Molecular pathology, classification, and diagnosis of sporadic human prion disease variants

Piero Parchi, Daniela Saverioni
IRCCS Istituto delle Scienze Neurologiche and Dipartimento di Scienze Neurologiche, Università di Bologna, Via Foscolo 7, 40123 Bologna, Italy

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Abstract

Human prion diseases are a unique group of transmissible neurodegenerative diseases that occur as sporadic, familial or acquired disorders and show a wide range of phenotypic variation. The latter has been attributed to the existence of distinct strains of the agent or prion, and to the genetic background of the host, namely the primary sequence of the gene encoding the prion protein, which is the site of mutations and polymorphisms. The characterization of distinct isoforms of the abnormal prion protein in the brain of affected patients, which has been shown to correlate with the disease phenotype, has recently led to the concept of molecular strain typing, in which the different prion protein isoforms or “types”, possibly enciphering the strain variability in their conformation, may serve as surrogate markers for individual prion strains. In sporadic Creutzfeldt-Jakob disease, the most common human prion disease, there are at least six distinct clinico-pathological disease phenotypes that largely correlate at a molecular level with two prion protein types with distinctive physicochemical properties and the genotype at the methionine/valine polymorphic codon 129 in the prion protein gene. Recent results of transmission studies indicate that five prion strains with distinctive biological properties can be isolated from these six disease variants. It has also been shown that about a third of sporadic cases show a mixed phenotype and the co-occurrence of prion protein types. The origin of prion strains and their co-occurrence as well as the mechanisms underlying the strain-specific neuronal targeting remain largely unexplained and their understanding constitute, together with the development of successful therapies and more sensitive and specific clinical biomarkers, the major challenges that this disease poses for the future.

Key words: CJD, FFI, neurodegenerative dementia, prion protein, polymorphism, phenotype, strain, transmission.

Introduction

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are rapidly progressive neurodegenerative disorders that affect most species of mammals. In humans, they comprise Creutzfeldt-Jakob disease (CJD) [25,77,78,132], Gerstmann-Sträussler-Scheinker disease (GSS) [59], and the recently described variable protease-sensitive prionopathy (VPSPr) [193]. Fatal insomnia (FI) [116], and kuru [111] are also usually reported as distinct disease entities, although recent studies have shown that their molecular features and transmission properties fall within the wide phenotypic spectrum of CJD [109,130,133].

Human prion diseases are clinically characterized by rapidly progressive neurological signs involving mul-
tiple systems and, neuropathologically, by spongiform change, neuronal loss, glial activation, and abnormal PrP deposition, which sometimes forms mature amyloid plaques. Unlike other neurodegenerative disorders characterized by protein misfolding and aggregation that usually either occur sporadically or are linked to pathogenetic single gene mutations, prion diseases can be also exogenously acquired. Furthermore, all forms of prion disease can be transmitted experimentally, irrespective of their apparent distinct aetiology. Like conventional infectious pathogens, the agent causing prion diseases, usually referred to as prion, naturally occurs in a variety of different strains: these are defined as natural isolates of infectious prions characterized by distinctive clinical and neuropathological features, which are faithfully recapitulated upon serial passage within the same host genotype [2].

Sporadic prion diseases, particularly sporadic CJD (sCJD), are the most common in humans, accounting for more than 80% of all cases and having an incidence of about 1.5 cases per million. Genetic forms (also called inherited or familial prion diseases) are of autosomal dominant inheritance, account for about 10% of prion cases, and are linked to mutations in the prion protein (PrP) coding sequence [33]. Accidental transmissions to humans are responsible for rare acquired forms such as iatrogenic and variant CJD (iCJD and vCJD, respectively) [20,184].

The pathogenesis of prion diseases is strictly related to a host-encoded glycoprotein of unknown function, the cellular prion protein (PrPc), which is encoded by the PRNP gene. Prion diseases do not develop or transmit in absence of PrPc expression, and are all characterized by tissue deposition of an abnormal, partially protease-resistant isoform (PrPSc) of PrPc [26,150]. PrPSc represents the only molecule thus far associated with infectivity, and according to the prion hypothesis, the essential, if not the exclusive, component of the transmissible agent [38,150]. PrPc misfolding leading to PrPSc formation involves consistent changes in the secondary structure of the protein with part of the α-helical structure turning into β-sheet [150]. The generated PrPSc aggregates and forms deposits throughout the brain that are associated with spongiform change, glial activation and neuronal loss.

The present review focuses on sporadic human prion diseases. After a brief analysis of aspects of the biology and molecular pathology of prions which are relevant to the understanding of the human disease, the distinctive features of the sporadic human disease variants or subtypes and the issues related to their diagnosis and classification are described.

Genetics and cell biology of the prion protein

In humans, the prion protein gene (PRNP) belongs to the Prm gene family, which also includes its paralogues Prmd (encoding Doppel) and Sprm (encoding Shadoo). It is located in the short arm of chromosome 20, spans 16 kb and contains two exons separated by an intron. The first exon contains untranslated sequences, while the second includes the open reading frame (ORF) encoding for PrPc. PrPc is constitutively expressed in the central nervous system (CNS), predominantly in neurons but also in glial cells, and in various non-neuronal tissues, particularly in leukocytes and other cell lines of the immune system. The fact that the PRNP sequence is conserved among species suggests that PrPc is involved in a key function that has developed early during evolution [43,103].

PrPc is translated in the rough endoplasmic reticulum (ER) as a 253-aminoacid peptide, which subsequently undergoes post-translational modifications in its transition through the ER itself and the Golgi apparatus. These modifications include the removal of a 22-aminoacid signal peptide at the N-terminal end, the formation of a disulphide bond and the addition of a glycosylphosphatidylinositol (GPI) anchor at the C-terminal end after the removal of the last 23 residues. Finally, complex oligosaccharides are attached at residues Asn 181 and Asn 197, although the addition of these glycans does not occur obligatory. As a consequence, three differently glycosylated forms of the protein, also termed glycoforms, are formed: the diglycosylated, monoglycosylated and unglycosylated PrPc. After crossing the ER and the Golgi apparatus, PrPc localizes at the cell surface where it is anchored by the GPI [65]. Both in the cell and at the cell surface, PrPc is associated with specific membrane lipid micro-domains, enriched in cholesterol and sphingolipids, called detergent-resistant membranes or lipid rafts. The membrane-spanning form of PrPc is in a dynamic equilibrium with an internalized form, which is recycled from the cell surface by endocytosis. During this inverse route, which is thought to be mediated by a specific subgroup of lipid rafts termed caveolae-like domains (CLD) [85,181], PrPc cleavage at residue 112 generates a truncated C-terminal fragment [41]. The biological significance of PrPc internalization is not known, although some evidence indicates that it is induced,
posed 

or apoptosis, neurite outgrowth, synapses formation 

Current evidence suggests that PrP C is involved in several regulatory mechanisms, like neuron survival and function, and myelinic fibres maintenance, and that these effects are possibly mediated by lipid rafts.

The structure of mature human PrP C comprises a poorly definite domain at the N-terminal end of the protein (which spans ~100 residues), a globular domain in the central portion (residues 125-228) and a short flexible C-terminal domain, ending with the GPI anchor (residues 229-230/231) [33]. The N-terminal domain contains the so-called repeat region (residues 51-91) which consists of a 9-aminoacid sequence followed by a tandem repeat of four copies of an 8-aminoacid sequence (PHGGGWGQ). This region is involved in copper ions binding [18,75]. The globular domain is composed of three α-helices and two antiparallel β-sheets, separated by short loops and kept together in their final tertiary structure by interactions between the exposed amino acidic lateral chains that are in close contact with each other when the protein is correctly folded [33].

PrP C physiological function remains largely elusive. Weissman et al. [27] made the important observation that PPRNP knock-out in mice do not significantly affect brain development and function, although some abnormalities were described later [158] in other PPRNP-null mice, who developed ataxia and Purkinje cell loss, due to a rearrangement in the regulatory sequences of the parologue gene Pmd encoding Doppel after PPRNP deletion [1]. Current evidence suggests that PrP C is involved in several regulatory mechanisms, like neuron survival or apoptosis, neurite outgrowth, synapses formation and function, and myelinic fibres maintenance, and that these effects are possibly mediated by the involvement of PrP C in signal transduction [1]. In this regard, PrP C expression, cross-linking or interactions with other proteins have been shown to affect the activity of several proteins involved in cell signalling pathways [1]. Based on the strong affinity of PrP C for copper and the effect of copper ions on the protein internalization into the cell, a role for PrP C in copper metabolism and in particular in its removal from synapses has also been proposed [71].

**PrP C-PrP Sc conversion and neurotoxicity**

The central event in prion disease pathogenesis is thought to be the formation of PrP Sc, an abnormal PrP conformer which preferentially accumulates in the CNS. According to the protein-only or prion hypothesis [149], PrP Sc formation works as a template-assisted process, in which PrP Sc (the template) drives PrP refolding into the pathogenic conformation [74,91].

