

# Incidence and spectrum of sporadic Creutzfeldt–Jakob disease variants with mixed phenotype and co-occurrence of PrP<sup>Sc</sup> types: an updated classification

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**Abstract** Six subtypes of sporadic Creutzfeldt–Jakob disease with distinctive clinico-pathological features have been identified largely based on two types of the abnormal prion protein, PrP<sup>Sc</sup>, and the methionine (M)/valine (V) polymorphic codon 129 of the prion protein. The existence of affected subjects showing mixed phenotypic features and concurrent PrP<sup>Sc</sup> types has been reported but with inconsistencies among studies in both results and their interpretation. The issue currently complicates diagnosis and classification of cases and also has implications for disease pathogenesis. To explore the issue in depth, we carried out a systematic regional study in a large series of 225 cases. PrP<sup>Sc</sup> types 1 and 2 concurrence was detected in 35% of cases and was higher in MM than in MV or VV subjects. The deposition of either type 1 or 2, when concurrent, was not random and always characterized by the

coexistence of phenotypic features previously described in the pure subtypes. PrP<sup>Sc</sup> type 1 accumulation and related pathology predominated in MM and MV cases, while the type 2 phenotype prevailed in VVs. Neuropathological examination best identified the mixed types 1 and 2 features in MMs and most MVs, and also uniquely revealed the co-occurrence of pathological variants sharing PrP<sup>Sc</sup> type 2. In contrast, molecular typing best detected the concurrent PrP<sup>Sc</sup> types in VV subjects and MV cases with kuru plaques. The present data provide an updated disease classification and are of importance for future epidemiologic and transmission studies aimed to identify etiology and extent of strain variation in sporadic Creutzfeldt–Jakob disease.

**Keywords** Prion protein · Brain mapping · Molecular typing · Neurodegeneration · Classification

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## Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are invariably fatal neurodegenerative disorders affecting humans and other mammals such as sheep, elk, and cattle [36]. These diseases are characterized clinically by a rapidly progressive neurological dysfunction and neuropathologically by spongiform degeneration, neuronal loss, gliosis, and sometimes by amyloid plaques, which combine in varying degrees of severity and regional distribution.

Biochemically, prion diseases are characterized by the conversion of a constitutively expressed cellular protein, the prion protein (PrP<sup>C</sup>) into an abnormally folded, insoluble, partially protease-resistant isoform (PrP<sup>Sc</sup>) [4, 8, 27]. Incubation of a brain homogenate from prion-infected individuals with proteinase K (PK) under conditions leading to a complete degradation of PrP<sup>C</sup> generates an N-terminal truncated form of PrP<sup>Sc</sup>, commonly referred to as PrP27-30, an established molecular marker for the disease [5, 38].

Sporadic Creutzfeldt–Jakob disease (sCJD), the commonest form of the human prion diseases, comprises a broad spectrum of clinico-pathological variants [30]. Prion strains are believed to be the main cause of TSE phenotypic diversity. Prion strains are defined as isolates that show distinct disease phenotypes upon transmission to syngenic animals, persisting on serial transmission [6, 15, 34]. In addition, the host genotype variability in the gene encoding PrP<sup>C</sup> (*PRNP*), as determined by polymorphisms and mutations, is another recognized causal factor for phenotypic heterogeneity [2, 12]. The hypothesis that PrP<sup>Sc</sup> itself may encode strain-specific properties has gained support over the years by the demonstration of distinct physico-chemical subtypes of PrP<sup>Sc</sup> that include differences in the level of glycosylation and in the size of proteinase K-digested PrP<sup>Sc</sup> fragments, possibly reflecting distinct protein conformations [1, 3, 9, 10, 14, 22, 28, 29, 32, 39, 41, 42]. Although the final proof that conformational variants of PrP<sup>Sc</sup> represent the biological basis of prion strains is lacking, the described physicochemical differences among PrP<sup>Sc</sup> types are increasingly used as surrogate markers of strain properties in a given host. Based on differences in gel mobility and N-terminal sequence of the core fragments (i.e. PrP27-30) generated by PK digestion, Parchi et al. [28, 29, 32] originally identified two major human PrP<sup>Sc</sup> types: type 1 with a relative molecular mass of 21 kDa and the primary cleavage site at residue 82, and type 2 with relative molecular mass of 19 kDa and the primary cleavage site at residue 97. The two PrP<sup>Sc</sup> types, in conjunction with the codon 129 genotype, significantly explained sCJD

phenotypic variability and provided a molecular basis for disease classification [7, 28, 30]. Six subtypes of sCJD with distinctive phenotypic features were identified, each resulting from a specific codon 129 genotype/PrP<sup>Sc</sup> type combination with two exceptions. MM1 and MV1 cases were phenotypically indistinguishable and, therefore, merged in one subtype (MM/MV 1), whereas, on the contrary, MM2 subjects were associated with 2 subtypes with distinctive histopathological features in the cerebral cortex and in the thalamus and designated accordingly (MM2-cortical or MM 2C and MM2-thalamic or MM 2T) [30]. By showing that at least some of these sCJD subtypes correspond to specific agent strain-host genotype combinations, preliminary transmission studies seem to confirm the biological basis of this classification [16, 17, 23, 42].

An important aspect of sCJD phenotypic variability, which has not yet been investigated thoroughly, concerns the existence of cases presenting more than one PrP<sup>Sc</sup> type and mixed histopathological features. This issue still represents a potential drawback for the widespread application of the current disease classification for diagnostic and epidemiologic purposes. Since the original report of this phenomenon [30], based on the analyses of a few samples for each brain, the co-occurrence of PrP<sup>Sc</sup> type 1 and 2 in a percentage varying between 12 and 44% of sCJD cases analyzed has been confirmed by a number of subsequent studies [13, 18, 20, 37, 40, 43]. In many of them, the question of how far the proportion of cases with concurrent PrP<sup>Sc</sup> types is a function of the sampling protocol employed was raised. More recently, two studies using novel antibodies recognizing an epitope between residues 82 and 96 (i.e. not detecting type 2) found that all type 2 samples show at least some associated protein migrating in the 21 kDa molecular weight range and concluded that PrP<sup>Sc</sup> type 1 coexists in all type 2 sCJD cases and that the number of cases with concurrent PrP<sup>Sc</sup> types is likely larger than previously thought [35, 45]. It was later shown, however, that the “type 1 selective” antibodies fail to discriminate properly between a *bona fide* PK-resistant PrP<sup>Sc</sup> core and the partially cleaved fragments generated during digestion due to the several PK cleavage sites included in the PrP<sup>Sc</sup> N-terminus, making difficult the interpretation of the results obtained [25].

To explore the issue of concurrent PrP<sup>Sc</sup> types in sCJD in depth, we carried out a systematic regional study in a large series of cases. To this aim, we applied a refined western blot assay with increased sensitivity for the detection of PrP<sup>Sc</sup> types coexistence [25] and correlated the molecular data with those obtained by clinical and pathological examinations.

## Materials and methods

### Case and tissue selection

This study was based on 225 cases with an established diagnosis of sCJD obtained after clinical, neuropathological, and molecular examination. Cases of genetic and acquired forms of CJD were excluded. The study was restricted to cases from which large amounts of both frozen and fixed brain tissue were available. 200 patients were obtained from a group of ~240 consecutive cases referred for diagnosis (~40 cases lacked sufficient frozen tissue), whereas the remaining 25 were specifically chosen to increase the number of cases with mixed phenotypes. More precisely, the 25 additional cases were selected based on the demonstration of a mixed synaptic and perivacuolar pattern of PrP deposition by PrP immunohistochemistry (see “Results”). 61 patients died in the USA between 1990 and 2001, and 164 in Europe (124 in Italy, 34 in Germany, and 6 in Belgium) between 1993 and 2007.

