Novel strain properties distinguishing sporadic prion diseases sharing prion protein genotype and prion type

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In most human sporadic prion diseases the phenotype is consistently associated with specific pairings of the genotype at codon 129 of the prion protein gene and conformational properties of the scrapie PrP (PrPSc) grossly identified types 1 and 2. This association suggests that the 129 genotype favours the selection of a distinct strain that in turn determines the phenotype. However, this mechanism cannot play a role in the phenotype determination of sporadic fatal insomnia (sFI) and a subtype of sporadic Creutzfeldt-Jakob disease (sCJD) identified as sCJDMM2, which share 129 MM genotype and PrPSc type 2 but are associated with quite distinct phenotypes. Our detailed comparative study of the PrPSc conformers has revealed major differences between the two diseases, which preferentially involve the PrPSc component that is sensitive to digestion with proteases (senPrPSc) and to a lesser extent the resistant component (resPrPSc). We conclude that these variations are consistent with two distinct strains in sFI and sCJDMM2, and that the rarer sFI is the result of a variant strain selection pathway that might be favoured by a different brain site of initial PrPSc formation in the two diseases.

It is now well established that the basic mechanism of prion diseases relies on the conversion of the normal or cellular prion protein (PrPC) to the abnormal and infectious conformer commonly identified scrapie PrP (PrPSc)1. A challenging aspect of this conversion mechanism is that it leads to the formation of heterogeneous PrPSc species commonly identified as strains2. Historically, strains have been defined as prion species that upon transmission to receptive hosts are associated with different disease phenotypes as determined by distinct incubation periods, histological lesions and PrP deposits with respect to type and topography3. Subsequently, strains were correlated to PrPSc species exhibiting distinct physicochemical properties, such as electrophoretic mobility, which were attributable to different conformations linked to any primary to quaternary structure variation4–8.

Human prion diseases are characterized by a marked phenotypic heterogeneity that has hampered their recognition and understanding9. Although this heterogeneity is due in part to the unique presence of three etiologies – sporadic, inherited and acquired by infection – the sporadic group alone includes at least seven phenotypes2,10. We proposed a classification of sporadic human prion diseases based on the pairing of the patient’s genotype at codon 129 of the PrP gene (the site of a common methionine (M)/valine (V) polymorphism) with the type, 1 or 2, of PrPSc (as determined by electrophoretic mobility of the protease resistant PrPSc core or resPrPSc)11,12. Individual 129 genotype - PrPSc type pairs consistently correlate and identify most sporadic prion disease phenotypes4,10,12. For example, sporadic Creutzfeldt-Jakob disease (sCJD) affecting 129MM patients and associated with PrPSc type 1 (identified as sCJDMM1) exhibits a clinical and pathological phenotype that significantly differs from

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the phenotypes associated with sCJDVV1 or sCJDMM2. However, this principle does not apply to all sporadic prion diseases.

Sporadic fatal insomnia (sFI, also identified as sCJDMM2T, for thalamic form), and sCJDMM2 (also identified as sCJDMM2C, for cortical form) are both associated with the 129MM genotype and PrPSc conformers of type 2, which display a similar resPrPSc profile in both diseases upon standard electrophoretic analysis. However, in spite of sharing molecular features, sFI and sCJDMM2 are associated with strikingly different clinical and histopathological phenotypes as well as prevalences (Supplementary Table S1). In sFI the major lesions are concentrated to the thalamus and consist of severe loss of neurons; in contrast, sCJDMM2 is characterized by severe spongiform degeneration made of distinctive large vacuoles that preferentially affect the cerebral cortex10,12. Bioassay studies indicate that the two diseases are associated with distinct prion strains13. Detailed studies of resPrPSc have shown consistent differences which, however, involve very minor fragments, raising questions as of whether these minor variations can bring about such drastic phenotypic differences14,15. Furthermore, systematic comparative studies of the physicochemical characteristics of the PrPSc associated with the two diseases are missing.

We searched for variations of PrPSc properties that could explain the striking phenotypic heterogeneity of sFI and sCJDMM2. We found major variations in the protease-sensitive component of PrPSc, while novel although less striking differences also emerged in resPrPSc.

Results
 Glycoform ratios and electrophoretic patterns of totPrPSc and resPrPSc. Comparative examination on Western blots (WB) of resPrPSc obtained from frontal cortex and enriched by sodium phosphotungstate (NaPTA) precipitation confirmed the lack of qualitative differences between sFI and sCJDMM2 electrophoretic profiles with regard to mobility and ratios of the three resPrPSc glycoforms (Fig. 1A,D).