PrP Sc is identical in its primary sequence to the PrP molecule from which it has originated, but its secondary structure is significantly different, since during PrP Sc formation, some α-helical structures turn into β-sheet [40,125,154]. The resulting abnormally misfolded isoform is distinguishable from the normal isoform because of (i) its insolubility in non denaturing detergents and (ii) its partial resistance to protease digestion that usually involves the C-terminal moiety beyond residues 80-100. Exceptions to this rule, however, particularly for the latter property are increasingly documented. Indeed, variable relative amounts of a protease-sensitive form of PrP Sc have been recently detected in a number of naturally occurring prion diseases [10,56,80,131,147].

It remains controversial exactly how and where in the cell the interaction and the conversion of PrP C to PrP Sc occur. Early studies pointed to the cell surface as the putative site of PrP Sc formation [16,39,175]. Subsequent reports, however, have suggested that also the ER plays a role in the conversion process, probably after PrP Sc is internalized [11]. In genetic prion diseases, in particular, the ER could be the primary site where the mutant PrP, already predisposed to a misfolded conformation, converts into PrP Sc [65]. There are also studies suggesting that PrP C-PrP Sc conversion may occur in CLDs or clathrin-coated pits [85,143,173,181]. Like other pathogens, prions might use lipid rafts to enter the cells and possibly to initiate and/or propagate the PrP C-PrP Sc conversion process that facilitates prion amplification. The role of lipid rafts in the conversion process is also suggested by the findings that both PrP C and PrP Sc are associated with them [7,8,17,117,175,176] and that the elimination of PrP C from them prevents PrP Sc formation [85,176]. More recent studies, however, have highlighted the possibility that lipid rafts favour the conversion by bringing together PrP Sc and PrP C, rather than by triggering PrP refolding [31]. Indeed, given their role in PrP C folding and stabilization of its conformation, lipid rafts may even prevent PrP C trans-conformation. According to this view, the conversion
PrP Sc must act as an informational molecule. Although the prion hypothesis and the strain phenomenon, protein-only hypothesis [54]. Indeed, to accommodate been traditionally seen as the major challenge to the passages in intermediate species with distinct

mary sequence (strains can be propagated in animals with the same

strain properties are clearly not encoded by PrP prion-

pearance, since neurons lacking PrP C are preserved from
der degeneration even if they neighbour the infected cells [106,107]. In this context, in addition to a PrP Sc gain of function mechanism, also a loss of function secondary to PrP C depletion [160] could be hypothesized as the molecular basis of neurodegeneration in prion diseases, even if this aspect remains poorly understood, due to the lack of conclusive data concerning the function of PrP C.

Prion strains and their molecular basis

It has been known for many years that mammalian prions, like conventional infectious agents, naturally occur in a variety of strains. The first demonstration of this phenomenon was obtained in goats experimentally inoculated with distinct scrapie isolates: some animals became drowsy, while others had a scratching syndrome [138]. Based on the length of incubation time following inoculation, the type and distribution of lesions, and the pattern of intracerebral deposition of PrP Sc, a variety of scrapie strains were subsequently identified after passage through inbred mouse lines [22,24,55,70]. The wide variety of scrapie strains have been traditionally seen as the major challenge to the protein only hypothesis [54]. Indeed, to accommodate the prion hypothesis and the strain phenomenon, PrP Sc must act as an informational molecule. Although the strain properties are clearly not encoded by PrP primary sequence (strains can be propagated in animals with the same PRNP genotype, and re-isolated after passages in intermediate species with distinct PRNP haplotypes), it has been clearly demonstrated that PrP Sc exists in a number of molecular subtypes that show differences in size and glycosylation pattern, degree of protease resistance, aggregation state, or conformational stability [12,13,46,127,129,136,155,169, 170,177, for a review of more recent studies see also 2]. According to many scientists in the field who believe in the prion hypothesis, these data represent a strong argument for the contention that the molecular basis of strain variation lies within the structure of PrP Sc.

Bessen and Marsh first embraced this idea when they showed that two strains of transmissible mink encephalopathy (TME), propagated in inbred Syrian hamsters, gave rise to PrP Sc molecules with distinct electrophoretic mobility and degree of resistance to protease digestion [13]. The two TME strain-specific PrP Sc were subsequently propagated in vitro through non-genetic mechanisms [12], leading to the conclusion that different conformers of PrP Sc may faithfully self-propagate and cause distinct clinico-pathological diseases [12]. Later, Caughey and colleagues, using infrared spectroscopy, provided also some direct evidence that the two distinct TME strains are associated with PrP Sc molecules that differ in conformation in regions with β-sheet secondary structure [37].

Data in support of the idea that, also in human pri-

on diseases, pathologically distinct phenotypes may result from different conformers of PrP Sc, came from the study of sCJD. Two types of PrP Sc (types 1 and 2) differing in relative molecular mass and glycoform ratio in conjunction with the codon 129 polymorphism, were shown to largely determine the clinico-pathological variability of sCJD [129,133]. By demonstrating the transmission of two human disease phenotypes associated with PrP Sc types 1 and 2, in syngeneic animals, Telling et al. [177] provided the first definitive demonstration of prion strain variation in humans. They inoculated brain homogenates from subjects affected by familial FI (linked to PrP Sc type 2) or familial CJD (linked to PrP Sc type 1) in transgenic (Tg) mice, and demonstrated not only a faithful reproduction of PrP Sc biochemical features of the original inoculum in the recipient animal, but also a different intracerebral distribution of lesions.

A rather indirect approach that has been increas-

ingly used in recent years to study the strain-specificity of PrP Sc structure, is based on the early work by Kocisko et al. [92], which analysed the progressive denaturation of PrP Sc in the presence of increasing concentrations of chaotropic salt guanidine hydrochloride (GdnHCl). Several authors used this approach to char-
acterize animal prion strains [58,142,155,178] and synthetic prions [97,98], and observed a correlation between PrPSc conformational stability (i.e. sensitivity to denaturation) on one hand, and the degree of infectivity or the converting activity of PrPSc on the other [38,58,142]. Along this line, Pirisinu et al. [146] have recently set up a novel assay which allowed them to distinguish between PrPSc isoforms related to distinct TSE variants, based upon the variable propensity to be denatured by GdnHCl measured through the change in solubilization rather than in the degree of resistance to protease digestion. By avoiding the protease-digestion step, the method may allow for the characterization of the so-called protease-sensitive PrPSc (sPrPSc), an abnormal isoform of PrPSc that is indistinguishable from PrP on the protease digestion assay despite retaining the abnormal conformation. sPrPSc is increasingly found to be associated to animal and human prion diseases: two recently characterized prion diseases, atypical scrapie [10] and protease-sensitive prionopathy [56], along with some phenotypic subtypes of GSS [80,131,147] have been shown to only accumulate sPrPSc together with protease-resistant N- and C-terminally truncated unglycosylated PrPSc fragments in the 7-14 kDa range. According to some authors, sPrPSc may contribute up to 90% of the whole PrPSc even in classical TSEs such as sCJD, thus calling into question the rationale on which TSE diagnosis is currently based (i.e. demonstration of protease resistant PrPSc) [49,156,178]. It has been also suggested that sPrPSc consists of aggregates of shorter size with respect to the protease-resistant PrPSc [137,179] and that, at least in some sCJD variants, the concentration and stability of these sPrPSc conformers correlate with the duration of clinical disease [86]. In addition to its quaternary structure (i.e. the size of aggregates) [137,179], the basis for the degree of protease resistance of PrPSc has been also attributed to its tertiary structure [118,147]. Whatever the case, it remains unknown what drives PrPSc to adopt a certain conformation, that in turn, would lead to a specific disease phenotype.

PrPSc glycosylation, beside protein conformation and, possibly, other factors, may also contribute to the molecular basis of strain variation. Indeed, the PrPSc molecules associated with distinct subtypes of human prion disease often show distinct glycoform ratios [115,127,136]. A recent example of this phenomenon, which has been highly publicized, is represented by the PrPSc associated with vCJD, which has a type 2 migration pattern, like other subtypes of CJD [127,136] but shows a glycoform ratio which is distinct from that seen in sporadic and iatrogenic CJD or kuru [46,127,136]. Since glycosylation is a co- translational process that is known to differ among cell types [94], the different glycoform ratios may reflect the involvement or the targeting of distinct neuronal populations, as a consequence of a strain-specific cellular tropism [50]. Alternatively, different prion strains may preferentially convert certain PrPSc glycoforms.

Although altogether all of the above cited studies provide strong evidence in favour of the contention that different PrPSc conformers underlie the concept of prion “strains”, the direct proof for this contention is not available as yet. Indeed, since these results are mainly indirect and the determination of the ultimate molecular structure of distinct PrPSc molecules is still lacking, questions and alternative interpretations of the data remain. For example, it is not known whether the distinctive properties of PrPSc directly reflect PrPSc conformation or are rather determined by interactions between PrPSc and other molecules. PrPSc is extracted from the brain in a highly aggregated state and the heterogeneity in size of the protein core may well reflect the quaternary rather than the secondary or tertiary structure of the molecule. For example, formation of PrPSc type 1 and type 2 may follow distinct ligand interactions of PrPSc. Similarly, the extent of conversion of each glycoform of PrPSc, which ultimately determines the glycoform ratio of PrPSc, at least in sporadic and acquired prion disease, may well represent a signature imparted by another informational molecule that interacts with PrP. Finally, the central question that still remains to be answered is how an identical primary sequence can drive different tertiary conformations in the prion protein, if no other informational molecule exists.