Brains were removed at autopsy and either one half or selected coronal sections of tissue, including all major brain structures and nuclei, were immediately frozen and stored at  $-80^{\circ}\text{C}$ . The remaining tissue was fixed in formalin and was used for neuropathological examination and PrP immunohistochemistry. Samples of frozen gray matter (between 50 and 100 mg) for protein analysis were obtained from the following regions: frontal (superior and middle frontal gyri), temporal (superior and middle temporal gyri), parietal (inferior parietal lobule), and occipital (calcarine cortex and lateral occipital gyrus) cortices, hippocampus (Ammon’s horn), limbic cortices (entorhinal cortex anterior, insular cortex, and cingulate gyrus), striatum (caudate and putamen nuclei), thalamus (medial and lateral nuclei), hypothalamus, brainstem (midbrain periaqueductal gray, pontine periaqueductal gray including locus coeruleus, medullar periventricular gray), cerebellum (hemisphere and vermis). The parietal cortex and the 3 samples from the brainstem were not available in 30 cases from Germany. Furthermore, 1 or 2 samples were occasionally lacking in some other cases [altogether 34 samples, mostly from the hypothalamus ( $n = 15$ ) and amygdala ( $n = 11$ )]. Sampling of frozen tissues was performed by the same investigator (PP) according to a defined protocol across the whole series of cases. Blocks of fixed tissue were taken from the opposite half of the brain and were used for histopathologic examination. Sampling of fixed tissues was performed by the same investigator (PP) according to a defined protocol in 160 cases. The remaining 65 cases were sampled in Munich (Germany) and Indianapolis (USA). In these Centers, which are both involved in CJD National surveillance and Brain banking, CJD brains are sampled extensively according to protocols,

which included all the areas that were of interest for this study. Sampling of both fixed and frozen tissue was performed twice in a subgroup of 10 codon 129 MM subjects.

### Protein analysis

Preparation of samples, Western blotting (WB), and PrP<sup>Sc</sup> typing were performed according to established methods [25, 26, 33]. In particular, for the sensitive detection of PrP<sup>Sc</sup> types 1 and 2 concurrence, we followed the method of Notari et al. [25]. All samples were homogenized in lysis buffer plus at pH 6.9, digested with proteinase K (PK) (Roche Diagnostics, specific activity by certificate of analysis: 47.9 U/mg) at a final concentration of 10 U/mL and run in a 6.5 cm long separating gel. In addition, selected samples from MV2 cases with kuru plaques (MV 2K) and MM or MV subjects showing mixed pathological features but only PrP<sup>Sc</sup> type 1 after the run in the 6.5 cm long gel were also run in a 15 cm long gel. To this aim, at least one sample from each cortical lobe was re-analyzed. All immunoblots were probed with the monoclonal antibody 3F4 with epitope at PrP residues 108–111 at 0.1  $\mu\text{g}/\text{ml}$  concentration (Signet Labs, MA, USA). In addition, immunoblots were probed with the monoclonal antibody 1E4 with epitope mapped at PrP residues 98–100 at 2  $\mu\text{g}/\text{ml}$  concentration (Sanquin Reagents, Amsterdam, The Netherlands) in the following cases: (a) all samples from VV1 cases; (b) 20 neocortical samples from 10 MM1 cases with pure synaptic PrP deposition, and (c) all samples from 30 MM or MV cases, which showed a mixed pattern of PrP deposition by immunohistochemistry and either no evidence or a very scanty and focal (1 or 2 areas) type 2 accumulation after immunoblotting with the 3F4 antibody. Finally, selected blots from MV 2K samples were also probed with SAF60 against PrP residues 157–161, at 0.4  $\mu\text{g}/\text{ml}$ .

We quantitatively calculated the ratio between type 1 and type 2 in the non-glycosylated PrP<sup>Sc</sup> after densitometric analyses of immunoblots probed with 3F4 using the AIDA Image Analyzer software (RayTest). This choice was in part driven by our experience indicating that deglycosylation is associated to a loss of definition in the type 1 (21 kDa) and type 2 (19 kDa) bands which makes their distinction and relative quantitation less reliable. Furthermore, given that it has been previously shown [28, 30] that the relative abundance of glycosylated versus non-glycosylated PrP<sup>Sc</sup> shows only relatively minor differences among the sCJD subtypes associated to either PrP<sup>Sc</sup> type 1 or PrP<sup>Sc</sup> type 2, we assumed that the analyses of the unglycosylated form would be representative of the whole protein. We tested this hypothesis by comparing densitometric analyses of non-glycosylated glycoforms versus deglycosylated preparations from representative regions of

MM cases with either a dominant type 1 or a dominant type 2. Overall, we found that, compared to the latter, the first approach is associated to a relatively minor overestimation of type 2 protein. In particular, the difference was almost negligible for the group of samples with dominant type 1 (69/31% on non-glycosylated isoforms vs. 72/28% after deglycosylation,  $n = 20$ ), and slightly more evident in the group of samples with dominant type 2 (29/71% on non-glycosylated isoforms vs. 37/63% after deglycosylation,  $n = 30$ ).

To express quantitatively with a single number the relative amount of type 1 and type 2 in a given case (total type 1 or type 2 “load”) we calculated the mean of the relative amounts of either type 1 or type 2, expressed in % of total PrP<sup>Sc</sup> signal, in the different brain regions examined.

#### Molecular genetic analysis

Genomic DNA from all subjects was used to amplify the coding region of *PRNP* in the polymerase chain reaction (PCR). *PRNP* open reading frame was amplified as previously described [11]. The PCR product was visualized on a 1% agarose gel to detect potential insertion mutations or deletions. Potential point mutations were revealed by dHPLC analyses. Mutations were also ruled out by direct sequencing of *PRNP* open reading frame in about 50% of cases. Finally, the codon 129 genotype was examined by digestion with the restriction endonuclease Nsp 1.

#### Neuropathology

Semiquantitative evaluation of spongiosis and gliosis was carried out in all cases by comparing hematoxylin and eosin stained sections obtained from the same 21 brain regions evaluated biochemically. Spongiosis was rated on a 0–4 scale (not detectable, mild, moderate, severe, and status spongiosis), whereas gliosis was scored on a 0–3 scale (not detectable, mild, moderate, and severe). A lesion profile for each patient was obtained by averaging the two scores.

For PrP immunohistochemistry, paraffin sections from formalin-fixed and formic acid treated blocks of the areas selected for neuropathologic examination were processed using the monoclonal antibodies 3F4, according to published protocols [30]. PrP deposits were described as diffuse or synaptic, perivacuolar, coarse or patchy and plaque-like.

#### Clinical analysis

Clinical data were available in most cases, and medical records always included at least one neurological examination. Duration of symptoms was calculated from the time

of presentation of neurological signs suggesting an organic cause. Prodromal symptoms were not taken into account. Clinical signs were classified “at onset” when observed within the first quarter of the mean duration of symptoms of the group to which the patient belonged (i.e. 1 month if the mean duration was 4).