However, differences were demonstrated when the proteinase K (PK) treatment of enriched samples was omitted, allowing for the examination of total PrPSc (totPrPSc) i.e. resPrPSc + PK-sensitive PrPSc (senPrPSc) = totPrPSc.
Total PrP^Sc revealed a significant variation in the ratios of the di-glycosylated, mono-glycosylated and un-glycosylated conformers that respectively showed the 56:28:16 ratios in sFI and 21:40:39 in sCJDMM2 (Fig. 1B,E). The possibility that the different glycoform ratios of totPrP^Sc reflected significant contamination of the NaPTA precipitate with PrPC was considered to be very unlikely in view of the different glycoform ratios exhibited by the PrP (presumably PrP^C) recovered in the NaPTA supernatant (Fig. 1C,F). Similar results were obtained from the medial thalamus, the cerebral region most affected in sFI (Supplementary Fig. S1).

Further characterization of totPrP^Sc and resPrP^Sc on two-dimensional (2-D) WB demonstrated marked differences in totPrP^Sc harvested from sFI and sCJDMM2 with regard to the number and gel mobility of the electrophoretic spots (Fig. 2A). With regard to totPrP^Sc, the 2-D polyacrylamide gel electrophoresis (PAGE) pattern was relatively simple in sFI, but quite complex in sCJDMM2, as it included PrP^Sc isoforms with wide ranges of relative molecular weights and isoelectric points. This disparity was especially prominent in the 6.0–7.0 isoelectric points (pI) range. In this region of the sCJDMM2 preparations, the spots corresponding to the three glycoforms were blurred by a smear comprised in the 40–20 kiloDalton (kDa) range. Furthermore, a prominent cluster of spots could be seen in the 19 kDa region while a weak but large, blurred spot occupied the ~50 kDa

Figure 2. Two-dimensional WB of PrP^Sc isoforms from sFI and sCJDMM2. TotPrP^Sc (A), resPrP^Sc (B), PNGase F-deglycosylated totPrP^Sc (C) and deglycosylated resPrP^Sc (D) were processed for 2-D WB following NaPTA precipitation with NaPTA in sFI and sCJDMM2 as indicated. (A) TotPrP^Sc shows a much more complex spot pattern in sCJDMM2 than in sFI, the pattern of which resembles that of PrP^C17. The patterns become much simpler and similar in the two diseases following treatment with PK (B), PNGase F (C) or both treatments combined (D). (Labels on the side of each WB indicate the three PrP glycoforms: D: Di-glycosylated; M: Mono-glycosylated; U: Un-glycosylated; PK 10 U/ml).
PrPSc nature of the ~53 kDa h.m.w. were confirmed following immunoprecipitation (IP) or in NaPTA preparations at pH 6.5, while the ~53 kDa component alone was barely noticeable in sFI (Fig. 3B). Presence, relative quantity and variations were very prominent in sCJDMM2 preparations, where they occupied a large area of the blot at approximately the presence of two large h.m.w. bands of ~53 kDa and ~90 kDa in sCJDMM2 while a single, less well represented high molecular weight (h.m.w.) area. Besides seemingly confirming the ratios of the three totPrPSc glycoforms observed in one-dimensional (1-D) WB, differences in pI were uncovered in the two diseases, due to the spanning over a wider range of pI for glycoforms of sCJDMM2 totPrPSc. Following PK treatment, the sCJDMM2 electrophoretic pattern became much simpler, matching that of sFI (Fig. 2B). In both diseases, resPrPSc spots conspicuously shifted toward the acidic region compared to those of totPrPSc. Patterns were even further simplified and variations between sCJDMM2 and sFI further reduced after deglycosylation of totPrPSc and resPrPSc with PNGase F, although minor differences persisted (Fig. 2C,D). Altogether, these 2-D WB findings confirm that the major heterogeneity between sFI and sCJDMM2 PrPSc, especially with respect to the ratios and pI of the glycoforms, is related to the totPrPSc species, pointing to senPrPSc as the component accountable for the diversity.

Figure 3. One- and 2-D WB of full-length totPrPSc immunoprecipitated from sCJDMM2 and sFI.