Given the analogy of some properties of prions with viruses and/or viroids and the overall need to fully clarify the well-known effects of co-factors on prion replication, attempts to demonstrate the PrPSc association with conventional nucleic acid and other molecules continue. In the most recent of such studies, Simonneau et al. [168] identified small RNA molecules as part of the TSE infectious particle. Previously, Safar et al. [157] also detected short nucleic acids (not exceeding 25 nucleotides) in association with PrP molecules, but ruled out the possibility that they could be prion specific. Irrespectively of whether or not they have a role in strain diversity, nucleic acids such as polyanions, but also polysaccharides, may well participate in amyloid formation, since amyloid plaques have been shown to contain sig-
significant amounts of both these molecules. Nucleic acids have also been demonstrated to affect amyloid protein conformation, by driving the final three-dimensional structure in the protein aggregates [104]. Despite no conclusive results are available for the role of nucleic acids associated with PrP\textsuperscript{Sc}, novel evidence indicating that prions, like viruses, “mutate” and adapt to the environment in which they replicate has been recently published. It has been found that when grown in cell cultures in the presence of swainsonine, a chemical compound which inhibits protein glycosylation, some strains become drug-resistant while others do not [102]. According to these authors, the process of mutation and selection that characterizes the different strains suggests that prions naturally exist in a variety of conformers, constituting ‘quasi-species’, from which the one replicating most efficiently in a particular environment is selected [45,53,101,167]. The mechanisms underlying this presumed PrP capability of assuming different conformations remain, however, to be elucidated.

**PrP\textsuperscript{Sc} types and the molecular basis of current sporadic human prion disease classification**

In human prion disease, it has been convincingly shown that there is a strong correlation between two PrP\textsuperscript{Sc} types with distinct physicochemical properties, named types 1 and 2, and the genotype at the methionine (M) / valine (V) polymorphic codon 129 of PRNP on one hand, and the pathological and clinical disease phenotype on the other [133,135]. PrP\textsuperscript{Sc} types 1 and 2 have been originally described in sCJD by Parchi et al. in 1996 [129] as two alternative forms of the protease-resistant, unglycosylated C-terminal core of the PrP\textsuperscript{Sc} molecule (Fig. 1). PrP\textsuperscript{Sc} type 1 has a relative electrophoretic mobility of 21 kDa and a primary cleavage site at residue 82 while PrP\textsuperscript{Sc} type 2 has a relative molecular mass of 19 kDa and a primary cleavage site at residue 97 [127,136]. Interestingly, PrP\textsuperscript{Sc} types 1 and 2 characterize all subtypes of CJD, i.e. sporadic, inherited or acquired by infection [127,136], thus suggesting a common mechanism of PrP\textsuperscript{Sc} formation that is independent from the apparently distinct aetiology of the disease.

The reliability of the molecular classification proposed by Parchi et al., based on two PrP\textsuperscript{Sc} types and 3 PRNP codon 129 genotypes (i.e. MM, MV, and VV) has recently been demonstrated in a collaborative study between seven laboratories involved in prion disease surveillance [134]. Following a detailed and standar-
be found in sCJD phenotypes sharing the same PrPSc type. For example, i) PrPSc type 2 from MV cases shows a unique doublet band that differs from PrPSc type 2 in MM and VV cases, and ii) type 1 PrPSc from VV cases migrates faster than type 1 PrPSc from MM1 and MV1 samples when PK digestion is performed at pH under 7.2 (Fig. 1). However, the recognition of these subtle differences between subtypes of type 1 and type 2 groups, respectively, was less satisfactory when tested among CJD surveillance laboratories, probably because it requires highly standardized gel electrophoresis protocols that are unsuitable for routine diagnostic needs [134].

Interestingly, PrPSc types 1 and 2 have also been found to co-occur in the same brain (Fig. 2). Following the original report of this phenomenon [133,151], a series of studies indicated that the prevalence of cases with co-occurrence of PrPSc types 1 and 2 varies between 12% and 44% [66,67,100,151,166,180]. To explain these findings, it has been proposed that the incidence of sCJD cases with both types 1 and 2 PrPSc reflects the extent of brain sampling and the sensitivity with which a minority type may be detected in the presence of larger amounts of the other protein fragment [66]. Pursuing this idea, subsequent studies, taking advantage of novel, putative type-1 [148,187] and type-2 [89] selective antibodies claimed that little amounts of type-1 PrPSc are found in all patients with sCJD type 2 and vice versa, that type-2 PrPSc is detected in all patients with sCJD MM1. However, the interpretation and relevance of these data for CJD classification and strain-typing was later contested, by showing that the PrPSc-selective antibodies recognize PrP fragments that do not match in physicochemical properties those detected by standard PrPSc typing [29,121]. For example, when relatively mild digestion conditions are used, the type 1 specific antibodies are incapable of distinguishing bona fide type 1 "core" fragment from the fragments obtained by an incomplete digestion of PrPSc type 2 N-terminus [29,121]. Similarly, as also discussed by the authors of the original study [89], the weak band recognized by the type 2-selective antibody may well represent a PrPSc fragment, truncated at residue 97, that remains embedded in the PrPSc type 1 aggregate, rather than bona-fide PrPSc type 2. Indeed, while the antibody recognizes a PrPSc type 2-like fragment in the cerebellum of MM1 subjects, the same fragment is not detected in subjects affected by sCJD MM 2C. Based on these considerations, we believe that the use of type 1 or type 2 selective antibodies alone is not recommended for strain typing studies in human prion disease.

The issues of incidence, diagnosis and phenotypic effects of the co-occurrence of PrPSc types have been recently re-examined comprehensively in a large series of sCJD cases [135]. To this aim, an extensive brain sampling and the most accurate methodology available for detection of the PrPSc type co-occurrence, which provides good sensitivity combined with absolute specificity (i.e. the distinction between bona fide types 1 and 2 “core” fragments from the fragments that are produced by incomplete protease digestion) has been applied [121]. The results obtained in a largely unselected series of 200 cases demonstrated an overall prevalence of PrPSc types 1 and 2 co-occurrence of 35% [135]. Although co-occurrence was detected in all codon 129 genotypes, PrPSc types 1 and 2 co-occurred more frequently in MM (43%) than in MV (23%) or VV (15%) subjects. The overall results indicated that the deposition of either type 1 or 2, when concurrent, is not random and always characterized by the coexistence of phenotypic features previously described for the "pure" subtypes. Indeed, most cases with a mixed phenotype, as defined in this
study [135], were also characterized by well identifiable distinctive histopathological traits indicative of the co-existence of two CJD variants within the same brain. Such a good correlation between biochemical and neuropathological features in our opinion, also supports the idea that the co-occurrence of PrPSC types in sCJD is not the rule, as the Western blot studies using the type 1 or type 2 selective antibodies would instead suggest, based on a rough evaluation. Given the importance of the recognition and correct classification of such cases for future epidemiological and transmission studies aimed to identify the aetiology and extent of strain variation in sCJD, a protocol of PrPSC typing has been proposed that is based on analysis of at least four critical regions such as the temporal, parietal and occipital neocortices, and medial thalamus [135].

Another aspect of molecular typing that has seen some developments in recent years concerns the characterization of novel truncated PrPSC species of lower molecular weight than PrPSC types 1 and 2. Unglycosylated PrPSC fragments of 7-8 kDa, truncated at both N- and C-terminal ends, were first identified in GSS [131,144,174]. Subsequently, in both sporadic and genetic forms of CJD, Capellari et al. [32] and Zou et al. [192] detected C-terminal fragments of about 12 and 13 kDa (PrP-CTF12/13), and showed that they are generated by a PrPSC cleavage at residues 162-167 and 154-156 respectively. Interestingly, the relative amount of PrP-CTF 13 varies between sCJD subtypes; in particular all PrPSC type 2-associated sCJD subtypes but the MM 2T show only traces of this fragment, whereas the peptide is present in a significant amount in MM1 cases and is particularly abundant in VV1 subjects [122]. Notari et al. [122] also identified a novel C-terminally-truncated PrPSC fragment showing an apparent molecular mass of either ~18.5 kDa (when associated with type 1) or ~17 kDa (when associated with type 2). This fragment shares the primary N-terminal sequence with either type 1 or type 2 but lacks the very end of the C-terminus together with the GPI anchor. Finally, a fragment with an apparent molecular mass of about 16 kDa, which is only generated in partially denaturing conditions, has been detected in sCJD MM1/MV1. Epitope mapping indicates that the fragment has an intact C-terminal end and is truncated in the region between residue 112 and residue 144. Taken together, these data suggest that each sCJD subtype can be associated with a specific profile of abnormal PrP fragments, possibly reflecting subtype-specific structural characteristics of the abnormal protein aggregate.