## Results

### PrP<sup>Sc</sup> detection and typing

In the present study, we tried to maximize the detection sensitivity of PrP<sup>Sc</sup> type co-occurrence in sCJD-affected brains. At variance with previous studies, we typed PrP<sup>Sc</sup> after a detailed topographic analysis of 21 brain regions and using the method recently described by Notari et al. [25], which has shown increased sensitivity for the detection of *bona-fide* concurrence of PrP<sup>Sc</sup> types 1 and 2. Furthermore, we probed selected immunoblots with 1E4, a recently characterized monoclonal antibody with a higher affinity for type 2 than for type 1 [44]. The latter approach allowed us an increased protein loading in the gel and a better identification of PrP<sup>Sc</sup> type 2 when present in relatively low amounts (Fig. 1a) in the protein types mixture.

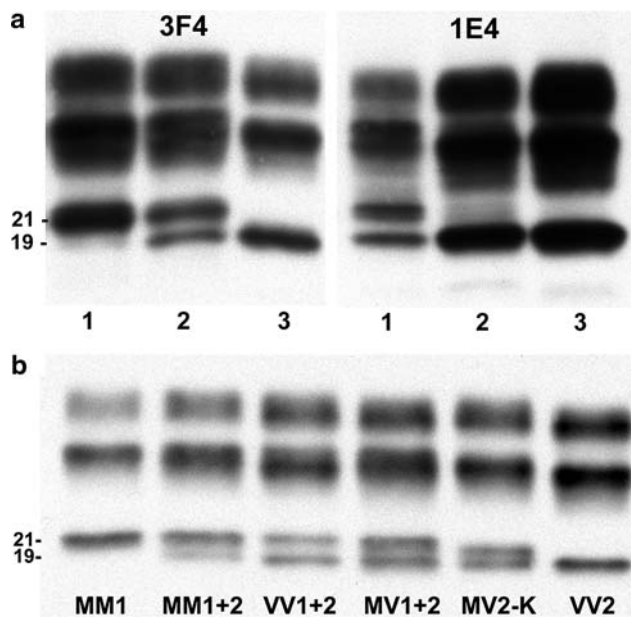
The results obtained in the largely unselected series of 200 cases after using both 3F4 and 1E4 antibodies are shown in Table 1. The overall prevalence of PrP<sup>Sc</sup> types 1 and 2 concurrence was 35% (32% when only the results with 3F4 were considered). The same incidence of mixed cases was also found in the two largest groups of subjects from Italy and USA when considered individually. Although the protein mixture was detected in each codon 129 genotype (Fig. 1b), PrP<sup>Sc</sup> types 1 and 2 co-occurred more frequently in MM than in MV or VV subjects. A PrP<sup>Sc</sup> profile comprising two distinct bands also characterized the MV2 subtype with kuru plaques (MV 2K) in many brain regions, but the relative size of the associated band (i.e. 20 kDa) distinguished these profiles from *bona-fide* types 1 and 2 concurrence (Fig. 1b), as previously shown [24, 25].

### Regional variation and relative “load” of PrP<sup>Sc</sup> Types 1 and 2 concurrence

#### MM subjects

The regional distribution of co-existing PrP<sup>Sc</sup> types 1 and 2 in MM subjects reproduced features previously observed in the pure MM1 and MM2-cortical (MM 2C) sCJD subtypes [30], although quantitatively the relative proportion of type 1 and type 2 accumulation in these cases varied extensively. Most cases had PrP<sup>Sc</sup> type 1 in all areas and PrP<sup>Sc</sup>





**Fig. 1** **a** mAb 1E4 shows a relative higher affinity for PrP<sup>Sc</sup> type 2 than for PrP<sup>Sc</sup> type 1. Western blot profiles of PrP<sup>Sc</sup> from 3 sCJD MM subjects showing various relative proportions of PrP<sup>Sc</sup> type 1 and type 2. Each sample has been loaded twice at the same concentration and probed either with 3F4 (*left*) and 1E4 (*right*). *Lane 1* sample with dominant type 1 and only 3–5% of type 2; *Lane 2* sample with 60/40% type1/type2 ratio; *Lane 3* sample with pure type 2. **b** Western blot profiles of PrP<sup>Sc</sup> (3F4 antibody) from sCJD subjects with concurrent PrP<sup>Sc</sup> types 1 and 2 carrying different codon 129 *PRNP* genotypes (*lanes 2–4*). PrP<sup>Sc</sup> type 1 from a MM1 case (*lane 1*), and PrP<sup>Sc</sup> type 2 from a VV2 case (*lane 6*) are included as controls. The PrP<sup>Sc</sup> profile associated with the sCJD MV2 phenotype with kuru plaques (sCJD MV2K) is shown in *lane 5*. The upper band of the PrP<sup>Sc</sup> doublet in *lane 5* migrates faster than the type 1 band. Frontal cortex homogenates were treated with PK and probed with 3F4. Approximate molecular masses are in kilodaltons

**Table 1** *PRNP* genotype and PrP<sup>Sc</sup> type in a unselected series of 200 sCJD cases

Type <sup>a</sup>	MM	MV	VV	Total
Type 1	67 (52)	2 (6)	2 (5)	71 (35)
Types 1+2 concurrence	56 (43)	7 (23) <sup>b</sup>	6 (15)	69 (35)
Type 2	6 (5) <sup>b</sup>	22 (71) <sup>b</sup>	32 (80)	60 (30)
Total	129	31	40	200

MV 1+2 includes 2 cases with kuru plaques and 5 without, MM2 includes 4 MM 2T, and 2 MM 2C cases, whereas MV2 includes 20 cases with kuru plaques and 2 without

% are expressed in brackets and refer to each single column

<sup>a</sup> Typing results refer to both 3F4 and 1E4 mAbs

<sup>b</sup> Phenotypically heterogeneous patient groups

type 2 detectable only focally in the cerebral neocortex or thalamus (Fig. 2a), whereas only a small group of subjects showed a predominant PrP<sup>Sc</sup> type 2 “load” (Fig. 2b). The two groups will be indicated as MM 1+2C and MM 2C+1 throughout the manuscript, MM 2C+1 being defined as a

case in which the mean total amount of type 2 (total type 2 “load”) expressed in % of total PrP<sup>Sc</sup> signal and calculated by averaging the results obtained from all brain regions examined, is higher than that of type 1 (i.e. >51%).

The detailed regional PrP<sup>Sc</sup> typing in the MM 1+2C group provided the following results. When 3F4 was used, 74% of cases showed type 2 in 7 or fewer of the 21 regions analyzed, and 42% of them in only 1 or 2 (Fig. 3). In the latter group, 3F4 detected the PrP<sup>Sc</sup> types mixture only in the cerebral cortex or thalamus in 37 of the 504 samples analyzed (Fig. 3). However, when 1E4 was used, the number of mixed samples in the cerebral cortex or thalamus raised about 2.6 times to 98 (data not shown). Noteworthy, the number of cases showing type 2 in only one or two areas dropped from 24 to 8. Furthermore, 1E4 detected PrP<sup>Sc</sup> type 2 in 6 out of 7 subjects in which 3F4 failed to demonstrate the type 2 protein despite its presence was strongly suggested by the results of PrP immunohistochemistry (see below). Finally, in the remaining samples from subcortical areas, 1E4 confirmed the results obtained with 3F4 and failed to reveal a significant type 2 accumulation.