Immunoprecipitation was carried out on NaPTA pellets with the mAb 8B4 to PrP N-terminus. (A) The 1-D WB shows h.m.w. bands (arrows) especially prominent in sCJDMM2 preparations, in addition to the three typical PrPSc glycoforms; (B) In 2-D WB, spots corresponding to the h.m.w. are dramatically represented in sCJDMM2 and barely detectable in sFI (arrows). Note that in these full-length totPrPSc preparations, low molecular weight fragments are lacking but the glycoform ratios reproduce those of the whole totPrPSc (Fig. 2A).

Characterization of full-length totPrPSc. To further investigate the complexity of the totPrPSc in sCJDMM2, we immunoprecipitated the full-length component of totPrPSc from NaPTA preparations using a monoclonal antibody (mAb) to the PrP N-terminal region (Fig. 3). The 1-D WB of the immunoprecipitate showed that the three full-length PrPSc glycoforms associated with sFI and sCJDMM2 reproduced the ratios previously observed on preparations of whole totPrPSc (Figs 3A and 1B,E). In addition, the eluate demonstrated the presence of two large h.m.w. bands of ~53 kDa and ~90 kDa in sCJDMM2 while a single, less well represented ~53 kDa band was detectable in sFI (Fig. 3A). On 2-D WB, the two ~53 kDa and ~90 kDa h.m.w. components were very prominent in sCJDMM2 preparations, where they occupied a large area of the blot at approximately pI 6.5, while the ~53 kDa component alone was barely noticeable in sFI (Fig. 3B). Presence, relative quantity and PrPSc nature of the ~53 kDa h.m.w. were confirmed following immunoprecipitation (IP) or in NaPTA preparations (i.e. omitting IP), using different elution conditions to exclude co-elution of the capturing mAb, and by probing with polyclonal antibodies to the C- and N-termini as well as with the conformational mAb OCD4 (Supplementary Figs S2 and S4B). The ~53 kDa and ~90 kDa h.m.w. components were found to be glycosylated and partially PK-sensitive; the ~53 kDa form was estimated to be 3–4 times better represented in sCJDMM2 than in sFI (Supplementary Fig. S2F–I).

When the 2-D WB patterns of whole and full-length totPrPSc from sCJDMM2 were compared, the pattern of the full-length totPrPSc isoform (presence of the ~53 kDa and ~90 kDa spots notwithstanding) appeared much simpler than that of the whole totPrPSc (which also comprised the truncated forms), especially in the pI region where the two h.m.w. components were located (Figs 2A and 3B). In contrast to those of sCJDMM2, totPrPSc 2-D patterns obtained from sFI were comparable, regardless of whether they exclusively represented the full-length totPrPSc isoform or the whole totPrPSc comprising full-length and truncated forms (Figs 2A and 3B). In conclusion, the 2-D study of full-length totPrPSc (i) confirms the presence in sCJDMM2 totPrPSc of significant amounts of ~53 kDa and ~90 kDa h.m.w. species, which appear to contain full-length totPrPSc; (ii) indicates that the PrPSc fragments that complicate the WB 2-D pattern of totPrPSc in sCJDMM2 originate from the h.m.w. components (Figs 2A and 3B); and (iii) confirms the variations of glycoform ratios and pI in sCJDMM2 and sFI, which are especially noticeable in the full-length un-glycosylated component (Fig. 3B).

Sedimentation properties of prion aggregates. PrPSc variations distinguishing sCJDMM2 and sFI were further searched with sedimentation equilibrium (SE) and sedimentation velocity (SV) centrifugations, which are commonly used to explore density and size of the PrPSc aggregates, respectively. Following SE, totPrPSc sedimentation profiles clearly differed between the two diseases: in sCJDMM2, over 70% of totPrPSc aggregates populated the sucrose high-density region of the gradient (bottom fractions), while in sFI, over 80% of the totPrPSc was recovered in a distinct peak located in a lower density region (fractions 3–8) (Fig. 4A). Remarkably, unlike those of sCJDMM2, sFI glycoform ratios varied significantly along the gradient (Supplementary Fig. S3). This variation resulted in a significant difference between the glycoform ratios recovered in the low- and high-density fractions in sFI, and in a difference in ratios between the two diseases in the low- but not in the high-density fractions (Supplementary Fig. S3B).
Following PK digestion of individual SE fractions, the signal of fractions 3–8 pertaining to sFI was no longer detectable, while the only sizable quantity of resPrPSc aggregates populated the high-density fractions in both diseases (Fig. 4B). Thus, PK treatment removed all significant differences between the two gradient profiles although minor differences remained in the glycoform ratios (Fig. 4B and Supplementary Fig. S5). In contrast, when PK digestion was carried out on the whole totPrPSc preparations before SE (rather than on the individual SE fractions), two distinct although overlapping aggregate populations were observed that involved fractions of lower density (fractions 8–18) in sFI than in sCJDMM2 (12–21) (Supplementary Fig. S6).