PrPSC heterogeneity also extends to the sugar moieties. As stated previously, the non-obligatory addition of one or two sugar chains results in three differently glycosylated isoforms (di-, mono-, or unglycosylated) in both PrPK and PrPSc. It has been recognized for a long time that the PrPK associated with different prion disease forms or subtypes may show distinct ratios of the three differently glycosylated form (the so-called glycoform ratio). These differences are widely used as a diagnostic tool for prion disease subtype recognition, especially between bovine spongiform encephalopathy (BSE) and natural scrapie in sheep, or between vCJD and sCJD or iCJD in humans [9,72,127,172]. In the large majority of CJD cases, PrPSc glycosylation is characterized by an over-representation of the monoglycosylated form. A rather major distinction with undoubted diagnostic relevance has been introduced to distinguish the above described “pattern A” from “pattern B” characterized by a predominance of the fully glycosylated form, the latter being found in vCJD [46,127] or in gCJD and fatal familial insomnia (FFI) linked to the E200K or D178N mutations, respectively [115,136]. However, finer significant differences in PrPSc glycoform ratio have also been described among CJD subtypes with either “pattern A” or “pattern B” using mono- or two-dimensional gel electrophoresis [73,126,127].

Subtypes of sCJD and their distinctive clinico-pathological features

Following the demonstration of the critical role of codon 129 genotype on sCJD susceptibility and phenotypic expression and the characterization of PrPSc types 1 and 2 [127], the clinical and pathological spectrum of sCJD has been completely re-examined. Based on a comprehensive and systematic analysis of molecular, clinical and pathological features, Parchi et al. [133] have originally described six molecular and corresponding clinico-pathological subtypes. Later studies have confirmed the basis of this classification [30,66,99,134,161]. Furthermore, the spectrum of phenotypes with mixed pathological features and the co-occurrence of PrPSc types, which accounts for about one third of all sCJD cases, have been recently more rigorously defined [29,135]. The main clinical and pathological features of both pure and mixed sCJD subtypes are summarized in Tables I and II.
<table>
<thead>
<tr>
<th>Disease variant</th>
<th>(%)</th>
<th>Age at onset (years)</th>
<th>Disease duration (months)</th>
<th>Early clinical features</th>
<th>CSF tests and DW-MRI findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM/MV1 40</td>
<td>70</td>
<td>4</td>
<td>Very rapidly progressive dementia with myoclonus. Ataxia in 50% of cases, visual signs and dysphasia in 25%.</td>
<td>• 14-3-3 and/or t-tau CSF tests are positive in almost all cases • Hyperintensity in striatum and cerebral cortex</td>
<td></td>
</tr>
<tr>
<td>VV2 15</td>
<td>65</td>
<td>6.5</td>
<td>Rapidly progressive ataxia sometimes preceded by transient diplopia or vertigo. Peripheral signs and fasciculation may occur</td>
<td>• 14-3-3 and/or t-tau CSF tests are positive in almost all cases • Hyperintensity in striatum and thalamus</td>
<td></td>
</tr>
<tr>
<td>MV 2K 8</td>
<td>65</td>
<td>16</td>
<td>Rapidly progressive ataxia and cognitive decline (either one may occur first)</td>
<td>• 14-3-3 and/or t-tau CSF tests are positive in about 70% of cases • Hyperintensity in cerebral cortex, striatum and thalamus</td>
<td></td>
</tr>
<tr>
<td>MM/MV 2C &lt; 1</td>
<td>68</td>
<td>20</td>
<td>Rapidly progressive cognitive decline</td>
<td>• 14-3-3 and t-tau CSF tests are positive in about 90% of cases • Hyperintensity in cerebral cortex</td>
<td></td>
</tr>
<tr>
<td>MM 2T &lt; 1</td>
<td>50</td>
<td>15.5</td>
<td>Insomnia, psychomotor hyperactivity, postural instability, diplopia, psychiatric signs</td>
<td>• 14-3-3 and t-tau CSF tests are negative in most cases • Negative DW-MRI; MRS may indicate thalamic gliosis [105]</td>
<td></td>
</tr>
<tr>
<td>VV1 &lt; 1</td>
<td>40</td>
<td>15.5</td>
<td>Rapidly progressive cognitive decline</td>
<td>• 14-3-3 and/or t-tau CSF tests are positive in almost all cases • Hyperintensity in cerebral cortex</td>
<td></td>
</tr>
<tr>
<td>MM/MV 1 + 2C 28</td>
<td>69</td>
<td>4</td>
<td>Depends on the relative amount of PrPSc type 1 vs. type 2. Most cases are similar to MM/MV1</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MM/MV 2C + 1 &lt; 2</td>
<td>65</td>
<td>18</td>
<td>Depends on the relative amount of PrPSc type 2 vs. type 1. Most cases are similar to MM/MV 2C</td>
<td>NA</td>
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<tr>
<td>MV 2K+C &lt; 2</td>
<td>NA</td>
<td>NA</td>
<td>Most cases are similar to MV 2K but with more prominent dementia than ataxia</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>MM 2T+C &lt; 1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>VV 2 + 1 &lt; 2</td>
<td>69</td>
<td>6.5</td>
<td>Similar to VV2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>VPSPr &lt; 2 in USA, 1% in Europe</td>
<td>129MM: 64,78</td>
<td>MM: 41,50</td>
<td>Behavioural and mood changes (more prominent in VV), language deficits, cognitive impairment, and motor signs, especially parkinsonism and ataxia (more prominent in MM)</td>
<td>• Preliminary data indicate that 14-3-3 and t-tau levels are not elevated in most cases [68,69,79,152,193] • Preliminary data indicate that DW-MRI is negative in most cases [68,69,79,152,193]</td>
<td></td>
</tr>
</tbody>
</table>

NA – no published data available; *From [133,135], unless otherwise specified. †From [34,48,61,161], unless otherwise specified. ‡From [112], unless otherwise specified.
<table>
<thead>
<tr>
<th>Disease variant</th>
<th>Distinctive neuropathologic features*</th>
<th>Amplification in vitro (PMCA [83], QuIC [140])</th>
<th>Transmission properties (animal model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM/MV1 Spongiform change with small (2-10 µm) vacuoles in neocortex, striatum, thalamus and cerebellar molecular layer (often only focal). Hippocampus and brainstem are remarkably spared. IHC: synaptic type of PrPSc staining in the cerebral cortex, basal ganglia and cerebellum</td>
<td>• PMCA: ++++/+++ for HuMM, ++/+ for HuMV, +/0 for HuVV substrates in MM1/MV1 • QuIC: rapid and efficient amplification with maximum reached within 15 h; not affected by codon 129 of substrate</td>
<td>• Distinct from MM 2C (bank voles) [119] • Distinct from MV 2K, VV2, MM 2C, and VV1 (Tg mice expressing human PrP) [15] • Distinct from VV2 and MV 2K (non-human primates) [130]</td>
<td></td>
</tr>
<tr>
<td>VV2 Spongiform change with medium size vacuoles in striatum, hippocampus, limbic cortex, thalamus, cerebellum (molecular layer), and midbrain. In the neocortex the spongiform change are confined to or predominate in the deep layers. IHC: Diffuse plaque-like PrPSc deposits and perineuronal staining in cortical layers 4 to 6</td>
<td>• PMCA: 0 for HuMM, 0 for HuMV, ++++ for HuVV substrates • QuIC: rapid and efficient amplification with maximum reached within 15 h; not affected by codon 129 of substrate</td>
<td>• Less efficient than MM1/MV1 and MM 2C (bank voles) [119] • Distinct from MM1/MV1, MM 2C, and VV1, but indistinguishable from MV 2K (Tg humanized mice) [15] • Distinct from MM1/MV1 but indistinguishable from MV 2K (non-human primates) [130]</td>
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<tr>
<td>MV 2K Unicentric amyloid plaques of kuru type in the granular layer of cerebellum. Other features are similar to VV2, but with more consistent plaque-like deposits</td>
<td>• PMCA: 0 for HuMM, + for HuMV, +++ for HuVV substrates • QuIC: rapid and efficient amplification with plateau phase within 15 h</td>
<td>• Less efficient than MM1/MV1 and MM 2C (bank voles) [119] • Distinct from MM1/MV1 but indistinguishable from VV 2 (non-human primates) [130] • Distinct from MM1/MV1, MM 2C, and VV1, but indistinguishable from VV2 (Tg humanized mice) [15]</td>
<td></td>
</tr>
<tr>
<td>MM/MV2C Spongiform change comprising relatively large confluent vacuoles associated to perivascular PrP deposition. Cortico-striatal distribution of pathology with relative sparing of brainstem and cerebellum</td>
<td>• PMCA: +++ for HuMM and HuMV, 0 for HuVV substrates • QuIC: long amplification times, with maximum reached within 60 h</td>
<td>• Distinct from MM1/MV1 (bank voles) [119] • Distinct from MM1/MV1, MV 2K, VV2, and VV1 (Tg humanized mice) [15]</td>
<td></td>
</tr>
<tr>
<td>MM 2T Moderate to severe atrophy of medial thalamic and inferior olivary nuclei in absence of definite spongiform change. Inconsistent or weak PrP deposition</td>
<td>• PMCA: ++ for HuMM, ++ for HuMV and 0 for HuVV substrates • QuIC: NA</td>
<td>• Distinct from MM1/MV1 and MM 2C (Tg mice expressing chimeric or human PrP) [109,114]</td>
<td></td>
</tr>
<tr>
<td>VV1 Spongiform change with medium-size vacuoles in the cerebral cortex and striatum and relative sparing of cerebellum. Faint, synaptic type of PrP deposition</td>
<td>• PMCA: 0 for HuMM and HuMV, +++ for HuVV substrates • QuIC: very long incubation times, with maximum not reached within 90 h</td>
<td>• Distinct from MM1/MV1, MM 2C, MV 2K, and VV2 (Tg humanized mice) [15]</td>
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</table>
The most common phenotype is the so-called MM/MV 1 type, which comprises about 40% of all sCJD cases, and is seen in subjects who are homozygous for methionine or heterozygous at codon 129 and have PrPSc type 1 [133,135]. The mean age at onset of symptoms is about 65 years and the average clinical duration 4 months. Signs at onset, which are often combined, vary and include cognitive decline in most cases, ataxia in approximately half of them, language dysfunction, visual signs of central origin, myoclonus or other involuntary movements, each occurring in about one fourth of patients. Neurological signs are unilateral at onset in about 25% of cases. Diffusion weighted (DW) magnetic resonance imaging (MRI) shows a signal increase in the basal ganglia in about 70% of cases, whereas the involvement of the cerebral cortex in at least three different regions is detected in about half of the patients [112]. The absence of hippocampal and thalamic signal increase is a distinctive feature of this subtype [112]. Periodic sharp-wave complexes (PSWCs) are recorded in the electroencephalogram (EEG) in about 80% of cases, usually within the first three months of symptoms [48,131].