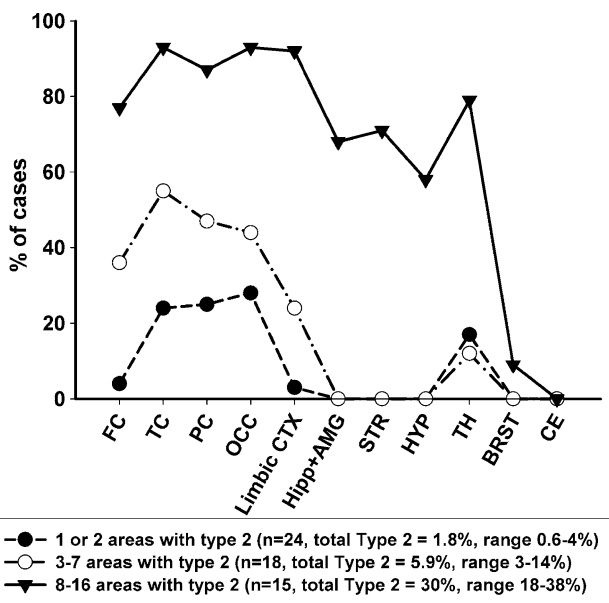
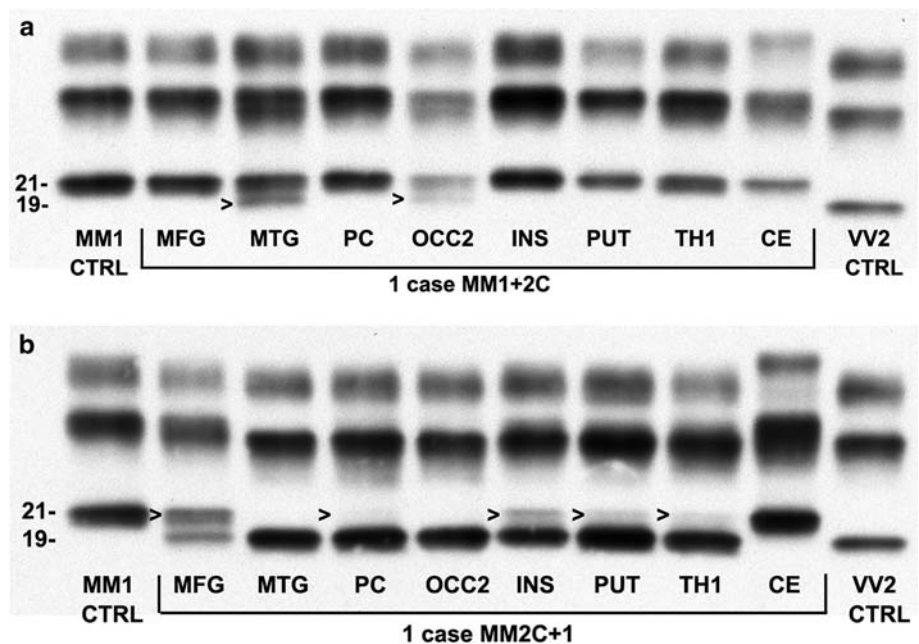
In the group of MM 1+2C with type 2 in up to 7 brain regions (i.e. 74% of cases), the amount of PrP<sup>Sc</sup> type 1 was higher than that of type 2 in 84% of the mixed samples, while type 2 had a stronger signal than type 1 in the remaining 16%. The mean total type 2 “load” in this group, calculated on immunoblots probed with 3F4, was 3.4% (range 0.6–14%).

The remaining 26% of MM 1+2C cases showed a more widespread PrP<sup>Sc</sup> type 2 accumulation involving from 10 to 16 different brain regions. In this group, PrP<sup>Sc</sup> type 1 was predominant over type 2 in 43% of the mixed samples, while type 2 had a stronger signal than type 1 in the remaining 57%. The mean total type 2 “load”, again calculated on immunoblots probed with 3F4 raised to 30% (range 18–38%).

The study of the regional protein distribution in MM 1+2C subjects showed that PrP<sup>Sc</sup> type 2 was overall more frequently seen in the cerebral cortex than in subcortical areas (Fig. 3). The temporal, parietal, and occipital neocortices were the cortical areas which preferentially accumulated the type 2 protein (Fig. 3). Among subcortical areas, the thalamus was by far the most frequent site of type 2 accumulation, whereas the brainstem and, to a greater extent, the cerebellum (Fig. 3) were the regions with the lowest frequency (1 and 0 cases, respectively) of type 2 detection.

At variance with MM 1+2C cases, in MM 2C+1 subjects type 2 was the only detectable protein species in some areas (Fig. 2b, Table 2) and its amount was higher than that of type 1 also in most areas where the two proteins coexisted (Table 2).

**Fig. 2** Western blot profiles of PrP<sup>Sc</sup> (3F4 antibody) from different brain regions of **a** one sCJD MM 1+2C subject showing the type 2 protein only focally in the cerebral cortex (*arrowheads*) and **b** one sCJD MM 2C+1 subject showing a widespread PrP<sup>Sc</sup> type 2 accumulation, a focal deposition of the type 1 protein (*arrowheads*) in the cerebral cortex, putamen, and thalamus, and a dominant type 1 in the cerebellum. Areas shown include the middle frontal gyrus (*MFG*), middle temporal gyrus (*MTG*), parietal cortex (*PC*), lateral occipital cortex (*OCC2*), insular cortex (*INS*), putamen nucleus (*PUT*), medial thalamic nuclei (*TH1*), and cerebellum (*CE*). Approximate molecular masses are in kilodaltons



**Fig. 3** Prevalence and regional distribution of PrP<sup>Sc</sup> type 2 in a series of 57 MM 1+2C subjects. PrP<sup>Sc</sup> type 1 was detected in all areas. Subjects are divided in 3 groups based on the number of areas with a detectable PrP<sup>Sc</sup> type 2 signal on WB. For each group, the % of type 2 positive samples in each brain region is shown. The mean total type 2 “load” for each group, as defined in “Materials and methods”, is given in brackets. FC frontal cortex, TC temporal cortex, PC parietal cortex, OCC occipital cortex, Limbic CTX limbic cortices, Hipp + AMG hippocampus+amygdala, STR striatum, HYP hypothalamus, TH thalamus, BRST brainstem, CE cerebellum

#### MV subjects

PrP<sup>Sc</sup> types 1 and 2 concurrence in MV cases was detected in two phenotypically distinct groups of subjects. The

absence or presence of kuru plaques best distinguished the two groups. In subjects lacking kuru plaques, the concurrence of PrP<sup>Sc</sup> types reproduced the features seen in MM 1+2C subjects. At variance with the MM group, however, the relative proportion of MV 1+2C and MV 2C+1 was significantly different. Indeed, only one case had type 1 in all brain regions and the concurrent type 2 in 5, two had 12 and 14 areas with a mixed molecular phenotype, respectively (but a predominant type 1 load), while the remaining 3 showed a predominant type 2 accumulation (Table 3).

PrP<sup>Sc</sup> types 1 and 2 concurrence was more difficult to assess in MV subjects with kuru plaques (i.e. MV 2K subtype) because of the presence of the 20 kDa band (Fig. 1), which we detected in virtually all samples from subcortical structures and in 75% of those taken from the neocortex (data not shown). Thus, a relatively weak 21 kDa signal cannot be considered proof of *bona-fide* PrP<sup>Sc</sup> type 1 concurrence, since it would also be compatible with a partially cleaved fragment of the 20 kDa PrP<sup>Sc</sup> core [25]. As a consequence, we considered the 21 kDa band a *bona-fide* type 1 only when its intensity was similar or higher than that of the 20 kDa fragment. Using these stringent criteria, we detected an undisputable type 1 signal in a least two cortical samples in only 2 of the 24 MV 2K cases analyzed (data not shown). Interestingly, at variance with the other MV 2K cases, the PrP<sup>Sc</sup> profile in these two cases included the so called 13 kDa CTF (C-terminal truncated fragment) (data not shown), which at variance with MV 2K is consistently found in both MM1 and VV1 cases [26].