Experiments on control cases indicated that totPrPSc recovered in P2 from sFI and sCJDMM2 and used for SE might have been contaminated up to 16% by PrPC, insoluble PrP (iPrP)17, or both (Cracco et al., unpublished data). Therefore, SE was repeated using totPrPSc P2 preparations generated under stringent conditions (indicated as stSE, see Supplementary Materials and Methods) and harvested from sCJDMM2 and sFI as well as from brains free of neurological diseases. Although following stSE the majority of sCJDMM2 and sFI totPrPSc aggregates populated the high-density region of the gradient, smaller but still significant differences in their respective distributions were also detected in the low-density area of the gradient: in sCJDMM2, only 4% of totPrPSc was recovered in fractions 5–10, whereas in sFI this component exceeded 14% (P < 0.05); a significant difference was also found in the high-density regions of the gradient (fractions 17–21, P < 0.05) (Fig. 5A). WB of stSE fractions 6–8 combined, which were harvested from brain equivalents of negative controls (presumably comprising PrPSc, iPrP or both) as well as from sFI and sCJDMM2, indicated that the contribution of PrPSc or iPrP (lane 1) was insignificant, excluding the possibility that the low-density peaks in sFI and sCJDMM2 (lanes 2, 3) were due to contamination (Fig. 5B). Furthermore, major SE characteristics of low- and high-density fractions (6–8 and 17–21), like PK sensitivity and variations of glycoform ratios observed along the gradient, were reproduced in stSE, indicating that totPrPSc exhibited similar qualitative features in SE and stSE gradients (Fig. 5C and Supplementary Fig. S3, and Cracco et al., unpublished data). It should also be noted that the stSE procedure caused a significant loss of totPrPSc to the S2 fractions, which impacted preferentially sFI and may explain the quantitative variations in aggregate distribution between the two SE methods (Cracco et al., unpublished data).

The possibility that in SE the low-density peak observed in sFI reflected contamination of totPrPSc with PrPC or iPrP (expected to populate similar gradient fractions and to be PK-sensitive) seemed also unlikely in view of the results of the IP experiments with mAb OCD4, the conformational antibody reacting with misfolded PrP species but not with PrPC16. Immunoprecipitation of PrP from the 4–8 and 17–21 SE fractions obtained from sFI and sCJDMM2 yielded significant quantities of PrP displaying glycoform ratios similar to those of the totPrPSc directly collected from the same SE and stSE fractions (Supplementary Figs S3 and S4). In contrast, OCD4 captured relatively negligible amounts of PrP, possibly iPrP, in S1 and S2 fractions generated from a negative case (Supplementary Fig. S4)17. Of note, h.m.w. components populated both low- and high-density fractions in sCJDMM2 but only the high-density fractions in sFI (Supplementary Fig. S4B).

Following SV centrifugation (1 hr, in 5–15% sucrose gradient), the sedimentation profiles were again different in the two diseases (Fig. 6). In sFI, virtually all totPrPSc aggregates were confined to the low-density fractions (fractions 1–5), while in sCJDMM2 only approximately 10% of totPrPSc distributed in that region, while nearly 70% was recovered in the highest density fractions of the gradient (Fig. 6A). Treatment with PK equalized the profiles since aggregates were exclusively recovered in the highest density fraction in both diseases, further adding to the notion that most PrPSc disparity resides in the senPrPSc fraction (Fig. 6B).

Solubility properties of totPrPSc and resPrPSc. Finally, both totPrPSc and resPrPSc associated with sCJDMM2 and sFI were further characterized by the conformational solubility and stability assay (CSSA) that assesses conformational stability based on the rate of PrPSc solubilisation at increasing concentrations of guanidine hydrochloride (GdnHCl)18,19. In sCJDMM2, totPrPSc and resPrPSc showed comparable solubility values; in sFI, while the totPrPSc solubility roughly matched that of sCJDMM2, resPrPSc was significantly less soluble than totPrPSc as well as the resPrPSc of sCJDMM2 (Fig. 7). Therefore, conformational stability is another feature differentiating resPrPSc in sFI and sCJDMM2.

