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ORIGINAL ARTICLE

Met_{166}-Glu_{168} residues in human PrP β2-α2 loop account for evolutionary resistance to prion infection

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Abstract

Aims: The amino acid sequence of prion protein (PrP) is a key determinant in the transmissibility of prion diseases. While PrP sequence is highly conserved among mammalian species, minor changes in the PrP amino acid sequence may confer alterations in the transmissibility of prion diseases. Classical bovine spongiform encephalopathy (C-BSE) is the only zoonotic prion strain reported to date causing variant Creutzfeldt-Jacob disease (vCJD) in humans, although experimental transmission points to atypical L-BSE and some classical scrapie isolates as also zoonotic. The precise molecular elements in the human PrP sequence that limit the transmissibility of prion strains such as sheep/goat scrapie or cervid chronic wasting disease (CWD) are not well known.

Methods: The transmissibility of a panel of diverse prions from different species was compared in transgenic mice expressing either wild-type human PrP^C (MDE-HuTg340) or a mutated human PrP^C harbouring Val_{166}-Gln_{168} amino acid changes (VDQ-HuTg372) in the β2-α2 loop instead of Met_{166}-Glu_{168} wild-type variants.

Results: VDQ-HuTg372 mice were more susceptible to prions than MDE-HuTg340 mice in a strain-dependent manner.

Conclusions: Met_{166}-Glu_{168} amino acid residues present in wild-type human PrP^C are molecular determinants that limit the propagation of most prion strains assayed in the human PrP context.

KEYWORDS
evolution, prion, PrP, resistance, strain

INTRODUCTION

Transmissible spongiform encephalopathies (TSE) or prion diseases are caused by the post-transcriptional conversion of the cellular form of the prion protein (PrP^C) into its accumulative abnormal isoform (PrP^{Sc}).[1] PrP^{Sc} can be discriminated from PrP^C by its detergent insolubility, tendency to aggregate and partial resistance to proteinase K digestion (PK) which produce a truncated fragment (PrP^{res}) mostly used as surrogate marker for prion detection.[1] Prion diversity exists in the form of distinct prion strains that show different features after inoculation of laboratory animals such as survival time, neuroanatomic target areas, patterns of PrP^{Sc} deposition in the brain and PrP^{res} biochemical properties.[2,3] This variability is thought to be enciphered within different PrP^{Sc} conformations.[4,5]

Juan Carlos Espinosa and Alba Marín-Moreno are contributed equally to this work.

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TSEs affect a large spectrum of mammalian species, including humans [affected by Creutzfeldt-Jacob disease (CJD) and kuru] and several human food chain species, such as sheep and goats (affected by scrapie), cattle [affected by bovine spongiform encephalopathy (BSE)], cervids [affected by chronic wasting disease (CWD)], and dromedary camels.[6–8] Thus, transmission of animal prions to humans constitutes a great concern for human health. Fortunately, prion transmission between species may be limited as a consequence of the transmission barrier phenomenon.[9] Eventual interspecies transmission of prion strains tends to be an inefficient process that shows partial attack rates and variable, often protracted, survival times. By contrast, intraspecies transmission of prions lacks any transmission barrier and is usually an efficient process showing 100% attack rates, stable survival times and maintenance of the biochemical properties of PrP^{res}. The transmission barrier is mainly driven at the molecular level by the identity between the primary sequences of host PrP^{C} and donor PrP^{Sc} but the inoculated prion strain may also influence the outcome. Thus, successful interspecies transmission seems to be determined by the conformational compatibility between host PrP^{C} and the misfolded strain-specific PrP^{Sc} conformers that are present within the infectious prion particles.[5] Given these considerations, "strain barrier" better describes the transmission barrier of a certain prion strain among two different species. Prion strain barriers can be evaluated using mice genetically engineered to express the PrP species to be assayed in the absence of endogenous mouse PrP.

