	12/902	25/508	12/902	1	0.00019998
14q11.2	6/122	0/902	22/508	0/902	2.56 x 10 <sup>-6</sup>	1.31 x 10 <sup>-10</sup>
16q12.2	4/122	13/902	27/508	13/902	0.130687	6.8 x 10 <sup>-5</sup>
16p11.2	0/122	0/902	9/508	0/902	1	0.00019998

**Table 19** Genetic loci associated with a genome-wide ( $<1 \times 10^{-8}$ ) or near genome-wide ( $1 \times 10^{-5}$ - $1 \times 10^{-8}$ ) statistically significant p value following data analysis in PLINK. The p values are noted for case/control association analysis for both sporadic and variant CJD. The table also shows the number of cases and controls where the duplication was detected.

### b) Validation of UK findings

Duplications on chromosomes 6, 7, 10, 12, 14 and 16 were validated by inspection in Illumina Genome Viewer. Chr 12 duplication was peri-centromeric and therefore less likely to be reliable. Chromosome 7p12.1 and 16q12.2 CNV duplications were also detected in inherited prion disease samples and were therefore less likely to represent susceptibility loci. Chr 10 duplication is located next to *GPRIN2* gene (Figure 34), a neural growth factor which has been well described whilst another gene *PPYR1*, pancreatic polypeptide receptor-1 is contained within the duplication.

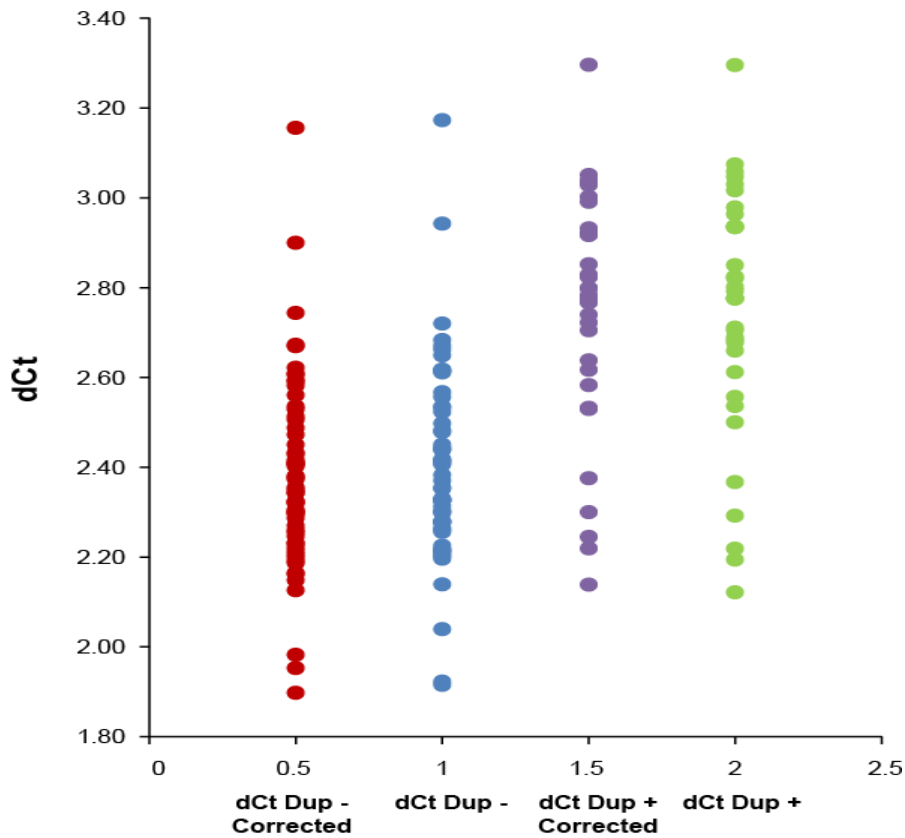


**Figure 34** shows an image of the chromosome 10q11.22 as visualized in the Illumina Genome Viewer. The first plot shows the control sample where the duplication is not present and the second plot shows a sample containing the duplication on chromosome 10.

*GPRIN2* and *PPYR-1* genes were genotyped using real time PCR. Chromosome 14 CNV duplication was genome-wide significant in both sporadic and variant CJD samples. This duplication has been associated with early age at onset in Alzheimer’s disease (Brouwers et al., 2006). Olfactory gene *OR11H12* which is contained within the chromosome 14 duplication was genotyped using real time PCR.

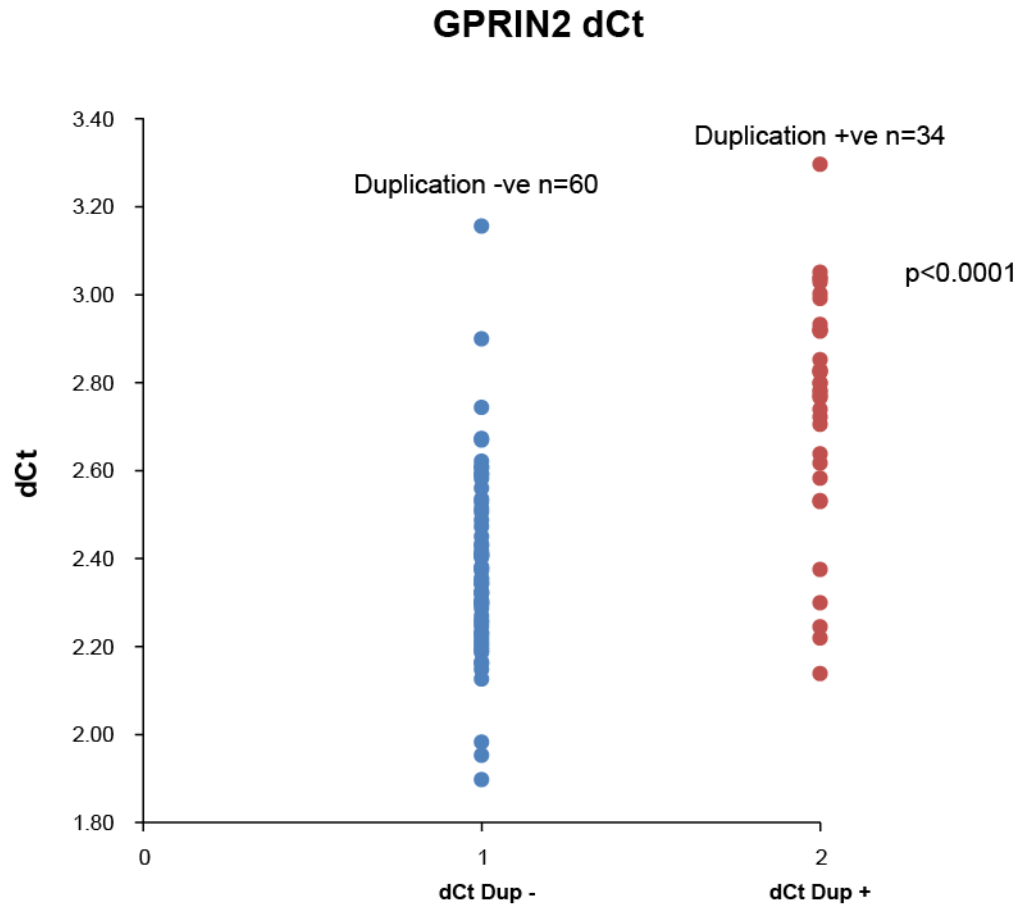
### c) Real time PCR in UK sporadic CJD

Figures 34-37 show the change in cycle number (dCt) in sCJD duplication positive samples compared with the duplication negative samples. Delta Ct values represent the number of additional cycles needed to achieve threshold amplification of a given DNA segment. Most primer sets that flank random regions of the genome display dCt values less than two. Figure 35 demonstrates comparison of dCt values for *GPRIN2* and *APP* duplication positive and duplication negative samples where *APP* duplication samples were used as controls. There is a clear difference between the 2 groups which is statistically significant. These data demonstrate that CNV duplications detected using CNV Partition and PLINK are also detected using real-time PCR thus, confirming our initial finding.



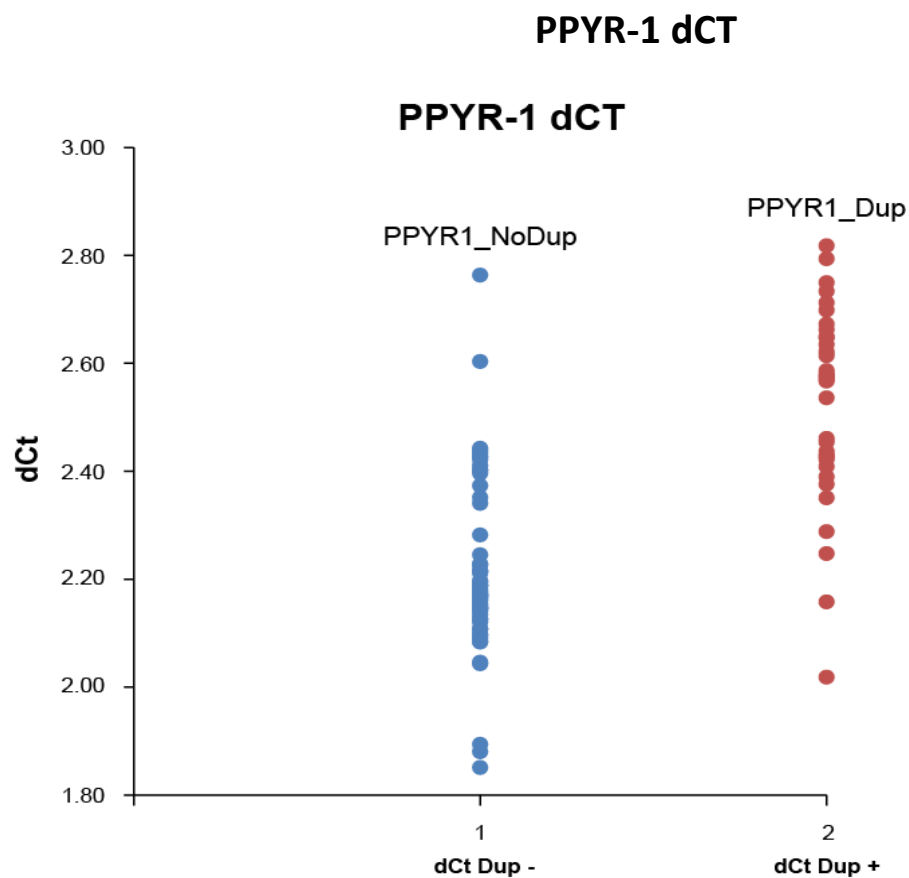
**Figure 35** shows a change in the cycle number (dCt) for GPRIN2 and APP duplication positive and duplication negative samples. There is a clear difference between the duplication positive and duplication negative samples. APP duplication positive samples were used as controls in this assay.

Figure 36 shows dCt values for sCJD patients with GPRIN2 dCt duplication compared to sCJD samples without the duplication. The graph shows that there are 2 distinct groups with a clear difference which is statistically significant.



**Figure 36** shows GPRIN2 dCt in sCJD samples with the CNV duplication compared to sCJD samples without the duplication. There is a clear difference between the 2 groups which is statistically significant. The data suggest that the duplication positive samples extend to include the GPRIN2 gene.

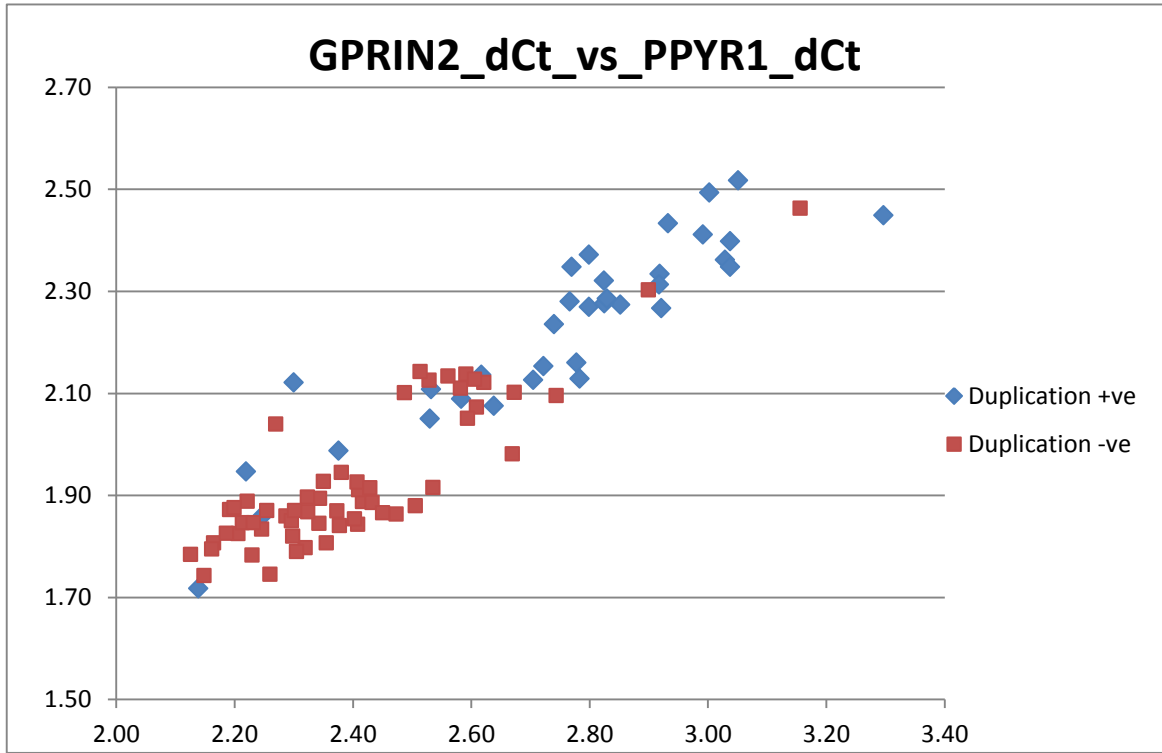
Chromosome 10 duplication is located next to GPRIN2 gene, whilst PPYR-1 is contained within the duplication. PPYR-1 gene was genotyped using real time PCR. Figure 37 shows PPYR1  $\Delta$  Ct in sCJD samples with the CNV duplication compared to sCJD samples without the duplication. There is a clear difference between the 2 groups which is statistically significant.



**Figure 37** shows PPYR1  $\Delta$  Ct in sCJD samples with the CNV duplication compared to sCJD samples without the duplication. There is a clear difference between the 2 groups which is statistically significant. The data suggest that the duplication positive samples extend to include the PPYR-1 gene.

Real time PCR was then performed for GPRIN2 and PPYR-1 duplication and their correlation was assessed. Figure 38 shows the correlation between the 2 assays which is good indicating that chromosome 10 duplication extends to include the GPRIN2 gene.





**Figure 38** shows samples with the GPRIN2 CNV duplication vs. PPYR1 CNV duplication. Samples where the duplication is present are marked in blue and samples where the duplication is absent are labeled in red. There is a good correlation between the 2 assays indicating that the chromosome 10 duplication extends to include the GPRIN2 gene. The duplication negative samples that appear to be in the duplication positive groups are probably contaminant or false positive. Similarly, the duplication positive samples that appear to be in the duplication negative group are likely to be false positives due to a 'noisy' test.

Furthermore, we analysed the clinical information of patients thought to be chromosome 10 and 14 duplication carriers, however they did not appear to have distinct clinical or pathological phenotypes, although in some cases the data was limited.

#### **d) CNV analysis in German sporadic CJD**

In order to confirm the above findings we performed a CNV analysis in the German sCJD patients. A total number of 635 German sCJD cases and 822 control samples were analyzed in the final project. The cases were analyzed on 670K Illumina Genome Studio platform and the controls on an older 550K Illumina platform. The 2 projects were merged using a unique sample sheet leading to a loss of a large number of CNV only probes. CNV analysis was performed in Genome Studio using CNV Partition and PLINK for case/control association analysis. The significant associations in the UK population were not replicated in the German cohort. The case/control association analysis in the German sCJD did not reveal any other significant associations.

The UK findings are intriguing as they are suggestive of potential novel susceptibility loci in prion disease. However, they do not replicate in the German cohort. One reason for this is that CNV Partition detects very small CNVs and may be more prone to false positive associations. Another explanation may be the difference in phenotype between UK and German cases due to the selection criteria. German sCJD patients were all neuropathologically confirmed cases with classic sCJD presentation which typically included a combination of rapidly progressive dementia, ataxia and myoclonus. The UK sCJD included both definite and probable cases making this patient group more phenotypically heterogenous. As further prion disease patients were recruited into the study the CNV analysis was repeated using the Penn CNV software on a larger sample set.

### 3.5.2 Copy number variants analysis using Penn CNV

CNV analysis using Penn CNV was performed on a larger sample set which included 1261 European patients (from Germany, France and UK) with prion disease and 5427 European controls. A total of 587 CNVs were detected in prion disease patients and 2786 in controls. All of the CNVs were found at <1% frequency in the control population. They included more than 20 markers. The size of CNVs were >100kb and they scored >50 with the PennCNV quality score. Any specific CNVs were also verified by inspection of B allele and LogR ratios in Illumina Genome Viewer. No statistically significant excess of deletions or duplications were identified. A total CNV burden, of sizes >100kb, >500kb or >1Mb were tested in each disease group and compared with the controls. These are summarized in the tables below. Table 20 shows the CNV analysis for deletions and duplications in UK sCJD. There was no statistically significant association for any of the CNV sizes as summarized in the table.

UK sCJD										
		Number	# CNVs per person	P	Number	# CNVs per person	P	Number	# CNVs per person	P
>100kb	Cases (n=583)	122	0.209		169	0.290		292	0.501	
	Controls (n=4632)	1190	0.257	0.983	1275	0.275	0.293	2471	0.534	0.838
>500kb	Cases	5	0.009		37	0.063		42	0.072	
	Controls	82	0.018	0.969	316	0.068	0.679	398	0.086	0.865
>1MB	Cases	0	0.000		14	0.024		14	0.024	
	Controls	29	0.006	1.000	87	0.019	0.244	116	0.025	0.589

**Table 20 Total CNV burden in UK sCJD compared to controls.** Total CNV counts, frequency and P value (uncorrected empirical test using PLINK) are shown in red where the nominal P value is <0.05. No category was statistically significant after correction for multiple testing.

Table 20 shows the CNV analysis for deletions and duplication in UK vCJD. This analysis included 114 cases and 4632 controls. Total number of 583 cases were analyzed and compared to 4632 controls. Deletions and duplication were separated according to their size which include CNVs >100kb, >500kb and >1MB. There was no statistically significant association in cases vs. controls in any of the groups tested.

UK vCJD										
		Number	# CNVs per person	P	Number	# CNVs per person	P	Number	# CNVs per person	P
<b>&gt;100kb</b>	Cases (n=114)	26	0.228		38	0.333		64	0.561	
	Controls (n=4632)	1190	0.257	0.736	1275	0.275	0.151	2471	0.534	0.358
<b>&gt;500kb</b>	Cases	1	0.009		7	0.061		8	0.070	
	Controls	82	0.018	0.863	316	0.068	0.664	398	0.086	0.741
<b>&gt;1MB</b>	Cases	0	0.000		3	0.026		3	0.026	
	Controls	29	0.006	1.000	87	0.019	0.364	116	0.025	0.549

**Table 21 Total CNV burden in UK vCJD compared to controls.** Total CNV counts, frequency and P value (uncorrected empirical test using PLINK) are shown in red where the nominal P value is <0.05. No category was statistically significant after correction for multiple testing.

A numerical excess of large duplications were also seen in UK sCJD and vCJD as shown in Tables 20 and 21 however, these changes were not statistically significant. Of the total 27 >1000kb duplications seen in European case samples, 3 overlap in a region of chromosome 16 (NCBI build 36, 15,387,380-16,197,033) comprising 7 genes, although this same region was involved in 6 large duplications in control samples. Due to differences related to platform and filtering we have not directly compared these frequencies with other studies.

## CNV analysis in PNG samples and controls

Further analysis was performed in elderly women resistant to kuru and compared to healthy Fore population. A total number of PNG cases included 122 patients and 273 controls. CNVs were separated into different groups according to their sizes (as discussed above). Total CNV burden which included CNV >100kb showed an association (p=0.046) following case/control analysis, however this was not statistically significant in consideration of the number of hypotheses tested. There was no other significant association in this disease group.

Elderly Women Resistant to Kuru vs Health Fore										
		Number	# CNVs per person	P	Number	# CNVs per person	P	Number	# CNVs per person	P
<b>&gt;100kb</b>	Cases (n=122)	<b>240</b>	<b>1.967</b>		263	2.156		503	4.123	
	Controls (n=273)	<b>480</b>	<b>1.758</b>	<b>0.046</b>	570	2.088	0.261	1068	3.912	0.102
<b>&gt;500kb</b>	Cases	0	0.000		16	0.131		16	0.131	
	Controls	3	0.011	1.000	61	0.223	0.989	69	0.253	0.997
<b>&gt;1MB</b>	Cases	0	0.000		9	0.074		9	0.074	
	Controls	2	0.007	1.000	42	0.154	0.991	44	0.161	0.996

**Table 22 Total CNV burden in elderly women resistant to kuru compared to healthy Fore.** Total CNV counts, frequency and P value (uncorrected empirical test using PLINK) are shown in red where the nominal P value is <0.05. Total CNV burden for CNVs >100kb was statistically significant in case/control analysis. There was no other statistically significant association.

In PNG population a 1% filter was not applied as the control population was small, and the population genetics of the Eastern Highland region has not been studied in detail. We found a nominally significant excess of deletions >100kb in the Elderly Women Resistant to Kuru group however this was not significant after correction for multiple testing.

## CNV analysis in German samples and controls

CNV analysis was repeated in German sCJD patients. This analysis included 564 cases and 795 controls.

CNVs were divided into groups according to their sizes ranging from >100kb, >500kb and >1MB. A

nominally significant excess of large duplications (>1000kb) were seen in the German sCJD study, at a

rate of 1.8% of cases vs. 0.5% of controls (p=0.033) however this was not statistically significant after

taking into account multiple testing (Table 23).

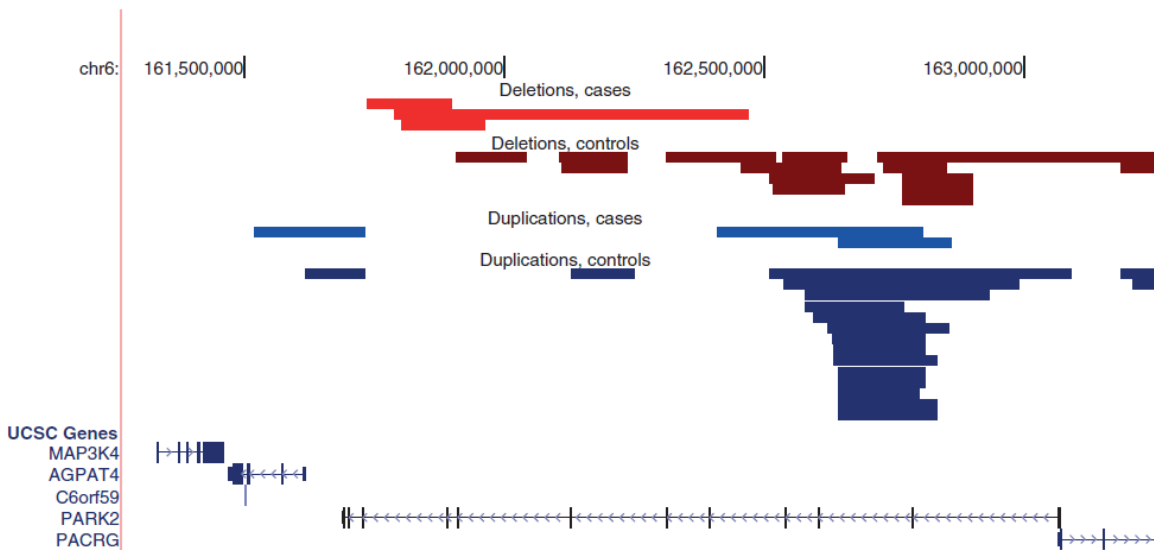
German sCJD		Deletions			Duplications			Deletions and duplications		
		Number	# CNVs per person	P	Number	# CNVs per person	P	Number	# CNVs per person	P
<b>&gt;100kb</b>	Cases (n=564)	103	0.183		124	0.220		231	0.410	
	Controls (n=795)	152	0.191	0.661	159	0.200	0.242	315	0.396	0.374
<b>&gt;500kb</b>	Cases	7	0.012		29	0.051		36	0.064	
	Controls	11	0.014	0.665	32	0.040	0.206	44	0.055	0.311
<b>&gt;1MB</b>	Cases	5	0.009		<b>10</b>	<b>0.018</b>		<b>15</b>	<b>0.027</b>	
	Controls	5	0.006	0.399	<b>4</b>	<b>0.005</b>	<b>0.033</b>	<b>9</b>	<b>0.011</b>	<b>0.039</b>

**Table 23 Total CNV burden in German sCJD patients compared to controls.** Total CNV counts, frequency and P value (uncorrected empirical test using PLINK) are shown in red where the nominal P value is <0.05. No category was statistically significant after correction for multiple testing.

## Association studies

For rare CNVs, no region showed a statistically significant excess of duplications or deletions in prion disease patients or elderly women resistant to kuru relative to their control group once multiple testing was taken into account. For common CNVs, similarly there were no statistically significant associations made. The top ranked nominal association was found in both the genome-wide and candidate gene studies: at the PARK2 gene (Figure 39) in UK sCJD vs. WTCCC controls ( $P=0.001$ ). CNVs in PARK2 (protein product Parkin) are known to cause autosomal recessive juvenile Parkinsonism, although there remains a debate about a causal role for structural variation in the heterozygous state (Kay et al., 2010; Kitada et al., 1998), which have been found in Parkinson's disease cases and controls overlapping exons 1-4, but in cases alone overlapping exons 5-12. 3 deletions overlapped exons 8 and 9 of PARK2 gene were found in sCJD patients. Two patients had a diagnosis of definite sCJD following autopsy, a third had a diagnosis of probable sCJD according to established clinical criteria which according to the recent literature predicts autopsy diagnosis with around a 98% probability (Zerr et al., 2009). All patients had a clinical phenotype typical of CJD without features of Parkinsonism. We went on to look for causal non-synonymous variants in PARK2 using exome sequencing data from 132 UK prion disease patients. No mutations were found.





**Figure 39** Structural variation at the PARK2 gene in sCJD. The top ranked nominal association was found in both the genome-wide and candidate gene studies: at the PARK2 gene in UK sCJD vs. WTCCC controls (P=0.001).

### CNVs overlapping loci of potential relevance to prion disease

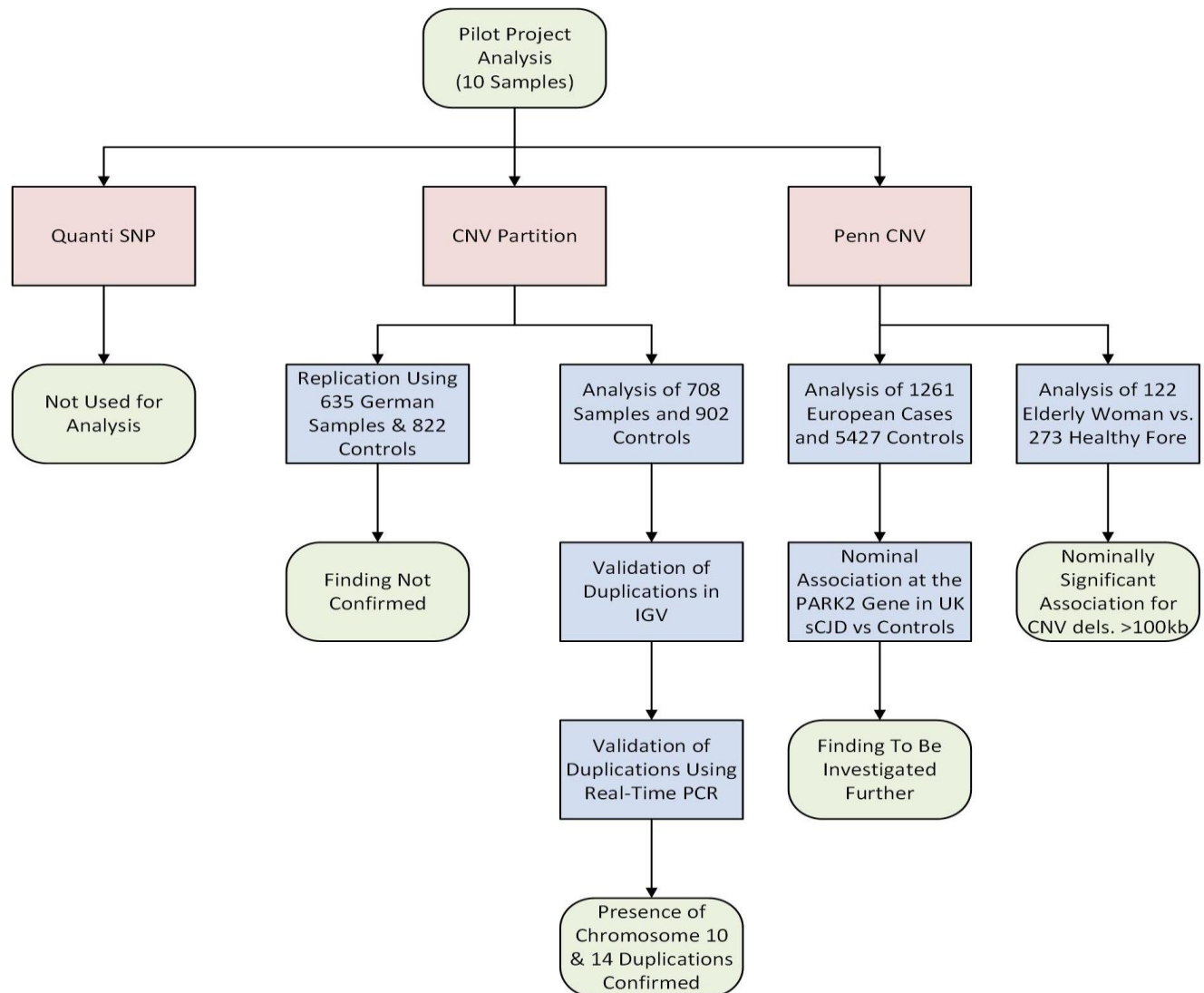
There are a number of genetic loci that have been reported to play a role in prion disease. These include *PRNP*, *RARB-THRB*, *STMN2*, *HECTD2*, *SPRN*, *ZBTB38-RASA*, *BACE*, *MTMR7*, *NPAS2*, *PLCD*, *CPNE8*, *SOD1*, *STCH* and *CTSD* (Akhtar et al., 2013; Beck et al., 2008; Kovacs et al., 2010; Lloyd et al., 2010; Lloyd et al., 2009; Mead et al., 2009a; Mead et al., 2011b; Sanchez-Juan et al., 2012). We extended our study to look for overlapping CNVs in these regions. We did not find any overlapping genes in more than one individual in a case or control group. One control individual, sample provided by the UK blood service for genotyping by the WTCCC was shown to have a 240kb duplication entirely overlapping *PRNP*. No prion disease patient had a CNV overlapping this gene. We went on to test for association at 100 candidate loci previously identified in studies of autism, mental retardation, epilepsy, schizophrenia. No statistically significant associations were identified except for the finding discussed above in *PARK2*.