Figure 4. Sedimentation equilibrium of totPrPSc and resPrPSc. TotPrPSc from sCJDMM2 and sFI were centrifuged at high speed for 19 hours in a 10–60% sucrose gradient. Identical volumes were collected from each fraction and processed for WB. Sedimentation profiles of totPrPSc (A) and resPrPSc (B). Note the distinct peak of totPrPSc low-density aggregates in sFI and the prevalence of high-density aggregates in totPrPSc from sCJDMM2 (A). In contrast, only high-density aggregates are demonstrated in resPrPSc (B) (PK 10 U/ml).
Discussion

We have uncovered significant variations affecting totPrPSc and, to a lesser extent, resPrPSc conformers that distinguish sFI and sCJDMM2. The variations appear to affect primarily the senPrPSc component of totPrPSc, as they drastically diminish when the totPrPSc preparations are digested with PK to reveal resPrPSc. The variations of totPrPSc involve a variety of properties, including (i) ratio and isoelectric point of the glycoforms, (ii) presence of two ~53 kDa and ~90 kDa h.m.w. prominent components and a plethora of low m.w. PrPSc fragments as well as (iii) existence of an aggregate population displaying distinct density and, possibly, size (Supplementary Table S2). The variations affecting primarily resPrPSc comprise (i) distinct densities of aggregates, which are detected when PK treatment is carried out before (rather than after) SE fractionation, and (ii) conformational stability (Supplementary Table S2). Furthermore, lower PK resistance of totPrPSc in sFI compared to that of sCJDMM2 has been reported by Saverioni et al.20. The finding that totPrPSc diversity in the two diseases appears to be mostly determined by the senPrPSc component may explain the enduring lack of recognition of significant PrPSc heterogeneity in these two diseases.

The observation that in sFI (but not in sCJDMM2) the glycoform ratio of totPrPSc is dominated by the di-glycosylated form and differs from that of resPrPSc argues that, in sFI, senPrPSc and resPrPSc preferentially target different PrPSc glycoforms for conversion21–25. It has recently been proposed that the sialic acid moiety of
the PrP<sup>C</sup> glycans impairs conversion to PrP<sup>Sc</sup>. Therefore, the dominance of the di-glycosylated (thus highly sialylated) isoform in sFI totPrP<sup>Sc</sup>, presumably involving the senPrP<sup>Sc</sup> component, might reflect a lower sialylation (or other variations) of PrP<sup>C</sup> glycans available for conversion to senPrP<sup>Sc</sup> in sFI, allowing for the conversion of even the PrP<sup>C</sup> di-glycosylated isoform. Alternatively, PrP<sup>C</sup> conversion to senPrP<sup>Sc</sup> might be less affected by sugar sialylation in sFI, or senPrP<sup>Sc</sup> and resPrP<sup>Sc</sup> glycoforms might have different turnovers. Regardless of the mechanism, the difference in glycoform ratios is significant considering the rising evidence that glycan representation, structure or both can affect strain characteristics<sup>21,25,26</sup>.

The high representation of the ~53 kDa and ~90 kDa h.m.w., and of the low molecular weight (l.m.w.) components in totPrP<sup>Sc</sup> from sCJDMM2, strikingly demonstrated in 2-D WB of totPrP<sup>Sc</sup>, is puzzling. Our preliminary characterization indicates that the two components share several physicochemical features, suggesting that they comprise a variety of PrP<sup>C</sup> glycans available for conversion to senPrP<sup>Sc</sup> in sFI, allowing for the conversion of even the PrP<sup>C</sup> di-glycosylated isoform. Alternatively, PrP<sup>C</sup> conversion to senPrP<sup>Sc</sup> might be less affected by sugar sialylation in sFI, or senPrP<sup>Sc</sup> and resPrP<sup>Sc</sup> glycoforms might have different turnovers. Regardless of the mechanism, the difference in glycoform ratios is significant considering the rising evidence that glycan representation, structure or both can affect strain characteristics<sup>21,25,26</sup>.