**Table 2** Regional relative amounts (in %  $\pm$  SD) of PrP<sup>Sc</sup> type 2 in MM 2C+1 subjects ( $N = 5$ )

Brain region	Type 1 only	Type 1 > 2	Type 2 > 1	Type 2 only
Neocortex	(0)	38 $\pm$ 11 (14)	70 $\pm$ 18 (46)	(40)
Limbic areas	(0)	41 $\pm$ 3 (16)	77 $\pm$ 9 (44)	(40)
Striatum	(0)	42 $\pm$ 8 (20)	78 $\pm$ 14 (70)	(10)
Thalamus	(0)	44 (10)	90 $\pm$ 7 (70)	(20)
Hypothalamus + Brainstem <sup>a</sup>	(21)	44 $\pm$ 4 (14)	75 $\pm$ 14 (43)	(22)
Cerebellum	(70)	38 (10)	61 $\pm$ 4 (20)	(0)

Numbers in brackets express the % of samples showing each of the 4 profiles in the listed brain regions

<sup>a</sup> Samples from the brainstem were not available in three cases

**Table 3** Regional relative amounts (in %  $\pm$  SD) of PrP<sup>Sc</sup> type 2 in MV 2C+1 cases ( $N = 3$ )

Brain region	Type 1 only	Type 1 > 2	Type 2 > 1	Type 2 only
Neocortex	(0)	48 (5)	84 $\pm$ 12 (47)	(48)
Limbic areas	(0)	48 (7)	80 $\pm$ 15 (73)	(20)
Striatum	(0)	33 $\pm$ 13 (50)	75 $\pm$ 14 (50)	(0)
Thalamus	(0)	46 (17)	83 $\pm$ 4 (83)	(0)
Hypothalamus + Brainstem	(8)	32 $\pm$ 10 (44)	65 $\pm$ 12 (25)	(0)
Cerebellum	(33)	7 (17)	(0)	(50)

Numbers in brackets express the % of samples showing each of the 4 PrP<sup>Sc</sup> profiles in the listed brain regions

### VV subjects

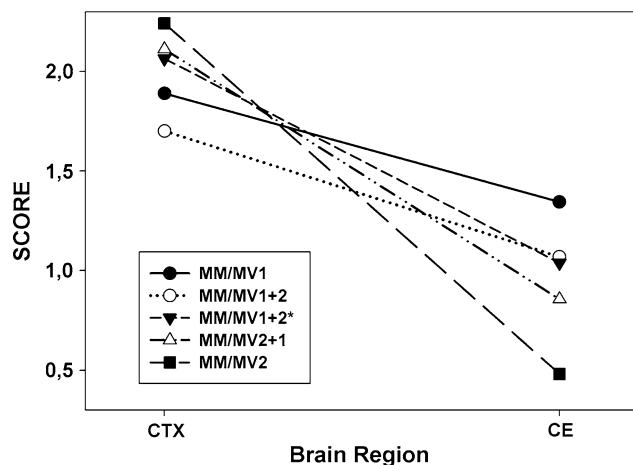
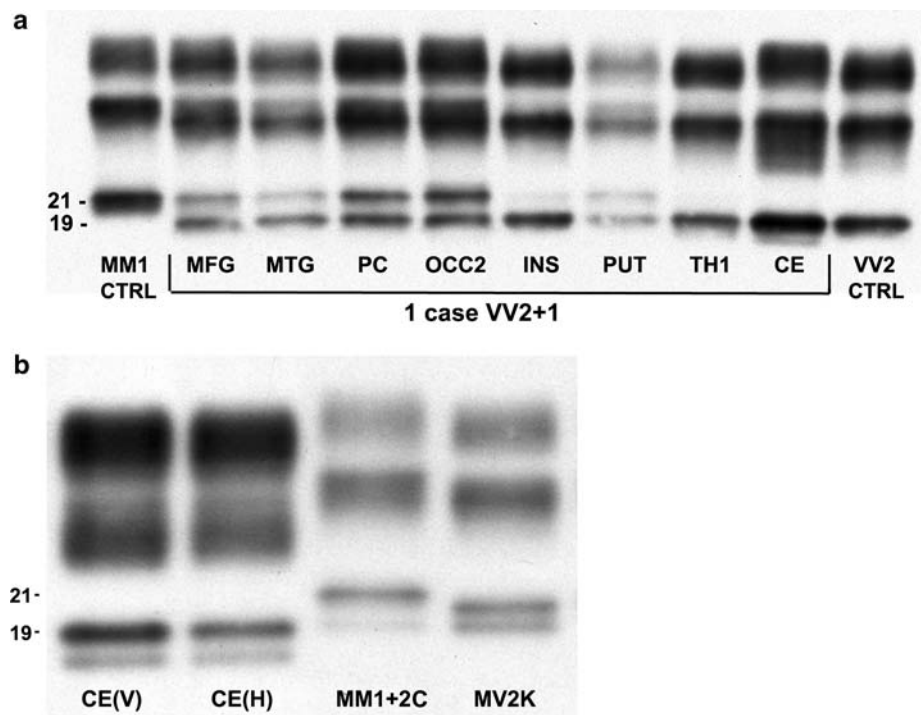
Six VV cases with concurrent PrP<sup>Sc</sup> types 1 and 2 were found. PrP<sup>Sc</sup> type 2 was always dominant and detectable in almost all samples. By contrast, type 1 was only present focally in the cerebral cortex, in the striatum or in both. In two cases, however, PrP<sup>Sc</sup> type 1 was seen in most samples from cerebral cortex and striatum and in a single case, it showed an even more widespread accumulation involving all the cerebral cortical samples, the striatum, amygdala, hippocampus, hypothalamus and thalamus (Fig. 4a). When concurring, type 2 was predominant over type 1 in 76% of samples, while type 1 had a stronger signal than type 2 in the remaining 24%. The mean total type 1 “load” in VV 2+1 was 9.4% (range 1.5–38%).

Although clearly distinguishable from a type 1 and 2 concurrence, also the PrP<sup>Sc</sup> type 2 profile in VV subjects was sometimes characterized by a doublet comprising a  $\sim$ 18.5 kDa fragment in addition to the typical 19 kDa band (Fig. 4b). These two bands concurred in the cerebellum in all cases, in the thalamus in 50% of them and in the midbrain in 18%. Since epitope mapping indicated that the 18.5 peptide, like the 19 kDa fragment, has an intact C-terminus (data not shown), the two fragments likely differ in their N-terminal PK cleavage site (i.e. the 18.5 fragment is further cleaved beyond residues 97–99).