Figure 40 summarizes the CNV analysis using different softwares and sample sets. The performance of each of the softwares QuantiSNP, CNV Partition and Penn CNV was compared using the 10 sample project. QuantiSNP was efficient in detecting large CNVs with a good confidence score however it was not efficient in detecting small CNVs. This software was not used for further analysis.

CNV Partition was efficient in detecting both large and small CNVs however it was more prone to false positive associations. This software was used in the initial analysis of 708 UK CJD samples and 902 WTCCC controls. Two duplications on chromosome 10 and 14 were found to have statistically significant association and were further confirmed by inspection in Illumina Genome Viewer and real-time PCR. The finding was not confirmed when the analysis was performed in the German population.

Penn CNV is thought to be reliable in detecting large CNVs and some smaller CNVs. It is less prone to false positive associations but there is a possibility that it would miss very small CNVs. This software was used in the analysis of 1261 European samples and 5427 controls. Nominally significant association of CNV deletion at PARK2 was detected using Penn CNV and candidate gene studies.

## Summary of CNV analysis performed in this study using different sample sets and softwares



**Figure 40** summarizes the CNV analysis using different softwares and sample sets. Ten sample project was initially analysed using Quanti SNP, CNV Partition and Penn CNV. Quanti SNP was not reliable in detection of small CNVs and was therefore not used in further analysis. CNV Partition was used in the analysis of 708 UK CJD samples and 902 WTCCC controls. Duplication on chromosome 10 and 14 were statistically significant in the case control association analysis. The finding was validated by inspection in Illumina Genome Viewer and confirmed using real-time PCR. CNV analysis was then performed on 635 German samples and 822 controls however the finding was not replicated in this sample set. As more samples became available the analysis was repeated using Penn CNV. 1261 European cases were compared to 5427 WTCCC controls. The case control association analysis revealed a nominally significant association at PARK2 gene in UK sCJD which was also detected using candidate gene analysis.

## V DISCUSSION

In this study we analyzed the clinico-pathological and genetic features in a large group of patients suffering from prion diseases. To this end we used complementary approaches including case studies, molecular pathology, candidate gene analysis and genome-wide copy number variants analysis. The results revealed unusual clinical presentations of variant CJD; relationship between heterozygosity at codon 219 and possible susceptibility to variant CJD; histopathological heterogeneity of prion diseases; and the relationship between neuropathological features and several candidate genes in CJD. The data obtained on the copy number variants appear to be conflicting and some suggestions to explain these findings are discussed. Taken together the data may contribute to better understanding of diagnostics and molecular epidemiology of CJD.

## **4.1 Phenotypical heterogeneity and susceptibility locus in variant CJD- clinical case reports**

### ***4.1.1 Atypical clinical presentation in two patients with variant CJD, correctly diagnosed following neuropathological analysis***

In this study we describe 2 patients who presented with clinical features consistent with sCJD but were later found to have variant CJD following neuropathological analysis. Phenotypic variability is demonstrated by an unusually old age at disease onset and sporadic-like MRI appearances. This finding highlights the variation in important illness characteristics and diagnostic investigations observed in prion diseases. Appleby et al (Appleby et al., 2009) studied the clinical variability in patients with sCJD and reported 5 clinical variants in sCJD based on the heterogeneity of clinical and investigation findings: the classic, Heidenhain, Oppenheimer-Brownell, cognitive and affective subtypes. It is likely that these atypical disease forms remain underdiagnosed and there may be further yet unidentified disease subtypes (Knight, 2006). Stoeck et al (Stoeck et al., 2008) argue that the heightened incidence of sCJD in recent years, observed across many different countries (Klug et al., 2013), may be associated with a shift in the clinico-pathological profiles. In their study patients of these subtypes were significantly older and showed a skewed male to female ratio when compared to published cases of sCJD-types or to patients from the 1996-2000 cohort. They argue that improved detection of rare sCJD subtypes may contribute to increased disease incidence.

Meissner et al (Meissner et al., 2009) have studied the brain MRI findings associated with each of the sCJD molecular subtypes. These included confirmed sCJD cases with codon 129 genotype (MM, MV, and VV), PrP<sup>Sc</sup> type, and fluid-attenuated inversion recovery (FLAIR) or diffusion-weighted brain imaging (DWI) from seven different countries. All MRI scans were assessed for signal changes according to a standard protocol encompassing seven cortical regions, basal ganglia, thalamus, and cerebellum. The

authors found that cortical signal increase and hyperintensities in the basal ganglia and thalamus were detected on MRI scans across all molecular subtypes of sCJD. This makes our finding of MRI features typical for sCJD in patients with vCJD even more relevant. We would argue that typical features may be present not only across different disease subtypes in sCJD but also patients with vCJD that may have previously been misdiagnosed as sCJD based on unexpected clinical and investigation findings. This finding provides further evidence for clinico-pathological heterogeneity and overlap between disease subtypes. It would be of interest to perform a retrospective clinico-pathological study on patients with sCJD and consider this possible unusual vCJD phenotype.

A further observation by Gelpi et al (Gelpi et al., 2013) described an atypical neuropathological phenotype of sCJD case in a 64 year old patient presenting with a 5 months of rapidly progressive dementia, psychiatric disturbance and language disorder. MRI showed atrophy, diffuse cortical hyperintensities and high signal in the left caudate nucleus on T2W and FLAIR images. No typical EEG changes were observed and 14-3-3 assay was positive. Western blot studies demonstrated the co-occurrence of PrP<sup>Sc</sup> types 1 and 2 in the frontal cortex and a relatively weak type 2 signal in the cerebellum. *PRNP* genotyping showed methionine homozygosity at codon 129. Neuropathological analysis showed classical CJD changes with small cortical foci of large confluent vacuoles and a relatively well preserved cerebellum. A striking feature was the presence of abundant kuru-type plaques in the cerebral cortex and subcortical white matter. Sparse kuru-type plaques were also seen in cerebellum, although only in white matter. This case shows a previously undescribed combination of histopathological features which preclude its classification according to the current phenotypic and molecular sCJD classification although this subclassification system is not rigid. The observation demonstrates that kuru-type amyloid plaques may also occur in sCJD cases with short clinical course and the co-existence of PrP<sup>Sc</sup> types 1 and 2. This finding represents a further example of phenotypic

heterogeneity as demonstrated by the clinico-pathological overlap between different prion disease groups.

Given the complexity of the molecular and neuropathologic factors inherent within the spectrum of prion diseases further studies of phenotypic variability are justified to allow early diagnosis, treatment and clinico-pathologic correlation. One of the challenges faced by clinicians is dealing with the variability of clinical symptoms, signs and investigation findings in prion disease, which may pose a serious diagnostic challenge as evidenced by the cases described. It may result in a delayed diagnosis and delayed inclusion in therapeutic trials when relatives/patients desire to do so. There are potentially other further cases that remain unreported and pose potential risks for recipients of blood products and patients undergoing surgery with the prion infected surgical instruments. In order to prevent these incidents and gain further accurate information about the potential risks an increase in autopsy rate and use of new blood test which has 100% specificity and 70% sensitivity for prion protein detection (Jackson et al., 2014) plays an important role.

#### ***4.1.2 Heterozygosity at codon 219 may represent a susceptibility polymorphism to variant CJD***

In this study we describe clinical and investigation findings of two patients with vCJD who were heterozygous at the polymorphic codon 219. Both patients were homozygous for methionine at codon 129. Heterozygosity at codon 219 is a common polymorphism in the healthy Japanese population and in populations in Oceania, South Asia, and the Middle East, but it has not been detected in Africans or Europeans (Beck et al., 2010; Mead et al., 2003; Soldevila et al., 2003). Eleven patients and controls with E219K all had Asian ethnicity or ancestry based on a heterogeneous diagnostic referral series totaling more than 1800 samples at the MRC Prion Unit. Recent data from a transgenic animal knock-in model suggest that the 219E and 219K proteins can convert to PrP<sup>Sc</sup> when challenged with sCJD or vCJD prions (Hizume et al., 2009). Human PrP 219K-expressing mice succumbed to vCJD with a shorter incubation time compared with human PrP 219E-expressing mice. The E219K heterozygous mice had a longer incubation time to vCJD than did either homozygous mouse. However, the present patients suggest that the heterozygous genotype at codon 219 is not protective against vCJD and may even confer increased risk. We do not know whether the PrP<sup>Sc</sup> seen on Western blot of tonsillar tissue in patient 2 includes PrP with 219K or whether this is solely derived from the 219E PrP. The E219K polymorphism occurs as a result of a substitution of negatively charged E by the positively charged K. Further studies are necessary to establish whether this charge change may be critical in permitting the conversion of PrP to its abnormal isoform in vCJD but not sCJD. Our seemingly paradoxical observations may be interpreted in the context of the conformational selection model of prion transmission proposed by Collinge (Collinge J, 1999) in 1999 and Collinge and Clark (Collinge and Clarke, 2007) in 2007. This model proposes that prion transmission occurs efficiently when there is an overlap between the infecting prion and the permissible conformations of the host PrP. In this circumstance, the 219K protein may not adopt the molecular conformations found in sCJD, resulting in strong resistance to this disease. However, in vCJD,



the model proposes that the 219K protein permits or even favors the bovine spongiform encephalopathy strain, resulting in no resistance to disease or even increased susceptibility.

Our finding points to a role of a genetic variant, other than codon 129, in susceptibility to vCJD. Further studies are necessary to confirm this finding however, as this polymorphism is rare in the populations exposed to BSE, it may not be possible to explore its significance in a large number of patients with clinical vCJD.

#### **4.2 Phenotypic heterogeneity- National Prion Monitoring cohort (NPMC) study**

In this study we confirm a key predictor of functional decline in rapidly progressive forms of prion disease, codon 129 subtype, enabling us to assess the potential gains from a stratified medicine approach to future clinical trials. Through use of natural history data collected in a prospective observational cohort study we have optimised a clinical trial model to increase power to detect clinically relevant effects, on a pre-mortality endpoint measuring functional decline, that is judged highly relevant by patients and carers. Our two key findings are, first, that genetic variation in the prion protein gene has a profound influence on the rate of functional decline in rapidly progressive human prion disease, and is therefore likely the essential factor on which to stratify a trial model. Second, that a combination of factors, including the use of a bespoke functionally-orientated rating scale, the use of frequent assessments by telemedicine, the rapidly progressive nature of prion disease, and the stratification by codon 129 contribute to the potential for remarkably powerful clinical trials.

Despite the fact that codon 129 was known to be an important modifier of susceptibility and phenotype of prion diseases (Pocchiari et al., 2004), we were surprised by the magnitude of the effects we observed on functional decline after diagnosis. The homotypic protein-protein interactions in *PRNP* homozygous prion disease patients may occur faster than heterotypic interactions in heterozygous individuals (Palmer et al., 1991) resulting in more rapid propagation of prions and/or generation of neurotoxic forms of abnormal PrP. Further to this, codon 129 is known to be an important strain selection factor (Collinge and Clarke, 2007). Prion strains, analogous to strains of bacteria or viruses, are associated with distinct types of misfolded PrP, and distinct and transmissible clinicopathological features. It is likely that some of the distinction between the rates of decline for codon 129 homozygotes are related to strain selection by codon 129. PrP<sup>Sc</sup> type, which is measure of abnormal PrP

conformation and strain, did provide some limited additional value beyond codon 129 alone, but was only able to be measured in ~40% of the cohort, retrospectively, seriously limiting its use as a factor in clinical trials. The lack of a role of PrP<sup>Sc</sup> type may relate to the fact that this is highly correlated with codon 129 genotype, and that, whilst autopsy rate in the study was high, missing data are substantial. We note that variation in the slopes of decline of codon 129 methionine homozygous patients was greater than for other genotypes, suggesting that strain diversity/permmissibility may be greatest in this genotype.

The last decade has seen the advent of genome-wide technologies which have driven the discovery of many risk factors in neurodegeneration (Singleton et al., 2010). Although examples are emerging (Cruchaga et al., 2010), phenotypic heterogeneity is less well understood, in part because the imperative to study large sample sizes has come at the expense of well characterised clinical cohorts. Clinical trials in common neurodegenerative diseases do not typically stratify by genetic factors. One exception is *APOE* in some studies of Alzheimer's disease immunotherapy, although this decision was primarily driven by an increased susceptibility to amyloid-related imaging abnormalities in *APOE4* carriers (Sperling et al., 2012). The potent genetic effects we observed in this study hints at the potential of using genetic modifiers to improve power in clinical trials in neurodegeneration more widely.

There are some limitations of this study. A concern about avoiding making a diagnostic error, and a sense that it is important to diagnose only treatable disorders, means that referral to specialist centres often occurs late in the clinical course of human prion disease, when confirmatory tests are complete. Consequently the majority of patients enrolled in our Cohort study had advanced neurodisability at enrolment. As a rough rule of thumb to simplify decision making and due to the rapidity of progression in sCJD, patients with an MRI brain suggestive of prion disease, who are either able to walk (even with

help) or speak some words should be referred promptly, particularly when clinical trials are active, as these patients are highly likely to have prion disease. Although this is by far the largest study of its type, we are still underpowered to detect the modifying effects of certain factors, particularly for rare combinations of PrP<sup>Sc</sup> type and codon 129 genotype which might be associated with atypical clinical courses.

Biomarker analysis is a highly active area of research in neurodegeneration and is a key component of the Cohort study. In prion disease however, where diagnostic accuracy (once referred) is high and progression rapid, the need for a surrogate marker of progression is less compelling than in slowly progressive disease syndromes. In the multivariate analysis, standard diagnostic MRI, EEG and CSF biomarkers did not add significantly to codon 129 subtype for predicting the slopes of functional decline. In our analysis, the number of samples for PrP<sup>Sc</sup> type was also very small which made it difficult to assess its role as a potential disease modifier. The analysis of quantitative MRI parameters which seem promising as predictors of the severity of microscopic tissue pathology is beyond the scope of this report (Siddique et al., 2010). The propensity of abnormal PrP seeds to trigger the polymerisation of recombinant PrP *in vitro*, for example by the PMCA or RT-QUIC reactions, have potential as highly responsive therapeutic biomarkers in CSF and will be important adjuncts to future trials.

Functional endpoints in other neurodegenerative diseases typically require several hundred or thousands of patients for adequately powered studies (Green et al., 2010). Here we have shown that using repeated measurements of a functional scale can reduce sample sizes by 10-30 patients, depending on the frequency of assessment. The functional scale also has advantages in that it is not affected by personal decisions regarding end-of-life care (including PEG feeding, antibiotics etc), which can add substantial variation obscuring treatment differences in small trials using a survival endpoint. It

is now accepted by some that common neurodegenerative disorders share fundamental mechanisms with prion disease, termed prion-like. Immunotherapeutics and small molecules have been developed which can clear prion infection from cultured neuronal cells, and cure infection or greatly impede it *in vivo* (Silber et al., 2013; Wagner et al., 2013; White et al., 2003). The prognosis for rapidly progressive prion disease is particularly severe and the unmet need is great, analogous to advanced cancer, which has proven to be a very effective test-bed for novel therapeutics. We hope that our study will modify the view of human prion disease as an orphan disorder futile for clinical study, into a tractable disorder for testing of therapeutics.

### **4.3 Histopathological heterogeneity**

As already discussed, spongiform change, neuronal loss, astrocytosis, glial proliferation and prion protein deposition represent the typical neuropathological changes associated with CJD.

Hyperphosphorylated tau and amyloid- $\beta$  accumulation are also present suggesting that prion diseases belong to a wider spectrum of neurodegenerative diseases that share common neuropathological pathways. In order to assess the role and possible interaction of different proteins associated with neurodegeneration we investigated the distribution and accumulation of amyloid- $\beta$ , prion protein and hyperphosphorylated tau in prion disease patients. We found that tau accumulation tends to co-localize with the prion protein deposition. Interestingly, amyloid- $\beta$  deposits are also present in these patients but total accumulation is significantly lower than the accumulation of either prion protein or hyperphosphorylated tau. The difference is particularly striking in the cerebellum where the amyloid- $\beta$  deposits appear to be virtually absent compared to the other two proteins. This finding highlights the importance of the interaction of the different proteins with the host tissues. The mechanism underlying the selective targeting of specific brain regions by different neurodegenerative diseases is an intriguing phenomenon. It is already known that cholinergic neurons of the cerebral cortex as well as the hippocampal neurons are affected in Alzheimer's disease (Francis et al., 1999), whereas dopaminergic neurons of the substantia nigra are targeted in Parkinson's disease (Sulzer and Surmeier, 2013). However, the reasons why other brain regions remain spared are still unknown (Jackson, 2014). A better understanding of the selective vulnerability of different tissues to neurodegeneration may allow development of targeted therapeutic approaches that would alter disease course and prevent progression. This selective vulnerability is thought to be determined by the interaction of different protein conformers with host microenvironments affected by a wide range of contributory factors. The interaction of misfolded proteins with the combination of cell-type –specific environments and brain-

region-specific neural features ultimately determine selective vulnerability. The spreading of the neurodegenerative process is likely controlled by a combination of opposing factors that facilitate or restrict spread of the disease. A better understanding of how these factors interact and contribute to the process of neurodegeneration is likely to play a key role in the discovery of therapeutics in prion diseases and other neurodegenerative disorders.

Another interesting finding is the statistically significant correlation between disease duration and total accumulation of hyperphosphorylated tau in prion disease patients. Spillantini et al (Spillantini and Goedert, 2013) argue that the conversion of soluble to insoluble filamentous tau protein is central to many human neurodegenerative diseases. Tau is an unfolded microtubule-associated protein that binds to and might have a role in the assembly and stabilization of microtubules. Because the assembly of tau is concentration dependent, regional variation in expression of the protein could favour its accumulation. Tau hyperphosphorylation seems to precede filament assembly, but whether this process is necessary or sufficient for filament assembly is unknown. It appears to take time and this may explain its positive correlation with disease duration i.e. increased tau accumulation correlates with increased disease duration and increased disease severity. Van Everbroeck et al (Van et al., 2002) have found that in the sCJD population determination of both 14-3-3 and tau protein concentrations are of a high diagnostic value. In their study they showed that high phosphorylated tau concentration in the cerebrospinal fluid is clinically correlated with a significantly shorter disease duration and early onset of akinetic mutism. These findings reconfirm the complex pathology observed in prion diseases and overlap between neuropathology of different disease groups. Whether there is a correlation between increased tau accumulation in the CNS tissue with more severe clinical picture in prion diseases remains to be established. This may be further evidence towards the interplay between the intra and extracellular hyperphosphorylated tau and different protein isoforms essential for the pathogenesis of

neurodegeneration that would provide a potential therapeutic target for prion diseases but possibly also other tauopathies.



#### **4.4 Candidate gene analysis vs. neuropathological analysis in prion diseases**

In addition to protein heterogeneity we investigated the role of several candidate genes known to be associated with the process of neurodegeneration. Their role in prion diseases is poorly understood or completely unknown. However, as they govern some of the processes common to prion and other neurodegenerative diseases we investigated their role in our cohort of sCJD patients.

As shown in the results we found *MAPT* gene to be associated with both increased deposition of prion and tau protein. This finding did not surpass multiple testing and will therefore need to be replicated. This finding if confirmed provides further evidence for disease overlap and common pathological pathways in different neurodegenerative diseases. The conversion of soluble to insoluble filamentous tau protein is central to many human neurodegenerative diseases collectively referred to as tauopathies. Therefore tau presents a potential therapeutic target with broad relevance. Because the assembly of tau is concentration dependent, regional variation in expression of the protein determined by the variation in *MAPT* gene could favor or restrict its accumulation (Spillantini and Goedert, 2013). Although some tau sites are more phosphorylated in the diseased than in the healthy brain, others are phosphorylated only in the diseased brain. Tau phosphorylation seems to precede filament assembly, but whether this process is necessary for protein aggregation remains unknown. Distinct brain regions and cell types are affected in different human tauopathies. These differences are partly due to different isoforms of filaments. Interestingly, all 6 isoforms are present in tau filaments in the brains of patients with Alzheimer's disease whereas filaments in patients with cortico-basal degeneration and progressive supranuclear palsy are made of four-repeat tau and tau filaments of patients with Pick's disease are made of three-repeat tau (Delacourte et al., 1996; Flament et al., 1991; Spillantini et al., 1997).

*MAPT* in populations of European descent is characterized by two haplotypes that result from a 900-kb inversion (H1) or non-inversion (H2) polymorphism. Inheritance of the H1 haplotype is a risk factor for progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and idiopathic Parkinson's disease (IPD) (Conrad et al., 1997; Houlden et al., 2001; Pastor et al., 2000). Subhaplotype H1c in a regulatory region in intron 0 of *MAPT* is associated with increased risk of PSP and CBD. The association of the H1 *MAPT* haplotype with PSP has a higher odds ratio than the association between *APOE4* and Alzheimer's disease. *APOE4* is the major risk factor allele for late-onset Alzheimer's disease (Corder et al., 1993). The H2 haplotype is associated with increased expression of exon 3 of *MAPT* in grey matter, suggesting that the inclusion of exon 3 is protective against PSP, CBD and PD. Thus tau isoforms containing exon 2 and 10 promote tau aggregation and exon 3-containing isoforms inhibit tau accumulation. Additional receptors for oligomeric amyloid- $\beta$  are likely to exist and it remains to be seen whether their protein aggregates can activate the same pathway.

Although most drug trials in Alzheimer's disease so far have focused on amyloid- $\beta$ , interest in tau-targeted treatments is becoming more relevant because of the discovery of the important role of tau in neurodegeneration and the difficulties associated with targeting of amyloid- $\beta$  to develop mechanism-based treatments for Alzheimer's disease. The pathway leading from soluble and monomeric to hyperphosphorylated insoluble tau is central to human tauopathies. Therefore, inhibition of aggregation and disassembly of tau aggregates are both promising therapeutic possibilities. The hyperphosphorylation of tau seems to be an early and crucial event in tau-mediated neurodegeneration. The amount of phosphorylation depends on the conformation of tau and on the balance between the activities of tau kinases and phosphatases.

If tau aggregates propagate between cells, they are likely to pass through an extracellular stage. The removal of extracellular tau aggregates by specific antibodies constitutes a potential mechanism-based treatment. Interference with the spread of tau aggregates at a preclinical stage might therefore prevent the appearance of clinical features in tauopathies but also other neurodegenerative disorders characterized by tau accumulation such as prion diseases.

#### **4.5 Copy number variants as susceptibility loci to prion disease**

In this study we report the first genome-wide analysis for risk conferred by structural variation in human prion diseases. They have not been previously studied in these disorders. CNVs in non-coding regions can have profound effect on human phenotypes by altering the copy number of an entire gene that is predisposed to dosage effect (Klopocki and Mundlos, 2011). CNVs can also result in position effects and cause long-distance effects as far as 1Mb from the translocation breakpoints. CNVs have an impact on the miRNA-mediated post-transcriptional network by modulating or predisposing to certain complex genetic diseases (Persengiev et al., 2013). Whereas single-nucleotide polymorphisms and their role in disease pathogenesis have been studied extensively, the role of structural genomic variants is still poorly understood. They have been associated with several neuropsychiatric disorders such as autism, schizophrenia and bipolar disorder. They have also been associated with PD and early onset AD which supports the possibility of the existence of CNVs-driven mechanisms in the process of neurodegeneration and brain aging.

In order to identify common variants that associate with complex diseases multiple genome-wide association studies have been performed. Some of the strongest effects of common variants have been found in late-onset diseases and in drug response. The major histocompatibility complex has also shown association with a variety of disorders. Although there have been some positive findings in neuropsychiatric genetics, common variation has explained little of the high heritability of these traits. In contrast, early studies of rare copy number variants have led rapidly to a number of genetic loci that strongly associate with neuropsychiatric disorders. It is likely that the use of whole-genome sequencing to investigate the role of variation in neuropsychiatric and neurodegenerative disorders will greatly advance our understanding of these conditions.

Maillard et al (Maillard et al., 2014) investigated the variation in brain anatomy in 16p11.2 deletion and duplication carriers. They showed that the number of genomic copies is negatively correlated to the grey matter volume and white matter tissue properties in cortico-subcortical regions implicated in reward, language and social cognition. The pattern of brain anatomy changes in carriers overlapped with the well-established structural abnormalities associated with autism spectrum disorders and schizophrenia. A combination of molecular, neuroimaging and clinical approaches will help a better understanding of contribution of genes to neuropsychiatric and neurodegenerative disorders by measuring their effect on local brain anatomy.

A recent review by Flaherty et al (Flaherty and Brennand, 2015) using patient-derived human induced pluripotent stem cells (hiPSCs) to model neuropsychiatric copy number variations argues that it has the potential to reveal the underlying disease mechanisms in schizophrenia. Genetic risk in schizophrenia is thought to be conferred by both common variants of relatively small effect and rare variants with high penetrance. Genetically engineered mouse models can recapitulate rare variants, displaying some behavioral defects associated with schizophrenia; however, these mouse models cannot recapitulate the full genetic architecture underlying the disorder. hiPSCs present an alternative approach for studying rare variants, in the context of all other risk alleles. Genome editing technologies, such as CRISPR-Cas9, enable the generation of isogenic hiPSC lines with which to examine the functional contribution of single variants within any genetic background. Studies of these rare variants using hiPSCs have the potential to identify commonly disrupted pathways in schizophrenia and allow for the identification of new therapeutic targets. It is possible that some of these techniques will be applicable in better understanding of role of CNVs in neurodegenerative diseases such as prion diseases.

CNVs encompass more nucleotide content per genome than SNPs thus their significance to genetic diversity is likely to be high. A genome-wide map of CNVs shows that between 6-19% of each individual's chromosomes exhibit CNVs. The mechanisms involved in the progress of brain aging and associated with neurodegenerative diseases are complex and are likely to be a result of disturbance of numerous genes. Thus, miRNAs dysregulation that would inevitably alter the expression of multiple genes might provide a better understanding for neuronal cell deterioration. Multiple factors participate in the control of miRNA expression. Persengiev et al (Persengiev et al., 2013) argue that CNVs-miRNA interactions are an important part of increased brain susceptibility to external and internal influences during the aging process. The authors highlight the urgency to annotate CNVs which can potentially alter miRNA expression and should be considered high-priority candidates in genotype-phenotypes mapping studies of brain-related disorders.

In this study we investigated the role of CNVs as possible novel susceptibility loci to prion diseases. The initial analysis revealed several CNV duplications with genome-wide significance on chromosomes 6, 7, 10, 12 and 14. The analysis was performed using CNV Partition which is a software supported by Illumina Genome Studio. The software was selected following analysis of the performance of three different softwares: Penn CNV, QuantiSNP and CNV Partition. CNV Partition was found to select the highest number of even very small deletions and duplications. This software has high sensitivity however it is potentially prone to making a higher number of false positive associations. In order to avoid these we selected a high confidence value and used stringent quality control criteria. The finding on chromosomes 10 and 14 were further confirmed using real-time PCR. This finding was not replicated when we tested the German CJD cohort and compared with the German control samples. The reason for this may be because the analysis of the German cohort was performed on an older platform which included only 550 000 probes that have not been enriched to detect CNVs. These were mainly SNP

probes and when compared to the 670K UK project contained a significantly lower number of CNV probes. It is possible that the German analysis was not sensitive enough to detect the significant associations observed in the UK CNV analysis.

The other explanation may be the patient selection criteria. German patients were all neuropathologically confirmed cases of classic sCJD with a rapidly progressive dementia, while the UK cases were more phenotypically heterogenous and included a number of probable sCJD patients. The UK and German patients were not matched for age, gender, duration and clinical presentation as this would have led to a significant reduction in sample size. However, majority of patients in both cohorts were in their 60ies at the time of presentation and had disease duration of several months. It may be that the CNV duplications detected in the UK population are associated with more atypical phenotypes and were therefore not replicated in the German case/control analysis.

Furthermore, CNV duplications on chromosomes 10 and 14 were not detected when the analysis was repeated on a larger number of UK cases and controls. The repeat analysis was performed using Penn CNV. This software has been widely used and is well described in the published literature. It is very sensitive for detecting large CNV deletions and duplications. It is less reliable for detecting small CNVs. It may be that the associations were not possible to detect due to the small sizes of the duplications. Otherwise, it is possible that the association detected using CNV Partition are spurious and for that reason were not replicated on a larger sample set or in a different population group.