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The high representation of the ~53 kDa and ~90 kDa h.m.w., and of the low molecular weight (l.m.w.) components in totPrP<sup>Sc</sup> from sCJDMM2, only, strikingly demonstrated in 2-D WB of totPrP<sup>Sc</sup>, is puzzling. Our preliminary characterization indicates that the two components share several physicochemical features, suggesting that they comprise a variety of PrP<sup>C</sup> glycans available for conversion to senPrP<sup>Sc</sup> in sFI, allowing for the conversion of even the PrP<sup>C</sup> di-glycosylated isoform. Alternatively, PrP<sup>C</sup> conversion to senPrP<sup>Sc</sup> might be less affected by sugar sialylation in sFI, or senPrP<sup>Sc</sup> and resPrP<sup>Sc</sup> glycoforms might have different turnovers. Regardless of the mechanism, the difference in glycoform ratios is significant considering the rising evidence that glycan representation, structure or both can affect strain characteristics<sup>21,25,26</sup>.
The disparity of senPrPSc aggregates that we observed between sFl and sCJDMM2, following fractionation by long and short sucrose density centrifugations, is also noteworthy since it relates to the current notion that aggregate profiles are good identifiers of distinct strains. The long (19 hrs.) SE centrifugations, thought to primarily assess density (mass/volume), showed that most totPrPSc co-distributes with low-density aggregates in sFl and with aggregates of high density in sCJDMM2. However, the detergent insoluble fractions used in this SE protocol included up to 16% contamination with non-PrPSc (PrPC and insoluble PrP). A more stringent SE protocol still demonstrated a significant difference between the distribution of sCJDMM2 and sFl low-density aggregates, although this protocol led to a loss of totPrPSc that predominantly affected senPrPSc in sFl. Furthermore, the dominant presence of senPrPSc in the sFl low-density fractions was further supported by the distinct glycoform ratios and by the immunoreactivity of the PrP recovered in these fractions using a conformational antibody that recognizes totPrPSc and resPrPSc but not PrPC. Although further study is needed, an appealing possibility is that the sFl low-density aggregates match the protease-sensitive small oligomers recently described and are associated with detergent-insoluble cholesterol-rich lipids originating from the PrPSc association with membrane rafts. Furthermore, association with lipids is thought to play a role in maintaining PrPSc conformation and controlling strain features.

Notably, the sFl (but not the sCJDMM2) glycoform ratios changed significantly along the gradient, regardless of the type of SE used, implying that in sFl the low- and high-density aggregates are composed of monomers that differ with respect to glycosylation. A further distinctive feature is that the h.m.w. components appeared to populate both low- and high-density fractions in sCJDMM2 but only the high-density fractions in sFl. Although caution has to be exercised because of the different representation of the h.m.w. components in the two diseases, this finding suggests that, in addition to representation, h.m.w. components differ also with respect to their participation in aggregate formation in sFl and sCJDMM2. Protease treatment of the individual fractions removed all differences between sFl and sCJDMM2, indicating that all low-density fractions 1–10 harboured senPrPSc while resPrPSc populated mostly the high-density fractions 11–21, in amounts directly related to the density of the fractions. However, when PK digestion of totPrPSc was carried out before SE fractionation (rather than on the individual fractions), two distinct, though overlapping, aggregate populations were observed in sFl and sCJDMM2, which accounted for over 80% of the entire resPrPSc aggregates in both conditions. The different resPrPSc aggregate distribution under this PK treatment condition likely denotes partial re-aggregation of resPrPSc that results in distinct aggregate populations in the two diseases. It also underlines the importance of the timing of PK treatment in SE.

Sedimentation velocity, which is supposed to separate aggregates by size, showed, like SE, that the great majority of totPrPSc aggregates populated the light fractions in sFl, while in sCJDMM2 the majority was recovered in the fractions of highest density. This sedimentation procedure generated an almost symmetrical bimodal profile due to the nearly complete separation of light fractions (1–5 in decreasing representation) from the 20–21 heavy fractions, suggesting that in our SV conditions aggregates appear similar in size in both diseases but are differently represented, with the smaller aggregates predominating in sFl. Following protease treatment of the individual fractions, aggregates were almost exclusively recovered in the densest fractions. However, in view of its minimalistic bimodal profile, our SV procedure might have failed to fully separate aggregates in sFl and sCJDMM2, making the interpretation of the sedimentation profile challenging. Nonetheless, SV experiments further confirm the dissimilarity of totPrPSc aggregates in sFl and sCJDMM2.