### Molecular-phenotypic correlations: the pathological phenotype

The pathological phenotype in MM/MV 1+2C and MM/MV 2+1C subjects displayed varying relative amounts of features as previously described in the pure MM/MV 1 and MM/MV 2C subtypes [30, 33]. The lesion profile in MM/MV 1+2C largely overlapped with that of pure MM/MV 1 cases, with the exception of the cerebellum, which was less affected relatively to the cerebral cortex (Fig. 5, and data not shown). Histopathologic hallmarks in these cases were (a) “morula-like”, sometimes referred to as “grape-like”, confluent foci of spongiform degeneration with large vacuoles in addition to the classic microvacuolation, and (b) a mixed synaptic/perivacuolar or coarse pattern of PrP deposition (Fig. 6a, b). With the exception of one case, in which, only the type 1 protein could be demonstrated by immunoblotting even after the 1E4 staining, in this group we found a 100% correlation between the large vacuoles and the perivacuolar staining on one hand and the PrP<sup>Sc</sup> type 2 detection by immunoblotting on the other. The extension of the perivacuolar or coarse PrP staining, co-localizing with the confluent vacuoles varied significantly from case to case and was overall more common in the cerebral cortex than in subcortical areas (Fig. 7). The temporal, parietal and occipital cortices were the most

**Fig. 4 a** Western blot profiles of PrP<sup>Sc</sup> (3F4 antibody) from different brain regions of the sCJD VV 2+1 subject with the more significant type 1 deposition. Areas shown include the middle frontal gyrus (MFG), middle temporal gyrus (MTG), parietal cortex (PC), lateral occipital cortex (OCC2), insular cortex (INS), putamen nucleus (PUT), medial thalamic nuclei (TH1), and cerebellum (CE). Approximate molecular masses are in kilodaltons. **b** Western blot profiles of PrP<sup>Sc</sup> doublets in sCJD VV2, sCJD MM 1+2 and sCJD MV 2K. Lanes 1 and 2 show the association of a ~18.5 kDa fragment with the typical 19 kDa type 2 band in sCJD VV2 cerebellum (CE, V = vermis, H = hemisphere). Approximate molecular masses are in kilodaltons



**Fig. 5** Relative severity of lesions (i.e. spongiform degeneration and astrogliosis) in the cerebral cortex (mean of scores in neocortical and limbic cortical areas) and cerebellum: comparison between MM/MV 1 ( $n = 65$ ), MM/MV 1+2C ( $n = 63$ ), MM/MV 2C+1 ( $n = 9$ ), and MM/MV 2C ( $n = 4$ ) sCJD groups. The *line slope* expresses the ratio between the degree of cortical and cerebellar pathology. Group MM/MV 1+2C\* includes only the cases within the MM/MV 1+2C group showing type 2 in at least 5 areas ( $n = 17$ )

affected cortical areas (Fig. 7). Among subcortical structures, the thalamus most often showed the perivacuolar staining, whereas the brainstem and to a greater extent the cerebellum (Fig. 7) were the areas with the lowest frequency.

The pathological phenotype of MM/MV 2C+1 largely overlapped to that of MM/MV 2C subjects. However, as a

distinctive feature from the pure MM/MV 2C phenotype, all these cases had a synaptic pattern of PrP staining in the cerebellum, which well correlated with the presence of PrP<sup>Sc</sup> type 1 on western blots.

The lesion profile and the pattern of PrP staining in all 6 VV subjects with concurrent PrP<sup>Sc</sup> types (VV2+1) were very similar to those of typical VV2 sCJD cases. As the only distinctive feature, the two cases with the most significant type 1 accumulation showed a less consistent laminar pattern of spongiform degeneration in the cerebral cortex and a milder cerebellar pathology when compared to VV2 cases with similar disease duration.

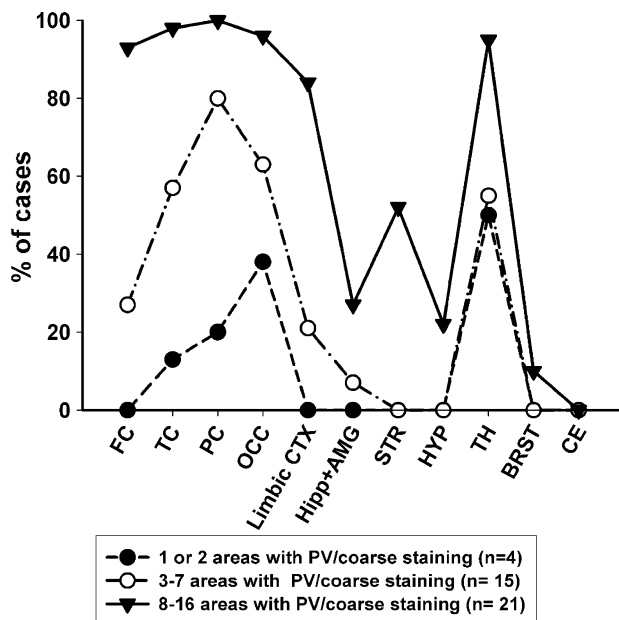
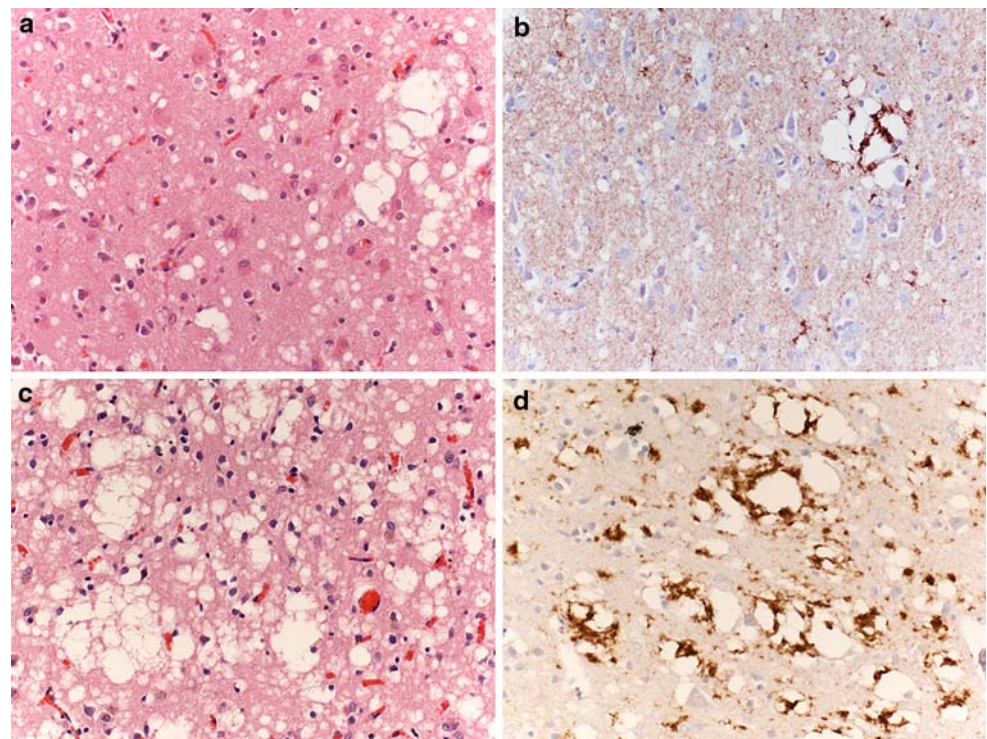
Lastly, the two MV 2K+1 cases had no clear distinguishing pathological features although they both showed a relatively mild pathology and the lack of a laminar pattern of spongiform degeneration in the cerebral cortex. Of notice, however, we observed a perivacuolar pattern of PrP<sup>Sc</sup> deposition co-localizing with the presence of larger vacuoles in 6 out of 25 of the sCJD MV 2K subjects (Fig. 6c, d) as well as in one MM2-thalamic (MM 2T) case. The regional distribution of the perivacuolar staining was not distinguishable from that observed in MM/MV 1+2C and mainly involved samples from the cerebral cortex (data not shown).