An interesting association at *PARK2* was the top ranked locus in the genome wide analysis however it did not surpass the threshold required for statistical significance. A role for heterozygous exon deletion in the etiology of Parkinson's disease remains unclear. In the most detailed population study to date CNVs overlapping exons 5-12 of *PARK2* were found in PD cases but not in controls. Our findings were

similar, in that we found 3 deletions of *PARK2* exons 8 and 9 in sCJD cases, but no CNVs overlapped these exons in the WTCCC control series. In order to further support an association at this locus we looked for evidence of replication in the other disease cohorts, and by testing for heterozygous causal SNPs in *PARK2* in prion disease remain speculative.

Mutations in the *PARK2* gene have been associated with juvenile Parkinsonism and there have been reports of the association with peripheral neuropathy (Vital et al., 2015). The autosomal recessive form of juvenile Parkinsonism is considered to be the most common form of familial PD and has been identified in European, North American, Turkish and Japanese families. It is known that the role of parkin protein in the brain is to break down misfolded proteins such as ubiquitin-protein ligase (Hattori, 2002). Although there are exceptions, the lack of Lewy bodies in parkin-mutated brains suggests its fundamental role in Lewy body formation. The elucidation of the exact role of parkin protein provides the mechanisms of the formation of Lewy bodies in common forms of sporadic PD. Therefore, it is important to detect the substrates for the parkin protein. Recently, several candidate substrates have been reported including CDCrel-1, synphilin-1, alpha-synuclein-22 (o-glycosylated alpha-synuclein), and Peal-receptor. The question is accumulation of which substrates are responsible for the nigral neuronal death in Park 2 linked brain. CNV deletion in this region provides further evidence for the overlap between related neurodegenerative conditions and possible common neuropathological mechanisms in disease pathogenesis as already discussed.

The possible role for altered expression of *PRNP* in resistance or susceptibility to prion disease has long been speculated since it was known that transgene copy number and the hemizygous state in mouse conferred strong effects on incubation time to mouse prions (Bueller et al., 1993), and suggestive haplotypic associations were found at the *PRNP* locus in sCJD (Mead et al., 2001). However, subsequent



larger GWA studies did not confirm the initial haplotype findings at *PRNP* (Mead et al., 2011b) and CNVs were not identified in a case series focusing on *APP* and *PRNP* (McNaughton et al., 2012). The identification of a duplication overlapping *PRNP* in a UK Blood Service control sample and not in cases is further evidence against the potential causal association of increased PrP expression due to *PRNP* duplication.

The absence of *PRNP* deletion in elderly women resistant to kuru is intriguing as this group experienced intense selection pressure during the kuru epidemic (Mead et al., 2009b). Potential explanations include the incompatibility of healthy human adult life with a half-dose of *PRNP*. Alternatively, the dominant-negative effects of amino acid changing variants of *PRNP*, such as 127V, may offer greater opportunities for selection to take place.

## **4.6 Future Work**

This study revealed several interesting and potentially important findings.

1. Heterozygosity at codon 219, a polymorphism common in the Asian populations is protective against sCJD but appears neutral or may even confer susceptibility to vCJD. Our finding was based on the 2 samples in a large referral series at the MRC Prion Unit due to its rarity in the UK population. It would be of interest to investigate the relevance of this polymorphism in susceptibility to vCJD in a larger population set exposed to the BSE epidemic. Other genetic loci with modest effects may play a role and have cumulative impact on the susceptibility and phenotypic heterogeneity in prion diseases.
2. We described 2 vCJD patients with unusual clinical presentation (in terms of age at presentation and MRI findings) who were correctly diagnosed following neuropathological analysis. Atypical clinical cases should be investigated not only to prevent misdiagnosis but also because they may be associated with novel genetic loci and PrP<sup>Sc</sup> strain types.
3. MAPTH1c haplotype appeared to play a role in both prion protein and hyperphosphorylated tau deposition. The finding provides evidence for possible link between prion disease and other neurodegenerative conditions and should be repeated in a larger sample set.
4. There was no strong evidence that CNVs play a significant role in susceptibility to prion diseases. As CNVs encompass such a large proportion of the human DNA and their role remains poorly understood further collaborative studies would be justified on a larger number of samples. In this circumstance a case-control analysis should be performed using the same platform for DNA analysis and software for data interpretation to prevent potential discrepancies due to probe

and analysis differences. If possible, the patients should be matched for age, gender and disease subtype although this may lead to a reduction of an already small sample set.

5. Our studies demonstrated that patient stratification and detailed assessments using MRC Rating Scale allowed collection of a comprehensive data set on disease progression in CJD patients. Therefore, only 90 patients would provide adequate study power to evaluate new therapeutic approaches in future clinical trials.

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## Tau, prions and A $\beta$ : the triad of neurodegeneration

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**Abstract** This article highlights the features that connect prion diseases with other cerebral amyloidoses and how these relate to neurodegeneration, with focus on tau phosphorylation. It also discusses similarities between prion disease and Alzheimer's disease: mechanisms of amyloid formation, neurotoxicity, pathways involved in triggering tau phosphorylation, links to cell cycle pathways and neuronal apoptosis. We review previous evidence of prion diseases triggering hyperphosphorylation of tau, and complement these findings with cases from our collection of genetic, sporadic and transmitted forms of prion diseases. This includes the novel finding that tau phosphorylation consistently occurs in sporadic CJD, in the absence of amyloid plaques.

### Cerebral amyloid, tau phosphorylation and cell death: how are they connected?

Human prion diseases represent a clinically and pathologically diverse group of neurodegenerative disorders

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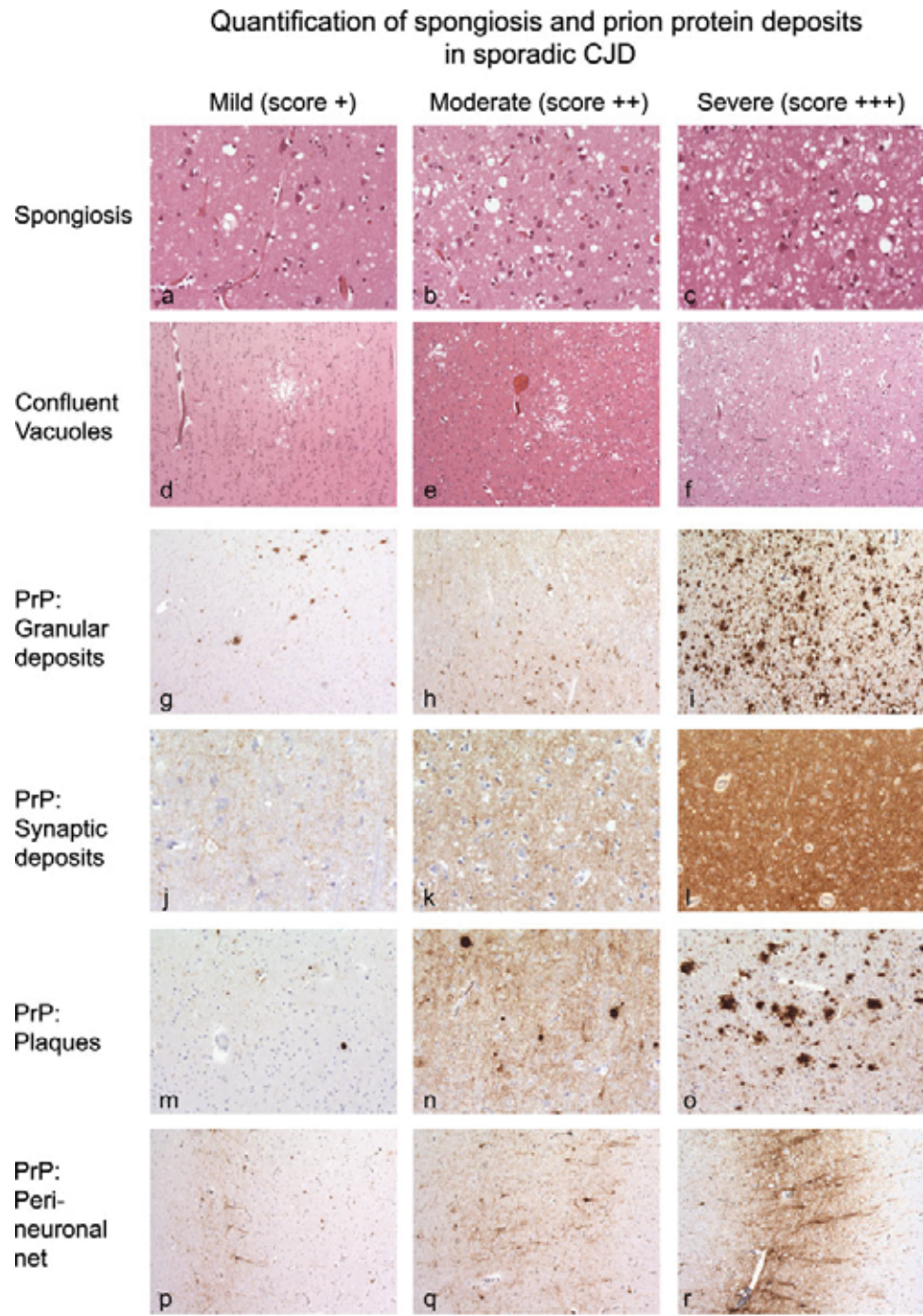
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including (1) sporadic Creutzfeldt–Jakob disease (sCJD), (2) inherited forms of prion diseases (inherited prion disease, IPD) such as Gerstmann–Sträussler–Scheinker syndrome (GSS) or fatal familial insomnia (FFI) and (3) acquired forms, such as variant CJD (vCJD), iatrogenic CJD (iCJD) and Kuru. We avoid the term “familial CJD”, as CJD was originally defined as a clinicopathological syndrome which is distinct from e.g. GSS or FFI. The term IPD links the different syndromes by pathogenesis and is therefore preferred.

According to the protein-only hypothesis [40], infectious prions are composed predominantly, if not entirely, of aggregates of misfolded, host-encoded, cellular prion protein (PrP<sup>C</sup>), commonly designated PrP<sup>Sc</sup> [83]. PrP<sup>Sc</sup> arises from normal prion protein (PrP<sup>C</sup>) through conformational conversion. The common neuropathological feature of prion diseases is a predominantly extracellular accumulation of PrP<sup>Sc</sup> in the central nervous system. Prion protein deposits are highly variable in their intensity (Fig. 1; Table 1), pattern, i.e. plaques (Fig. 1m–o), perineuronal labelling (Fig. 1p–r), synaptic deposition (Fig. 1j–l) and formation of coarse granular deposits (Fig. 1g–i) and their distribution within the CNS. These deposits correspond biochemically to amyloid, i.e. aggregates of protein with high content of  $\beta$ -sheets. The deposits are accompanied by spongiform change with neuronal vacuolation and degeneration (Fig. 1a–f) and astrocytic and microglial reaction, all of which can vary considerably within the CNS and between different individuals.

Whilst prion diseases can be readily transmitted via various routes and between species, the most common A $\beta$  amyloidosis is transmissible at a considerably lower efficiency, and has to date been limited to experimental settings [29]. Another well recognised CNS amyloidosis, familial British dementia (FBD) has not been experimentally tested for transmissibility.

**Fig. 1** Spongiform changes and PrP deposition in sporadic prion disease: severity of spongiform changes, ranging from mild to moderate and severe, with diffuse, homogenous spongiform changes (**a–c**) and patchy confluent vacuolisation (**d–f**). Patterns and intensities of commonly observed prion protein deposits: **g–i** coarse granular deposits of prion protein, which may form confluent aggregates. **j–l** Synaptic PrP, ranging from mild to severe, and **m–o**, formation of plaques in sporadic CJD. All patterns and intensities are used in the scoring scheme to compare with tau burden in our series; 160  $\mu\text{m}$  (**a–c**, **m–o**), 300  $\mu\text{m}$  (**d–f**, **p–r**), 80  $\mu\text{m}$  (**j–l**)



In the last few years, a striking number of epidemiological, neuropathological, and biochemical similarities between prion diseases and Alzheimer's disease have been identified, in particular the fact that there are interactions between the two proteins [41, 59, 74] and between the signalling pathways involving both proteins.

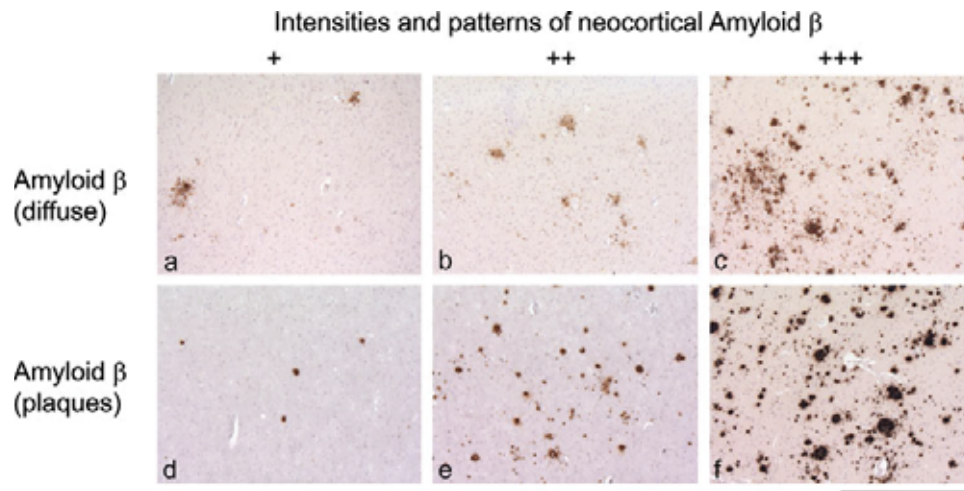
Epidemiologically and clinically, both disorders are dementing illnesses that mainly occur sporadically, but can also occur as familial forms. Histopathologically, both are

characterised by the deposition of an extracellular amyloid that is produced by neurones. In both diseases, there is formation of amyloid oligomers and ultimately also of solid amyloid aggregates in the brain (Figs. 1g–o, 2a–f; Table 1): both amyloid proteins can accumulate diffusely (Fig. 2a–c), or they can exhibit prominent and widespread deposition of dense amyloid plaques with a diameter of more than 200  $\mu\text{m}$  (Fig. 2d–f), and both elicit a considerable astrocyte and microglial reaction, variable neuronal

**Table 1** Description of features used to score pathological findings

Feature	Pattern	Score 1	Score 2	Score 3
Spongiform degeneration		Fine, diffuse (Fig. 1a)	Intermediate (Fig. 1b)	Severe; formation of large, partly confluent vacuoles (Fig. 1c)
Prion protein	Granular	Occasional disseminated granules, or infrequent patches. Can be associated with synaptic PrP (see Fig. 1g)	Frequent disseminated and partly confluent granules. Often associated with synaptic deposits (see Fig. 1h)	Very dense, often confluent granules. Can dominate the entire grey matter (see Fig. 1i)
	Synaptic	Low density or patchy (Fig. 1j)	Intermediate, diffuse (Fig. 1k)	Strong, diffuse (Fig. 1l)
	Plaques	Low frequency (Fig. 1m)	Intermediate frequency (Fig. 1n)	High density/frequency (Fig. 1o)
	Perineuronal net	Occasional patches or very fine, delicate decoration of neurones. Typically deep cortical layers. (Fig. 1p)	Contiguous network of perineuronal labelling. Mini plaques often present (Fig. 1q)	Dense network of perineuronal labelling. Often small or medium sized plaques. Also combined with synaptic deposits (Fig. 1r)
A $\beta$	Diffuse	Rare diffuse A $\beta$ deposition (Fig. 2a)	Frequent diffuse deposits (Fig. 2b)	Very dense, often widespread diffuse deposits (Fig. 2c)
	Plaques	Equivalent to CERAD low (Fig. 2d)	Equivalent to CERAD intermediate (Fig. 2e)	Equivalent to CERAD high (Fig. 2f)
Tau phosphorylation	Prion protein associated	Very occasional stubs or rod-like inclusions <75 per 10 HPF (Fig. 3a)	Moderately frequent density of rod-like inclusions (75–500/10 HPF (Fig. 3b)	Frequent deposition of rod or stub like inclusions (>600/10 HPF), often forming coarse granular aggregates (Fig. 3c)
	A $\beta$ associated	Rare threads (Fig. 3d)	Occasional tangles, threads if intermediate density (Fig. 3e)	Frequent tangles and dense meshwork of threads (Fig. 3f)

Reference is given to figures that illustrate the respective pathological feature; the score “0” is not specifically mentioned, as it is regarded as self-explanatory



**Fig. 2** Intensities of A $\beta$  deposits: **a–c** diffuse protein deposits without formation of plaques (ranging from mild to severe). **a** Score 1 (mild) describes occasional patchy deposits, occasionally seen on low power magnification. **b** Moderate, score 2 describes deposits that are seen in <50% of adjacent low power fields. **c** An example of

heavier diffuse amyloid beta burden, indicating a presence in most of the low power field on a cortex section. Dense core amyloid beta plaques, approximately corresponding to CERAD sparse (**d**), intermediate (**e**) and high (**f**). Scale bar 300  $\mu$ m (**a–f**, **g–i**)

loss, and occasionally deposition of amyloid in or around vessels walls.

Biochemically, both diseases are characterised by aggregation of a protein that is encoded and expressed by the host. It was recently suggested that A $\beta$ 42 may act through a PrP receptor [41, 59]. Experimental data suggest that there is a functional link between PrP<sup>C</sup> and A $\beta$  processing: (1) knockdown of PrP<sup>C</sup> in N2A cells increases A $\beta$  levels in vitro, (2) PrP knockout mice as well as scrapie-infected mice show increased A $\beta$  level and (3) PrP<sup>C</sup> overexpression reduces A $\beta$  formation by downregulating the APP cleaving enzyme  $\beta$ -secretase [77].

Recent genetic evidence also links prion disease to Alzheimer's disease, in that the APOE-E4 allele, a well-established risk factor for AD, also may increase the risk for sporadic CJD [57], but surprisingly may delay onset of inherited prion disease with the P102L mutation [92]. A detailed discussion of the similarities between CJD and AD, the relationship between codon 129 polymorphism and a model of A $\beta$ 42 action through PrP<sup>C</sup> receptor are given in a review by Gunther and Strittmatter [41].

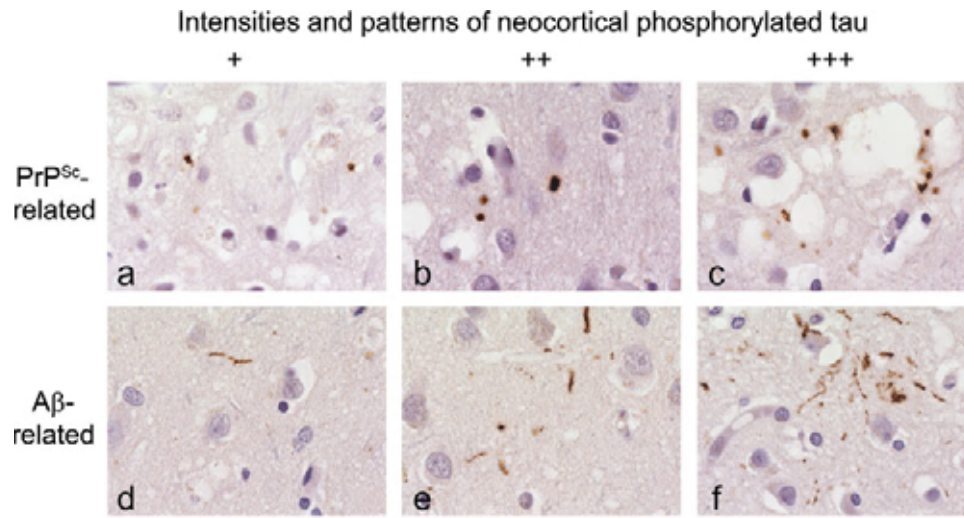
### The amyloid cascade hypothesis

According to the amyloid cascade hypothesis, proposed by Hardy and Higgins [44] the increased production or decreased clearance of amyloid beta (A $\beta$ ) peptides results in the accumulation of the hydrophobic A $\beta$ 40 and A $\beta$ 42 peptides with subsequent aggregation and formation of insoluble plaques. This induces a cascade of deleterious changes, such as neuronal death and eventually causes Alzheimer's

disease. Since then, this hypothesis underwent several transformations due to the accumulating data supportive of or inconsistent with the theory [81]. The current version assumes a toxic role of soluble prefibrillar oligomers based on the several in vivo and in vitro experiments [37, 42]. The results contradicting these findings [61, 86, 90] and the recognised experimental artifacts [7] make it more difficult to elucidate their genuine role in disease development. However, a number of recent studies in transgenic mice have further strengthened the concept of the amyloid cascade hypothesis: intracerebral injection of A $\beta$  seeds trigger the aggregation of endogenous A $\beta$ : intracerebral inoculation of APP23 transgenic mice with brain homogenates from Alzheimer's patients or with brain extracts from aged APP23 transgenic mice elicits a marked anticipation of the disease in young APP23 mice [69]. This finding can be interpreted as prion-like transmission or as seeding process. The latter is a more likely scenario, as implantation of small steel wires coated with minute amounts of A $\beta$ -containing brain homogenate into the brain of APP23 transgenic mice triggered significant deposition of A $\beta$  in the CNS, whilst peripheral inoculation of these mice with A $\beta$  did not seed in the CNS [29]. It may be argued that the presence of a prion receptor, but not of an "A $\beta$ " receptor in peripheral tissues, such as nerve endings or immune cells.

### Cerebral amyloid and tau hyperphosphorylation: what is the trigger?

A prominent feature of cerebral amyloidoses is the induction of tau hyperphosphorylation. This is very well



**Fig. 3** Amyloid beta or PrP induced pattern of tau phosphorylation differ: **a–c** the range of PrP induced tau deposits. The scores of tau correspond to the scores of PrP shown in Fig. 1, i.e. tau score 1 is typically seen in areas with PrP score 1 and dense tau deposits (scores 2 or 3) is typically seen in areas with PrP scores 2–3. **d–f** In contrast,

A $\beta$  induces a threaded tau phosphorylation pattern, with elongated dystrophic neuritis but very little stub- or rod-tau. All examples are from the frontal or parietal cortex, and correspond to diffuse beta deposits scores 1–3. *Scale bar 25  $\mu$ m (a–f)*

established for Alzheimer's disease (AD), but is also a prominent feature in the rare FBD and has been sporadically described for prion diseases mostly in the context of the presence of plaques, such as in vCJD or in many inherited forms. A prominent feature of AD is the accumulation of hyperphosphorylated tau within or in the vicinity of cortical amyloid deposits (Fig. 3d–f). Importantly, this direct association of tau pathology in areas of A $\beta$  accumulation has to be separated from a probably independent process of tau phosphorylation that starts in the entorhinal cortex and spreads over the limbic system and finally extends into neocortical regions. This latter process has been characterised in detail and was formalised in a staging system developed and defined by Braak and Braak [10, 11]. The clinical dementia correlates better with the Braak stage than with the deposition of A $\beta$ .

FBD is an autosomal dominant, neurodegenerative disorder, presenting with dementia, spastic tetraparesis, and cerebellar ataxia, also known as Worster–Drought syndrome [68, 82, 85, 94]. Similar to Alzheimer's disease, in particular its inherited forms, the neuropathological hallmarks of FBD include extensive cerebral amyloid angiopathy (CAA), cerebellar degeneration with severe CAA and parenchymal amyloid plaques. There are also hippocampal amyloid plaques as well as neurofibrillary tangles, and white matter degeneration similar to that seen in Binswanger's disease [50]. Deposition of hyperphosphorylated tau in FBD is indistinguishable from that in AD, both immunohistochemically and ultrastructurally.

In prion disease, hyperphosphorylation of tau has been described, but it is not a well-known or well-characterised

feature. Not much is known about the relationship between disease duration, *PRNP* codon 129 genotype, glycoform, histological manifestations and the degree of tau phosphorylation. Several studies have reported the deposition of hyperphosphorylated tau in small series of sporadic, familial and variant forms of prion diseases. These reports highlight the role of prion amyloid plaques as an essential prerequisite to elicit tau phosphorylation and raise important questions related to the mechanism responsible for tau phosphorylation. A detailed discussion of these reports is given below.

#### **A $\beta$ , PrP and tau: their connection to cell cycle and cell death**

Several pathways are thought to play a role in neurodegenerative processes, some of which are unusual suspects. A number of cell cycle proteins have recently been implicated in neurodegenerative processes. CDK5, GSK3 $\beta$ , and pAkt are all well-characterised mediators of growth, survival and inhibitors of neur(on)al differentiation. All are now also linked to the family of neurodegeneration—prion protein, A $\beta$  and tau phosphorylation.

*CDK5*, a serine-threonine kinase, is a cell cycle protein that is also responsible for processes, such as axonal guidance, cortical layering and synaptic structure/plasticity, and it is mainly expressed in postmitotic neurones [18, 78]. Dysregulation of CDK5 has been implicated in neurodegeneration for some years [17, 18, 20, 71, 78, 88] and it is now likely to be involved in abnormal phosphorylation



of tau. In keeping, CDK5 inhibition alleviates tau phosphorylation and cytoskeletal lesions [96].

*GSK3 $\beta$*  has recently been identified as a likely candidate directly to phosphorylate tau and mechanistic links between *GSK3 $\beta$* , tau [60, 84] and A $\beta$  [45, 46, 51] have been established. A less well described, but mechanistically appealing connection has also been made to PrP, which makes the hypothesis of cell cycle related proteins and neurodegeneration attractive [79].

Another pathway that has been implicated in neurodegeneration involves *PTEN/pAkt*. The tumour suppressor PTEN antagonises the phosphorylation of Akt, hence downregulation of PTEN increases the phosphorylated, active form of Akt (pAkt), which has pro-survival, pro-proliferation effects and counteracts apoptosis and cell differentiation. However, whilst this function of pAkt is important and relevant for cells capable of self-renewal and proliferation, i.e. the developing CNS, it is different for quiescent/postmitotic cells, such as neurons, where constitutive activation of Akt can cause neurodegeneration [67], including abnormal phosphorylation of tau, mediated by CDK5 in a *GSK3 $\beta$*  independent fashion [56, 71]. Whilst the role of A $\beta$  triggering tau phosphorylation is well established [9, 38, 62, 75], a recent study also showed that tau phosphorylation is transmissible too, which may have wide ranging implications for the concept of the involvement of A $\beta$  as the sole trigger for hyperphosphorylated tau in Alzheimer's disease [23].

Experimental evidence for the connection of tau, A $\beta$  and PrP comes from work of from Perez et al. [79]. Using PrP 106–126 peptides, a widely used paradigm to test prion toxicity in vitro, *GSK3 $\beta$*  mediated tau phosphorylation was induced. Other studies provide compelling evidence that CDK5, PrP and A $\beta$  are mechanistically connected and involved in neurodegeneration [64, 65].

### The PrP–tau connection (I): inherited prion diseases and phosphorylation of tau

Inherited prion diseases display a wide spectrum of pathological deposition of prion protein. The formation of conspicuous and well-demarcated amyloid plaques is typically seen in inherited forms with codon P102L (Figs. 4m, 5c) P105L, and A117V mutations (Figs. 4n, 5d), whilst D178N or E200K (Figs. 4o, 5e) mutations show less well-defined plaque pathology. Other mutations, such as octapeptide repeat insert (OPRI) mutations present histologically with a unique striping pattern of the cerebellum (Fig. 4k, l).

Reports of tau pathology in inherited prion diseases consistently describe marked dystrophic neurites with hyperphosphorylated tau, accentuated in the vicinity or

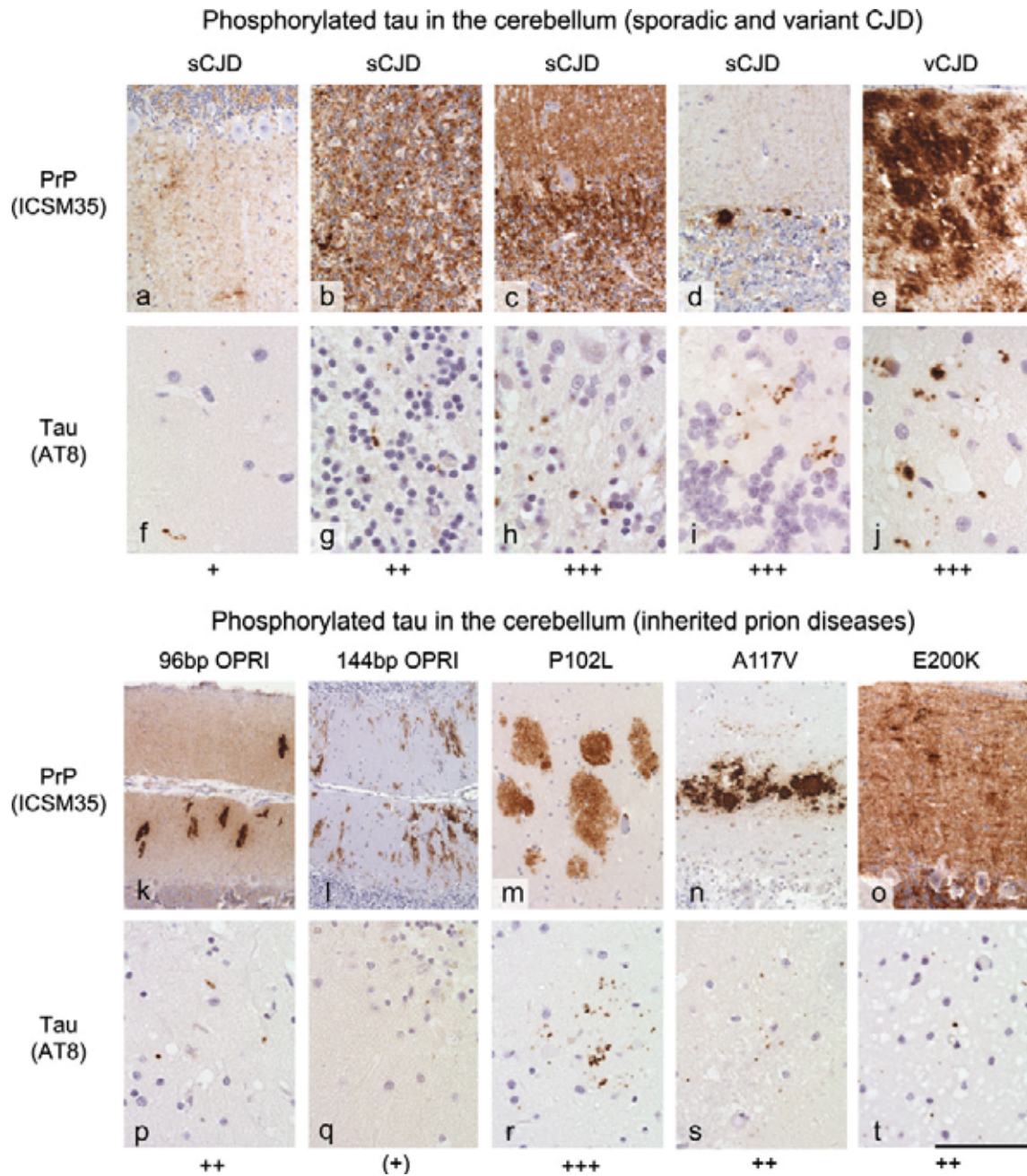
located within amyloid plaques. The first reports were those of classical GSS with the *PRNP* P102L mutation [5, 8, 34, 35, 73, 89]. Later studies of P102L GSS with detection of abundant phospho-tau concluded that this may be an effect of PrP-mediated phosphorylation rather than a A $\beta$  related effect, as there were only minimal A $\beta$  deposits seen [52]. However, it may be argued that this latter study detected tau phosphorylation in the entorhinal cortex which has formed independently from the prion amyloid deposition, in the context of Alzheimer's disease corresponding to Braak and Braak stage I. Other mutations associated with the clinical phenotype of GSS (A117V mutation [91]), or P105L [53, 95] reported similar findings. In vitro experiments with a prion protein peptide carrying the A117V mutation decreased the rate of microtubule formation more efficiently than wild-type PrP106–126. This was thought to be related to the displacement of tau, where A117V mutation is more efficient at inhibiting microtubule formation [13].