Sedimentation velocity studies of PrPSc have recently been carried out in sCJD subtypes by Saverioni et al. The SV profiles of sCJDMM2 and sFl in this study profoundly differ from ours especially due to the underrepresentation of the low-density component in sFl. However, Saverioni et al. carried out SV fractionation on PrPSc preparations (P3) which were purified under stringent conditions by performing two rounds of over 2-hour high speed centrifugations, and sonication. Nonetheless, a significant difference in aggregate distribution was observed between sFl and sCJDMM2.

The major finding obtained with the conformational solubility and stability assay was that while the stabilities of totPrPSc were similar in both diseases, the resPrPSc stability was significantly higher in sFl not only when compared with that of the corresponding totPrPSc but also with respect to the stability of resPrPSc in sCJDMM2. A possible mechanism of the enhanced stability of sFl PrPSc following PK treatment is re-aggregation of resPrPSc promoted by PK treatment, which is different in the two diseases and results in increased stability of resPrPSc in sFl but not in sCJDMM2. This mechanism is consistent with the drastically different SE aggregate profiles engendered when PK digestion is performed on the totPrPSc before SE (a condition similar to that of the CSSA procedure) as opposed to the PK digestion of the SE-generated individual fractions. Alternatively, assuming that the stability of totPrPSc represents the average of the stabilities of both senPrPSc and resPrPSc components, the high stability of the resPrPSc even without PK treatment might compensate for the very low stability of senPrPSc. Should this be the case, stabilities of both senPrPSc and resPrPSc associated with sFl would differ from those of sCJDMM2. Regardless of these interpretations, the CSSA test uncovers an additional unexpected disparity of resPrPSc in sCJDMM2 and sFl. Stability data comparable to ours have been reported for sCJDMM2 PrPSc in previous studies, along with the finding that, in sCJDMM2, senPrPSc is more stable than resPrPSc.

A bioassay study by Moda et al. showing that sCJDMM2 and sFl have quite distinct transmission properties also supports the diversity of the strains associated with these two diseases according to the classic definition of strain. On the other hand, the notion that distinct strains generally associate with distinct phenotypes and vice versa is supported by the finding that following bioassay or direct characterization no major differences were observed in totPrPSc and resPrPSc from sCJDMM1 and sCDMV1, two subtypes of CJD that, despite the different genotype at codon 129, show no significant phenotypic disparity. These considerations raise at least two key questions: (i) Which of the PrPSc variations in sFl and sCJDMM2 do encrypt the basic phenotypic differences of these two diseases? (ii) How can these variations be compatible with the current concepts of strain formation and evolution? Although future studies will provide more precise answers to the first question, the different representations of major components of totPrPSc like the individual glycoforms and the dimers, the differing...
stabilities of resPrPSc and the different aggregate profiles of both totPrPSc and resPrPSc point to the presence of fundamental differences in the two diseases involving PrPSc tertiary and quaternary structures, or the modalities of the PrPSc to PrPSc conversion, both of which can likely specify distinct phenotypes. Concerning the compatibility of our findings with current prion strain notions, it is widely accepted that prion strains initially form as a spectrum of conformers, which are then prioritized through a process of Darwinian selection, with the dominant component triggering the disease and imparting its phenotypic characteristics. Low compatibility of all strains with PrPSc may require a conformational change of the PrP initially converted or lead to the targeting of selected PrPSc conformers to allow propagation. Caution must certainly be used in applying these notions (largely acquired from animal and cell experimentation) to sporadic human prion diseases in which the initial PrPSc is formed de novo, in the absence of an exogenous strain serving as template, and seemingly in circumscribed brain regions. Nevertheless, the coexistence of significant amounts of PrPSc types 1 and 2 in about 40% of 129MM cases, combined with the finding that the disease phenotype reflects the ratio of the two types, is consistent with the presence of a selection process resulting in the co-existence of a dominant strain with sub-strains also in human sporadic prion diseases. Other remarkable features of human sporadic prion diseases are that the variety of phenotypes and the major PrPSc species with which they are associated, regularly recur with very similar features and consistent prevalences despite the apparent lack of template. Furthermore, as mentioned above, the PrP genotype, determined by the methionine/valine polymorphism at codon 129, is a strong determinant of the prion strain. For example, in sCJD approximately 86% of the affected subjects who are methionine homozygous (sCJDMM) carry exclusively or predominantly PrPSc type 1, while the remaining 14% have PrPSc type 2 as the exclusive or prevalent type. Accordingly, the sCJDMM1 phenotype accounts for about 86% of all cases of sCJDM while sCJDMM2 and sFI phenotypes account for up to 11.9% and 1.8%, respectively. According to the spectrum hypothesis, it is tempting to speculate that all three PrPSc strains (i.e. type 1MM; type 2MM and type 2MM sFI variant) are initially present in sCJDMM and sFI patients, but PrPSc type 1 is preferentially selected while the sFI strain very rarely is.