#### Molecular-phenotypic correlations: clinical features

MM/MV 1+2C and MM/MV 2C+1 subjects showed a significant difference in both disease duration and frequency of symptoms at onset according to the relative load



**Fig. 6** **a** A “grape-like” focus of spongiform degeneration with large confluent vacuoles surrounded by typical spongiform degeneration with fine, relatively small vacuoles in sCJD MM 1+2C (hematoxylin and eosin  $\times 200$ , frontal cortex); **b** focal perivacuolar staining surrounded by a synaptic pattern of PrP immunoreactivity ( $\times 200$ ) in the cerebral cortex of a sCJD MM 1+2C; **c** spongiform degeneration with large vacuoles (hematoxylin and eosin  $\times 200$ ) and **d** perivacuolar PrP deposition ( $\times 200$ ) in the cerebral cortex of a sCJD MV 2K



**Fig. 7** Regional distribution of the coarse/perivacuolar pattern of PrP<sup>Sc</sup> deposition in 40 MM/MV 1+2C cases divided in 3 groups according to the number of regions showing the perivacuolar pattern in individual cases. For each group, the % of cases revealing the perivacuolar pattern in each brain region is shown. *FC* frontal cortex, *TC* temporal cortex, *PC* parietal cortex, *OCC* occipital cortex, *Limbic CTX* limbic cortices, *Hipp + AMG* hippocampus+amygdala, *STR* striatum, *HYP* hypothalamus, *TH* thalamus, *BRST* brainstem, *CE* cerebellum

of the two protein types (Tables 4, 5). Clinical signs in MM/MV 1+2C with a limited PrP<sup>Sc</sup> type 2 deposition (up to 5 brain regions involved) did not significantly differ from those of MM/MV 1, but the disease duration became significantly longer and cerebellar signs less frequent at onset with increasing type 2 load. Thus, MM/MV 2C+1 subjects showed both a disease duration and relative frequency of symptoms at onset consistent with those of sCJD MM/MV 2C. Age at onset and disease duration in VV 2+1 subjects were consistent with those of sCJD VV2, but the relative frequency of cognitive decline at onset was higher than in VV2 cases, although the difference was not statistically significant, probably because of the low number of VV 2+1 cases. Last, clinical features in the 2 MV 2K+1 subjects were within the spectrum of the MV 2K group.

## Discussion

Previous studies have addressed the issue of PrP<sup>Sc</sup> types 1 and 2 co-occurrence in sCJD. Most of them raised the question of the influence of the number of cases and brain areas analyzed and emphasized the possibility that the co-occurrence of PrP<sup>Sc</sup> types 1 and 2 is underestimated [13, 18, 20, 30, 37, 40, 43]. On the other hand, the use of a novel, potentially very sensitive approach, later shown to have pitfalls related to the detection of unspecific bands

**Table 4** Demographic characteristics and classification of patients

sCJD groups	MM/MV 1	MM/MV 1+2C	MM/MV 2C+1	MM/MV 2C	MV 2K <sup>a</sup>	VV 1	VV 2+1	VV 2
No. cases	66	63	9	4	26	5	6	39
Age at onset (years)	70.1 (48–86)	68.6 (42–89)	65.3 (53–72)	67.8 (61–75)	65.4 (48–81)	39.3 (24–49)	69.3 (59–85)	64.5 (45–83)
Duration* (months)	<b>4.0 (1–24)</b>	<b>4.0 (1–26)</b>	<b>18.1 (6–33)</b>	20.0 (12–36)	15.8 (5–48)	15.3 (14–16)	6.5 (3.5–13)	6.3 (3–18)

<sup>a</sup> Includes 2 cases with MV 2K+1

\*  $p < 0.001$  between MM/MV 1+2C and MM/MV 2C+1 or between MM/MV 1 and MM/MV 2C+1 (Wilcoxon rank sum test)

**Table 5** Symptoms at disease onset (in %) in the sCJD subgroups

sCJD Groups	MM/MV 1	MM/MV 1+2C	MM/MV 2C+1	MM/MV 2C	MV 2K <sup>a</sup>	VV 1	VV 2+1	VV 2	
No. of cases	66	63	17 <sup>b</sup>	9	4	26	5	6	39
Cognitive	74	71	76	89	100	54	100	66	25
Gait or limb ataxia <sup>§</sup>	<b>51</b>	<b>28</b>	<b>12</b>	<b>0</b>	0	85	0	100	100
Visual central	32	27	35	22	0	4	0	0	0
Aphasia	15	19	18	33	25	4	20	0	0
Myoclonus	8	0	0	0	0	0	0	0	0
Psychiatric	12	11	17	0	0	0	0	0	2
Pyramidal	6	0	0	0	0	0	0	0	0
Sensory	2	3	0	0	0	12	0	0	5
Other dyskinesias	3	2	6	0	0	8	0	0	0
Oculomotor	2	3	0	0	0	0	0	0	18

<sup>a</sup> Includes 2 MV 2K+1 cases

<sup>b</sup> Includes only the cases in which PrP<sup>Sc</sup> type 2 was detected in at least 5 areas

<sup>§</sup>  $p < 0.005$  between MM/MV1 and MM/MV 1+2C with more than 5 areas with type 2 and  $p < 0.002$  between MM/MV 1 and MM/MV 2C+1 (Fisher exact test)

generated by partially digested PrP<sup>Sc</sup> fragments [25], likely led other investigators to overestimate the incidence of the concurrent PrP<sup>Sc</sup> types [35, 45]. Thus, the overall results on the phenomenon of the coexistence of molecular and clinico-pathological sCJD subtypes are at present inconclusive with respect to incidence, effect on disease phenotype and criteria for disease classification. To contribute to the full understanding of these issues, in the present study, we combined a systematic analysis of several brain regions in a large series of case including all codon 129 genotypes and the rarest phenotypes with the use of a refined methodology for the detection of the PrP<sup>Sc</sup> type concurrence, which provides good sensitivity combined with high specificity [25].

After screening about 4,200 samples from a largely consecutive series of 200 cases, we estimated that PrP<sup>Sc</sup> types 1 and 2 coexist in about 35% of sCJD cases, which is overall consistent with figures from some of the previous studies [13, 37, 43] in which the number of cases and areas analyzed were significantly lower. This finding supports the idea that PrP<sup>Sc</sup> types co-occurrence involves a relevant but limited group of sCJD subjects and indicates that the incidence of the phenomenon had not been significantly underestimated.

As far as the characteristics of the CJD population with mixed phenotypes are concerned, our data show that the PrP<sup>Sc</sup> types 1 and 2 co-occur more frequently in the MM than in the MV and VV genotypes. More specifically, the large majority of sCJD cases with concurrent PrP<sup>Sc</sup> types combines features of the MM and MM 2C sCJD subtypes, in variable proportions. Most commonly, in such cases, the MM1 phenotype is predominant over the MM 2C phenotype, but the opposite situation also rarely occurs. The latter results significantly differ from those obtained in most previous studies. Indeed, Head et al. [13] mainly found a focal type 1 co-occurrence in MM and MV subjects with dominant type 2, Schoch et al. [40] detected the mixed protein types mostly in MV2 cases showing the type 1 only focally in subcortical areas, and Uro-Coste et al. [43] mainly detected a random co-occurrence of type 1 in MV or VV cases with dominant type 2. Given that only our study was based on a large series of consecutive cases, we attribute such heterogeneity of previous results to case selection biases, although methodological differences may also have contributed [43].