OPRI mutations, such as 96 bp [21], or 144 bp inserts [22] into the N-terminal octarepeat region, are characterised by a unique pattern of immunoreactivity for PrP, which is oriented perpendicularly to the cerebellar surface (Fig. 4k, l), also show a marked tau phosphorylation, which co-localises with PrP deposits. A case report of a 216 bp OPRI mutation instead showed a pattern different from those with a shorter insert, with the formation of large amyloid plaques, again co-localising with hyperphosphorylated tau [27].

Finally, the stop mutation 145X [33] with formation of plaques and cerebral amyloid angiopathy (“PrP-CAA”) showed similar results with tau co-localising to plaques.

### The PrP-tau connection (II): tau phosphorylation in sporadic and variant CJD

Following the observation of hyperphosphorylated tau in inherited prion diseases with remarkable tropism to amyloid plaques, but not in sCJD, several studies analysed this phenomenon further: Giaccone et al. [36] compared tau phosphorylation patterns of AD and vCJD and found the same three bands of 68, 64 and 60 kDa in an immunoblot probed for tau. Morphologically, hyperphosphorylated tau co-localised with florid plaques. However, it was also found that unlike in AD, there was no detectable soluble hyperphosphorylated tau in vCJD. In contrast to the findings that we report in our series (see below), their study did not detect hyperphosphorylated tau in brains of sCJD patients [36]. A study comparing the ultrastructure of prion amyloid in GSS and vCJD [87] came to a similar conclusion, but in addition found that dystrophic neurites containing hyperphosphorylated tau occurs in sCJD with small plaques. It was concluded that plaque-forming prion



**Fig. 4** Prion protein-triggered tau phosphorylation in the cerebellum: *upper row* deposition of abnormal PrP; sporadic CJD with synaptic PrP deposition (**a–c**) or with small plaques (**d**). **e** vCJD with heavy PrP burden with diffuse deposits as well as plaques. The corresponding tau phosphorylation is shown below (**f–j**): it is approximately proportional to the PrP<sup>Sc</sup> burden and is also closely associated with plaques. However, an exceptionally strong tau deposit is consistently seen in vCJD cerebella (**i**). Inherited forms of prion disease show variable amounts of p-tau deposits, which do not correlate with the

PrP burden and also do not correlate with the pattern (plaque or synaptic): The pattern of abnormal PrP deposition is distinct for each mutation. 96 and 144 bp OPRI form a characteristic striping pattern perpendicularly to the cerebellar surface (**k**, **l**) with moderate tau labelling in the 96 bp OPRI and very little tau in the 144 bp OPRI. The extent of tau phosphorylation appears to be independent of the plaque load, with P102L and A117L cases showing similar tau burden as in the E200K which features synaptic PrP deposition. *Scale bar* 120  $\mu$ m (**a–e**, **n**, **o**); 250  $\mu$ m (**k**, **l**, **m**) and 40  $\mu$ m (**f–j**, **p–t**)

diseases are capable of generating phospho-tau deposits, but forms with synaptic PrP deposits may be incapable to do so [87].

To examine this further, we examined 79 brains from patients with sporadic CJD and compared them to 12 cases with inherited prion disease and 5 vCJD cases. We can

demonstrate for the first time that there is indeed presence of hyperphosphorylated tau in sCJD with synaptic (Figs. 1j–l, 6i, j) or pericellular PrP, notably also in the absence of plaques (Figs. 3a–c, 6a, b, e, f). When considering with the previous reports, we detected a very substantial formation of phospho-tau positive rods in five cases of vCJD, including two cases of blood transfusion related transmission (Fig. 4j). In those cases with concomitant A $\beta$  amyloid pathology (mild,  $n = 14$ ), we found both patterns, namely granular, rod-shaped PrP-induced tau inclusions (Fig. 3a–c) and thread-shaped A $\beta$  related formations (Fig. 3d–f). Cases of inherited prion disease (P102L, A117V, D178N, E200K, 96 and 144 bp octarepeat insert) show accumulation of tau directly associated with prion protein plaques (Figs. 4p–t, 5f–j).

### The PrP-tau connection (III): neocortical PrP<sup>Sc</sup> and pTau correlate in sporadic and in inherited forms of prion diseases

In our series of sporadic, inherited and variant forms of prion diseases ( $n = 79$ ), we found a correlation of prion protein burden (Fig. 1) and the formation of rod or stub-shaped tau deposits in the neocortex (Fig. 3; Tables 2, 3). Previously, the ability of prion protein to hyperphosphorylate tau was thought to be associated with the presence of amyloid plaques, as seen in vCJD, GSS or certain forms of sCJD. We show here in a large cohort, that indeed synaptic PrP is directly associated with the presence of minute deposits of hyperphosphorylated tau. Regions in which prion protein deposits across the entire thickness of the cortex also show the presence of tau rods in the same area (Fig. 6i, m). Other cases where the burden of abnormal prion protein is restricted to deeper cortical layers show a distribution of tau rods congruent with prion protein accumulation and neurodegeneration (Fig. 6a, e). In sCJD, deposition of tau significantly correlates with the intensity of prion protein load (correlation coefficient  $r = 0.69$ ,  $p < 0.01$ ), but does not correlate with disease duration ( $r = -0.037$ , n.s.). Importantly, the size of tau granules in sCJD with homogenous synaptic PrP was smaller than that in sCJD with formation of large PrP granules.

To exclude that the tau phosphorylation was induced by coexisting A $\beta$  amyloid, we stratified for presence or absence of A $\beta$  amyloid (Tables 2, 3). Almost half of the cases ( $n = 36$ ) were entirely free of any form of A $\beta$ , a small number showed patchy, mild and diffuse deposits of A $\beta$  (corresponding to Fig. 2a) ( $n = 13$ ) and the remainder showed more intense diffuse A $\beta$  and/or core plaques (see Fig. 2b–e) ( $n = 27$ ). The morphology of hyperphosphorylated tau associated with abnormal prion protein is remarkably distinct from that elicited by A $\beta$ , in particular

in cases of synaptic PrP deposition (Fig. 3). Prion protein related tau hyperphosphorylation shows as short stub- or rod-like structures (Fig. 3a–c). The most subtle deposition forms small rod- or stub-like punctate inclusions (granules). Their shape resembles granules seen in argyrophilic grain disease [31]. They do not extend to fibrillary or “neuritic” tau, whilst the most subtle deposition of A $\beta$ -associated tau fibrils occurs in the form of thin, single neuritic threads in the cortex (Fig. 3d). The next stage of A $\beta$ -induced tau phosphorylation is a more frequent presence of fibrils (Fig. 3e), and finally the A $\beta$ -induced tau pathology amounts to a delicate network of dystrophic processes (Fig. 3f), including neurofibrillary tangles. Amyloid plaques are generally surrounded by a small corona of dystrophic neurites, which are well known and have been frequently described in the literature. The temporal and entorhinal cortex was excluded from the analysis because this area shows a tau pathology that emerges independently from A $\beta$  or prion protein pathology, and is likely to represent an independent pathogenic process, which may explain the findings of Ishizawa et al. [52]. It is possible, but difficult to prove whether phosphorylation of tau in this area may be enhanced by the deposition of prion protein.

Those forms of prion diseases, which show labelling of perineuronal networks and small plaques [87], show tau rods in the vicinity of the perineuronal nets and around small plaques. In our series, microplaques in sCJD (Fig. 4d) as well as inherited forms with prominent plaques (Fig. 5) showed an obvious accumulation of tau at a higher density (Fig. 6e–h) forming short processes resembling dystrophic neurites. However, these intraplaque and periplaque neurites were still more granular than those associated with A $\beta$  plaques. Importantly, this phenomenon was observed in the absence of any A $\beta$  pathology. The most straightforward explanation of PrP-associated tau phosphorylation is a simple dose-dependent direct toxicity whereby PrP amyloid is directly involved in the process. Alternatively, a critical level of toxic species may be produced during the conversion process, which is thought to involve a number of conformational intermediates or side products during prion conversion and propagation, variably named as PrP\* [1] or PrP<sup>L</sup> (lethal) [24]. These toxic (by-) products, may directly or indirectly trigger pathways mentioned above and hence contribute to tau phosphorylation. However, considering the kinetics of abnormal protein accumulation in prion diseases [24], and the relative abundance of prion amyloid in comparison to hyperphosphorylated tau, makes the latter a likely side effect rather than a main trigger of prion neuropathogenesis. However, the dissection of the events involved in tau phosphorylation may well be the key to understand prion neurotoxicity.

**Table 2** Summary of all cases analysed in this study

Cases included in the study												
	MM			MV			VV			NA		$\Sigma$
sCJD	31			10			12			5		<b>58</b>
IPD	8			5			1			2		<b>16</b>
vCJD	5			0			0			0		<b>5</b>
$\Sigma$	<b>44</b>			<b>15</b>			<b>13</b>			<b>7</b>		<b>79</b>
PrP deposition in the frontal cortex												
	No A $\beta$			Very mild Diffuse A $\beta$ (A1)			Moderate diffuse A $\beta$ (A2) and or Plaques (P1-3)					
score	1	2	3	1	2	3	1	2	3			
sCJD MM	3	8	4	1	1	0	2	6	4		<b>29</b>	
sCJD MV	1	1	0	0	2	0	2	4	0		<b>10</b>	
sCJD VV	0	2	1	2	2	0	1	2	2		<b>12</b>	
sCJD ND	1	1	1	0	1	0	0	0	1		<b>5</b>	
IPD	2	1	6	1	1	1	0	0	3		<b>15</b>	
vCJD	0	1	3	0	1	0	0	0	0		<b>5</b>	
$\Sigma$	<b>7</b>	<b>14</b>	<b>15</b>	<b>4</b>	<b>8</b>	<b>1</b>	<b>5</b>	<b>12</b>	<b>10</b>		<b>76</b>	
Tau deposition in the frontal cortex												
	No A $\beta$			Very mild Diffuse A $\beta$ (A1)			Moderate or severe diffuse A $\beta$ (A2, A3) and / or Plaques (P1-3)					
score	1	2	3	1	2	3	1	2	3	4		
sCJD MM	4	9	1	1	1	0	2	6	3	1	<b>28</b>	
sCJD MV	0	2	0	2	0	0	1	3	1	1	<b>10</b>	
sCJD VV	0	2	1	0	2	1	1	3	1	0	<b>11</b>	
sCJD ND	0	3	0	0	0	1	0	1	0	0	<b>5</b>	
IPD	1	2	5	0	1	1	0	2	1	0	<b>13</b>	
vCJD	0	0	4	0	0	1	0	0	0	0	<b>5</b>	
$\Sigma$	<b>5</b>	<b>18</b>	<b>11</b>	<b>3</b>	<b>4</b>	<b>4</b>	<b>4</b>	<b>15</b>	<b>6</b>	<b>2</b>	<b>72</b>	
Tau deposition in the cerebellum												
	No A $\beta$											
score	0	1	2	3								
sCJD MM	1	15	9	0							<b>25</b>	
sCJD MV	0	5	4	0							<b>9</b>	
sCJD VV	0	1	8	2							<b>11</b>	
sCJD ND	0	1	3	0							<b>4</b>	
IPD	3	5	4	0							<b>12</b>	
vCJD	0	0	0	5							<b>5</b>	
$\Sigma$	<b>4</b>	<b>27</b>	<b>28</b>	<b>7</b>							<b>66</b>	

All PrP, tau and A $\beta$  scores were obtained using the scoring scheme shown in Figs. 1, 2, and 3. In case of multiple concurrent PrP patterns, the highest individual score (e.g. granular score 2 and synaptic score 1 = final score 2) was used. No cases with cerebellar A $\beta$  were included in this study. A score 4 for frontal cortex tau was given in cases that clearly exceeded the intensity shown in Fig. 3. One case (sCJD VV) scored 0 for tau and was omitted. All other cases had a tau score of 1 or greater. Cases with a tau score of 4 (exceeding the density shown in Fig. 3) are also shown sCJD MM, MV, VV sporadic CJD with PRNP codon 129 genotype, IPD inherited prion disease

### Phosphorylation of tau in the cerebellum: an underestimated feature?

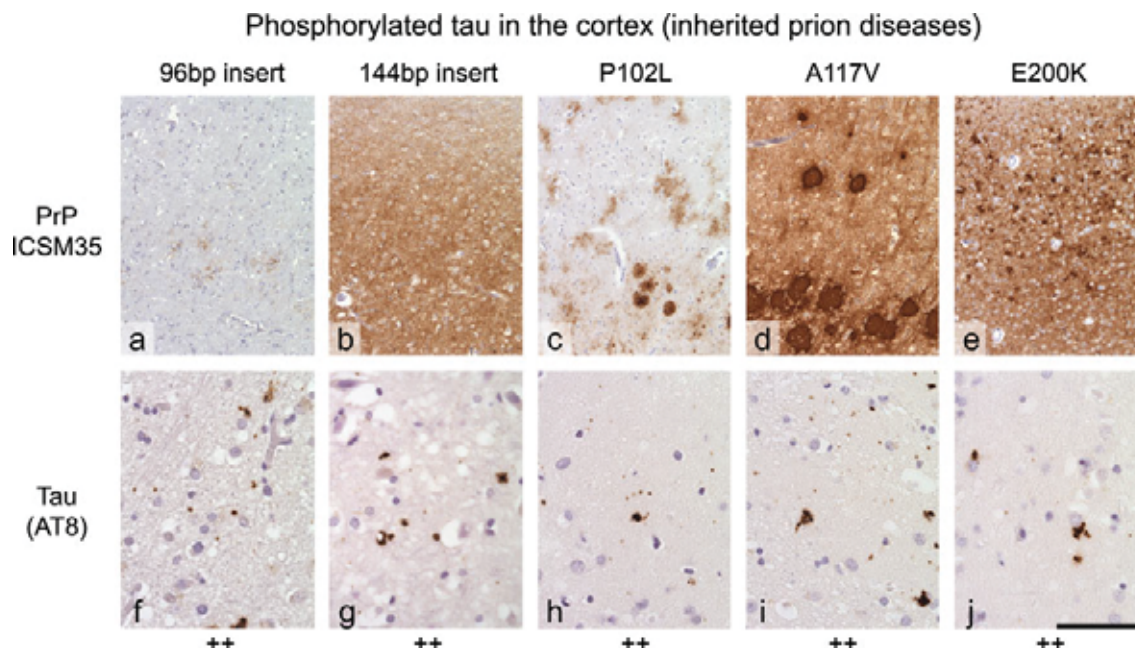
In our series of sporadic, inherited and variant forms of prion diseases, we detected formation of rod-shaped tau deposits in the molecular and granular layers. Some forms of sporadic prion diseases form abundant small plaques,

alongside with synaptic PrP, which is associated with marked periplaque hyperphosphorylation of tau. The same observation is made in inherited prion disease with the predominant formation of cerebellar plaques, such as P102L GSS, where tau is associated with, but not limited to, plaques. We show here that sporadic forms trigger tau phosphorylation in the cerebellum in a “load-dependent”

**Table 3** Statistical analysis of relationship between prion protein deposition, tau phosphorylation and disease duration

Parameter 1	Parameter 2	Number of cases	<i>R</i>	Significance level
Tau (frontal cortex), no A $\beta$ amyloid	PrP deposits: the strongest score was taken into account	28	0.69	<0.01
Tau (frontal cortex), no A $\beta$ amyloid	Duration of illness	28	-0.037	n.s.
Tau (cerebellum), all prion diseases	PrP deposits	69	0.50	<0.01
Tau (cerebellum), all prion diseases	Duration of illness	63	0.063	n.s.
Tau (cerebellum), sCJD only	PrP deposits	52	0.39	<0.01
Tau (cerebellum), sCJD only	Duration of illness	49	-0.16	n.s.

Parameters 1 and 2 were correlated in Pearson's test (n.s. not significant)



**Fig. 5** PrP-induced tau phosphorylation in cortex of inherited prion diseases: *upper row* deposition of abnormal PrP in the frontal cortex; there is considerable variability of the intensity of PrP<sup>Sc</sup> burden and some forms are characterised by distinct patterns of plaque formation, as described before. The *lower row* shows tau deposits corresponding to the area depicted above. A, 96 bp OPRI mutation with almost undetectable PrP<sup>Sc</sup> load, resulting in tau phosphorylation similar to

the 144 bp OPRI (f, g), despite its significantly higher PrP<sup>Sc</sup> load (b). c This case with a P102L mutation shows predominantly diffuse deposits and only very few plaques, as compared to the very heavily plaque-forming A117V case (d), both showing similar tau hyperphosphorylation (h, i). Another case with no plaque formation, E200K (e), shows a tau load similar to all other cases (j). Scale bar a–e 240  $\mu$ m, f–j 60  $\mu$ m

fashion (Fig. 4; Table 3), where cases with a relatively low cerebellar burden of abnormal PrP show fewer rod-shaped tau positive inclusions (Fig. 4a, f) than those with an intermediate (Fig. 4b, g) or high PrP load (Fig. 4c, h). This correlation is statistically significant for sCJD (correlation coefficient  $r = 0.39$ ,  $p < 0.01$ ) which increases when all forms of prion diseases are included ( $r = 0.50$   $p < 0.01$ ). Tau phosphorylation in the cerebellum occurs in the molecular layer (Fig. 4f), granular layer (Fig. 4g) and in the Bergmann glia/Purkinje cell layer (Fig. 4h, i). vCJD is characterised by a particularly heavy deposition of abnormal PrP in the neocortex and the cerebellum (Fig. 4e), again there is a significant and dense deposition of hyperphosphorylated tau in all areas (Fig. 4j, molecular layer).

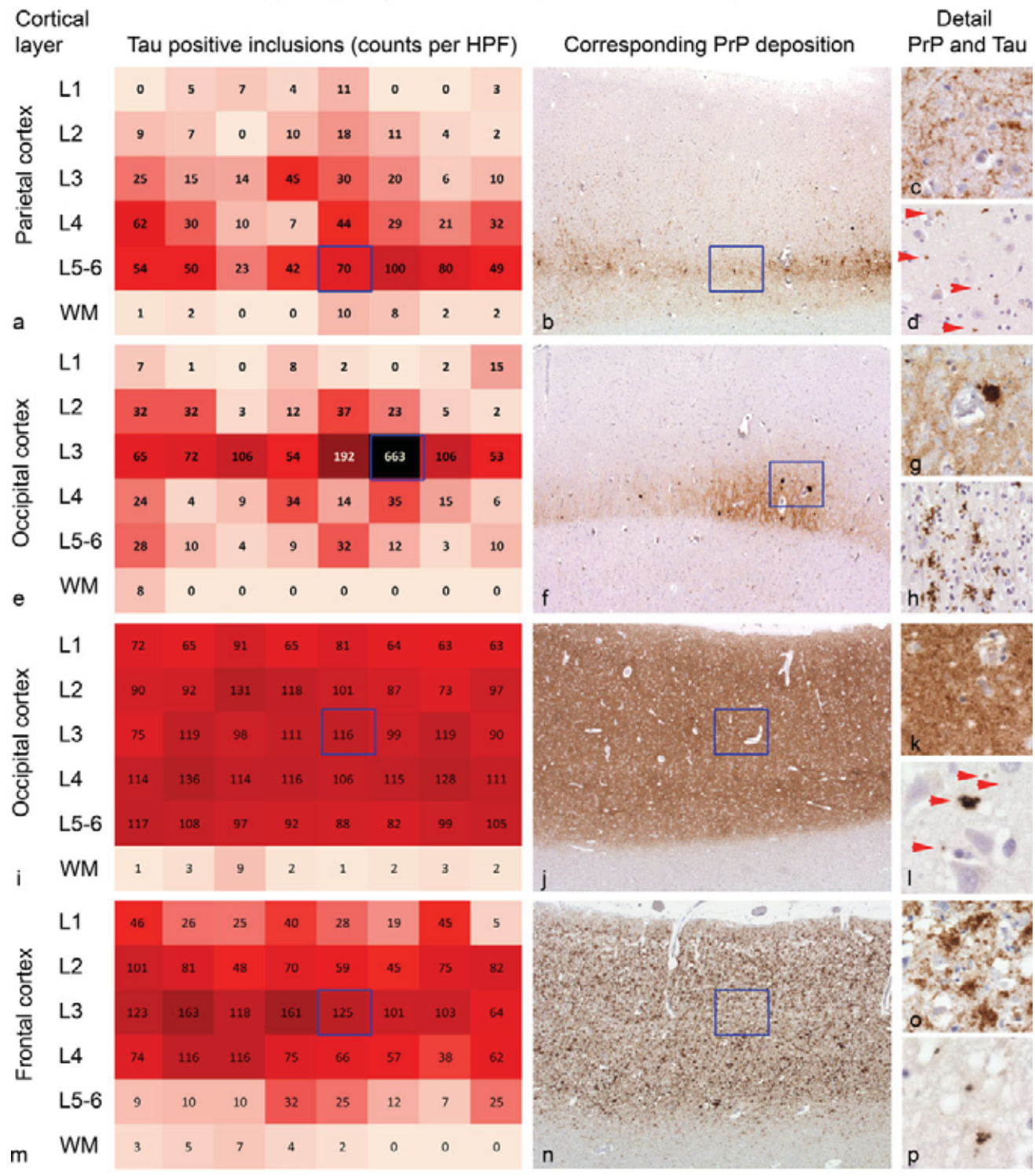
**Fig. 6** PrP deposits are congruent with hyperphosphorylated tau rods. *Left column* (heat maps) (a, e); i, m numbers of tau rods or granules were determined per high power field on a section in the cortex and a small strip of adjacent subcortical white matter (immunostaining for AT8 Tau). Each field shows the count and a colour coded representation, (heat map). *Middle column* b, f, j, n a parallel tissue section, stained for abnormal PrP shows deposits corresponding to the tau heat map. *Right column* c, d, g, h, k, l, o, p high power magnification of the areas indicated by a blue square in the heat map and in the overview of the PrP labelled section (corresponding to ca 25% of the blue square). Arrows in the tau labelled section indicate very small granules (d, l); c perineuronal net pattern of abnormal PrP, no plaque formation and moderate density of tau particles (d), g more intense PrP deposition and formation of small plaques, which elicit heavier tau deposition (h). The homogenous synaptic deposit (j) corresponds to a homogenous distribution of tau, which appears in very small granules (i). The coarse granular PrP deposits (n, o) are associated with larger, better discernible tau granule (p). Scale bar b, f, i 1,500  $\mu$ m, c, d, g, h, k, l 140  $\mu$ m

When comparing the cerebellar tau in brains with 96 bp OPRI, 144 bp OPRI, P102L, A117V and E200K mutations (Fig. 4k–t), we found that (1) plaque and synaptic PrP elicit a similar degree of tau hyperphosphorylation, but (2) varies between genotypes, which is

particularly evident when comparing with 96 and 144 bp OPRI (Fig. 4s, t).

These findings can be interpreted as follows: (1) within one type of PrP (e.g. sCJD prion protein) the degree of tau phosphorylation is likely to be dose (or load-) dependent,

### Tau phosphorylation corresponds to PrP deposits



as shown in Fig. 4a–c and f–h; whilst (2) each genetic mutation is able to elicit a response that is specific to a given mutation, suggesting that the PrP load and tau phosphorylation may not be comparable between different mutations (Fig. 4p–t). The relatively small number of inherited forms investigated here does not allow for robust statistical analysis. It has been argued that the long duration may contribute to the extent of tau phosphorylation, as vCJD and GSS show significantly higher tau load and have longer incubation times than sCJD. We show here that there is no correlation between the duration of the illness and the degree of tau phosphorylation. Prion protein load appears to be the main factor triggering tau phosphorylation.

The capacity of cerebellar neurons to hyperphosphorylate tau has only recently been recognised and is probably generally underestimated. Primary tauopathies, such as progressive supranuclear palsy (PSP) are examples of the capacity of cerebellar Purkinje cells [55, 80] or neurones of the dentate gyrus [93] to hyperphosphorylate tau. In patients with PSP or corticobasal degeneration, the clinical phenotype of cerebellar ataxia is directly associated with the progressive accumulation of hyperphosphorylated tau in Purkinje cells [55]. Another disease featuring tau phosphorylation in cerebellar neurones is Niemann–Pick disease where defects in the intracellular trafficking of exogenous cholesterol causes neurodegeneration. Neurofibrillary tangles in the cortex of Niemann–Pick brains are morphologically similar to those in AD. In the cerebellum, there is a marked deposition of phospho-tau in the dentate nucleus and granular layers of the cerebellum [66], but a remarkable absence of neurofibrillary tangles (NFT) is noted [17]. Several mouse models have addressed this [18] and whilst accumulation of hyperphosphorylated tau in cerebellar granule or Purkinje neurones can be successfully achieved in several mouse models (stress-induced [76], Niemann–Pick disease type C [18], pAkt-mediated tau phosphorylation [71]), no model has yet achieved formation of NFT, highlighting a specific pathway or cellular machinery required for NFT formation that is absent in the cerebellum [18]. A transgenic mouse expressing human P301L mutant tau did not show cerebellar tau at all [63].

One of the reasons that cerebellar tau phosphorylation is widely underestimated is the presence of abundant cortical phospho-tau in the neocortex in Alzheimer's disease whilst it is strikingly absent from the cerebellum, due to the lack of significant A $\beta$  accumulation in the cerebellum. If at all, A $\beta$  deposits in the cerebellum only at late stages of the disease process. Only few studies have demonstrated A $\beta$  deposition in the cerebellum [4] forming diffuse deposits, but no plaques with dystrophic neurites [58]. Cases of familial AD can show a significant A $\beta$  burden in the cerebellum, and can form deposits of various shapes and sizes

[32]. In the same series, cases with sporadic AD showed diffuse and granular deposits, and may, therefore, have represented a group with high overall A $\beta$  load [4]. More commonly, vascular A $\beta$  may cause cerebellar haemorrhages [26] or infarctions [19]. Familial AD cerebella accumulate mutant A $\beta$ 42 [54, 72], whilst in sporadic AD, these deposits are composed of A $\beta$ 40 amyloid in humans [70] as well as in experimental models [48].

Most studies including recent multicenter studies of the neuropathology of AD have not systematically examined tau hyperphosphorylation in the cerebellum [2, 3]. A recent biochemical and confocal imaging study has demonstrated co-localisation of tau and A $\beta$  in synaptosomes of all brain regions, including the cerebellar samples, which showed the lowest levels within the CNS [30].

In contrast to Alzheimer's disease where cerebellar A $\beta$  is essentially absent, most sporadic, inherited and transmitted forms (iatrogenic CJD, variant CJD and Kuru) of prion disease are characterised by a substantial prion protein deposition in the cerebellum (Fig. 4e). In sCJD, cerebellar prion protein is often seen as synaptic, homogeneous deposit in the molecular layer and to a lesser extent, in the granular layer (Fig. 4a–d). Other typical patterns are characterised by small granular deposits, which may become confluent to form microplaques. Kuru, an acquired prion disease in humans, transmitted by oral uptake during mortuary feasts in the Fore linguistic group in Papua New Guinea, is clinically characterised by cerebellar ataxia, rather than dementia, and shows a marked involvement of the cerebellum, with formation of dense plaques of variable sizes [12, 43].

Iatrogenic prion disease can be caused by a wide variety of procedures, mostly due to the transmission of CJD prions contained in contaminated growth hormone derived from human cadavers, or by implantation of contaminated dura mater grafts [15, 39], transmission of CJD prions during corneal transplantation [28, 47], contaminated electroencephalographic (EEG) electrode implantation or surgical operations using contaminated instruments or apparatus [14]. The pattern of prion protein deposition is characterised by synaptic PrP and formation of small and medium sized plaques in neocortex and in the cerebellum.

Finally, vCJD in the UK and other countries, caused by human exposure to BSE prions from cattle (Collinge et al. 1996; Bruce et al. 1997; Hill et al. 1997; Collinge 1999; Asante et al. 2002), is characterised by extensive plaque formation including the cerebellum.