This phenotype shows the classical pathology of CJD: spongiform change with small (2-10 µm) round to oval vacuoles, predominantly located in the neuropil (Fig. 3A).

![Image](https://via.placeholder.com/150)

Table II. Cont.

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>Spongiform change with medium-size vacuoles in the cerebral cortex, striatum and thalamus.</td>
<td>NA</td>
</tr>
<tr>
<td>MM + 2C</td>
<td>Spongiform change with large-vacuoles in the cerebral cortex, striatum and thalamus.</td>
<td>NA</td>
</tr>
<tr>
<td>MM + 1C</td>
<td>Spongiform change with small-vacuoles in the cerebral cortex, striatum and thalamus.</td>
<td>NA</td>
</tr>
</tbody>
</table>

Periodic sharp-wave complexes (PSWCs) are recorded in the electroencephalogram (EEG) in about 80% of cases, usually within the first three months of symptoms [48,131].

The second most common phenotype comprises about 15% of sCJD cases and includes subjects that are homozygous for valine at codon 129 and show PrPSc type 2 (VV2) [133,135]. The mean age at onset and clinical duration are 60 years and 6.5 months, respectively. Clinically, VV2 patients show prominent gait ataxia at onset, while dementia often occurs later in the course of the illness. Prominent myoclonus is absent in about one third of the cases, and most patients also lack PSW discharges on EEG. DW-MRI shows involve-
**Fig 3.** Distinctive histopathological features of sporadic human prion disease subtypes. **A**) Typical spongiform change characterized by small, fine, microvacuoles in the neuropil of sCJD MM1 (H&E stain, occipital cortex). **B**) Synaptic pattern of PrP deposition in cerebellum of sCJD MM1. A delicate diffuse staining is seen in the molecular layer while in the granular cell layers, the cerebellar glomeruli are stained. **C**) Plaque-like PrP deposits in the granular and molecular layers of the cerebellum in sCJD VV2. These plaque-like deposits are not visible in routine H&E or PAS-stained sections. **D**) Perineuronal PrP<sup>Sc</sup> staining in the deep cortical layers of sCJD VV2. **E**) Unicentric amyloid plaque of the kuru-type in the cerebellar granular layer in sCJD MV 2K (H&E, stain). Plaque-like PrP deposits in the granular and molecular layers of cerebellum (**F**) and cerebellar white matter (**G**) in sCJD MV 2K. Spongiform change characterized by relatively large confluent vacuoles (**H**) (H&E stain), with perivascular PrP<sup>Sc</sup> staining (**I**) as typically seen in MM 2C, and MM/MV 1 + 2C sCJD cases. Typically, dense, coarse deposits of PrP are seen surrounding confluent vacuoles. The cerebellum in MM 2C is either PrP negative or shows a focal patchy/coarse staining. **J**) Target-like pattern of PrP deposition in the caudate nucleus of a MV case with VPSPr. **K**) Florid plaques in the cerebral cortex of vCJD. All PrP immunostaining is with mAb 3F4. All pictures have the same magnification (200x), except for panel E (400x).
ment of the basal ganglia and thalamus, with the highest frequency among the sCJD subtypes. In contrast, the cerebral cortical signal increase is usually limited and most frequently found in the limbic cortex rather than in the neocortex. Pathologically, the VV2 subjects show moderate to severe spongiform change and gliosis with variable neuronal loss in the grey matter of limbic structures, striatum, thalamus, hypothalamus, cerebellum and brain stem. In contrast, the neocortex is often spared, particularly in cases with a relatively rapid course (i.e. less than 6 months). The spongiform change in the cortex is often laminar and mainly involves the deep layers. Immunostaining is characterized by the presence of plaque-like, focal PrP deposits (Fig. 3C) that are not visible with routine staining procedures and do not contain PrP amyloid, since they are Congo red and thioflavin S negative. Another distinctive immunostaining feature is the strong reaction around some neuronal perikarya (e.g. perineuronal staining) (Fig. 3D), while the neuropil shows the synaptic pattern. Moreover, cases with a less than one-year duration are characterized by laminar distribution of the immunostaining in deep cortical layers corresponding to the spongiform change. The distribution of PrPSc, like the distribution of the spongiform change, is affected by the duration of the disease. In cases with a less than 5-month duration, PrPSc is present in relatively large and similar amounts throughout the brain except for the neocortex where it is present to a limited extent. This distribution is quite different from that of MM1 and MV1 subjects in which PrPSc is most abundant in the neocortex. In cases with over 1-year duration, PrPSc also markedly increases in the neocortex so that it becomes homogeneously distributed in all grey matter regions [133,135].

The third most common phenotype affects about 8% of cases, and comprises the kuru plaque variant, which is linked to MV at codon 129 and PrPSc type 2 (MV 2K) [133,135]. The mean age at onset and clinical duration are 59 years and 18 months, respectively. This subtype shows striking similarities with the VV2 phenotype, but is characterized by a significantly lower progression rate. Indeed, a long clinical duration of disease exceeding two years is not infrequent in this type and may raise diagnostic difficulties at the clinical stage. Clinically both ataxia and dementia are prominent. Typically, there are no PSWCs on EEG recording. DW-MRI shows, like in the VV2 type, a quite constant involvement of basal ganglia and thalamus. In particular, the thalamic signal increase is most frequently observed in the pulvinar, sometimes giving rise to a typical “pulvinar sign” [190], followed by the mediadorsal nuclei and the anterolateral nuclei. Involvement of the cerebral cortex is less frequent and most often includes the frontal lobes and cingulate gyri. The distinctive features of this type are cerebellar kuru plaques (Fig. 3E), while other pathological changes are similar to VV2 with more consistent plaque-like deposits (Fig. 3F, G). Kuru plaques are round, dense, PrPSc-positive amyloid plaques, which, in contrast to plaque-like deposits, are visible on routine haematoxylin-eosin (HE) staining (Fig. 3E). They are regularly found in the molecular and granular layers of the cerebellum but are only rarely seen in other cerebral structures [133,135].

Two distinct phenotypes affect subjects who are homozygous for methionine at codon 129 and show PrPSc type 2 [133,135]. The first (MM2-cortical or MM 2C) comprises about 1% of the sCJD population. The mean age at onset is 65 years, and the average disease duration 17 months. Clinically, these subjects present with cognitive impairment, while sustained myoclonus, visual signs and typical EEG changes are usually absent. Cerebellar signs are mild or absent, even late in the course of the disease. A widespread DW-MRI cortical signal increase, which typically includes the temporal lobes, is characteristic of this subtype. Basal ganglia involvement is instead rather limited, whereas, compared to the MM1 type, a thalamic signal increase occurs more frequently in these cases. The lesion profile of MM 2C subjects is similar to that of the MM1 or MV1 group, with the exception of the cerebellum that lacks significant spongiform change despite the long duration of symptoms. Moreover, the spongiform change typically consists of large and coarse vacuoles (Fig. 3H). PrP immunohistochemistry shows a coarse pattern of staining (Fig. 3I), which is often called perivascular because of its prevalent localization at the rim of the large vacuoles. Recently, this phenotype was also described in at least one MV subject [135].