The preferential brain region where the initial PrPSc to PrPSc conversion takes place might also play a role in strain selection and PrPSc properties, especially when the influence of the 129 polymorphism is expected to have no discriminating role as in sCJDMM2 and sFI. Indirect evidence points to the thalamus as the locale of the earliest lesions in sFI, while the severe involvement of the cerebral cortex as for histopathology and PrPSc accumulation argues that the brunt of the conversions process in sCJDMM might take place in the cortex. To deepen the current understanding of the early events in the pathogenesis and phenotypic determination in sporadic prion diseases it would be important to gain insight into how the brain locale (i.e. the biological environment) of the initial PrPSc to PrPSc conversions is chosen and the role it plays in strain diversity.

Materials and Methods

See Supplementary Materials and Methods for a list of reagents and antibodies used, and for description of the following: molecular genetics, prevalence, clinical and histopathological evaluations, methanol and methanol-chloroform precipitations, deglycosylation by PNGase F, IP with 8B4, antibody co-elution, IP with the original NaPTA precipitation protocol 7 was modified to improve PrP solubilization and reduce PrP contamination in the precipitate. Homogenates were cleared by 5 × 75 sec. cycles with high-energy cell disrupter Mini-Beadbeater-16 (BioSpec), allowing 1 min interval at 4°C between each cycle. Solubilization was optimized by freezing the samples between cycles 3 and 4.

Sodium phosphotungstate precipitation. The original NaPTA precipitation protocol 7 was modified to improve PrP solubilization and reduce PrP contamination in the precipitate. Homogenates were cleared by 5 min 500 × g centrifugation at 4°C. 10% cleared homogenates, mixed 1:1 with a solution 16% sarkosyl NL in 2X D-PBS without CaCl2 and MgCl2 at pH 7.4, were well vortexed. For further details refer to Supplementary Materials and Methods.

Proteinase K digestion. Samples in LB pH 8.0 were incubated at 37°C for 1 hour with PK (58 U/mg specific activity, 1 U/ml equal to 17.2 μg/ml PK) that was adequate to efficiently digest both PrPSc and senPrPSc (data not shown). The reaction was stopped by the addition of 3 mM PMSF. Unless otherwise indicated, PK digestion was performed in samples harvested from the same brain equivalents.

Determination of total protein concentration. Pierce BCA Protein Assay Kit was used following the manufacturer’s instructions with minor modifications.
Conformational stability and solubility assay. CSSA was performed as originally described18 with minor modifications19. For further details refer to Supplementary Materials and Methods.

Statistical analysis. Unpaired, two-tailed Student’s t-test was used, after determination of equal or unequal variance between the samples. The level of statistical significance was indicated: *P < 0.05; **P < 0.01; ***P < 0.001.

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Acknowledgements

The authors thank the patients’ families, the CJD Foundation and all the members of the NPDPSC, in particular Mses. Yvonne Cohen, Diane Kofskey, Miriam Warren, Katie Glisic, Janis Blevins and Mr. Aaron Foutz. We also are grateful to Dr. Gianluigi Zanusso for assistance with 2-D data and Ms. Laura Grossi for manuscript editing. This study was supported by National Institutes of Health Grants R01 NS083687 and P01 AI106705, and The Charles S. Britton Fund (to P.G.); in part by CDC Grant U51 CK000100 (to J.G.S.), the Intramural Research Program of the NIAID (to B.C.) and Grant P30 AG010133 (to B.G.).

Author Contributions

Conceived and designed the experiments: P.G., L.C., S.N., B.C., I.C. and J.G.S. Performed the experiments: L.C. Performed clinical review: B.S.A. Performed histopathological examinations and made final diagnoses: P.G. and M.L.C. Contributed materials: M.-S.S., S.G.C. and W.-Q.Z. Analyzed the data: P.G., L.C., S.N., B.C., I.C. and J.G.S. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Cracco, L. et al. Novel strain properties distinguishing sporadic prion diseases sharing prion protein genotype and prion type. Sci. Rep. 7, 38280; doi: 10.1038/srep38280 (2017).

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