## Conclusion

The capacity of disease-associated PrP to trigger phosphorylation of tau has been discovered sequentially. Early

reports have described this phenomenon in obvious cases, where abundant plaques were present. Increasing awareness and understanding of this phenomenon and refinement of immunohistochemical and biochemical techniques have subsequently triggered additional studies, extending the observation to variant CJD and plaque-forming sporadic prion diseases. In parallel, the recognition of prionopathies and prion-like mechanisms as a concept for neurodegenerative disease pathogenesis has triggered a wealth of comparative experiments which led to the discovery of similarities and functional relationships between A $\beta$  and prion protein. Tau takes part in this process and we have highlighted the evidence that may represent a mechanism of amyloid toxicity. Although the relationship between amyloid toxicity and tau phosphorylation appears straightforward in our cohort of sCJD cases, the issue may be more complicated in inherited prion diseases. Other parameters that were not systematically addressed in our study are genetic (tau haplotype, *PRNP* codon 129 genotype) or demographic factors (e.g. age of onset). Our data presented here underpin the concept of amyloid-triggered tau phosphorylation, further contribute to the understanding of the relationship between prion amyloid and tau toxicity and set the scene for future research on larger cohorts. Furthering the study of pathways involved in tau phosphorylation may also be the key to understand prion neurotoxicity.

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RESEARCH ARTICLE

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# MRI findings are often missed in the diagnosis of Creutzfeldt-Jakob disease

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## Abstract

**Background:** Establishing a confident clinical diagnosis before an advanced stage of illness can be difficult in Creutzfeldt-Jakob disease (CJD) but unlike common causes of dementia, prion diseases can often be diagnosed by identifying characteristic MRI signal changes. However, it is not known how often CJD-associated MRI changes are identified at the initial imaging report, whether the most sensitive sequences are used, and what impact MRI-diagnosis has on prompt referral to clinical trial-like studies.

**Methods:** We reviewed the MRI scans of 103 patients with CJD referred to the National Prion Clinic since 2007 and reviewed the presence of CJD-associated changes, compared these findings with the formal report from the referring centre and reviewed the types of sequence performed.

**Results:** In sCJD we found CJD-associated MRI changes in 83 of 91 cases (91% sensitivity). However, the referring centres documented CJD-associated MRI changes in 43 of the sCJD cases (47% sensitivity). The most common region not documented by referring centres was the cortex (23 of 68 sCJD cases), but there was a statistically significant discrepancy in all regions ( $p < 0.0001$ ). Patients in whom MRI abnormalities were missed by the referring hospital were more advanced at the time of recruitment to a clinical trial-like study ( $p = 0.03$ ).

**Conclusions:** CJD-associated MRI changes are often not documented on the formal investigation report at the referring centre. This is important as delay makes enrolment to clinical trials futile because of highly advanced disease. If a diagnosis of CJD is suspected, even if the initial imaging is reported as normal, a specialist MRI review either by an experienced neuroradiologist or by a prion disease specialist unit could facilitate earlier diagnosis.

**Keywords:** Prion, MR imaging, Creutzfeldt-Jakob disease, Dementia

## Background

Creutzfeldt-Jakob disease occurs as a sporadic, iatrogenic and genetic fatal neurodegenerative disorder [1]. With the exception of genetic types, CJD can only be definitively diagnosed by tissue examination, usually brain biopsy or at autopsy. However, using clinical and investigative criteria, WHO sought to define the likelihood that a patient has CJD. The original criteria include dementia with variety of neurological signs, the presence of 14.3.3 proteins in the CSF and characteristic EEG with pseudo-periodic complexes. More recently MRI criteria have been added giving a sensitivity and specificity >90% for

probable CJD [2,3]. These changes comprise alterations on diffusion weighted (DWI) and FLAIR images in the basal ganglia, thalamus and cortex [4,5]. However these changes can be subtle and easily missed by a clinician unfamiliar with such a rare disorder. With the development of potential therapies to alter the natural history of the disease early diagnosis is essential to give the maximum chance of salvaging functional brain tissue [6]. As MRI is the most widely available non-invasive test assisting diagnosis, it is important that clinicians are aware of the imaging features. At present it is not known how often the MRI abnormalities are reported by radiologists in cases of definite CJD. We therefore reviewed images from 133 consecutive patients referred to the National Prion Clinic and compared the report from the referring clinician and our own specialist radiologists; 103 were

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suitable for the study of whom 70% had an autopsy or cerebral biopsy.

## Methods

### Experimental procedures

#### Patients

In 2004 the Chief Medical Officer wrote to UK neurologists requesting that all patients with suspected CJD are referred to both the National CJD Surveillance Unit in Edinburgh and the National Prion Clinic (NPC) based at the National Hospital for Neurology and Neurosurgery (NHNN), London. All patients underwent PRNP analysis for genetic mutations. MRI scans from all visited patients are requested by the NPC. The scans studied in this work were from patients who were referred to the NPC by their local physician with full consent and the patients were enrolled into either the clinical trial PRION-1 or the on-going National Prion Monitoring Cohort study. Both studies were approved by the Eastern Medical Research Ethics Committee and are compliant with the Helsinki Declaration.

#### MRI evaluation and reporting

**NPC MRI review** The first MRI scan were reviewed by one of two senior neuroradiologists (H.H. and J.S.) at The National Hospital for Neurology and Neurosurgery (NHNN and the NPC). The radiologists were aware of the suspicion of CJD at the time of reporting but were not aware of the results of any biopsy or post mortem results at the time of reporting. The available MRI sequences were reviewed together as the sensitivity of individual sequences was *not* being compared. Three types of high signal intensity lesions were accepted as CJD-associated MRI changes on DWI and/or Fluid Attenuated Inversion Recovery (FLAIR) and/or T2-weighted sequences:

1. Lesions in the striatum (caudate or putamen or both)
2. Lesions in the thalamus including the pulvinar
3. Lesions along the cortical ribbon (cerebral or cerebellar)

**External MRI formal report review** All external reports were reviewed for whether:

1. The MRI was normal
2. The MRI differential diagnosis included CJD
3. CJD-associated abnormalities were specifically documented in the caudate, putamen, thalamus or cortex

#### Exclusion criteria and classification

All patients in whom a clinical diagnosis other than CJD was suspected, or those who did not fulfil the criteria for

CJD were excluded, as were all scans before 2007 when specific scan review was not routine. Patients who were referred to the NPC with a suspected diagnosis of inherited prion disease (IPD) were also excluded, as prion gene sequencing resulted in a definite diagnosis and MRI rarely shows any signal change with the majority of mutations. Two cases with the E200K mutation and one case with the P102L mutation detected at a later stage were included as the clinical picture at the time was indistinguishable from sCJD.

The remaining patients were categorised by CJD type with all available investigations; those with sporadic CJD were further sub-categorised at the time of initial referral as: possible, probable or definite using the WHO criteria. We then re-classified the sCJD patients using the modified criteria suggested by Zerr et al. [3] for both the referred MRI report and then our own MRI impression to see whether our MRI interpretation altered the classification of these patients. Where available, the scales data was documented, based on a composite functional scale derived from the Barthel ADL index and Clinical Dementia Rating Scale Sum of Boxes.

#### Statistical analysis

The patient population characteristics and the comparison of the presence of CJD-associated MRI images between NPC and referring centre were assessed using the Chi2 test (GraphPad Prism).

## Results

We obtained 133 MRI scans from 113 patients referred to the NPC between 01.03.2007 and 24.05.2010 with a suspected diagnosis of CJD. 30 scans were excluded: 21 cases did not fulfil the WHO diagnostic criteria for CJD or were thought clinically to be due to a different cause; the referring report could not be obtained in 9 cases.

A total of 103 reported scans were available for review of which 56% were from males. The eventual diagnosis was sCJD in 91 cases with a median age of onset of 65 years (3 possible, 35 probable and 53 definite). Two cases of iatrogenic CJD (iCJD) from cadaver sourced growth hormone were identified (both 41 years old when symptomatic), 3 patients with inherited prion disease and 7 patients had vCJD with a median age of onset of 22 (2 probable and 5 definite) (Table 1).

In sCJD, we found characteristic increases in MRI signal intensity in 83 of the 91 cases at NPC review, a sensitivity of 91% for MRI in the diagnosis of CJD in this series (Table 1). CJD-associated changes were not detected in 8 a total of cases, 2 of whom did not have a DWI (2 definite sCJD). The remaining 6 cases had a DWI sequence performed (two definite, three probable and one possible sCJD) (Table 1). The referring centres, however, described CJD-associated MRI changes in 43 of

**Table 1 The characteristics and MRI report comparison of 103 CJD patients**

	Final diagnosis (n)				Median age of Onset (min-max)	No. DWI sequences (%)	No. cases with "CJD-associated changes" on MRI (%)		No. cases from neurological centre (%)	No. cases with unreported changes from neurological centre (%)
	Possible	Probable	Definite	Total			NPC review	Referring centre		
sCJD	3	35	53	91	65 (38–85)	75 (82)	83 (91)	43** (47)	55 (60)	19 (43%)
vCJD		2	5	7	22 (20–53)	6 (86)	7* (100)	7 (100)	5 (71)	0
iCJD		2		2	41 (0)	1	2	0	2 (100)	2 (100)
IPD			3	3		1			-	-
E200K			2		67 (63–70)	-	2	0	0	-
P102L			1		61 (0)	-	1	1	0	-

The scans and corresponding reports of 103 CJD patients were reviewed. The population comprised 91 sCJD, 7 vCJD, 2 iCJD and 3 IPD patients. The number of scans with DWI is shown as is the incidence of CJD-associated changes as noted by NPC and referring centre.

\*= 2 of the 7 patients had MRI changes normally associated with sCJD.

\*\*= p<0.0001 compared with NPC opinion of CJD-associated changes using Chi<sup>2</sup> with 1df and Yates' correction.

the 91 sCJD cases (sensitivity of 47%), with unreported CJD-associated MRI changes in 40 of the 83 sCJD cases found to have MRI changes at NPC review (p<0.0001 compared with our identification of similar changes using Chi<sup>2</sup> with Yates' correction and 1 degree of freedom). In cases with unreported CJD-associated MRI changes, there was no significant difference between cases referred from a neurological centre or general hospital (Table 1). Occasionally CJD-associated MRI changes were commented upon but thought to be calcification of the basal ganglia, but more commonly there was no mention of critical thalamic/cortical signal changes.

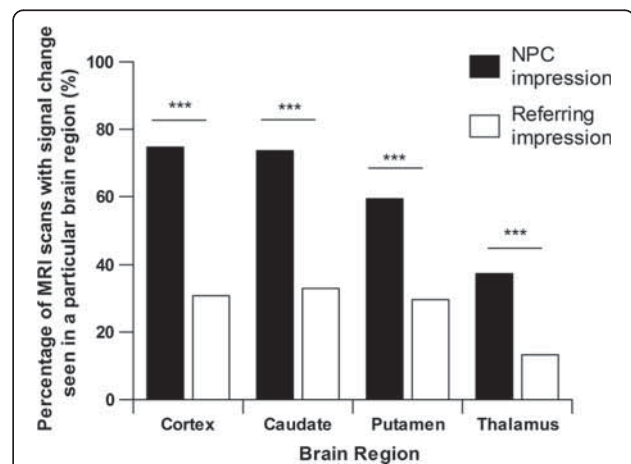
To further investigate whether referring centres identified CJD-associated MRI changes we reviewed specific locations of increased MRI signal in sCJD cases (Figure 1). We found that the most common sites for CJD-associated MRI changes were cortex (74%) (Additional file 1: Figure S1B and S1C) and caudate (73%) with thalamus being the least affected (37%) (Figure 1). The most common region not documented by referring centres was the cortex (23 of 68 sCJD cases), but there was a statistically significant discrepancy in all regions (p<0.0001 Figure 1).

All reports of vCJD were concordant with our own. Interestingly, two definite cases did not have a pulvinar signal increase greater than that of the basal ganglia and were initially classified as sCJD. In contrast, both iCJD cases were initially reported to have had normal scans (Table 1, Additional file 1: Figure S1A). When the diagnosis was categorised according to the modified criteria suggested by Zerr et al. [3] at the time the patient presented to the NPC, using the MRI report and our own MRI impression, we found that the diagnosis changed from possible to probable sCJD in only two cases as all other patients with negatively reported scans had either characteristic EEG changes or positive CSF 14-3-3 protein (data not shown).

DWI sequences were performed in 83 of the 103 MRI studies (79%) (Table 1). In the 20 patients for whom

DWI was not performed, CJD-associated MRI changes were detected in 16 MRI studies. In all the MRI studies, FLAIR and T2-weighted sequences were present.

A subset of the cohort which included 62 patients were rapidly reviewed by the National Prion Clinic team and scales data obtained based on a composite functional scale derived from the Barthel ADL index and Clinical Dementia Rating Scale Sum of Boxes. Where the MRI abnormalities were detected by the referring hospital (n=40), patients had a significantly higher (less advanced disease) (p=0.03) median functional score of 5/35 (range 0–31) compared to median MRC score of



**Figure 1 The MRI scans of 91 patients with sCJD were reviewed and compared with the corresponding report from the referring centre.** The overall sensitivity of MRI scans found by the NPC was 91% compared with 47% from the referring centre (p<0.0001). The NPC found that 74% of cases had cortical high signal, 73% of cases had changes in the caudate, 59% of cases had changes in the putamen and 37% in the thalamus. The region that there was the most discrepancy between the two impressions was the cortex but there was a significant difference in all regions evaluated (p<0.0001).

2/35 (range 0–27) when the MRI abnormalities were missed by the referring hospital (n=22).

## Discussion

We have shown that MRI signal changes are often not identified on initial scan at referring hospitals in patients with CJD. Signal abnormality characteristically occurs in the basal ganglia and thalamus [7-9] but this was reported in only about half the referred patients and cortical abnormalities were reported in even fewer patients. Missed diagnosis was associated with a more advanced clinical state at enrolment in clinical-trial like studies.

There are a number of reasons why the MRI signal abnormalities were not reported by the referring clinicians. Firstly, the rarity of CJD means radiologists, even in a neurological centre, have not seen many cases and will be unfamiliar with the characteristic MRI findings. Secondly, it is possible that CJD at the time of original scanning was not suspected, or was not mentioned on the original request for MRI scanning, which would make the radiologist consider a much wider range of possibilities. The heightened suspicion of CJD at referral to our centre is highly likely to be a factor in the increased ascertainment. It is possible that after joint multidisciplinary meetings with added clinical information, new interpretations of the scan were not updated on the actual MRI report.

Thirdly, abnormality can be difficult to detect when the MRI is degraded by movement artefact, a common phenomenon in confused patients with CJD. In these situations, it is even more imperative to perform a DWI sequence which is relatively short compared to other MRI sequences and can provide good quality information in an uncooperative patient. Despite the inclusion of DWI sequences in many routine MRI protocols, we found that DWI was only performed in 79% of MRI studies. When a patient is uncooperative, performing the DWI sequence earlier in an MRI protocol may be more helpful than persisting in acquiring good quality images in other sequences.

Finally, the MRI abnormalities may have been detected but dismissed as artefact and not reported. Increased signal abnormality in the neocortex was seen in 17% of controls subjects in a study by Young et al. [4] which could lead to caution in interpretation of DWI findings by many radiologists. However, visualisation of the DWI-trace image in conjunction with the Apparent Diffusion Coefficient (ADC) map where corresponding decreased signal is seen (Additional file 1: Figure S1D), can confirm initial suspicion of DWI abnormalities. Performing an additional DWI sequence at a high b-value such as  $b=3000 \text{ s/mm}^2$  can increase confidence when DWI signal abnormality is suspected [10]. DWI using a standard b-value of  $1000 \text{ s/mm}^2$  and a high b-value of  $3000 \text{ s/mm}^2$  are routinely performed as part of the MRI Protocol for

the diagnosis of prion diseases at our institution. Asymmetrical cortical or deep grey nuclei involvement can also increase confidence in abnormal signal interpretation. Nevertheless cortical ribboning must be interpreted with caution; this particularly applies to use of machines with field strength higher than 1.5 T used in the present study.

We found evidence of a less advanced clinical state once patients were referred to our centre in patients where the MRI abnormalities were detected by the referring centre compared to those where the MRI abnormalities were initially missed. At present a high proportion of CJD patient present to specialist centres with very advanced neurodisability (often within a few days of death) when clinical trials are futile. MRI is usually one of the initial investigations when a patient is admitted which could allow a clinical diagnosis to be made at an earlier stage, avoiding uncertainty amongst patient relatives and the need for further unnecessary investigations. We believe that in patients where the initial MRI findings were missed by the referring centre, further investigation delayed referral leading to a more advanced clinical state at specialist referral.

## Conclusion

MRI is a useful tool in the diagnosis of CJD but the characteristic changes are often not identified at initial scan in the early stages of disease. This does not imply an adverse criticism of the radiologists who have probably never, or rarely seen, an MRI from a patient with CJD and to whom the diagnosis has not been raised as a possibility. However CJD MRI findings have been reported in the literature for over a decade and it is important if experimental therapeutics are to be successfully tested in human trials. If a diagnosis of CJD is suspected, even if the initial imaging is interpreted as normal, specialist MRI review either by an experienced neuroradiologist or by a prion disease specialist unit could facilitate earlier diagnosis.

## Additional file

**Additional file 1: Figure S1.** Selection of 4 cases from the 40 cases where CJD-associated MRI changes were missed at the referring centre. (A) A probable iCJD (growth hormone treated) patient: axial T2W images demonstrates hyperintensity within the basal ganglia and thalamus bilaterally; (B) A "probable" sCJD patient: axial DWI images show cortical hyperintensity in the frontal cortex bilaterally; (C) A "definite" sCJD patient: axial DWI shows hyperintensity in the head of caudate nucleus and occipital cortex bilaterally; (D) A "definite" sCJD patient: axial ADC map demonstrates restricted diffusion in the head of caudate and putamen bilaterally.

## Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

CC, PR, SM, JC and HH designed the study. CC, AT, AL, PR and SM collected data. The raw data was analysed by HH and JS. The data was then cleaned and analysed further by CC, HH, PR and SM. Statistical analysis was performed by CC, HH and SM. Figures were written by CC and HH with review by AT and AL. The paper was initially drafted by CC, PR, SM and HH before internal presentation and analysis by all contributing authors. The final paper was revised by CC, PR, SM, JC and HH. JC is responsible for the overall content as guarantor. All authors read and approved the final manuscript.

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# The Medical Research Council Prion Disease Rating Scale: a new outcome measure for prion disease therapeutic trials developed and validated using systematic observational studies

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Progress in therapeutics for rare disorders like prion disease is impeded by the lack of validated outcome measures and a paucity of natural history data derived from prospective observational studies. The first analysis of the UK National Prion Monitoring Cohort involved 1337 scheduled clinical assessments and 479 telephone assessments in 437 participants over 373 patient-years of follow-up. Scale development has included semi-quantitative and qualitative carer interviews, item response modelling (Rasch analysis), inter-rater reliability testing, construct analysis and correlation with several existing scales. The proposed 20-point Medical Research Council Prion Disease Rating Scale assesses domains of cognitive function, speech, mobility, personal care/feeding and continence, according to their relative importance documented by carer interviews. It is quick and simple to administer, and has been validated for use by doctors and nurses and for use over the telephone, allowing for frequent assessments that capture the rapid change typical of these diseases. The Medical Research Council Scale correlates highly with widely used cognitive and single item scales, but has substantial advantages over these including minimal floor effects. Three clear patterns of decline were observed using the scale: fast linear decline, slow linear decline (usually inherited prion disease) and in some patients, decline followed by a prolonged preterminal plateau at very low functional levels. Rates of decline and progress through milestones measured using the scale vary between sporadic, acquired and inherited prion diseases following clinical expectations. We have developed and validated a new functionally-oriented outcome measure and propose that future clinical trials in prion disease should collect data compatible with this scale, to allow for combined and comparative analyses. Such approaches may be advantageous in orphan conditions, where single studies of feasible duration will often struggle to achieve statistical power.

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**Keywords:** prion; cohort; rating scale; outcome measure; CJD

**Abbreviations:** CJD = Creutzfeldt-Jakob disease; MRC = Medical Research Council

## Introduction

The prion diseases are a group of rare neurodegenerative conditions for which no proven disease-modifying treatment is available. They are characterized by templated misfolding of the normal cellular prion protein (PrP<sup>C</sup>) into abnormal disease-associated forms (generally referred to as PrP<sup>Sc</sup>) but they may have genetic, idiopathic or acquired aetiologies. There is remarkable clinical heterogeneity both within and between these different aetiological disease types (Collinge, 2001).

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, 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<sup>C</sup>) has now reached an advanced stage (Mallucci *et al.*, 2002, 2003, 2007; White *et al.*, 2003; Nicoll and Collinge, 2009; Nicoll *et al.*, 2010; Klohn *et al.*, 2012). However, if the promise of these agents is to be realized, it is vital that trial methodology is optimized and a number of challenges specific to prion disease trials are overcome. Even a highly effective therapeutic agent may fail to produce conclusive results without an optimal trial design.

The Medical Research Council (MRC) PRION-1 trial demonstrated that a large clinical trial in a single country is feasible, but was limited by the lack of a validated measure of clinical progression (Collinge *et al.*, 2009). 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 (Mead *et al.*, 2011). However, a number of unresolved issues remained: no single scale captured progression across the full range of functional physical and cognitive domains affected by any of the individual categories of prion disease; patients could not be visited frequently enough to capture the very rapid decline that 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; and the patient sample was too small to allow reliable analysis of important aetiological or severity-stratified subgroups (Mead *et al.*, 2009).

With the aim of developing a single, functionally-orientated and validated outcome measure, tailored to the particular demands of a prion disease clinical trial, we modified and combined elements of three rating scales that showed relative strengths in the PRION-1 analysis and are well established in other neurological

settings: the Modified Barthel Activities of Daily Living Index (Barthel) (Mahoney and Barthel, 1965; Collin *et al.*, 1988), the Clinical Dementia Rating Sum of Boxes (Morris, 1993), and the Glasgow Coma Score (Teasdale and Jennett, 1974). We carried out semi-quantitative interviews to assess patients' relatives and carers on which manifestations of prion disease are of greatest concern to them, and ensured that these were reflected in the domains assessed by our scale. We assessed reliability of use over the telephone (allowing much higher assessment frequency), and used Rasch analysis (Hobart *et al.*, 2007) to refine an outcome measure that had the most favourable statistical properties when used in this patient group. Finally, we illustrate the natural history of symptomatic patients in the National Prion Monitoring Cohort study and/or PRION-1 trial using the MRC Prion Disease Rating Scale, and performance relative to existing scales.

## Materials and methods

### Patient referral, clinical diagnosis and enrolment

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) (Collinge *et al.*, 2009) and the National Prion Monitoring Cohort study (subsequently 'Cohort study', 2008–2012). Collinge *et al.* (2009) provides 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 prion disease (sporadic CJD, variant CJD, iatrogenic CJD, and inherited prion disease). 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 variant CJD (recipients of implicated whole or leucodepleted blood transfusion notified by the Health Protection Agency). A small group of healthy control subjects were also recruited (friends or relatives without pathogenic *PRNP* mutations or other known risk factors). 'At risk' individuals and healthy control subjects 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 (J.C., S.M. and/or P.R.) within a week of enrolment, or prior to enrolment if there was clinical uncertainty. Probable sporadic CJD diagnosis was made according to World Health Organization 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  $\geq 2$  cortical regions) (Zerr *et al.*, 2009). Patients not meeting criteria for sporadic CJD were enrolled if this was thought to be the most likely diagnosis at review by a panel of senior NPC clinicians. Probable variant CJD diagnosis was made according to World Health Organization criteria (WHO, 2001). Inherited prion disease was diagnosed by gene test. Iatrogenic CJD was diagnosed using sporadic CJD 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:

Stratum 1: Symptomatic patients with sporadic CJD, variant CJD, iatrogenic CJD and forms of inherited prion disease likely to have rapid clinical progression (due to 4-octapeptide repeat insertion, E200K, D178N, E211Q or V210I mutations); Stratum 2: Symptomatic patients with inherited prion disease likely to have slow progression (those with 5 and 6-octapeptide repeat insertion, P102L, P105L, Q212P, A117V or Y163X mutations); and Stratum 3: At-risk and healthy control individuals.

Patients in Stratum 1 had face-to-face follow-up assessments initially every 6–8 weeks. If clinical progression proved to be slower than the expected significant deterioration over this interval (i.e. minimal or no change by overall clinical impression over 6–8 weeks), study physicians could decide to reduce follow-up frequency for subsequent assessments (up to a maximum interval of 24 weeks). Patients in Strata 2 and 3 had follow-up assessments every 6 to 12 months. The following rating scales were administered at study assessments: Modified Barthel Activities of Daily Living index (Mahoney and Barthel, 1965; Collin *et al.*, 1988), Clinical Dementia Rating Sum of Boxes (Morris, 1993), Glasgow Coma Score (Teasdale and Jennett, 1974), Rankin (Rankin, 1957), and Mini-Mental State Examination (Folstein *et al.*, 1975). In September 2009 an initial 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 1 also had telephone assessments every 1 to 2 weeks between follow-up assessments. These were discontinued if there was minimal change between consecutive assessments (at worst possible score).

## Survey of carer priorities

The Barthel Activities of Daily Living Caregiver Interview Summary form was used to gather semiquantitative 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 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.

## Development pathway for novel rating scale

Building on and extending the analysis of the pre-existing scales in PRION-1 (Mead *et al.*, 2011), an iterative approach was taken to develop and refine a novel scale for use in prion disease. 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, the need to include a range of both physical and cognitive domains, 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 between treating clinicians and nurses, 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 on the first two initial scale version assessments post September 2009 per individual, to ensure that the analysis was not unduly affected by a small minority of patients with many assessments, and to permit testing the function of the scale over time. Rasch analysis was used to see whether the initial scale fitted this model using Rasch Unidimensional Measurement Model 2030 software. The partial credit variant of the polytomous model was chosen to reflect multiple successively ordered response categories of varying difficulty (Andrich *et al.*, 2008). We took the following recommended (Tennant and Conaghan, 2007) iterative steps to optimize the fit of our scale to the Rasch model: (i) exploring item–person interactions (to examine the degree to which the Guttman pattern was achieved) and item–trait interactions using  $\chi^2$ -based fit statistics; (ii) rescoring items that demonstrated disordered thresholds (i.e. an item's scoring categories do not progress in a logical order); (iii) removing the most poorly fitting items, or combining items into super-items where appropriate; (iv) examining local dependencies between items that could confound the assumption of unidimensionality of the scale, and dropping items when misfit was still apparent; (v) investigating differential item functioning for gender, age, time of assessment (first versus second assessment), assessor (doctor versus nurse administering scale) and mode of assessment (telephone versus face-to-face). We also analysed a random sample of later follow-up assessments to evaluate whether there was differential item functioning for the selection of the follow-up visit to include in the main analysis; (vi) 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; and (vii) 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 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 also used scores derived from the individual component scales (Barthel, Clinical Dementia Rating Sum of Boxes, and Glasgow Coma Score) prior to September 2009 (Tools was scored as missing) for individuals who did not have two scores post-September 2009.

## Results

### Patients

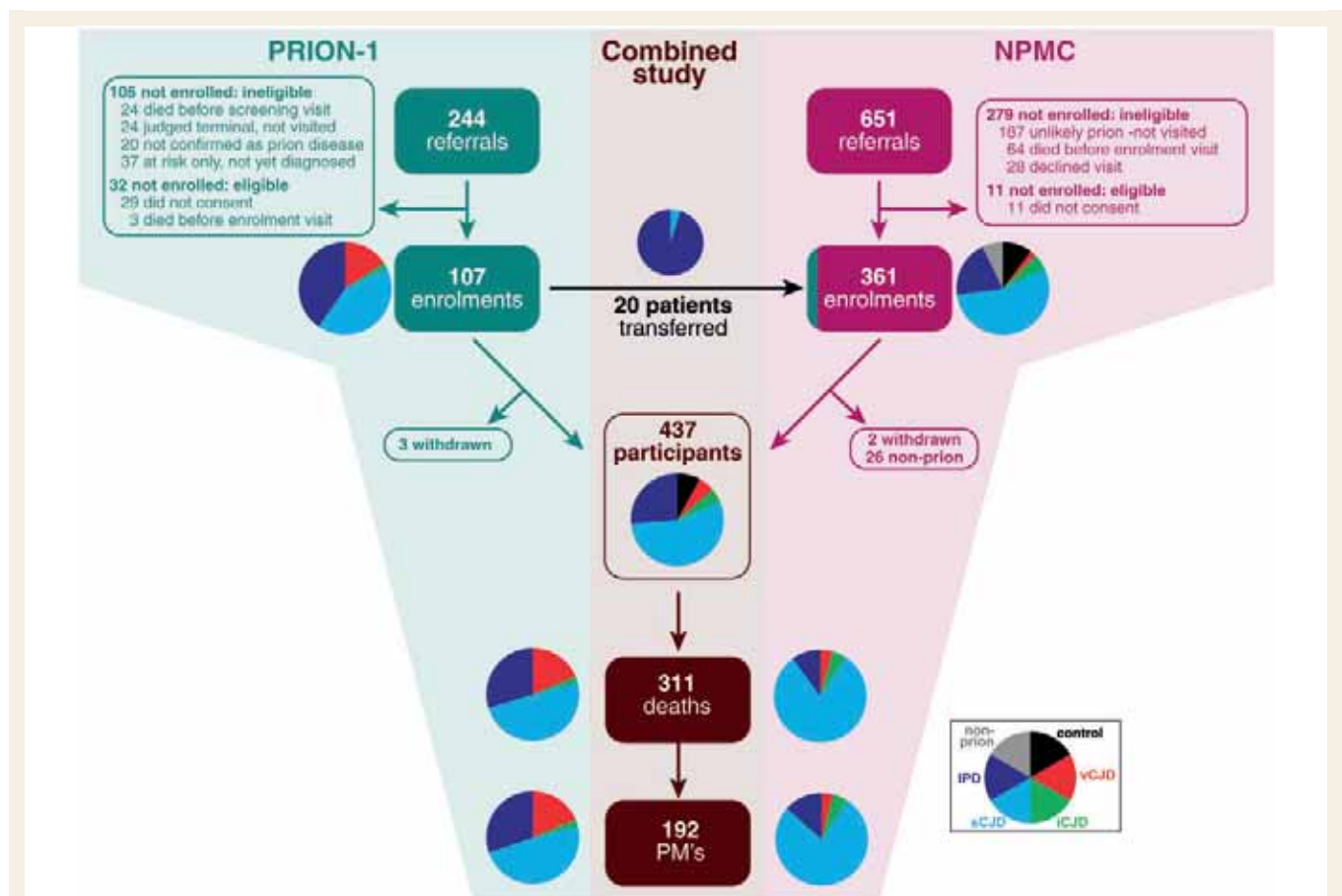
Four hundred and thirty-seven participants consecutively enrolled into the Cohort and/or PRION-1 studies (up to April 2012) were included in the analysis; 240 patients with sporadic CJD, 25 with variant CJD, 12 with iatrogenic CJD, and 81 with symptomatic inherited prion disease (three, seven and 19 patients with 4, 5 and 6 octapeptide repeat insertion, respectively, seven with A117V mutation, four with D178N, eight with E200K, 26 with

P102L, one patient each with E211Q, P105L, Q212P and V210I mutation and three with Y163X) together with 34 individuals at risk of inherited prion disease, 10 at risk of variant CJD, and 35 healthy control subjects. Three hundred and eleven patients died during the study, 192 (62%) of these underwent post-mortem examination, which confirmed the diagnosis of prion disease in all cases. Eighty-nine (20%) had diagnosis confirmed in life by gene test (all 81 inherited prion disease) or tissue biopsy ( $n = 8$ ) some also with post-mortem examination. One hundred and fifty-five (35%) were diagnosed using clinical and investigation findings alone.