Since subjects with mixed PrP<sup>Sc</sup> types represent a significant proportion of the sCJD population, show distinctive phenotypic features, and potentially represent a

**Table 6** Nomenclature and classification of sCJD subtypes

Nomenclature <sup>a</sup>	% <sup>b</sup>	Distinctive histopathologic features
Pure subtypes		
MM/MV 1; VV2; MV 2K; MM/MV 2C; MM 2T; VV1;	65	Previously established [30] (for a summary see Table 8 in [30])
Mixed subtypes		
MM/MV 1+2C	26	As in MM/MV 1 but with clusters of large vacuoles associated to perivacuolar and coarse PrP deposition mainly in cerebral cortex or thalamus
MM/MV 2C+1	2	As in MM/MV 2C but with synaptic-type PrP staining in the molecular layer of the cerebellum
VV 2+1	3	Virtually indistinguishable from VV2
MV 2K+1	1	Virtually indistinguishable from MV 2K
MV 2 K+C	3	As in MV 2K but with clusters of large vacuoles associated to perivacuolar and coarse PrP deposition mainly in cerebral cortex
MM 2 T+C	<1	As in MV 2T but with clusters of large vacuoles associated to perivacuolar and coarse PrP deposition mainly in cerebral cortex

<sup>a</sup> It is largely based on codon 129 *PRNP* genotype, which can be either methionine (M) or valine (V) and the PrP<sup>Sc</sup> type (1 or 2 according to Parchi et al. [28, 29]). Since both MM 2 and MV 2 groups are associated to 2 distinct phenotypes, these are further defined with a third parameter (*capital letter*) referring to distinctive histopathological features: *K* kuru type amyloid plaques, *C* predominant cortical pathology with confluent vacuoles and perivacuolar PrP staining, *T* prominent thalamic pathology with atrophy

<sup>b</sup> Percentage of total consecutive sCJD cases ( $n = 200$ ) investigated

distinct subtype in terms of biological relevance, it is important that they are properly identified and are added as new subtypes in the current sCJD classification (Table 6).

Despite the emphasis on molecular features of current sCJD classification, it has become increasingly clear that PrP<sup>Sc</sup> typing alone, when limited to a single or even a few brain samples, fails to provide an accurate classification in a significant proportion of cases. This is mainly related to the focal nature of the “mixed features” in many sCJD cases with PrP<sup>Sc</sup> types 1 and 2 concurrence. Indeed, we would have misclassified the disease subtype in about 27.5% (using 3F4) of cases with MM or MV genotype, if we had analyzed PrP<sup>Sc</sup> only in the frontal cortex, the area more commonly used for typing worldwide. For the same reason, discrepancies may arise when PrP<sup>Sc</sup>-typing and PrP immunohistochemistry are performed from individual samples taken from opposite hemispheres or even adjacent cortical gyri. However, our study shows that the regional deposition of either type 1 or type 2 when concurrent is not random and that a relatively limited number of critical brain structures must be assessed to reach an accurate classification. Furthermore, our results further underline the importance of applying both molecular and neuropathological assessment for sCJD subtype classification. In this regard, the lack of detection of PrP<sup>Sc</sup> type 2 in a minority of MM subjects, despite the presence of a mixed synaptic and perivacuolar pattern of PrP deposition, indicates that when

type 2 is very focal or limited in amount, histopathologic examination is more sensitive in identifying such cases than PrP<sup>Sc</sup> typing, at least when only the 3F4 antibody is used. Given the very strong correlation in MM subjects between PrP<sup>Sc</sup> type 2 detection and the large “grape-like” vacuoles and the perivacuolar pattern of PrP deposition on histopathologic examination, which is in line with results previously obtained in other studies [18, 37], we propose that these cases are classified as MM 1+2C or MV 1+2C even without the final proof of type 2 detection by western blot. Alternatively, PrP<sup>Sc</sup> typing using the antibody 1E4 was in our hand as sensitive as the histopathologic examination in the detection of cases with very focal type 2.

In the light of the present results, the most important regions to be assessed pathologically include the cerebral cortex from each of the 4 lobes, the striatum, hippocampus, thalamus and cerebellum. The cerebellum, in particular, is critical for the recognition of the synaptic pattern of PrP deposition as marker of PrP<sup>Sc</sup> type 1 concurrence in the cases with dominant type 2.

Taken together, our data indicate that a protocol including the neuropathologic assessment of the eight brain regions mentioned above and PrP<sup>Sc</sup> typing in four critical regions such as the temporal, parietal and occipital neocortices, and medial thalamus is strongly recommended for a reliable sCJD group classification addressing the issue of mixed phenotypes. Indeed, by applying this protocol



instead of examining all 21 brain regions, we would have reached the same classification of cases in the present series.

We also wish to underline the importance of identifying correctly the sCJD cases with mixed features for transmission purposes. Indeed, the question of whether the concurrence of PrP<sup>Sc</sup> types 1 and 2 in CJD reflects a co-infection by two prion strains related to specific undiscovered human genotypes, or determined by epigenetic factors remains unanswered and will largely rely on transmission studies in which the careful selection of samples will be of critical importance. Concerning this critical question, we find intriguing that the large, confluent vacuoles and the perivacuolar pattern of PrP<sup>Sc</sup> deposition, we originally linked to sCJD MM 2C are also found in a subgroup of MV 2K subjects in addition to MM/MV 1+2C. In addition, we have described here the same morphological features in one case of fatal insomnia (i.e. the MM2-thalamic subtype or MM 2T) which adds to two previously reported cases [19, 30, 31]. Thus, it seems that large confluent vacuoles and the perivacuolar pattern of PrP<sup>Sc</sup> deposition may be found in sCJD associated with all phenotypes linked to MM or MV at codon 129. Although this observation remains difficult to interpret at present, it appears relevant for our future understanding of the molecular basis and the extent of strain variation in sCJD. In any case, our observation strongly suggests that the phenomenon of mixed phenotypes in sCJD goes beyond PrP<sup>Sc</sup> types 1 and 2 coexistence and also involves subtypes which shares the same PrP<sup>Sc</sup> type. This, in turn, further underlines the importance of combining histopathological assessment and biochemical PrP<sup>Sc</sup> typing for sCJD subtype characterization.

The present data also show that the association of two PrP27-30 fragments, which does not represent a bona-fide type 1 and 2 concurrence, may also be a feature of some sCJD cases. Thus, the PrP27-30 profile in VV2 cases in the cerebellum, thalamus and midbrain is sometime characterized by a doublet comprising a 18.5 kDa in addition to the typical 19 kDa band, while the western blot profile of PrP27-30 in the MV 2K cases appears almost invariably characterized by the association of two PrP<sup>Sc</sup> core fragments including a classic 19 kDa type 2 band and a slower migrating band of about 20 kDa. Although these profiles truly represent concurrent PrP<sup>Sc</sup> fragments, and the 20 and 18.5 kDa fragments likely reflect specific PK cleavage sites, the 20 and 18.5 kDa bands are distinguished from the type 1 and type 2 fragments because, at least to date, they were never detected independently from types 1 and 2, and are not markers of specific clinico-pathological phenotypes. Knowledge of these regional variations is nonetheless important to avoid misinterpreting a PrP<sup>Sc</sup> profile as novel when only one brain region is analyzed [21].

Finally, the results obtained from the analyses of lesion profiles and clinical features in the subgroups of sCJD cases with mixed features deserve further comment. By showing that the relative “load” of each of the two PrP<sup>Sc</sup> types significantly correlates with disease duration, the relative frequency of certain symptoms, and the ratio between cortical and cerebellar pathology, our study provides further strong evidence for the PrP<sup>Sc</sup> type being a major biological determinant in human prion disease.

In conclusion, the present data add to our knowledge of the prevalence and phenotypic spectrum of the sCJD variants with mixed molecular and pathological features, provide an updated molecular classification of the disease subtypes and will serve for future epidemiologic and transmission studies aimed at disclosing the etiology and extent of strain variation in sCJD.

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**Conflict of interest statement** The authors declare that they have no conflict of interest.

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