The second phenotype linked to PrPSc type 2 and methionine homozygosity also affects about 1% of the sporadic human prion disease population. This variant (MM2-thalamic or MM 2T) is virtually indistinguishable from FI [116,128], and there is now convincing evidence that both the sporadic MM 2T variant and FI are caused by the same prion strain (see below). Clinically, insomnia with inability to initiate and maintain sleep, frequent arousals, and enacted dreams is often an early and dominant symptom. Motor signs are also part of the clinical phenotype and may include diplopia, dysarthria, dysphagia, pyramidal signs and gait abnormalities. Myoclonus, spontaneous and evoked, is always found.
As a rule, there are no PSWCs on EEG. MM 2T patients usually show no signal alterations on DW-MRI [64] and this can be attributed to the absence or limited extent of spongiform changes in the basal ganglia, thalamus, and cerebral cortex [108]. However, two recent studies [63,105] have documented, by MR spectroscopy, a metabolic pattern indicating gliosis restricted to the thalamus, which may be quite specific to the MM 2T phenotype. The diagnostic sensitivity of 14-3-3 and total tau detection in the cerebrospinal fluid (CSF) is also unrewarding, since in most cases, the CSF levels of both proteins do not reach the threshold for a positive test. Neuropathologically, there is prominent atrophy of the thalamus and inferior olive with only minor pathologic changes in other areas of the brain. Spongiform change may be absent or focal and is virtually limited to the cerebral cortex. The relative preservation of the striatum is also unique among sCJD subtypes. PrPSc IHC may be positive only in the entorhinal cortex.

Finally, a rare phenotype that affects about 1% of the sCJD population is linked to PrPSc type 1 and homozygosity for valine at codon 129 [133,135]. The VV1 subjects show a mean age at onset of about 40 years, which is by far the youngest among sCJD subtypes, and a mean duration of 15 months. Although VV1 subjects identified to date are limited in number, they appear to show a quite homogeneous and consistent phenotype, both clinically [113] and pathologically [133,135]. Symptoms at onset include progressive dementia, often of the fronto-temporal type, which may evolve for some months without significant motor signs, such as ataxia or myoclonus. In addition, a typical EEG is absent. The pathology in these subjects predominately affects the cortico-striatal regions, while other subcortical structures, including the cerebellum, are relatively spared. Routine PrP immunohistochemistry shows a faint punctuate staining in the cerebral cortex, despite the severe spongiform change.

**sCJD types with “mixed” molecular and pathological features**

**MM/MV 1+2C type**

The MM/MV 1+2C variant is by far the most common mixed type of sCJD. As many as 43% of all MM cases turned out to be mixed MM 1+2C cases on very close inspection [135]. This includes cases in which the large and confluent vacuoles are only found in very restricted regions of the cerebral cortex or the thalamus making the demonstration of PrPSc type 1 and 2 co-occurrence by Western blot very difficult. Given the rarity of the other mixed phenotypes, this is the only subtype in which clinical correlations were drawn. It was found that both disease duration and frequency of symptoms at onset in MM/MV 1+2C subjects vary according to the relative load of the two protein types. Thus, while the clinical phenotype in MM/MV 1+2C with a focal PrPSc type 2 deposition (up to 5 brain regions involved) do not significantly differ from those of the MM/MV 1 phenotype, the disease duration becomes significantly longer and cerebellar signs less frequent at onset with increasing type 2 load.

Similarly, the histopathology in this variant shows varying relative amounts of features previously described in the pure MM/MV 1 and MM/MV 2C subtypes. More precisely, this type is characterized by confluent foci of spongiform change with large vacuoles interspersed with the classic microvacuolation, and by a mixed synaptic/perivacuolar or coarse pattern of PrP deposition. There is a significant quantitative variation of these combined features from case to case which are overall more common in the cerebral cortex than in subcortical areas. The temporal, parietal and occipital lobes are the most affected cortical areas, whereas among subcortical nuclei, the thalamus most often shows the perivascular or coarse staining. Similarly, the lesion profile in MM/MV 1+2C largely overlaps with that of pure MM/MV 1 cases [135].

**VV 2+1 type**

At variance with patients carrying the M allele, the occurrence of mixed phenotypic features in VV cases is essentially based on the demonstration of PrPSc type 1 and 2 co-existence by immunoblotting. Indeed, the lesion profile and the pattern of PrP staining in the rare VV 2+1 type show features that are largely overlapping with those of typical VV2 sCJD cases. As the only distinctive feature, two cases with a significant type 1 accumulation showed a less consistent laminar pattern of spongiform change in the cerebral cortex and a milder cerebellar pathology when compared to VV2 cases with similar disease duration [135].

**MV 2 K+C and MM 2 T+C types**

The remaining two neuropathologic types with mixed features are also rare [135]. Furthermore, they are only identifiable histopathologically since they are characterized by the co-occurrence of two phenotypes.
that are both associated with PrP^Sc type 2. The first of these mixed types comprises MV cases showing both kuru plaques in the cerebellum and confluent vacuoles associated with a perivascular and coarse focal PrP^Sc deposition in the cerebral cortex. According to the most recent nomenclature this type has been defined as MV 2 K+C [135].

The second of these mixed types has been named MM 2 T+C and has been to date reported in only 2 individuals carrying MM at codon 129. It is characterized by the co-occurrence of thalamic and inferior olivary atrophy coexisting with confluent vacuoles and the perivascular and coarse PrP^Sc pattern of deposition in the cerebral cortex [135].

“Atypical” sporadic human prion disease: Variably protease-sensitive prionopathy (VPSPr)

A few thousand cases of sporadic prion disease have been diagnosed to date by surveillance centres worldwide and the vast majority of them fit the classification system described above. Only a few exceptions were noted. Most of such “atypical” cases were diagnosed in the USA and are now recognized to belong to a novel form of human sporadic prion disease designated VPSPr [193], while the remaining ones stand at the moment as a small group or a case report awaiting further observations and characterizations [88,189].

To date, a total of 30 cases of VPSPr have been published [68,69,79,152,193]. The disease can apparently affect all 3 codon 129 genotypes, although the expression of valine seems to increase susceptibility. Among the reported cases, 19 were VV at codon 129, 8 MV, and 3 MM. Symptoms at onset included mental behaviour-loss, depression, cognitive impairment, and motor signs, especially Parkinsonism and ataxia. The mental signs are prominent in the codon 129 VV patients whereas motor signs are more common in the MV and MM patients. In the latter groups, myoclonus was also described. The analysis of protein 14-3-3 in the CSF is negative and cerebral DW-MRI shows no abnormalities in most cases [68,69,79,152,193].

Pathologically, the spongiform change, which is especially seen in neocortical and subcortical regions of the cerebral cortex, consists of relatively large vacuoles, which are more prominent in the codon 129 MV and VV genotypes than in the MM subjects. Amyloid microplaques containing PrP are often present in the cerebellar molecular layer in both VV and MV subjects. The pattern of PrP immunostaining in both VV and MV subjects consists of target-like rounded formations made of clusters of granules, which are often interspersed within a background of fine granules (Fig. 3). In contrast, MM cases show a PrP immunostaining pattern that resembles the plaque-like pattern described in VV2 sCJD.

The Western blot profile of abnormal PrP is by far the most distinctive feature of VPSPr [193]. After treatment with proteases, PrP^Sc forms a striking, ladder-like, electrophoretic profile including at least 4 bands. Furthermore, abnormal PrP shows a variable degree of protease resistance according to the codon 129 genotype. It is protease-sensitive in subjects with VV, whereas it shows a degree of resistance comparable to some sCJD types in MV or MM subjects at codon 129.

Characterization of human prion strains by experimental transmission

Prion strains, defined as isolates that cause distinct disease phenotypes upon transmission to syngeneic animals, which persist on serial transmission, are believed to be the main cause of phenotypic diversity and are therefore also the main candidates to explain the heterogeneity of human prion disease.

Compared to the number of strain typing transmission studies conducted with the scrapie agent since the early 1970s, the characterization of human prion strains has been significantly delayed. Early studies of non-human primates aimed to prove the transmissibility of human prion disease but lacked the characterization of the isolated strains [21]. Furthermore, transmission of sCJD to wild-type mice did not often result in clinical disease [23], whereas transmissions to bank voles have only recently proved more efficient in terms of clinical outcome, although mainly for sCJD isolates linked to the M allele (MM1, MV1, and MM 2C) [119]. To facilitate CJD transmission studies, various lines of transgenic mice have been produced that express full-length or chimerical human and mouse PrP genes [72,87,93].

In the late 1990s, most transmission studies were prompted by the BSE crisis. This has led to the demonstration that vCJD and the large majority of BSE cases are caused by the same prion strain, which is distinct from prions found in sCJD, iCJD and kuru [23,72,95]. At about the same time, the first characterization of human sporadic prion isolates was accomplished in transgenic mice. Inocula from a single sporadic Fi (i.e. MM 2T) case
produced disease characteristics that differed from those induced by sCJD MM1 as well as gCJD E200K-129M, V210I-129M inocula [109].