Figure 1 illustrates enrolment, stratification, follow-up and drop-out from the studies. Ninety-seven per cent 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 recognized prion disease in the UK. Less than 1.5% withdrew from the studies. Baseline characteristics are shown in Table 1.

### Scale development

Table 2 summarizes the scale development process. Ranked symptoms and impairments reported by caregivers were grouped into functional domains. The most frequently recorded were within



**Figure 1** Study profile. iCJD = iatrogenic CJD; IPD = inherited prion disease; NPMC = National Prion Monitoring Cohort; PM = post-mortem; sCJD = sporadic CJD; vCJD = variant CJD.

**Table 1** Baseline characteristics at enrolment

	All patients	sCJD	IPD	vCJD	iCJD	At risk	Control
<b>Enrolled</b>	<b>437</b>	<b>240</b>	<b>81</b>	<b>25</b>	<b>12</b>	<b>44</b>	<b>35</b>
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
<b>Rankin</b>							
Number assessed	<b>430</b>	<b>238</b>	<b>79</b>	<b>23</b>	<b>11</b>	<b>44</b>	<b>35</b>
Asymptomatic (0)	75	0	0	0	0	41	34
No or slight symptoms (1/2)	29	4	18	2	3	1	1
Moderate disability (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
<b>Barthel index</b>							
Number assessed	<b>424</b>	<b>238</b>	<b>76</b>	<b>20</b>	<b>12</b>	<b>43</b>	<b>35</b>
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
<b>MMSE</b>							
Number assessed	<b>385</b>	<b>205</b>	<b>72</b>	<b>19</b>	<b>11</b>	<b>43</b>	<b>35</b>
Median (IQR)	12 (0–27)	0 (0–10)	18 (5–24.8)	16 (12–21.5)	15 (1.5–27)	30 (29–30)	30
<b>CDR</b>							
Number assessed	<b>353</b>	<b>178</b>	<b>69</b>	<b>21</b>	<b>9</b>	<b>42</b>	<b>34</b>
Median (IQR)	11 (2–18)	18 (12–18)	8 (3–15)	11 (5.5–16)	10 (4–18)	0	0
<b>GCS</b>							
Number assessed	<b>399</b>	<b>232</b>	<b>64</b>	<b>20</b>	<b>10</b>	<b>38</b>	<b>35</b>
Median (IQR)	14 (10–15)	11 (9–14)	15 (11–15)	15 (13.8–15)	14 (13.3–15)	15	15
<b>MRC Scale</b>							
Number assessed	<b>388</b>	<b>212</b>	<b>67</b>	<b>18</b>	<b>12</b>	<b>44</b>	<b>35</b>
Median (IQR)	9 (2–19)	3 (1–8.8)	16 (5.5–19)	13 (9–17.5)	11.5 (5–17.3)	20	20

Interquartile ranges (IQR) are shown. 'At risk' and 'Control' groups did not contribute directly to development of the novel scale.

CDR = Clinical Dementia Rating Sum of Boxes; GCS = Glasgow Coma Score; iCJD = iatrogenic CJD; IPD = inherited prion disease; MMSE = Mini-Mental State Examination; sCJD = sporadic CJD; vCJD = variant CJD.

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, based on that proposed in Mead *et al.* (2011), consisted of slightly modified versions of all subcomponents of the Modified Barthel Activities of Daily Living Index, the memory, orientation and judgement/problem-solving subcomponents of the Clinical Dementia Rating Sum of Boxes, the best verbal response subcomponent of the Glasgow Coma Score and a novel subcomponent assessing ability to use tools (15 items, 35 thresholds) (Mead *et al.*, 2011). 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 patients with prion disease 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 min. In addition to this, an approximation of the initial scale could be calculated from Barthel, Clinical Dementia Rating Sum of Boxes and Glasgow Coma Score 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 h of each other on 50 occasions, over the telephone by a nurse within 24 h of a face-to-face assessment by a doctor on two occasions, and by both doctor and nurse attending the same visit on two 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 rescaling; and orientation, which was subsequently merged with memory.

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 ( $\chi^2 = 166.16$ ,  $df = 30$ ,  $P < 0.0001$ ). As Rasch analysis relies on the assumption that there is a single construct being measured by all items in a scale, including patients with different disease types that are known to vary widely in their typical clinical progression, it is

Table 2 Summary of scale development

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

plausibly impossible to establish a fit to the Rasch model using the whole data set. As our primary objective was to develop an outcome measure tailored to sporadic CJD, all other disease groups were excluded and the analysis repeated using 205 records from 132 patients with sporadic CJD (all of those in whom the initial scale had been administered). Fit improved somewhat, but was still unsatisfactory ( $\chi^2 = 77.01$ ,  $df = 30$ ,  $P < 0.0001$ ) 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 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) (Tennant and Conaghan, 2007). These changes are summarized in Table 2. Item dependencies were identified between grooming and bathing; transfers and mobility; toilet and stairs; bathing, orientation and memory. We created two 'super-items' 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 3. Once item

**Table 3** Final MRC Prion Disease Rating Scale

Item	Category criteria	Score
<b>Bowel function</b>	At least one episode of incontinence in last 7 days	0
	Continent for last 7 days	1
<b>Bladder function</b>	Always incontinent or catheterized	0
	Continent or occasional accidents	1
<b>Toilet use</b>	Fully dependent	0
	Needs some help	1
	Independent	2
<b>Bathing</b>	Fully dependent or needs some help	0
	Independent	1
<b>Feeding</b>	Unable or NG/PEG/RIG fed (takes nothing by mouth)	0
	Needs help but can swallow (even if unsafe)	1
	Independent	2
<b>Transfers and mobility</b>	Bedbound, unable to sit	0
	Can sit, but cannot mobilize or transfer without help (from person or walking aid)	1
	Can transfer or mobilize independently or both	2
<b>Stairs</b>	Unable	0
	Needs help	1
	Independent	2
<b>Best verbal response</b>	Mute	0
	Incomprehensible sounds	1
	Single words	2
	Sentences, but difficulty in finding words, uses incorrect words or is often disoriented/confused	3
	Normal conversation	4
<b>Memory and orientation to surroundings</b>	Shows no awareness of surroundings or any evidence of memory	0
	Evidence of retaining some highly learned material (e.g. recognizing 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
<b>Judgement and problem solving</b>	Unable to show any judgement or problem-solving	0
	Able to show some judgement or problem-solving, even if this is severely impaired	1
<b>Use of tools</b>	Unable to use any tools or objects	0
	Able to use some tools or objects, with help if necessary	1

NG = nasogastric; PEG = percutaneous endoscopic gastrostomy; RIG = radiologically inserted gastrostomy.

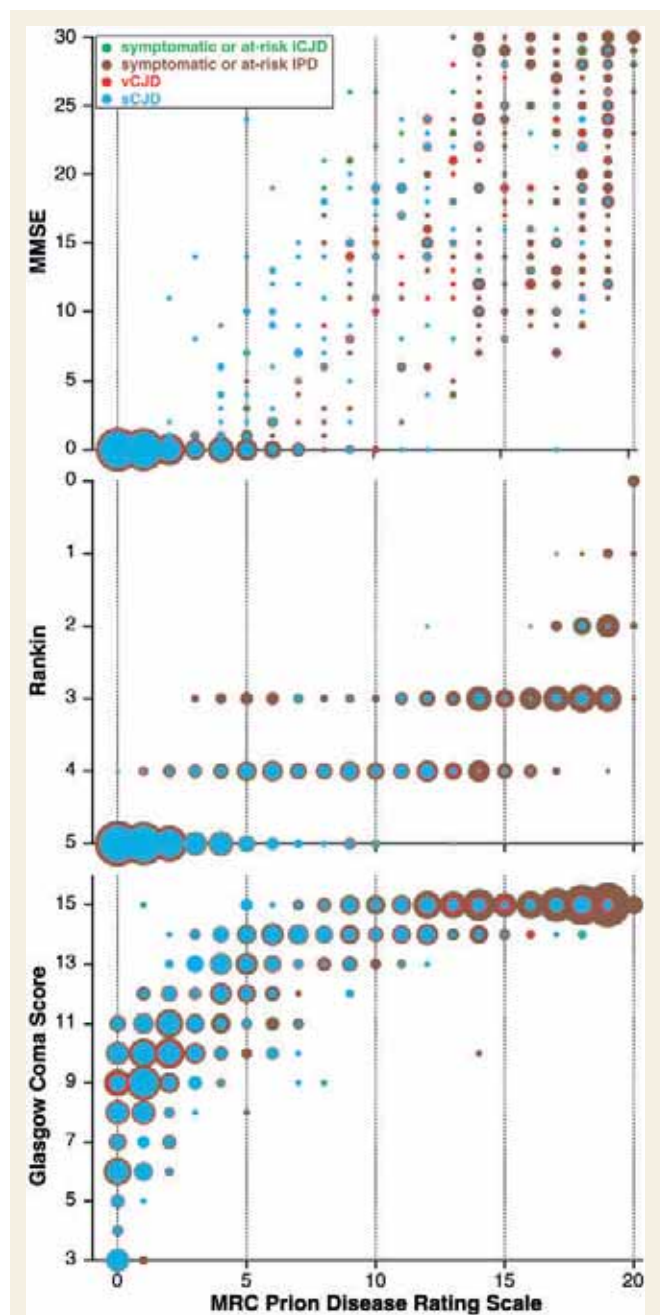
rescoring, appropriate ordering and local dependencies were addressed, items were dropped if fit was still not achieved.

A revised scale including all of these modifications, termed the MRC Scale, demonstrated a good fit ( $\chi^2 = 25.19$ ,  $df = 22$ ,  $P = 0.29$ ). The mean fit residual for all 11 items in this scale was  $-0.498$  with a standard deviation (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 all items across all person factors confirmed the data to be free of differential item functioning when Bonferroni probability adjustment for multiple testing was applied, with gender, age quartile, assessor (doctor or nurse), assessment mode (face-to-face or telephone) included as person factors.

Principal components analysis demonstrated that the greatest variance in the data existed between the most positively loaded items; those assessing speech and cognitive functions (speech, memory/orientation, judgement and use of tools) and the most negatively loaded items which 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 sporadic CJD disease progression.

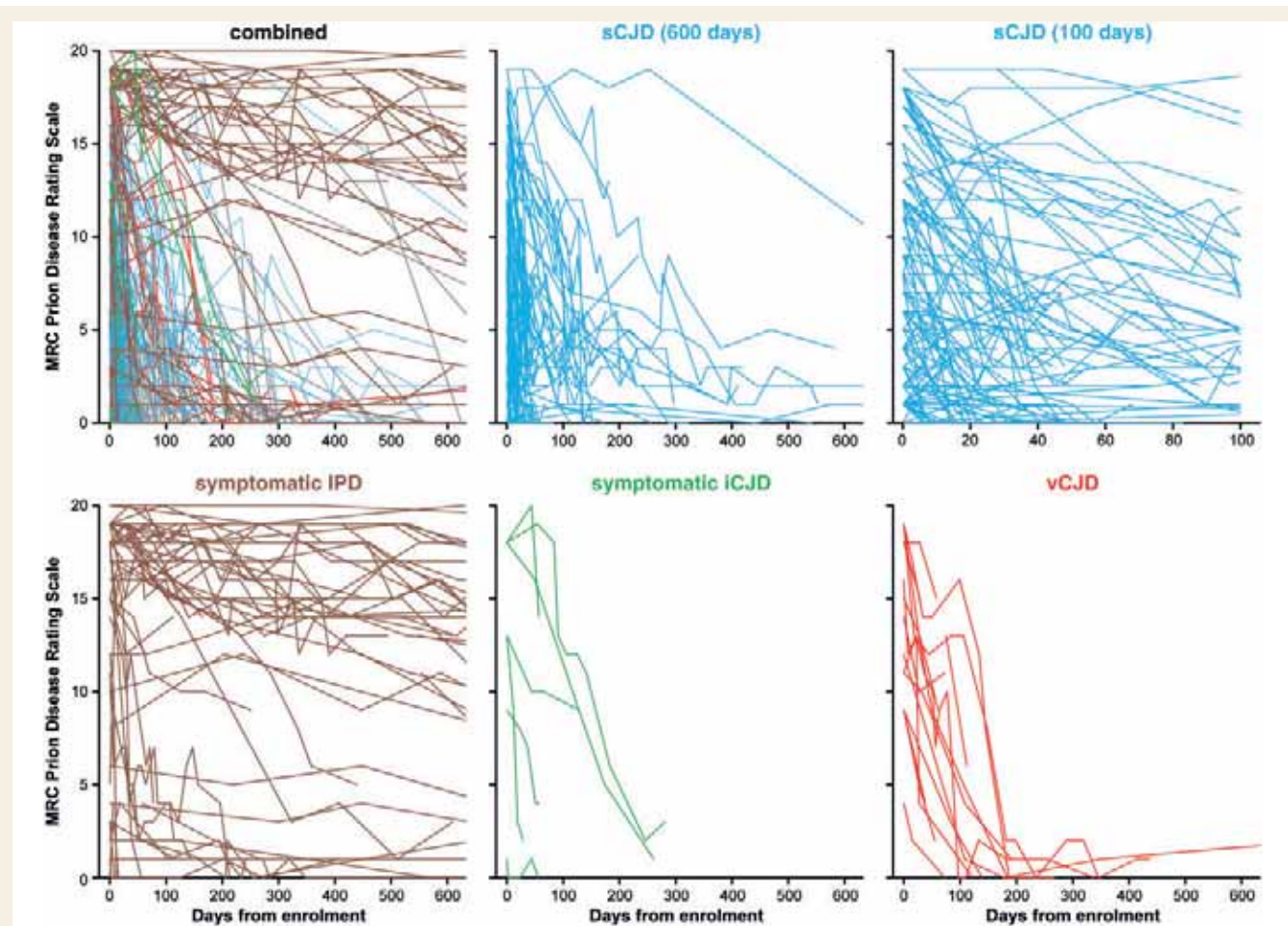
Including earlier data derived from the original component scales (Barthel, Clinical Dementia Rating Sum of Boxes, and Glasgow Coma Score) along with the data collected using the initial scale allowed a total of 380 scales in 239 patients with sporadic CJD to be analysed. The overall fit to the Rasch model was less good but remained acceptable ( $\chi^2 = 33.75$ ,  $df = 22$ ,  $P = 0.052$ ), 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. Testing the MRC Scale's fit in all Stratum 1, rapidly progressive patients, including a rapidly progressive subset of inherited prion disease along with sporadic CJD, also demonstrated acceptable fit to the model ( $\chi^2 = 30.14$ ,  $df = 22$ ,  $P = 0.11$ ). Unsurprisingly, testing fit for patients with inherited prion disease alone and for all symptomatic patients (all disease groups including Stratum 2) continued to result in a poor fit due to the heterogeneity of clinical syndromes that comprise inherited prion disease (such as the distinction between the late cognitive features of Gerstmann-Sträussler-Scheinker syndrome, and early predominant cognitive decline in 6-octapeptide repeat insertion prion disease).

The final format of the rating scale is shown in Table 3. The inter-rater reliability in administration between doctors and nurses of the final scale as a whole was excellent (interclass correlation coefficient =  $0.96$ ). Figure 2 shows the correlation of the MRC



**Figure 2** 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. These plots illustrate the relative absence of floor effect with the MRC Scale when compared with the Rankin or the Mini-Mental State Examination (MMSE) scales, but not the Glasgow Coma Score, as patients with minimum/worst score on the Mini-Mental State Examination and Rankin can still be distinguished with the MRC Scale. In addition these illustrate the multidimensionality of the inherited prion disease (brown) group in that some patients decline in function with normal Mini-Mental State Examination (typically patients with Gerstmann-Straussler-Scheinker disease) and others decline in Mini-Mental State Examination with high levels of function (typically 6-octapeptide repeat insertion mutation patients). iCJD = iatrogenic CJD; IPD = inherited prion disease; sCJD = sporadic CJD; vCJD = variant CJD.





**Figure 3** Trajectories of change in patients up to 600 days post-enrolment for all patients (combined), sporadic CJD (sCJD), inherited prion disease (IPD), iatrogenic CJD (iCJD) or variant CJD (vCJD) only. Three broad patterns are seen: a slow decline in inherited prion disease patients, a rapid and somewhat variable decline in all aetiological groups, and a pattern of decline followed by a preterminal plateau at low levels of function in all aetiological groups. All sporadic CJD 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 patients with inherited prion disease.

Scale with Mini-Mental State Examination, Rankin and Glasgow Coma Score 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 Mini-Mental State Examination or Rankin can still be separated by the novel scale. Whilst Glasgow Coma Score has the ability to separate patients scoring zero on the current scale (Glasgow Coma Score ranging 3–11), these distinctions are difficult to interpret clinically and would require a clinical examination.

## The natural history of prion diseases

Figure 3 shows individual patient trajectories for the MRC Scale over time, colour-coded by disease type. Patients in Stratum 2 have been included for comparison purposes with the caveat that the MRC Scale is not measuring a single progression construct in these patients. 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 patients with sporadic CJD) and slow decline over years (almost exclusively patients with inherited prion disease). A slightly less rapid decline is observed in patients with sporadic CJD who are heterozygous at *PRNP* codon 129, patients with variant CJD and iatrogenic CJD and some patients with inherited prion disease (Figs 3 and 4). In all groups, decline measured with the MRC Scale generally appears to be linear.

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

Figure 5 illustrates the typical progression of a patient with sporadic CJD 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 our clinical experience of these patient groups and are consistent with the validity of the MRC Scale.

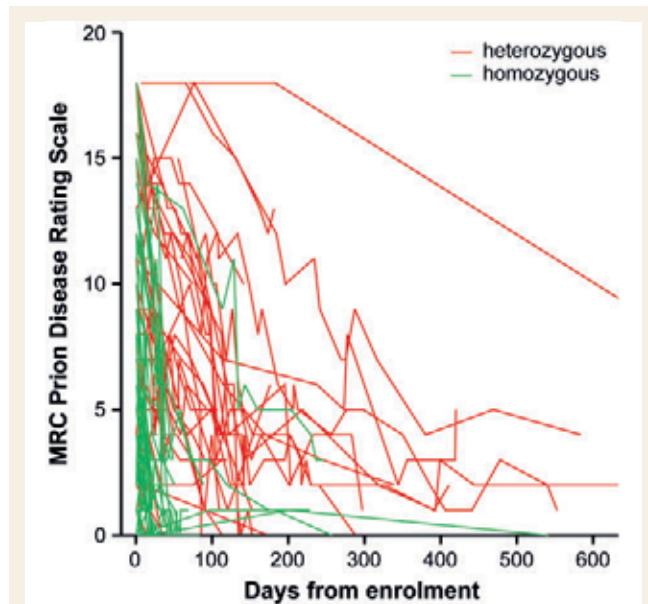
## Discussion

In this paper we have sought to overcome a fundamental obstacle 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

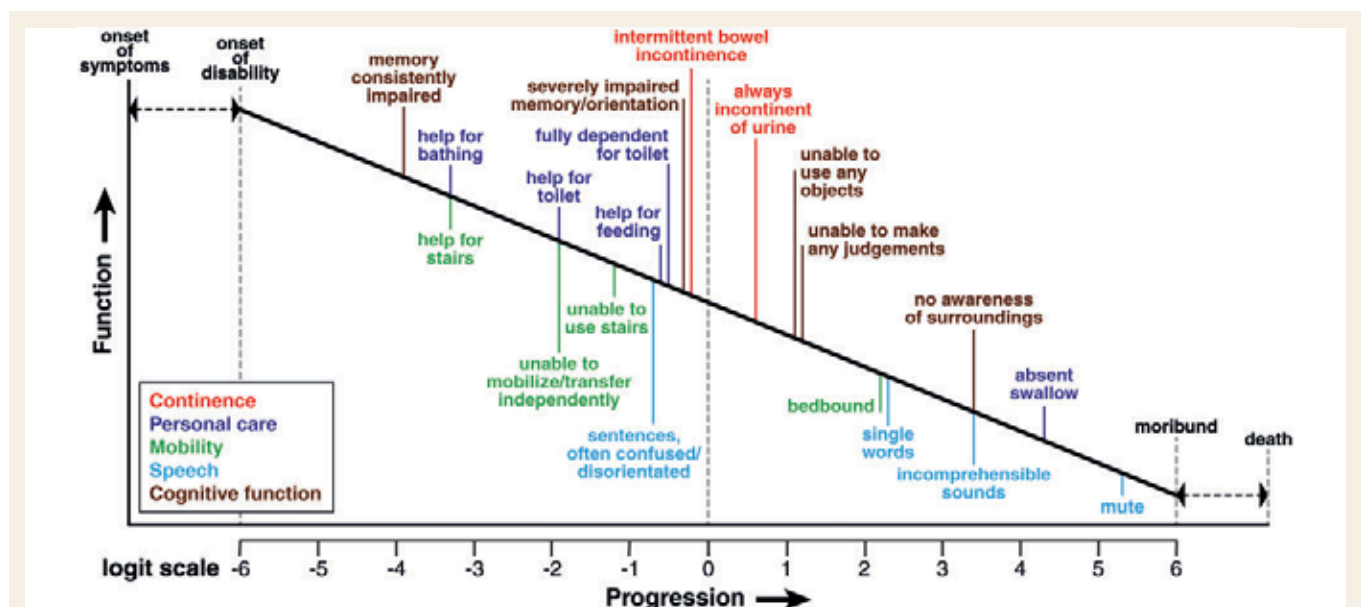
complementary approaches, and taking into account analysis of rating scales data from the PRION-1 trial (Mead *et al.*, 2011), we have developed, refined and validated a bespoke rating scale in the context of the Cohort study, the largest prospective clinical study of the natural history of prion disease. Our outcome measure aims to maximize the likelihood of future trials giving a clear answer on therapeutic efficacy. This study also illustrates that the advanced neurodisability at referral of patients with sporadic CJD 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.

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 before, at or after reaching the end stages of disease progression (e.g. due to aspiration pneumonia). Existing rating scales, which are well validated in other neurological settings, are far less well suited to prion disease. For example, the Mini-Mental State Examination and Alzheimer's Disease Assessment Scale – cognitive subscale, routinely used as an outcome measure in Alzheimer's disease clinical trials, suffer from a marked floor effect (Mead *et al.*, 2011) and fail to capture the profound physical impairments that are fundamental features of these diseases and may be present despite preserved cognitive function. Cognitive decline is itself a fundamental feature of prion diseases, and we have included items assessing cognitive function in a way that is more robust in this population, by assessing carer-reported level of function.

The remarkable clinical heterogeneity of prion disease, combined with its rarity, represents a major challenge to trial design. Very inclusive enrolment criteria will maximize patient numbers,



**Figure 4** Trajectories of patients with sporadic CJD 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.



**Figure 5** Schematic of the pattern of decline in a patient with sporadic CJD that would be most consistent with the Rasch model. Progression is represented by the logit scale used in the Rasch analysis reflecting the relative difficulties of the thresholds that comprise the MRC Scale. 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.

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 that 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 sporadic CJD and changes in patients with some slowly progressive forms of inherited prion disease has not been possible. Patients with inherited prion disease are highly variable in clinical presentation with predominantly mobility or predominantly cognitive progression. It is likely that the detection of subtle changes required to define the onset of disease in inherited prion disease will require analysis of neuropsychological testing that is ongoing in the Cohort study. Our findings suggest that, for maximum efficiency, the main group that should be targeted for future trial recruitment should be those Stratum 1 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 (Collinge *et al.*, 2009).

While we have tried to design a scale that reflects the priorities of patients and their carers, a compromise must be struck with methodological concerns. Our 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 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 our outcome measure, but we are currently investigating them using other methods (e.g. use of the Neuropsychiatric Inventory), and these data will be reported elsewhere. 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.

We have built up a large and detailed natural history data set, with the intent 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 data set can be made available to other physicians conducting clinical trials, and we encourage research groups worldwide to take advantage of this in planning clinical trials. To enable this direct comparison of results from different studies, we propose that the MRC Prion Disease Rating Scale be adopted as a standard outcome measure for prion disease clinical research.

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. Demonstrating a disease-modifying effect of a therapeutic agent in prion disease may therefore lead to insights with wider implications.

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## Conflict of interest

J.C. is a director and shareholder of D-Gen Ltd, an academic spin-out company working in the field of prion disease diagnosis, decontamination and therapeutics. No other author has a conflict of interest.

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RESEARCH

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# Filamentous white matter prion protein deposition is a distinctive feature of multiple inherited prion diseases

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## Abstract

**Background:** Sporadic, inherited and acquired prion diseases show distinct histological patterns of abnormal prion protein (PrP) deposits. Many of the inherited prion diseases show striking histological patterns, which often associate with specific mutations. Most reports have focused on the pattern of PrP deposition in the cortical or cerebellar grey matter.

**Results:** We observed that the subcortical white matter in inherited prion diseases frequently contained filamentous depositions of abnormal PrP, and we have analysed by immunohistochemistry, immunofluorescence and electron microscopy 35 cases of inherited prion disease seen at the UK National Prion Clinic. We report here that filamentous PrP is abundantly deposited in myelinated fibres in inherited prion diseases, in particular in those with N-terminal mutations.

**Conclusions:** It is possible that the presence of filamentous PrP is related to the pathogenesis of inherited forms, which is different from those sporadic and acquired forms.

**Keywords:** Creutzfeldt-Jakob disease, Inherited prion disease, White matter, *PRNP* gene mutation, Octapeptide repeat insert mutation, OPRI, Gerstmann Sträussler Scheinker Syndrome, GSS, Transgenic mice, Axons, Myelin basic protein

## Background

Human prion diseases represent a clinically and pathologically diverse group of neurodegenerative disorders which invariably progress to a fatal outcome. Prion diseases comprise sporadic forms (Creutzfeldt-Jakob disease (sCJD)), inherited prion diseases (IPD) (sometimes also classified as familial CJD (fCJD), Gerstmann-Sträussler-Scheinker Syndrome (GSS) and fatal familial insomnia (FFI)), and acquired forms with variant CJD (vCJD), iatrogenic CJD and kuru. The common neuropathological characteristics of human prion diseases are intraneuronal vacuolation with neuronal loss (spongiform degeneration)

in the grey matter, reactive astrocytosis and microglia activation as well as a highly variable accumulation of abnormal prion protein (PrP<sup>Sc</sup>), which represents misfolded forms of normal cellular prion protein (PrP<sup>C</sup>). The patterns of PrP<sup>Sc</sup> deposition in prion diseases are well characterised at immunohistochemical and ultrastructural levels [1,2] and most observations focus on pathological changes in the grey matter. Involvement of the cerebral and cerebellar white matter in sCJD has been described and suggested to be a primary or secondary process in panencephalopathic type CJD [3,4]. Cerebellar white matter PrP deposition is a prominent feature in the “VV2” subtype type [5]. It also occasionally occurs in other forms of prion diseases, where it is thought to be secondary to cortical degeneration and neuronal loss. The presence of PrP plaques in the white matter has been described in various plaque-forming types of CJD including variant CJD [6] and cases of sCJD with methionine homozygosity

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at codon 129 of the *PRNP* gene (*PRNP* 129MM) [7]. In a series of 20 autopsy cases of CJD, white matter deposition of abnormal PrP was examined in four cases by immunohistochemistry and transmission electron microscopy [8]. Abnormal PrP accumulation in the white matter is also seen in sheep with atypical scrapie [9] as well as in scrapie-infected Syrian hamsters where an intense white matter PrP<sup>Sc</sup> signal was seen in histoblot studies. This was thought to support the hypothesis that PrP<sup>Sc</sup> is transported along axons [10].

Having observed conspicuous filamentous deposits of abnormal PrP in the white matter in autopsy material from patients with inherited prion disease attending the National Prion Clinic and examined at autopsy between 2004 and 2012, we have subsequently analysed our cases systematically for the presence of white matter PrP deposits. We distinguished the patterns of small granular deposits or small plaques that are known for sCJD from a fine, thread like pattern that was seen in inherited cases. The present study describes the frequency, intensity and incidence of this pattern in a series of 35 cases of inherited prion diseases, comprising octapeptide repeat insertions (4OPRI, 5OPRI and 6OPRI) and *PRNP* point mutations of the codons 102, 117, 178, 200 and 210. In addition we give a brief overview of the salient PrP pathology that has been described previously in these inherited forms [11]. We have deliberately omitted a detailed description and discussion of other features such as spongiform degeneration, neuronal loss, gliosis, and the glycoform patterns, as these have been extensively reported elsewhere.

## Methods

### Research governance, post mortem examination and tissue preparation

Ethical approval for these studies was obtained from the Local Research Ethics Committee of UCL Institute of Neurology/National Hospital for Neurology and Neurosurgery. Informed consent to use the tissue for research was obtained. Clinical data, disease history, *PRNP* mutation and *PRNP* codon 129 status were available for all cases. Autopsies were carried out in a post mortem room designated for high risk autopsies. Brains were removed and stored in buffered formalin. The frontal, temporal, parietal and occipital cortex and the cerebellum were dissected during the post mortem procedure and immersed in 10% buffered formalin for up to one week, immersed into 98% formic acid for one hour and postfixed for 24 h in 10% buffered formalin. Tissues were then processed through graded alcohols and embedded in paraffin wax. In a small subset of cases, a small tissue block was prepared from the frontal cortex and white matter and immersed into glutaraldehyde for preparation of semithin resin section and electron microscopy. Frozen tissue of frontal brain and

cerebellum was collected routinely and banked. The remaining brains were stored permanently in 10% formalin and additional brain regions were dissected and processed to paraffin blocks at later time points.

### Antibodies and immunohistochemistry

In this study we used anti-PrP ICSM35 (D-Gen Ltd, London, UK, raised in *Prnp*<sup>0/0</sup> mice against recombinant human PrP as previously described [12];) and anti PrP KG9 TSE Resource Centre, Roslin Institute University of Edinburgh [13]. ICSM35 recognises residues 93–102 and KG9 binds to residues 140–180 of human PrP. To detect abnormal PrP deposition, mounted sections were treated with 98% formic acid for 5 min, placed on an automated Ventana Discovery staining machine, heated to 95°C in 2.1 mM Tris–HCl, 1.3 mM EDTA, 1.1 mM sodium citrate, pH 7.8, for 30 min, digested for 16 min with a low concentration of protease (Protease 3, 0.02 U/ml alkaline protease, order no 760–2020 Ventana Medical Systems), incubated in Superblock for 10 min, then exposed to the primary antibody followed by biotinylated anti mouse IgG secondary antibody and visualised using the iView detection kit Haematoxylin was used as counterstain.

### Immunofluorescence

Immunofluorescent dual labelling was carried out on selected, representative frontal lobe sections of cases with 4OPRI, 6OPRI, P102L, A117V, D178N, E200K mutations and sCJD 129MM, MV, and VV. For dual labelling and co-localisation of abnormal PrP in white matter structures we applied the same antigen retrieval conditions as above. Then we dispensed the antibody ICSM35 (1:1500) together with either anti-Neurofilament (NF200, SIGMA N5389; 1:100) or anti-Myelin Basic Protein (SMI94, Covance, 1:250) followed by the secondary antibodies labelled with fluorochromes Alexa 488 (goat anti Mouse IgG1) or Alexa 546 (goat anti mouse IgG2b). Images were taken on a ZEISS LSM510Meta Laser Scanning confocal microscope, using a Plan-Apochromat 63×/1.4 Oil objective. All images were taken at a scanning resolution that matched the optical resolution of the lens (pixel size corresponding to 99.6 nm on the section).

### Transmission electron microscopy

Tissue blocks of no more than 2 mm<sup>3</sup> were immersed in 2.5% glutaraldehyde, treated with formic acid for 24 h and following postfixation on glutaraldehyde, processed for resin (Epon) embedding. Resin sections were stained with Toluidine blue and suitable regions were selected for electron microscopy. Ultra-thin sections were stained with lead citrate and examined in a Jeol 100-CXII electron microscope. Images were recorded on a 4Megapixel SIS Megaview digital camera.

### Western blotting and glycotyping

Brain tissue homogenates (10% w/v) from frozen samples of frontal cortex were prepared in Dulbecco's phosphate buffered saline lacking  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  ions using tissue grinders or by serial passage through needles of decreasing diameter. Aliquots (typically 20  $\mu\text{l}$ ) were removed and proteinase K added from a 1 mg/ml stock solution (prepared in water) to give a final concentration in the sample of 50  $\mu\text{g}/\text{ml}$ . Following incubation at 37°C for 60 min, samples were centrifuged at 16100 g for 1 min before termination of the digestion by the addition of an equal volume of 2 × SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% v/v glycerol, 4% w/v sodium dodecyl sulphate, 4% v/v 2-mercaptoethanol, 0.02% w/v bromophenol blue) containing 8 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride and immediate transfer to a 100°C heating block for 10 min. Samples were centrifuged at 16100 g for 1 min prior to electrophoresis in 16% tris-glycine gels (Invitrogen). Gels were electroblotted onto PVDF membrane (Immobilon-P; Millipore) and subsequently blocked in PBS containing 0.05% v/v Tween-20 (PBST) and 5% non-fat milk powder for 60 min. After washing in PBST, the membranes were incubated with anti-PrP monoclonal antibody 3F4 (0.2  $\mu\text{g}/\text{ml}$  in PBST) before washing in PBST (60 min) and incubation with alkaline-phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich Prod. No. A2179) diluted 1:10,000 in PBST. After washing (1 h with PBST and 2 × 5 min with 20 mM Tris pH 9.8 containing 1 mM  $\text{MgCl}_2$ ) blots were incubated for 5 min in chemiluminescent substrate (CDP-Star; Tropix Inc) and visualized on Biomax MR film (Kodak). Control samples (of known PrP<sup>Sc</sup> type) were analysed in parallel and run on the same blots to enable assignment of molecular strain type according to the London classification [14,15].

### Examination and quantification

We analysed 35 autopsy cases of inherited prion disease seen at the National Prion Clinic, NHNN and these were compared with 26 cases with sCJD (9 cases with codon 129 MM, 10 with MV and 7 with VV polymorphism). The inherited cases included the following *PRNP* gene mutations (numbers of cases in brackets): 4 octapeptide/96 base pair repeat insertion [4OPRI] (3), 5OPRI/120 bp (1), 6OPRI/144 bp (4), P102L (10), A117V (4), D178N (3), E200K (9) and V210I (1).

Brains were examined in the following areas: Frontal, temporal, parietal, occipital and cerebellar cortex. More regions were available for some of the brains, sometimes with very extensive sampling, but we restricted our analysis to the above-mentioned representative areas of subcortical white matter. Using a semi-quantitative scoring scale (Table 1), all features were recorded for the cortical areas and the cerebellum. Intensity and the

**Table 1 Description and definition of the quantification of the filamentous or granular depositions**

Score (granules or threads)	Number of inclusions (High power field (HPF) with a 40x objective)
0	Less than 1 inclusion per 4 HPF
1	More than 1 inclusion per 4 HPF, up to 5 inclusions per 1 HPF
2	5-20 inclusions per 1 HPF
3	More than 20 inclusions per 1 HPF

Scoring scheme used to semiquantitatively assess the frequency and density of granular or filamentous inclusions in DAB stained paraffin sections on a LEICA DM2500 with a 40x HCX Pan Achromat objective.

deposition pattern of PrP were recorded for the cortical grey matter and the subcortical white matter, as well as the cerebellar molecular and granular layers and the cerebellar white matter. The following features were observed and recorded: in the cortical grey matter, there was synaptic labelling and deposition of plaques, in addition filamentous PrP was present in the white matter fibres extending into the cortex. In the white matter, we recorded small granules as well as thin threads of variable length, which depended on the orientation of the white matter tracts. The scores 0-3 reflect the abundance of PrP deposits (threads or granules), which is listed in Table 1. The quantity and the pattern of cortical prion pathology was recorded for all cases as shown in Table 2.

### Statistical analysis

The quantity score of thread pathology was transformed into numerical values (- = 0, + = 1, ++ = 2, +++ = 3. 4OPRI, 5OPRI and 6OPRI cases; and D178N, E200K, V210I cases, were grouped together to allow group sizes exceeding 3. The null hypothesis was the assumption of no difference between groups. Table 3 shows the results of the statistical analysis.

### Results

35 cases of inherited prion disease were analysed, scored for PrP staining patterns and compared with 26 cases of sCJD. We observed changes in the grey and white matter that were similar to previously published patterns of abnormal PrP deposition. The specific findings and individual white matter staining pattern for each case with mutations in the *PRNP* open reading frame, relevant clinical data, codon 129 genotype, western blotting results, and cortical PrP pattern, are summarized in Table 2 and subsequently in this paragraph. In addition, a detailed description of the findings is given below. PrP<sup>Sc</sup> molecular strain typing analysis was performed for about half the cases (Table 2). Where determined, molecular strain types were consistent with those published previously [15] however some brain samples were scored negative for PrP<sup>Sc</sup> (Table 2). The apparent absence of detectable

**Table 2 List of all cases analysed for white matter PrP in this study**

Figure	Case no	Mutation	Codon 129 status	PrP <sup>Sc</sup> molecular strain type*	Gender	Age at death	Disease duration (mo)	Amount of PrP <sup>Sc</sup> in WM		PrP pattern WM T = Threads D = Dots		Other pathology	
								FB	Cer	FB	Cer	Cortical PrP pattern	Spongiosis
	1	V210L	MV	2	M	69	5	+	—	T, D	—	S1, PN1, D2	+
	2	E200K	MM	2	M	65	4	+	—	D	—	S2, GR1, PN1	+
	3	E200K	MV	ND	M	67	24	+	—	D	—	S1	+
	4	E200K	MM	ND	F	57	6	++	+	D	D	S1, PN1	+
Figure 2D-F, Figure 4A, B	5	E200K	MV	2/3	F	45	38	+++	+	T, D	D	S3, GR2	+++
	6	E200K	MM	ND	M	61	6	++	+	D	D	S2, GR2, PV2	+
	7	E200K	MM	2	M	65	5	—	—	—	—	S1, PN1	++
	8	E200K	MM	2	M	65	9	+	—	D	—	S1	+
	9	E200K	MM	2	F	69	4	+	+	D	—	S2, PN1	++
	10	E200K	MM	ND	M	47	19	++	++	T, D	T, D	S2, D1, PV2	+
	11	D178N	MM	Negative	M	60	7	+++	+	D	D	P3, D2	o
Figure 2A-C, Figure 3I, J	12	D178N	MV	ND	M	60	24	++	—	D	—	S2, GR1, P1	+++
	13	D178N	MV	ND	F	51	20	—	—	—	—	S1, GR1	++
	14	A117V	MV	Negative	M	43	6	+++	++	T	D	S2, P3, D3	o
	15	A117V	WV	Negative	M	40	46	+++	++	T	D	S2, P3	++
Figure 1M-P, Figure 3G, H	16	A117V	MV	Negative	M	47	47	+++	—	T	—	S1, P3	o
Figure 5C, D	17	A117V	MV	ND	F	40	98	+++	+	T	T, D	S2, P3	++
	18	P102L	MV	Negative	M	53	64	++	++	T	T, D	P3, D2	o
	19	P102L	MM	1	M	58	53	+	+	T	D	P3, D2	++
	20	P102L	MM	ND	M	64	24	—	+	—	T, D	S1, P1	o
Figure 1I-L	21	P102L	MM	ND	M	59	49	+++	+++	T, D	T, D	P3, D2	++
	22	P102L	MV	2	F	64	33	+++	++	D	D	S2, P3, D2	++
	23	P102L	MV	ND	M	61	50	+++	+++	T, D	T, D	S2, P3, D1	+++
	24	P102L	MM	2	F	65	64	+	++	T, D	T, D	S1, P3, D2	++
Figure 3E, F	25	P102L	MM	Negative	F	62	48	++	+	D	D	S2, P3, D1, PV1	+
	26	P102L	MM	Negative;8 kDa	M	52	155	+	+	D	D	P2	o
	27	P102L	MM	ND	F	66	81	+	+++	D	T, D	S1, P3	++
	28	6OPRI	MV	Negative	F	49	8	+++	+	T	D	S1	o



**Table 2 List of all cases analysed for white matter PrP in this study (Continued)**

Figure 1E-H	29	6OPRI	MV	Negative	F	46	0	+++	+++	T	T, D	S2, P3, D3	o
	30	6OPRI	MM	ND	M	44	118	+++	+++	T, D	D	S3, GR1, P1, D1	+
Figure 3C, D, Figure 5A, B	31	6OPRI	MM	ND	F	45	72	+++	++	T	T, D	S1, PN1	+
	32	5OPRI	MM	ND	M	57	323	+	+	T, D	T, D	S1	+
	33	4OPRI	MM	ND	F	56	7	+++	+	T	T, D	S3, GR1	+
Figure 1A-D, Figure 3A, B	34	4OPRI	MM	ND	M	73	14	++	++	T, D	T, D	S1	+++
	35	4OPRI	MM	2	F	62	5	++	++	T	T, D	S1, GR1, PN1	++

\*Proteinase K-digested PrP fragment size according to the London classification [14,15].

ND, not done.

All case numbers (first column) are cross-referenced to Figures 1, 2, 3, 4, 5 and 6. The columns on the right describe the pattern of PrP deposition in the white matter. The semi quantitative density scores of white matter PrP are represented in column 8. mo: months, M: male/methionine, F: female, V: valine, WM: white matter, FB = Forebrain, Cer = Cerebellum, T = Threads, D = Dots. The two columns on the right describe the pattern and intensity of cortical PrP deposition as described in [11] and the degree of spongiform changes. S = synaptic, PN = perineuronal net, GR = Granular; P = well demarcated plaques; D = diffuse plaques; and PV = perivascular deposits. The number indicates the semi-quantitative intensity or density of the respective feature.

**Table 3 Calculation of the 1 way ANOVA**

Group	Number of subjects	Mean	Standard deviation
1 (OPRI)	8	2.5	0.88
2 (P102L)	10	1.7	1.53
3 (A117V)	4	3	0
4 (E200K, D178N, V210I)	13	1.4446	0.7697

protease-resistant PrP is a relatively common feature of some forms of inherited prion disease and in part relates to sampling variation and in particular the region of brain studied [15]. Typically we examined only a single sample (~200 mg) of frontal cortex.

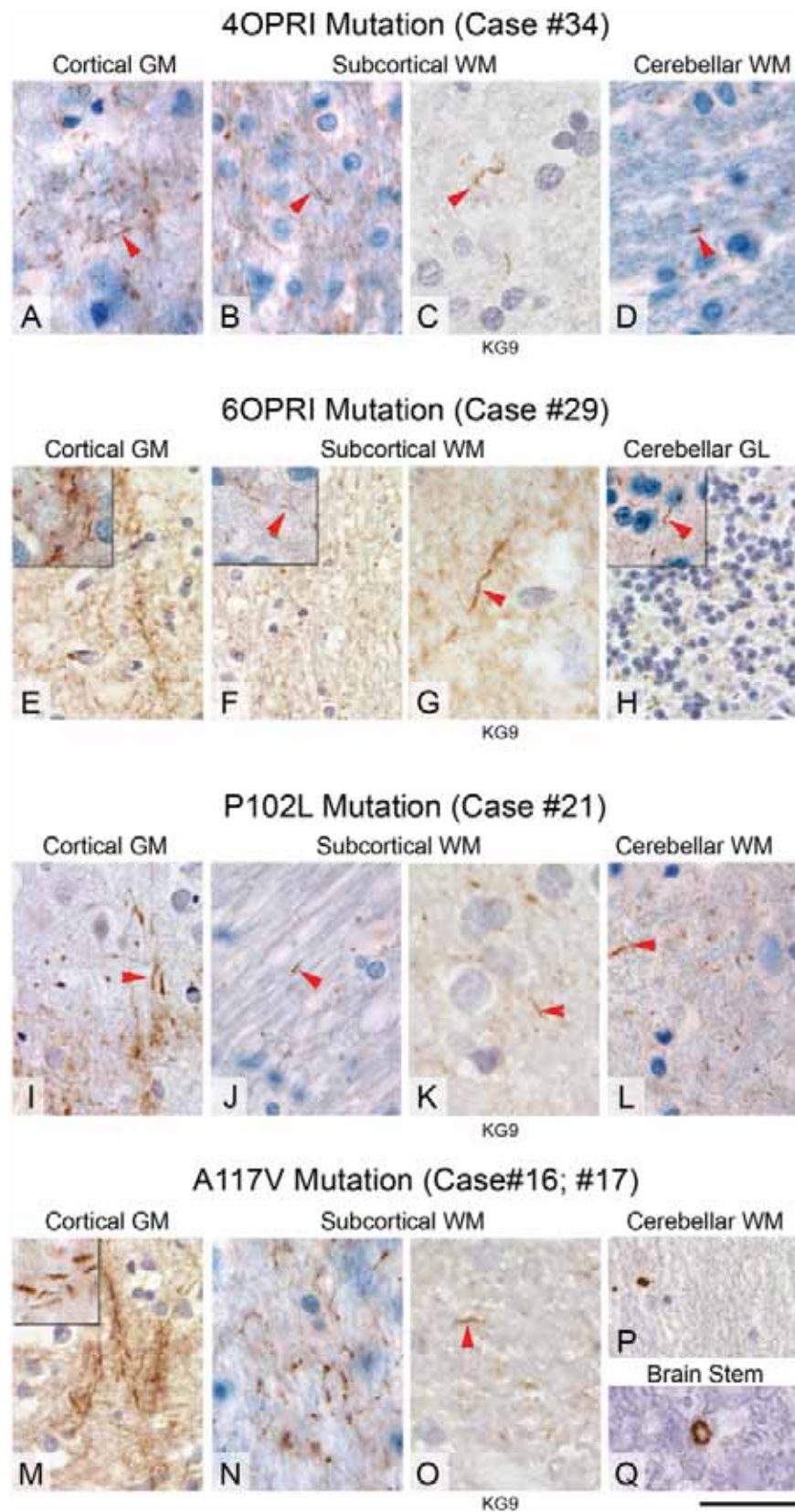
The white matter of brains with inherited CJD prion disease show in the majority of cases filamentous PrP deposition in the frontal, temporal parietal and occipital regions of cerebral, subcortical white matter and to a much lesser extent in the cerebellar white matter, and the brain stem (Table 2, Figures 1, 2, 3, 4, 5, 6). Cortical areas that contain significant amounts of abnormal synaptic or plaque PrP, tend to show more abundant filamentous white matter whilst a low cortical PrP load is more often associated with very little or no filamentous PrP in subjacent white matter tracts. Table 4 and Figure 7 show the distribution of white matter filaments in Case #17. In this case, the strongest labelling is seen in frontal and temporal lobes, basal ganglia, followed by parietal and occipital regions, and the least frequent labelling in the cerebellum and pons. Occasionally we found circular PrP deposits in cross sections (Figure 1Q), suggestive of a localisation on myelin sheaths. In addition, there was fine granular or dot-like positivity in almost all cases, including sCJD of all three codon 129 genotypes (MM, MV and VV). When the cases with filamentous inclusions are plotted onto a diagrammatic representation of the *PRNP* open reading frame, we observed that the strongest and most consistent filamentous white matter deposits were in cases with N-terminal mutations (Figure 6), with octarepeat inserts, and A117V mutations, followed by P102L mutations. These filaments were much less frequent in brains with C terminal D178N, E200K and V210I mutations. The intensity was unrelated to disease duration or codon 129 genotype (Table 2) but appeared to mirror cortical PrP load. The two groups with marked formation of threads (4OPRI, 6OPRI and A117V) comprise younger patients (40-50ys); while patients with other mutations were more than 50 years old with the exception of two patients with an E200K mutation, of whom one showed an unusual clinical phenotype with very long disease duration (#5). The brain in this case showed filamentous white matter PrP. One way ANOVA confirmed that N terminal mutations are more likely to accumulate filamentous abnormal PrP than the white matter of C

terminal mutant brains (Significance level  $p = 0.012$ ). Instead, no filamentous, but variable granular PrP deposits are seen in cases of sCJD, with no intensity difference between the different polymorphisms on codon 129 (Table 5). Double labelling immunofluorescence studies confirm the presence of PrP filamentous material in the white matter. In keeping with light microscopy observations, the most abundant filaments are seen in 4- and 6OPRI, and A117V mutations, whilst P102L, D178N, E200K and V210L mutations are less often associated with filamentous PrP in the white matter. The filamentous nature and the localisation at light microscopy level are suggestive of axonal accumulation. To determine the precise location of the filaments in the white matter, we used double-labelling immunofluorescence and detection by confocal laser microscopy. There was virtually no overlap between Alexa 488 labelled neurofilaments and Alexa 546 labelled PrP, suggesting that the filamentous deposits are not in the axoplasm. Instead there were PrP threads that appeared to flank and run in parallel to axons, suggesting a localisation directly adjacent to the axoplasm of myelinated fibres. Indeed further co-localisation studies with Alexa 488 conjugated antibodies against myelin basic protein confirmed that these filamentous PrP deposits shows robust co-localisation of PrP threads with myelin sheaths of all sizes. Longitudinal sections of myelinated fibres occasionally contain PrP threads on either side of the centrally located axons (Figure 3). Sections of dual labelled axons show a co-localisation of PrP with MBP, sparing the central axon (Figure 3). In contrast, samples of sCJD contain granular deposits outside the myelin sheath but no filamentous PrP (Figure 4), in keeping with the observations by light microscopy.

In the following paragraphs we describe PrP deposits in cortical grey matter, followed by changes in the subcortical white matter, cerebellar grey and white matter changes and the co-localisation of abnormal PrP with myelin and axonal structures. Finally, the results of the dual labelling experiments are described. Type and intensity of the white matter deposits (none, dot like/granular or filamentous) with relation to the mutation are also summarised in a graphic representation in Figure 7. This figure also gives a reference to the cases in Table 2.

**4OPRI [96 base pair insert] (1 male, 2 female); Figure 1A-D, Figure 3A, B**

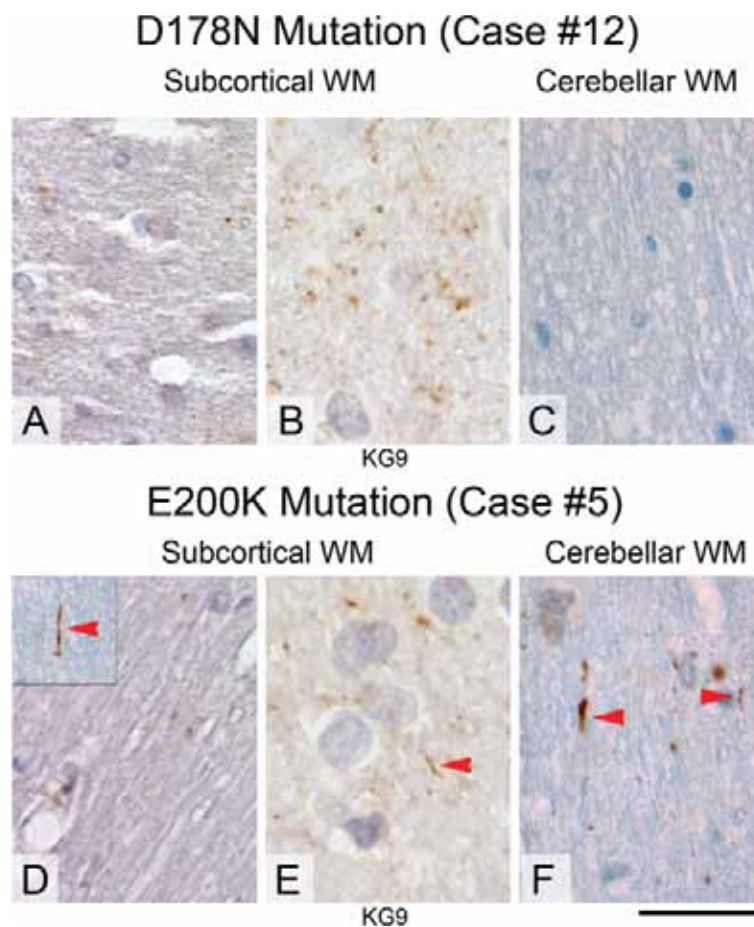
The mean age for this group is 63.7 years (56–73) (Cases #33-35). The cerebral grey matter of these cases shows both axonal threads and granular synaptic stain (Figure 1A). The white matter also shows threads and dots of abnormal PrP (Figure 1B,C). The cerebellar cortex displays the classical “striped” or “tigroid” pattern of PrP depositions, perpendicular to the cerebellar surface and the Purkinje cell layer as reported previously



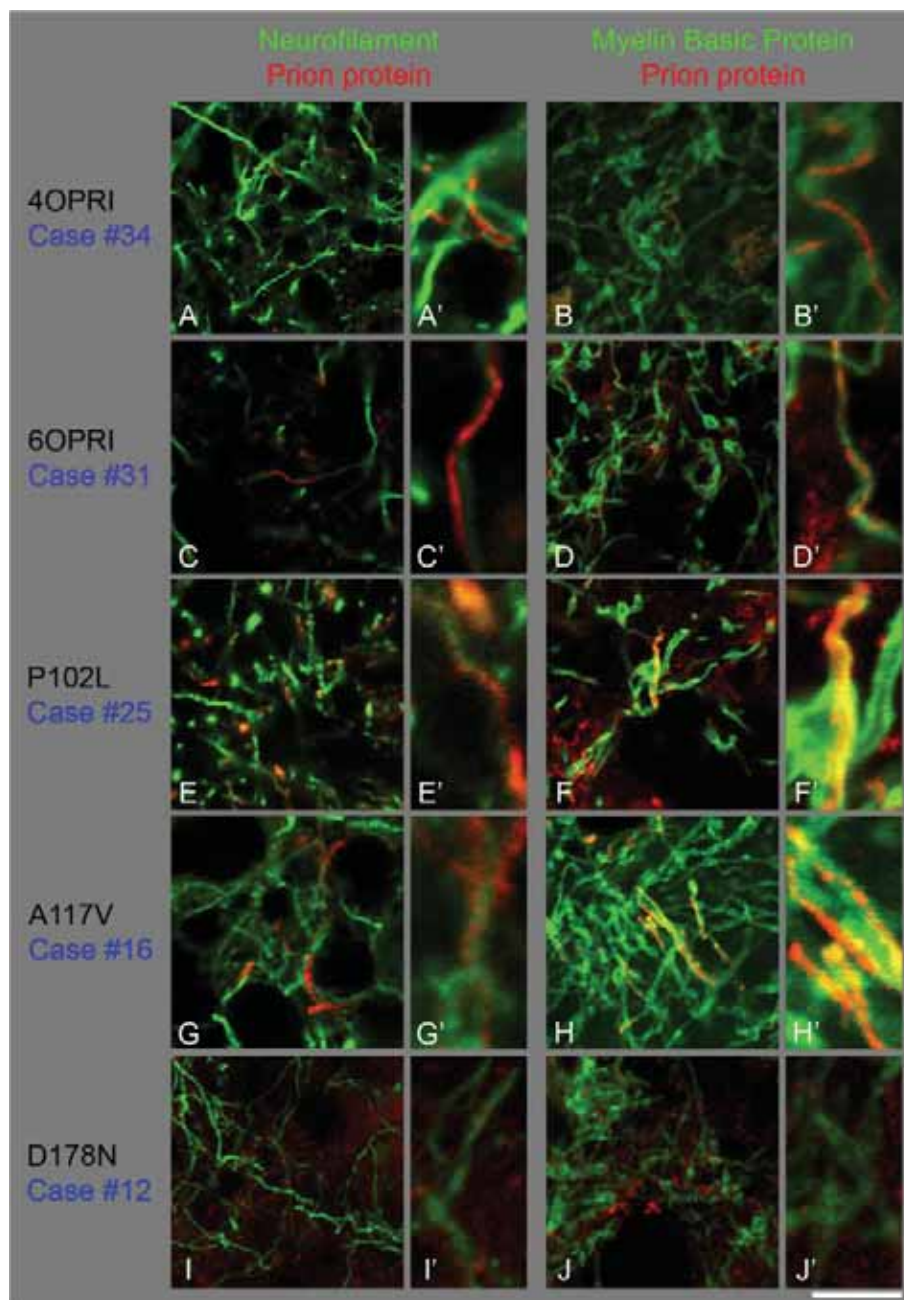
**Figure 1** (See legend on next page.)

(See figure on previous page.)

**Figure 1** Threads of PrP positive deposits in inherited prion diseases with 4 and 6 octapeptide repeat insert mutation, P102L (Gerstmann Sträussler-Scheinker syndrome), and A117V mutation. **AD**, 4OPRI mutation with abundant threads, which form a dense crisscrossing network (corresponding to myelinated fibres) in the cortex (**A**) and forming multiple, thin parallel threads in the subcortical white matter, detectable with two antibodies, ICSM35 (**B**) and KG9 (**C**). Arrowheads in **B** and **C** point to accentuated threads. In the cerebellum (**D**), the PrP-containing filaments are thicker, shorter, and less abundant than in the cortex. The 6OPRI mutation (**E-H**) is characterised by abundant threads in the cortical grey matter I, subcortical white matter (**F**, ICSM35, **G**, KG9), and in the cerebellar granular layer (**H**). **I-L**, Both forebrain and cerebellum with *PRNP* P102L mutations show white matter threads of abnormal PrP in the subcortical (**J**, ICSM35 and **K**, KG9) or cerebellar (**L**) white matter. Arrows **I-L** show short threads of PrP deposition. Small myelinated fibres extending into the cerebral cortex also show threads of abnormal PrP (arrowhead in **I**) alongside with large amyloid plaques (not shown) and synaptic PrP. **M-Q**, A117V mutation (Case #16 M-P and Case #17, **Q**) with abundant cortical threads (**M**), corresponding to myelinated cortical fibres. The inset shows several parallel threads. Also the subcortical white matter is rich in PrP positive threads (**N**, ICSM35 and **O**, KG9). The cerebellum of the same case lacks filamentous inclusions, whilst small granular deposits are often observed (**P**). The brain stem of case #17 shows cross sections of myelinated fibres with occasional circular PrP-positive structures, corresponding to PrP containing myelin sheaths. Scale bar corresponds to 20  $\mu$ m in **P**, 40  $\mu$ m in the insets of **E**, **F**, **H** and **M**, to 80  $\mu$ m in **C**, **G**, **H**, **O** and to 140  $\mu$ m in all other panels.