Preliminary data concerning other sCJD subtypes became available later [93,119] but only very recently, the results obtained by a re-evaluation of the National Institutes of Health series of prion disease transmitted to non-human primates and by more comprehensive experimental transmissions to transgenic mice have significantly contributed to the issue of the extent of strain variation in sporadic human prion disease and provided answers to the crucial question of how the current classification relates to different strains of sCJD [15,90,130].

The results of these studies indicate that five out of six of the neuropathologic and molecular “pure” types of sCJD defined by Parchi et al. [133,135], behave indeed as different strains of the agent after transmission. Most importantly, sCJD MM1 and MV1 isolates have identical transmission properties, which significantly differ from those of sCJD VV2 or MV 2K. Furthermore, sCJD MM 2C, sCJD MM 2T and sCJD VV1 subtypes behave differently from each other and from the other isolates after transmission to Tg mice. However, at variance with the sCJD MM1/MV1 and VV2/MV 2K strains, only one or two cases of sCJD MM 2C, MM 2T, and VV 1 have been examined, with the assumption that transmission characteristics of a single case will be representative of the particular subgroup. Thus, the results obtained for these rare subtypes, although clear and somehow expected, await confirmation.

Very recently, preliminary results of the transmission of VPSPr of VV 129 genotype to Tg mice expressing human PrP129-V, have shown that VPSPr is indeed infectious, although VPSPr prions transmit less efficiently than sCJD VV2 prions [57]. Inoculated animals developed PrP plaques and the typical ladder-like PrP Sc profile of VPSPr, but did not show signs of clinical disease or prominent spongiform change.

**About the presumed origin of sporadic prion disease**

The origin of prion strains in a naturally occurring disease such as sCJD, remains difficult to explain. According to the leading scientists who believe in the protein only hypothesis, sCJD results from the random occurrence of a somatic mutation in PRNP or a stochastic spontaneous conversion of PrPc into PrPSc [124, 150,182]. The main argument in favour of this hypothesis is that CJD has a uniform worldwide occurrence, with only limited evidence [60] of changing incidence over the years, and no convincing geographic clustering, except for areas with large numbers of familial cases. Thus, epidemiological studies do not depict the characteristic of a transmissible disease. In addition, no environmental risk factors have been definitely proven, although surgical procedures, at least in a study, have been significantly associated with the development of sCJD [47].

What is less discussed and publicized, however, is the fact that there are also features of the sporadic prion disease that do not so easily fit the scenario defined for this disorder by the prion hypothesis. For example, if the origin of sCJD is indeed stochastic, one would expect to observe a high phenotypic heterogeneity of the disease with no tendency to produce well defined patterns with regard to type and distribution of lesions. In contrast, only two major types of PrPSc, and a limited number of clinico-pathological phenotypes that are highly consistent among subjects are found. This indicates that events leading to the formation of PrPSc in sCJD are few, and stereotyped rather than random or stochastic.

Genetic data concerning strain-specific susceptibility are also intriguing. It is well established that codon 129 of PRNP modulates the susceptibility to sCJD [124, 159,185]. This notion is based on the findings that codon 129 MM and, possibly VV genotypes are more prevalent among sCJD cases than in the general population, whereas MV heterozygotes are almost three times less prevalent. The relative protection given by the heterozygous status is usually explained within the protein only model of prion replication [124]. According to this model, PrPc conversion into PrPSc would occur most favourably in individuals with two identical copies of the PrPc (i.e. homozygotes MM or VV), rather than in heterozygotes [124]. It has been recently found, however, that there are at least two distinct groups of heterozygous sCJD subjects, showing distinct disease phenotypes [133,135]. Interestingly, the heterozygotes linked to PrPSc type 1 are less numerous than those with type 2, despite the fact that type 1 exceed type 2 by a ratio of 2 to 1 in the homozygotes [133]. Thus, the degree of protection given by the heterozygous genotype changes according to whether PrPc converts to type 1 or to type 2. This observation appears difficult to elucidate in terms of a spontaneous PrPc to PrPSc conversion, whereas it is more readily explained by differences in allelic interaction between
two distinct prion strains. It is also intriguing that codon 129 M and V alleles seem to have a quite different behaviour in terms of strain susceptibility and associated phenotypes. Indeed, at least 5 different strains have been linked to the M allele, whereas only 2 have been associated with the V allele to date. If indeed the prion strains are encrypted by differences in protein conformation or misfolding, how can a single amino acid change at codon 129 cause such a difference between the M and V allele in terms of protein misfolding?

Moving to a different issue, recent data have shown that the frequency distribution of age at onset in sCJD is not uniform [133], and certainly does not increase exponentially after a certain age, as it would be logical if a stochastic event, particularly a somatic mutation, would be the trigger of the disease. Instead, there are significant differences in age at onset among different sCJD variants [133,135], which more likely reflect age-related differences in the susceptibility to distinct strains of the CJD agent.

Finally, it appears also difficult to conciliate the spontaneous origin of sCJD with the notion that in all animal prion diseases a horizontal transmission of the disease has been demonstrated. This also, considering the clinicopathologic variability of sporadic human prion diseases, mirrors that of animal diseases, since in both the basis of phenotypic heterogeneity has been shown to depend on both agent strain variation and the genetic variability of the host.

Taken together, all these characteristics of sporadic prion disease are difficult to explain by theories attributing the origin of the disease to random, stochastic events. As a consequence, the hypothesis that at least some variants of sporadic prion disease may represent acquired disorders should not be completely dismissed. It would be important, in particular, that further epidemiological studies are conducted not considering sCJD as a single entity, but rather distinguishing among the different variants, in order to search for possible spatial or geographic clustering and potential subtype-specific risk factors. In this respect, Jansen et al. [80] have recently reported a higher proportion of MV heterozygous sCJD patients compared to previous studies in Caucasian, whereas the VV homozygous patients were relatively underrepresented. Although not conclusive, this finding is overall intriguing and deserves consideration and future follow-up.

Laboratory test for the pre-mortem diagnosis of sporadic human prion disease

Detection of surrogate markers in the CSF

At present the definitive diagnosis of sCJD can only be obtained through the demonstration of PrPSc in affected tissues. Pre-mortem diagnostic criteria assessing diagnostic probability, originally introduced for epidemiological purposes, have been recently updated [191]. They rely on clinical features and the results of cerebral DW-MRI and laboratory assessment of surrogate markers, such as 14-3-3 [76] and total tau (t-tau) [123] proteins in CSF.

14-3-3 proteins are a group of cytosolic polypeptides with regulatory functions, which are released in the CSF during neuronal damage. As a consequence, not only CJD but also other pathological conditions characterized by a rapid course and neuronal damage, such as viral, paraneoplastic or Hashimoto encephalitis, a recent stroke, metabolic encephalopathy such as Wernicke, epilepsy, or even rapidly progressive AD or Lewy body dementia may lead to a (false) positive test [28,42,161]. Overall, the test specificity for sCJD has been reported to vary from 74% to 96%, depending on type of controls used for the comparison [28,42,161]. Indeed, the specificity of this test increases significantly if cases with a positive standard MRI and/or those showing an inflammatory pattern in the CSF are excluded. Thus, 14-3-3 CSF detection represents a significant supportive diagnostic tool only in the appropriate clinical context.

Recently, attempts have also been made to improve the sensitivity and/or the specificity of the test by modifying the methodology of protein detection. Gmitterová et al. [61] developed an improved ELISA technique allowing a better quantification of the protein compared to the analysis by immunoblotting, but did not obtain any improved sensitivity. In another study, Satoh et al. [165], by using a semi-quantitative Western blot and various antibodies to specifically detect distinct 14-3-3 isoforms, showed that the specific detection of the γ-isofrom may increase the test sensitivity without influencing significantly its sensitivity.

Beside 14-3-3 the microtubule associated protein tau is also largely used as a surrogate CSF marker for the pre-mortem diagnosis of sCJD, having showed a comparable, or even higher, sensitivity and specificity than 14-3-3 [34,123,161,164]. Indeed, current routine CSF tests in most laboratories involved in CJD diagnosis include both 14-3-3 and t-tau detection since the com-
bination of the two results is thought to increase diagnostic sensitivity. The latter is still a critical issue for the clinical diagnosis of sporadic prion disease, given the high degree of heterogeneity of the disease. Unfortunately, the more the disease subtype is atypical and difficult to recognize clinically, and worse the CSF tests perform. Proteins 14-3-3 and t-tau are markedly elevated in sCJD MM1/MV1 and their measurement provides a diagnostic pre-mortem test with a reported sensitivity that ranges from 91% to 100% [34,48,61,161]. Similarly, the reported diagnostic sensitivity of 14-3-3 and t-tau detection is quite high also for the VV2 (from 84% to 100%) [34,48,61,161] and the VV1 subtypes, although for the latter the data are based on a few cases only, given the rarity of this CJD type. The diagnostic sensitivity of the CSF tests is significantly lower for both the MM 2C (from 61% to 78%) and MV 2K (from 57% to 89%) subtypes [34,48,61,161] and becomes inconsistent (lower than 50%) in sCJD MM 2T and VPSPr [56,161].

Other markers for neuronal damage (neuronal specific enolase, a glycolytic enzyme, and S-100 beta protein, a glial protein) have also been tested during the past years, but currently they provide no added value to the test based on 14-3-3 and/or t-tau detection [161].

Finally, concerning the differential diagnosis with other neurodegenerative dementias such as AD, which is the most frequent cause of a false positive test based on 14-3-3 or t-tau among the non-prion neurodegenerative dementia, the analyses of both t-tau/ phosphorylated tau ratio and Aβ-amyloid levels have been reported to be useful [188].

In conclusion, despite the lack of specific tests to detect the disease-associated PrP in body fluids, the constellation of clinical symptoms and signs together with the results of EEG, cerebral DW-MRI, 14-3-3 and t-tau CSF and PRNP genotype examinations have significantly increased the overall specificity and sensitivity of sCJD in vivo diagnosis. The interpretation of the results of CSF investigations, however, requires significant attention and knowledge about the clinical conditions that may lead to false positive results, and most importantly, about the existence of atypical forms of sporadic prion disease that are often associated with a negative CSF or DW-MRI examination. Thus, it remains very important for epidemiological and public health issues that all cases with atypical dementia are followed to autopsy.

Protein misfolding cyclic amplification (PMCA)

In the early 1990s Kocisko et al. [91] developed the original in vitro prion protein conversion assay by showing that partially purified and GdnHCl denatured brain PrPSc is able to convert protease-sensitive PrPC isolated from cultured cells in a test tube. Although instrumental in gaining a better understanding, the mechanisms by which PrPSc can be converted to PrPSc, this assay [91] suffered from a number of limitations, particularly the relative inefficiency of the reaction. To overcome this shortcoming, the ability of PrPSc to convert in vivo native PrP into PrPSc has been more recently exploited in a variety of detection techniques, where the signal from in vitro generated abnormal PrP is amplified through cycles of sonication or shaking in the presence of an excess of PrPSc or recombinant PrP (rec-PrP) as a substrate [6,171].

In the PMCA, a sonication step is alternated to an incubation step in a variable number of rounds to favour PrPSc disaggregation and increase the conversion rate [35,153]. In the serial PMCA (sPMCA), the reaction product is cyclically diluted into fresh substrate after each round, in order to maximize the conversion rate [14,36].

It has been shown that PMCA throughput varies considerably according to the reaction conditions, which implicates a role for molecular co-factors [35]. Recently, for example Yokoyama et al. [186] demonstrated that the amplification efficiency of cell-PMCA for vCJD PrPSc increased to more than 100-fold per round in the presence of heparin, whereas the same effect is not seen when sCJD or hamster-adapted 263K scrapie are used as seed. Thus, these results suggest that each prion strain requires a specific pool of co-factors for PrPC conversion to be optimized.

Another important variable affecting PMCA efficiency concerns the similarity between the seed and the substrate. In sCJD, for example, PMCA efficiency tends to increase when the codon 129 genotype of the seed matches that of the substrate, although exceptions to this rule are seen. Indeed, 129VV PrPSc seems to exclusively convert 129VV PrPC, while 129MM PrPSc converts, with decreasing efficiency, 129MM PrPC, 129MV PrPC or 129VV PrPC (the latter, however, only with the MM1 PrPSc substrate). Finally, 129MV PrPSc appears to convert either 129MM or VV PrPC, depending on the PrPSc type to which it is associated (type 1 or 2): PrPSc MV1 converts with the best efficiency PrPC MM, while PrPSc MV2 converts with the best efficiency PrPC VV [83]. Given that PrPSc type 1 in sCJD is preferentially found...
to be associated with the MM 129 genotype, whereas type 2 is preferentially matched to the VV genotype, the converting activity of 129MV PrPSc seems to reproduce this peculiar association. It has also been shown that PMCA amplification reproduces with high fidelity the original PrPSc type of the seed both in terms of fragment size and glycosylation ratio [82-84].

The theory behind the so-called prion in vitro conversion on which PMCA is founded, postulates that both PrPSc and PrPc are required for PrPSc amplification to occur. At variance with this scenario, however, it has been shown that PrPc conversion may also occur spontaneously in the presence of poly-A+ RNA, and that this newly generated PrPSc is infectious like its natural counterpart [51]. Other authors have also underlined the lack of specificity of PMCA, thus confirming this consistent drawback of the technique. Despite this significant limitation, some scientists continue to use PMCA to increase PrPSc levels in order to make it detectable in body fluids such as CSF and blood that are accessible for pre-mortem, non-invasive diagnostic assays.

The basic PMCA format has also been changed over the years by introducing technical changes that would simplify the technique or make it more efficient. rPrP-PMCA [3], for example, employed a recombinant PrP substrate expressed in bacterial cells to overcome the difficulties and high expenses related to PrPc recovery from human or transgenic mice brain tissue or from cell cultures. Furthermore, PMCA with teflon beads (PMCAb) has been shown to increase consistently the reaction efficiency with respect to the original format [62]. Beads are thought to help sonication in breaking PrPSc fibrils into smaller fragments, thus increasing the PrPc amount that undergoes conversion from 10% up to 100% in a single 24-hour round.

**Quaking-induced conversion (QuIC)**

In this technique, as in rPrP-PMCA, recombinant human PrP expressed by E. Coli is used as the substrate to be seeded by PrPSc, although sonication is replaced by shaking at a relatively high temperature.

QuIC has ultimately gained more interest than PMCA as a detection technique with diagnostic applicability since it is apparently free from the potential drawbacks of the PMCA approach like the time taken, the complexity of the substrate and reliance on sonication, which is difficult to standardise [4].

Real-time (RT) QuIC is a recent adaptation of QuIC in which the seeded conversion of hamster recombinant PrP (rPrP) into aggregates of PrPSc is monitored in real time [183]. Thioflavin T (ThT) is included in the reaction and binds to the aggregated PrPSc causing a change in the ThT emission spectrum which is monitored using fluorescence spectroscopy. The reaction is performed in multiwell microplates, thus allowing the analysis of 96 samples at a time.

This improved QuIC format was inspired by the so-called amyloid seeding assay [44] in which polymerization of rPrP into amyloid fibrils is “seeded” by partially purified preparations of prions without any shaking or sonication and detected by monitoring the changes in the fluorescence emission of thioflavin T, when binds amyloid fibrils.

RT-QuIC seems to be a very promising tool for PrPSc detection in biological fluids. In this regard, two recent studies [5,110] have shown that CSF samples from patients with neuropathologically confirmed sCJD, at variance with non-CJD controls, can “seed” the RT-QuIC reaction to produce a sustained increase in ThT fluorescence. The first study reported that positive RT-QuIC responses were seen in 29 out of 34 (85%) CSF samples from patients with neuropathologically confirmed sCJD and in none of 14 control patients (100% specificity). Noteworthy, RT-QuIC was able to discriminate sCJD affected patients carrying all three PRNP codon 129 genotypes [5]. The second study involved 56 CSF samples from neuropathologically confirmed sCJD patients and 53 from CJD suspected patients that were subsequently found to have an alternative diagnosis. In this study, RT-QuIC showed 91% sensitivity and 98% specificity, compared to 91% sensitivity and 55% specificity for 14-3-3 detection performed on the same samples, thus demonstrating a relevant increase in specificity, without loss in sensitivity, for RT-QuIC with respect to the 14-3-3 test [110]. Both these studies suggest that RT-QuIC may lead to a significant improvement in the pre-mortem sCJD diagnosis and encourage further investigation upon larger patients’ cohorts and across a larger number of laboratories. RT-QuIC analysis appears to have similar sensitivity but markedly more specificity than those of both CSF 14-3-3 and tau protein detection. However, the numbers are too small to draw any firm conclusions and further prospective studies are needed to determine whether these figures hold true when larger numbers of patients are investigated. Furthermore, issues such as reproducibility and specificity must be particularly investigated, also in the interlaboratory setting, given the strict dependency of the RT-QuIC on the recombinant substrate. The use of the latter should be carefully investigated in regard to
the potential problem of spontaneous generation of PrP aggregates, an issue that has been disclosed with PMCA but not as yet fully explored in the RT-QuIC.

In another study, Peden et al. [140] have addressed the question of whether PrPSc derived from different CJD variants (including both sCJD and vCJD) could affect the amplification efficiency of RT-QuIC, as it was demonstrated with PMCA. They found no substantial decrease in the assay throughput when the 129 genotype between seed and substrate did not match. Moreover, similar amplification efficiency for hamster rec-PrP was observed for all sCJD phenotypes with the only exception of sCJD VV1 and MM2, which appeared slightly less permissive to amplification. At variance with sCJD, vCJD PrPSc showed a poor amplification, even using a homologous 129M human rec-PrP substrate. This diversity was apparently not determined by inhibiting factors, but rather by conformational characteristics of vCJD PrPSc.

Overall, these data suggest that RT-QuIC may be better standardized than PMCA, at least for sCJD variants, which makes RT-QuIC the current best candidate technique for the development of a pre-mortem CSF diagnostic test for sCJD with better sensitivity for all variants. In addition, a better understanding of the factors differentially affecting PMCA and RT-QuIC could also help to better elucidate the PrPSc amplification process in vivo.

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