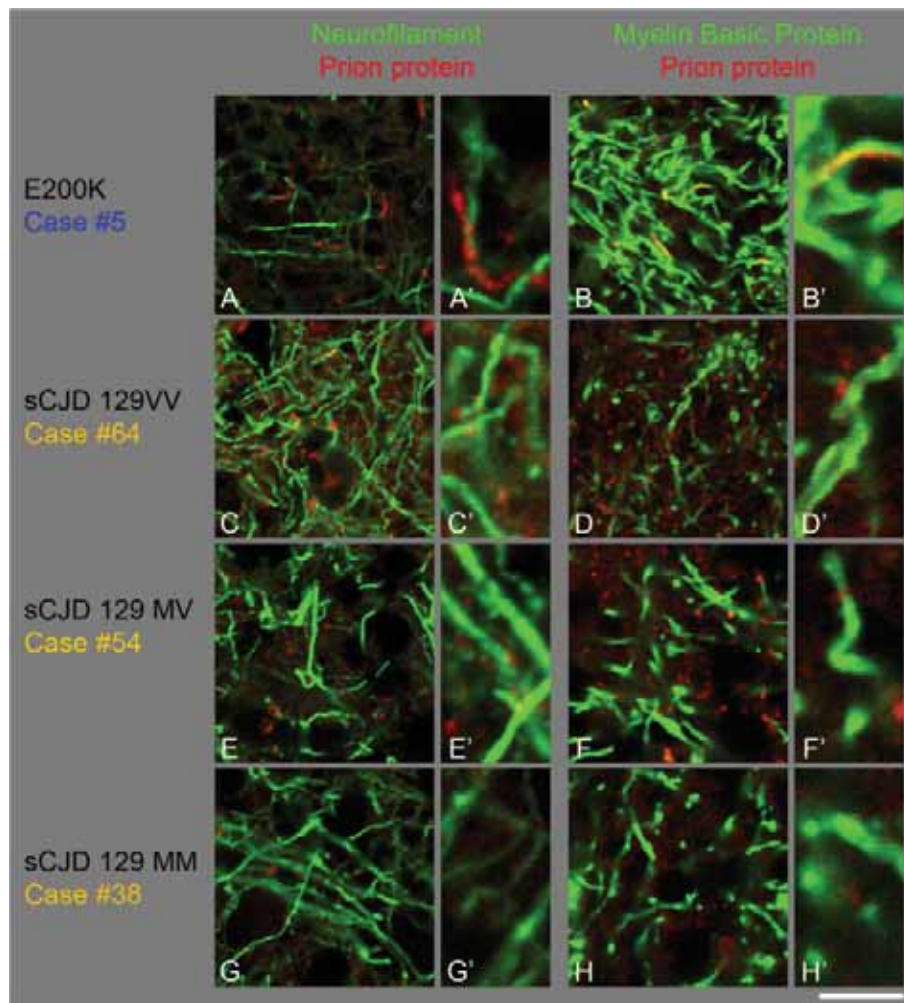
**Figure 2** Patterns of PrP deposits in brains with *PRNP* D178N and E200K mutations. **A-C**, there are small dot like inclusions in the white matter of a case with D178N mutation (**A**, **B**, ICSM35 and KG9), no cerebellar threads (**C**). **D-F**, E200K mutation with sparse threads in the subcortical white matter (**D**, ICSM35 and **E**, KG9) and the cerebellar white matter (**F**). The inset in (**D**) shows a rare straight PrP positive filament and the arrowhead points at a short stub or dot of abnormal PrP. The cerebellar white matter (**F**) of the same case shows occasional thicker, straight filaments of abnormal PrP (arrowheads). Scale bar corresponds to 140  $\mu$ m in (**D**), to 80  $\mu$ m in **B**, **E** and to 40  $\mu$ m in all other panels.



**Figure 3** Co-localisation studies of abnormal PrP (Alexa 546, red) with Neurofilament (A, C, E, G, I) or with myelin basic protein (B, D, F, H, J), (green, Alexa 488) in the white matter of the frontal lobe. A, B, 4OPRI mutation shows the neurofilament signal being located beside abnormal PrP (A, A'), whereas there is an overlap between MBP and PrP signal (B, B'). C, D; 6OPRI with filamentous PrP being localised next to Neurofilament (C, C') but directly congruent with MBP (D, D'). E, F there is little white matter PrP in this example of a P102L mutation, whilst a case with an A117V mutation (G, H) shows abundant white matter PrP, which shows PrP immunoreactivity next to neurofilaments, (G, G') and co-localises with MBP, resulting in yellow signal (H, H'). The more C-terminal mutations, such as D178N show mostly granular PrP deposits and no strong filamentous PrP (I, J). Scale bar 16µm for A-J and 4 µm respectively for A'-J'.

[11,16] and the white matter comprises rare to moderately frequent dots and thread-like staining (Figure 1D). Filamentous deposits were seen with both antibodies (Figure 1B, ICSM35 and 1C, KG9). All three 4OPRI cases show consistent axonal labelling in cerebral grey and

white matter as well as cerebellar white matter, in a pattern and intensity similar to that observed in the 6OPRI group [11,17]. Co-localisation studies (Figure 3A,B) with PrP (red) and neurofilaments (green) show occasional parallel course of PrP threads and axons (Figure 3A,A'),



**Figure 4** Co-localisation studies of abnormal PrP (Alexa 546, red) with Neurofilament (A, C, E, G) or with myelin basic protein (B, D, F, H), (green, Alexa 488) in the C-terminal E200K mutation and in sCJD control cases (frontal cortex). A, B, E200K mutations shows rare filamentous PrP, co-localising with the myelin sheath (B, B') but not detectably with axonal neurofilament (A, A'). All sCJD cases (129VV, C, D; 129MV, E, F; 129MM, G, H) show variable amounts of granular PrP which does not co-localise with neurofilaments (C, E, G) or Myelin basic protein (D, F, H). Scale bar: 16  $\mu$ m for A-H and 4  $\mu$ m respectively for A'-H'.

while both longitudinal and transverse sections of myelin sheaths confirm the presence of the threads within the myelin structure Figure 3B,B).

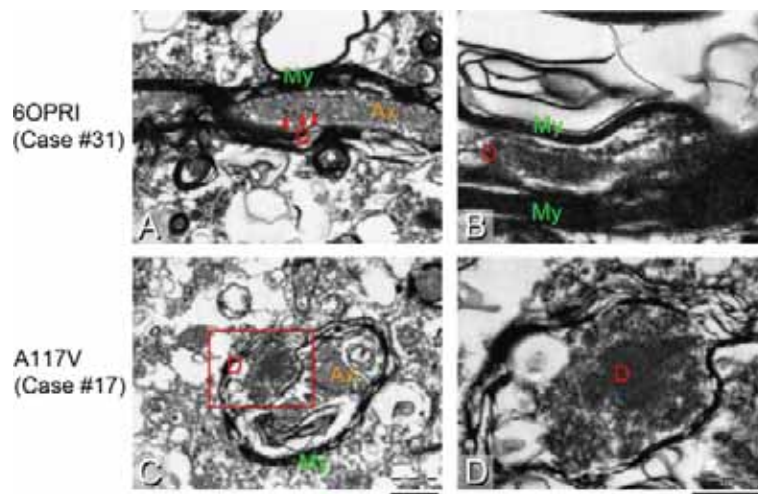
#### 5 OPRI [120 bp insert] (1 male)

The age of this patient was 57 years, with disease duration of several years (Case #32). The onset of his illness was difficult to determine as he gradually developed neurological symptoms in his forties which superimposed a pre-existing personality disorder with aggressiveness and outbursts. The brain showed PrP deposits that are similar to sporadic sCJD and the characteristic striping pattern, which is generally seen in the cerebellar cortex with 4OPRI and 6OPRI mutations, was absent. The cerebral

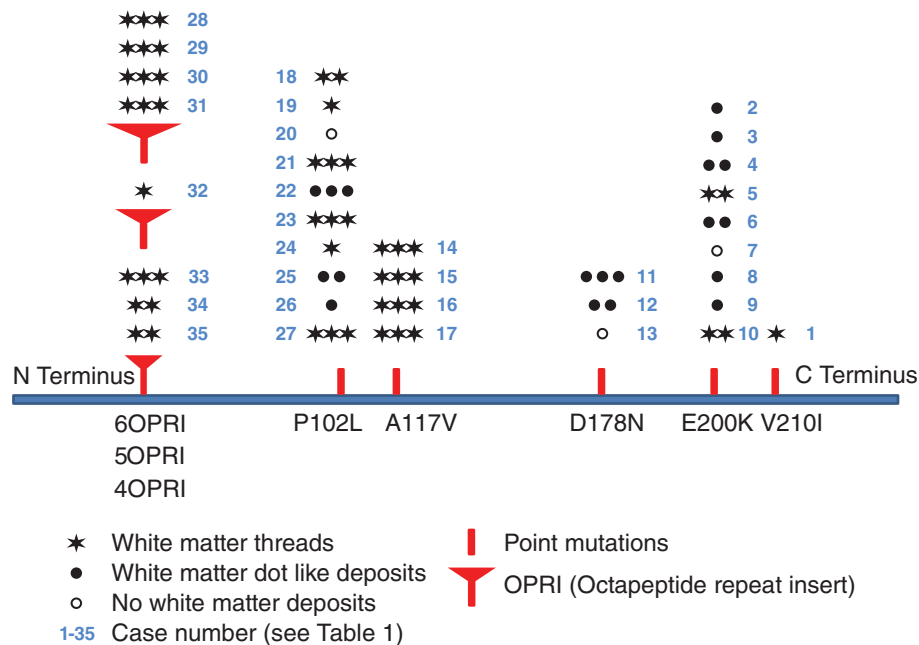
white matter showed subtle white matter threads and there was no white matter PrP in the cerebellum.

#### 6OPRI [144 bp insert] (1 male, 3 female); Figure 1E-H, Figure 3C,D, Figure 5A,B

The mean age for this group is 46 years (44–49) (cases #28-31). In all cases the cerebral grey matter contains abundant axonal threads (Figure 1E), as well as a granular synaptic stain and scattered microplaques. These threads are present in the myelinated fibres that radiate from the white matter into the cortex. The subcortical white matter contains PrP positive threads (Figure 1F, ICSM35 and 1G, KG9) and to a lesser extent, fine granules. The cerebellar molecular layer shows the classical, distinct “stripy” or “tigroid” pattern with an orientation



**Figure 5** Electron microscopy of two cases with abundant white matter deposition of PrP amyloid in the frontal cortex. **A, B**, Case #31 with 6OPRI mutation shows small granular deposits in the axoplasm of a longitudinal section of a myelinated axon, and more substantial fibrillar electron dense deposits in a myelin sheath, that has become divided by these inclusions, raising the possibility of amyloid. **C, D**, Case #17 with an A117V mutation shows a cross section of a myelinated fibre with the axon (Ax) being separated from the para-axonal inclusion that led to a splitting of the myelin (My) sheath. The red box indicates the region shown in **D** with a characteristic electron dense deposition, raising the possibility of amyloid. Scale Bars: 1 µm (**A, C**), 0.5 µm (**B, D**).



**Figure 6** Schematic representation of the deposition of white matter filamentous PrP in relation to the mutation: the horizontal bar represents the *PRNP* open reading frame with the N terminus on the left and the C terminus on the right. OPRI and point mutations are indicated with red symbols. At each mutation, the cases with white matter threads (filaments) are indicated with stars; the number of stars indicates the abundance of deposits (corresponding to the score in Table 2). For cases where no filaments are seen, white matter dot-like deposits are indicated by filled circles and cases with no white matter deposits are symbolised by open circles. The blue number next to the symbols corresponds to the case numbers in Table 2, all figures and in the text). The position of the mutations on the schematic representation is not exactly to scale.

**Table 4 Regional distribution of filamentous PrP in a brain with A117V mutation (Case #17): semiquantitative scoring of white matter PrP filaments**

Case #17: areas analysed	Density of filamentous PrP in white matter tracts in the region
Anterior frontal F1-F2	+++
Posterior frontal Superior frontal gyrus and pre motor cortex	++
Temporal T1-T2	+++
Temporal T2-T3	+++
Tip of temporal lobe	+++
Parietal	++
Occipital	++
Anterior cingulate	+++
Hippocampus	+++
Amygdala	+++
Basal ganglia, ant.	+++
Basal ganglia, mid.	+++
Basal ganglia, post	++
Thalamus with subthalamic nucleus	+
Upper midbrain	+ / ++
Lower midbrain	+
Pons	+
Lower medulla	n.a.
Cerebellum Vermis	+
Cerebellum with dentate	-
Cerebellum hemisphere	(+)

The graphical representation is shown in Figure 7.



**Figure 7 Regional distribution of filamentous PrP in a brain with A117V mutation.** (Case #17): the intensity of the white matter filaments is represented in shades of red, the most intense representing a strong deposition (frontal, parietal and temporal brain) and there is a decreasing gradient towards the posterior lobes, the cerebellum and the brain stem, where fewer filaments were observed. The corresponding semi-quantitative evaluation is listed in Table 6.

perpendicularly to the cerebellar surface as described before [11,18-20]. This pattern is a hallmark of octapeptide insert mutations and unlikely to be related to the filamentous deposits. Threads are also seen in the cerebellar granule layer (Figure 1H) and the cerebellar white matter shows variable amounts of dot- and thread-like staining. Case #30 shows a staining pattern that varies from the other cases with a 6OPRI mutation, in that the grey matter displays granular synaptic positivity without thread labelling and the white matter shows a predominance of granular labelling and fewer threads. In conclusion, this group shows consistent, strong filamentous positivity in both, grey and white matter (Figure 3), similar to the group with 4OPRI mutation (see above). The cerebellar white matter shows thread pathology, a feature also seen in cases with P102L and 4OPRI mutations. Co-localisation studies show a striking overlap of PrP with myelin sheaths, whilst colocalisation with axons was below detection limits, confirming that PrP threads are located extra-axonal, within the sheath (Figure 3C,D). However, ultrastructural studies of case #31 show granular protein deposits also in the axoplasm but, in keeping with immunofluorescence studies, more substantial deposits in the myelin sheath, which appears displaced and split by the amyloid aggregates (Figure 5A,B).

**P102L (6 male, 4 female); Figure 11-L, Figure 3E, F**

The mean age for this group is 60.8 years (53–66) (cases #18-27). The cerebral grey matter of these cases contains frequent core- and multicentric plaques with diffuse granular synaptic pattern as described before [21,22] and occasional threads composed of abnormal PrP (Figure 11). The subcortical white matter contains scattered plaques of small size with variable fine granular and thread-like positivity (Figure 1J, ICLM35 and 1K, KG9). The cerebellar cortex shows plaques and large granular aggregates. The white matter contains variable plaques, granules and short threads (Figure 1L). In the majority of cases with P102L mutation, a small, and at most moderate amount of filamentous labelling can be observed in the white matter and less dense axonal positivity in the grey matter. In more than two thirds of the cases filamentous labelling is also present in the cerebellar white matter. In immunofluorescence studies however, nearly no filamentous material was detected (Figure 3E,F).

**A117V (3 male, 1 female); Figure 1M-P, Figure 3G,H, Figure 5C,D**

The mean age for this group is 43 years (40–47) (cases #14-17). The grey matter contains frequent granular plaques with diffuse, moderate-to-strong granular synaptic labelling as well as strongly labelled short (Figure 2M inset) and long filamentous white matter deposits (Figure 2M). In all three cases the white matter shows frequent and intense thread-



**Table 5 Summary of intensity scores in sporadic CJD**

sCJD, codon 129 genotype	Scores for granular PrP (number of cases)				Significance
	Score 0	Score 1	Score 2	Score 3	
129MM	1	3	3	2	MM vs. MV: 0.23 (n.s.)
129MV	3	4	2	1	MV vs. VV: 0.72 (n.s.)
129VV	2	2	2	1	MM vs. VV: 0.48 (n.s.)

The left columns indicated the genotype of the codon 129 polymorphism, the subsequent columns the number of cases with the indicated scores (0–3) and the right columns the statistical analysis of the scores between the different codon 129 genotypes. No statistically significant difference in the intensity scores are seen between cases with codon 129 MM, MV or VV.

like positivity with sparse fine granules and plaques (Figure 1N, ICLM35 and 2O, KG9). The cerebellar cortex mainly contains patches of plaques and the white matter shows occasional plaques with none or occasional dot-like PrP deposits as described before [23,24]. In conclusion, this group displays a strong, dense filamentous PrP labelling in the cerebral white matter, similar to the 6OPRI and 4OPRI cases (see above), with significant filamentous PrP extending to the myelinated axons in the cortical grey matter and no cerebellar threads. A cross section of the pons (Figure 1Q) show occasional circular PrP deposits, strongly suggestive of a localisation in the myelin sheath. Immunofluorescent labelling confirms extensive co-localisation of PrP threads in the sheaths of myelinated fibres, and no axonal PrP. The dual labelling of PrP with neurofilaments demonstrates no colocalisation of the signal (Figure 3G, G'), whilst there is frequent and robust dual labelling of myelin sheaths and filamentous PrP (Figure 3H, H'). All A117V cases strongly accumulate filamentous PrP and it is generally very easy to detect filaments in the white matter of A117V cases. Ultrastructural studies of case #17 show substantial deposits of amyloid in the myelin sheath (Figure 4C,D).

**D178N (2 male, 1 female); Figure 2A-C, Figure 3I,J**

The mean age for this group is 57 years (51–60); cases #11-13. Both patients showed clinical CJD phenotype. Here, the cortical grey matter shows diffuse, weak to moderate fine granular synaptic stain with plaques of variable size. The subcortical white matter shows a wide variability (none to very frequent) of fine granular positivity but no thread like labelling (Figure 2A, ICSM35 and 3B, KG9). The cerebellar cortex shows patchy synaptic stain and granular aggregates with variable amounts of condensed granular PrP, occasionally forming microplaques, and the subcortical white matter has very rare or PrP dot-like positivity (Figure 2C). Overall these cases show little PrP labelling in the white matter and no thread-like PrP deposits. No threads were detected by immunofluorescence, confirming the observations by light microscopy (Figure 3I,J).

**E200K (6 male, 3 female); Figure 2D-F, Figure 4A, B**

The mean age for this group is 60 years (45–69) (cases #2-10). The cortical grey matter shows diffuse, granular or

fine dot-like synaptic staining that ranges from weak to strong intensity, similar to that in sCJD. In addition, there was cortical perineuronal PrP deposition with variable amounts of microplaques, and there are rare filamentous PrP deposits. The subcortical white matter contains variable amounts (sparse to very frequent) of fine granular positivity (Figure 2D) and occasional white matter threads (Figure 2D, inset, ICSM35, and 2E, KG9). The cerebellar cortex shows patchy synaptic staining and granular aggregates with or without microplaques. The cerebellar white matter shows rare granular positivity and threads in some cases (Figure 2F). In one case (case 5 in Table 2) the cerebellar white matter showed frequent filamentous staining as well as dot-like positivity (Figure 2E, Figure 4A,B). This patient displayed an unusual clinical phenotype (younger age and significantly longer disease duration) compared to the other patients with the same mutation, which may explain the difference to the patients with older age and shorter disease duration. Overall, the PrP staining patterns in brains with the E200K mutation are similar to those observed in sCJD [25]. Among the cases with *PRNP* gene mutations, this is the only group that shows perineuronal PrP labelling. Double labelling immunofluorescence of one case (#5) shows filamentous PrP deposits. There is co-localisation of PrP in MBP positive myelin sheaths whilst PrP in axonal structures is below the detection limit (Figure 4A,B).

**V210I (1 male)**

In this single case (#1) with this mutation, the findings are very similar to those in E200K mutations. We found only cerebral PrP<sup>Sc</sup>, some of which formed fine threads but no cerebellar PrP<sup>Sc</sup>. The cortex shows synaptic labelling with a homogenous distribution across all cortical layers, similar to that of sCJD.

**Sporadic CJD (26 cases), Figure 4C-H**

26 cases with codon 129 polymorphism MM (9), MV (10) and VV (7) were examined. All cases showed variable amounts of granular PrP deposits, ranging from none to frequent, corresponding to a score from 0–3 (see Tables 5 & 6). Across all cases, no difference was seen in the intensities of granular white matter PrP deposition between codon 129 genotypes MM, MV and VV. Double labelling

**Table 6 List of sporadic CJD cases included in the control group**

Codon 129	Gender	Age (years)	Disease duration (Months)	Frequency of granular PrP deposits in the frontal subcortical white matter (intensity score)
MM	M	75	2.5	0
MM	M	76	3	2
MM	F	66	6	3
MM	M	89	13	1
MM	F	81	3	1
MM	M	62	3	2
MM	F	74	5	2
MM	F	63	3	3
MM	F	74	3	1
<b>Average MM</b>	<b>M:F = 4:5</b>	<b>73.3</b>	<b>4.6</b>	<b>1.67</b>
MV	F	73	7	1
MV	F	67	2	1
MV	F	70	10	0
MV	M	75	2	1
MV	M	62	11	0
MV	M	63	17	0
MV	M	56	11	2
MV	M	62	3	2
MV	M	77	12	3
MV	M	68	3	1
<b>Average MV</b>	<b>M:F = 7:3</b>	<b>67.3</b>	<b>7.8</b>	<b>1.1</b>
VV	M	69	9	0
VV	F	57	3	1
VV	F	72	5	1
VV	F	81	9	2
VV	F	81	3	0
VV	M	83	3	2
VV	M	61	2	3
<b>Average VV</b>	<b>M:F = 3:4</b>	<b>72</b>	<b>4.8</b>	<b>1.29</b>

Cases with Codon 129MM, MV and VV alleles were used. The table also lists the age at death, duration of illness and the density of granular PrP in the white matter. The case numbers are cross-referenced in Figure 4.

immunofluorescence of PrP with axons or myelin sheaths shows no co-localisation of granular white matter PrP with either structure in all three codon 129 genotypes. Instead there is frequent granular PrP, which is immediately adjacent to the myelinated fibres (Figure 4C-H).

## Discussion

Our study shows that filamentous white matter PrP deposition correlates with the localisation of the *PRNP* mutation

and to some extent with the age of the patients. Previous studies have described PrP plaques in the CNS white matter in various forms of prion diseases including vCJD [6] and some cases of 129MM sCJD [7] and in the cerebellum of 129VV patients [26]. A study of 20 autopsy cases of CJD (not further specified), revealed PrP<sup>Sc</sup> deposition in both cerebral and cerebellar white matter in 4 cases [8]. Immunohistochemical detection of abnormal PrP showed structures described as “arrays adjacent to ‘myelinic’ fibres and as clumps adjacent to oligodendrocyte nuclei”, and transmission electron microscopy showed that they were associated with “dense lysosomes in oligodendroglial perikarya and in their processes”. These inclusions were characterized as “finely fibrillary, paracrystalline, amorphous, or densely osmophilic material”. The authors concluded that white matter involvement in spongiform encephalopathy may be due to direct modifications of oligodendroglial cells associated with abnormal metabolism of PrP [8]. In inherited prion diseases, white matter plaques have been described before, and typically present as small, well circumscribed amyloid deposits [21,24].

In animals, PrP accumulation in the white matter has been described in sheep with atypical scrapie, with two staining patterns; a ‘globular’ type, forming ring- or oval-shaped deposits prominent in the spinocerebellar tracts, reticular formation, cerebellar and cerebral white matter, and other white matter tract areas and a ‘punctate’ type consisting of small circle or elongated tear-drop deposits prominent in several grey matter areas, e.g. the reticular formation, substantia nigra, fimbria hippocampi, and amygdala [9]. Again these features are distinct from the filamentous immunoreactivity in human inherited prion disease. Experimental models of prion disease have added only little insight into the possible pathogenesis of white matter PrP deposition. In a study of scrapie infected Syrian hamsters [10], an intense PrP<sup>Sc</sup> signal was found in the white matter, and was thought to support the hypothesis that PrP<sup>Sc</sup> is transported along axons, but the histoblot technique had a resolution insufficient for the subcellular localisation of the deposits. Studies using the mouse adapted Fujisaki prion strain reported white matter destruction and deposition of abnormal PrP in white matter tracts [8,27], without being more specific regarding the PrP deposition pattern. The CJD Fujisaki strain was derived from a brain that now is regarded as a case of P102L GSS [27].

Several transgenic mouse models of inherited prion disease have been generated but no specific white matter pathology has been reported. A transgenic mouse model expressing a nine-octapeptide insertion mutant mouse PrP (9OPRI) fused with enhanced green fluorescent protein [28] exhibited a striking white matter accumulation of abnormal PrP with intra-axonal mutant PrP as linear filamentous aggregates in central and peripheral axons, suggesting disruption of axonal transport [28].

Several models of the human P102L feature plaques but no white matter PrP [29-34]. Transgenic mice expressing a murine *Prnp* A116V (corresponding to the human *PRNP* A117V) mutation [35] on a *Prnp*<sup>0/0</sup> background displays predominantly plaque pathology, chiefly in the cerebellum. Mice expressing the homologue of the human D178N (129V) mutation showed motor dysfunction, alteration of spatial working memory and abnormal EEG pattern and sleep disturbances [36], histologically punctate, micro-plaque like deposits, and ultrastructurally a swelling of the endoplasmic reticulum of cerebellar granule neurons. A model of the E200K mutation [34,37] showed no spontaneous disease even after 900 days. Inoculation with human E200K -129VV brain yielded extensive plaques while an E200K 129MM inoculum yielded a predominantly synaptic pattern, but no white matter pathology was observed [34].

Previous studies of the transport of PrP along axonal pathways in animal models have concluded that transport of infectious prions uses mechanisms distinct from fast axonal transport [38], in contrast to PrP<sup>C</sup> [39]. It has been suggested that prion spread occurs via non-canonical mechanisms [40,41], for example in a domino-like manner along PrP<sup>C</sup> expressing nerve membranes or Schwann cells [42]. It is possible that an impairment of axonal transport or clearing mechanisms leads to deposition in the myelin sheath of N-terminal mutant PrP. Whilst no quantitative (and comparative) data exist for the amount of PrP<sup>C</sup> in CNS oligodendrocytes and axons, experimental data of the peripheral nervous system suggest that Schwann cells express negligible levels of *Prnp* mRNA, and that expression of axonal PrP is required for Schwann cell myelin maintenance [43]. It is possible but remains speculative that a similar mechanism, involving transfer of PrP from the axon to the myelin sheath is relevant for the maintenance of CNS myelin. It is possible that soluble forms or micro-aggregates of PrP, produced in the neuronal body, move by passive or active axonal transport. Our ultrastructural studies provide evidence of granular protein deposits in the axoplasm, possibly representing abnormal PrP, which may eventually accumulate in myelin sheaths. Indirect evidence for this suggestions comes from the observation that the most abundant filamentous white matter PrP is localised in the vicinity of cortical areas that contain significant amounts of abnormal synaptic or plaque PrP, whilst a low cortical PrP load is more often associated with very little or no filamentous PrP in subjacent white matter tracts.

Filamentous PrP deposits are not observed in sCJD. A possible explanation is the different pathogenesis, in that all neuronal cells express mutant PrP and that all cells carrying the mutation may synchronously convert mutant PrP into the abnormally folded form of PrP. The variability between cases with the same mutation (see [22]) may be explained by a variable capacity to clear abnormal PrP, or a differential propagation of disease-related isoforms, as

for example shown for the P102L mutation, where at the molecular level three isoforms of protease-resistant PrP with divergent physicochemical properties can be propagated [22]. The difference between cases with different mutations can be explained by different propensities to form deposits, as well as a region-specific propensity of cell populations to accumulate abnormal PrP even within the same family. It could also be argued that the initiation of the disease as well as the mechanism of cell-to-cell transmission in sporadic prion diseases is different from inherited forms. A remarkable observation is a very strong bivariate correlation between age of the patient at death and the density of threads in the white matter ( $p = 0.001$ ). A regression model even favours age over mutation type, that is, age is a stronger determinant of the density of threads than the mutation itself (patients with more threads die younger). However, this correlation is explained by the fact that age at death correlates with the mutation.

Functional studies are required to further elucidate the mechanism of PrP deposition in the myelin sheath, and the propensity of N-terminal mutations to form such inclusions.

## Conclusion

We report here the presence of white matter deposition of abnormal PrP in inherited prion diseases. Strikingly, abnormal PrP appears to deposit in the myelin sheath of myelinated axons. The filamentous inclusions are strongest in cases with N-terminal mutations (OPRI, A117V) and less intense or absent in mutations toward the C-terminus (D178N, E200K). Areas that are most affected are in the frontal, temporal and parietal lobes and less filaments are seen in the occipital lobes, the cerebellum and the brain stem.

## Competing interests

JC is a Director and JC and JDFW are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination and therapeutics. D-Gen markets the ICSM35 antibody used in this study. SB carries out a large scale screening study for detection of abnormal prion protein in archival appendix samples. This study was commissioned in a competitive tender by the UK Health Protection Agency. The other authors declare no conflicts of interest.

## Authors' contributions

LR, IM and SB carried out the histological analysis and collated the data. JML and MG prepared tissue sections and stains. JDFW, JL and RD carried out the protein analysis and western blotting. AL, SM, PR and JC recruited, clinically examined and consented the patients. AL and SM carried out the genetic analysis. SB, SM, JC and JDFW wrote and critically read the manuscript. All authors read and approved the final manuscript.

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