Only one prion strain has ever been reported to overcome the human transmission barrier. Classical BSE (C-BSE), that started an epidemic in UK cattle during the 1980s and soon extended to other European countries, is the causative agent of variant Creutzfeldt-Jacob disease (vCJD) in humans as consequence of dietary exposure to C-BSE contaminated tissues.[10–12] vCJD is responsible for at least 178 deaths in the UK[13] and its estimated prevalence within British population is high, as suggested by the prevalence of vCJD PrP^{Sc} in human appendixes (493 cases per one million population).[14] vCJD has only been confirmed in individuals homozygous for Met at residue 129 of human PrP[15] with one exception of heterozygosity (Met/Val) at this codon[16] proving the special importance of this polymorphism for prion transmission in the human PrP context. Another two BSE phenotypes, with pathology and epidemiology different from C-BSE, were observed after the EU active surveillance implementation in 2001. PrP^{res} biochemical properties from these cases differed from the ones of C-BSE on Western blot (WB) in terms of the protease-resistant fragment size and the ratio of glycoforms. These atypical forms were termed L-BSE or H-BSE (L-Low or H-High) according to the apparent molecular mass of the unglycosylated WB band of the PrP^{res}.[17,18] L-BSE has a higher zoonotic potential than C-BSE in transgenic mice overexpressing Met_{129} human PrP variant[19–22] but no transmission was observed in transgenic mice overexpressing the Val_{129} human PrP variant.[22] H-BSE was unsuccessfully transmitted in both models.[19,22] As observed in the case of C-BSE and L-BSE and the 129 Met/Val dimorphism in human PrP, only one amino acid change may drastically alter the susceptibility to prion strains. Moreover, heterozygosity at 129 codon of human *PRNP* gene has been associated with prolonged survival in individuals exposed orally to kuru, thus a strong balancing selection at the *PRNP* 129 locus during the evolution of modern humans has been proposed.[23]

The zoonotic potential of other animal prion strains has not yet been definitely proved. However, a study using transgenic mice overexpressing human PrP was done for several scrapie isolates and the transmission efficiency of some of them was comparable to that of C-BSE.[24] Nevertheless, despite the human alimentary exposure to scrapie, epidemiological studies failed to identify any clear link between scrapie and TSE occurrence in humans.[25,26] CWD, which elk, deer, reindeer and moose, have spread extensively throughout North America,[27] has been also detected in South Korean ranched elk,[28] and Norwegian and Finnish wild animals.[29–31] CWD prions are highly infectious and readily transmitted among cervids even through environmental exposure,[32,33] causing extraordinarily high prevalence that can exceed 90% in captive deer.[34] Humans, wild animals and livestock species like cattle, swine and sheep are likely exposed to CWD through consumption and/or contact with CWD-contaminated products/materials. An active surveillance of more than 17,000 US residents revealed that around 20% of them hunt cervids and more than two-thirds have consumed venison,[35] but a clear risk of developing a TSE through human dietary exposure to CWD has not been found.[36] Experimental transmissions of CWD to transgenic mice expressing human PrP suggest that a strong strain barrier for CWD exists in humans.[37–39]

The role of other human PrP residues, apart from the codon 129 polymorphism, in the human transmission barrier for animal prions remains unclear. When the human PrP sequence is compared to the ones of other proven prion susceptible mammals like sheep, cattle, elk or macaque (Figure 1), amino acid changes are observed at the 166 and 168 positions. While humans harbour Met_{166} and Glu_{168}, most mammal species show Val_{166} and Gln_{168} (according to the human amino acid sequence). 166 and 168 amino acid positions are located in the β2-α2 loop of PrP^{C}.[40] Accumulating evidence suggests that changes in these region are deeply related to alterations in prion strain susceptibility and pathogenicity.[38,41–46] For instance, in the sheep *PRNP* gene, a polymorphism described in the 171 position (equivalent to 168 position in human PrP) was associated either to scrapie susceptibility (Gln_{168}) or resistance (Arg_{168}).[47] Gln to Arg substitution triggers a secondary structure change in the β2-α2 loop that modifies the connectivity of this region with other PrP^{C} structures.[48]

The conservation of Val_{166} and Gln_{168} PrP amino acids in species able to propagate different prion strains supports investigating the role of Val_{166}-Gln_{168} to Met_{166}-Glu_{168} substitution in the propagation of different prion strains within the human PrP^{C} context. For that purpose, we compare the susceptibility/resistance to a panel of different prion strains of two transgenic mouse models expressing similar levels of wild-type (MDE-HuTg340) or Val_{166}-Gln_{168} (VDQ-HuTg372) human PrP^{C}.

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Human      -GWGQG-GGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGLGGYMLGSAMS 135
Gorilla    .................................................. 135
Macaque    ...........N..H.......S........................... 135
Elk        G....-....................V....................... 135
Cattle     G....-.....G..............V....................... 135
Sheep      G....-...S................V...........S........... 135

Human      RPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDEYSNQNNFVHDCVNITIK 185
Gorilla    ................................Q................. 185
Macaque    ..L....N...........Y..........V.Q................. 185
Elk        ..L....N...........Y..........V.Q.N...T.........V. 186
Cattle     ..L...........................V.Q...............V. 186
Sheep      ..L....N...........Y..........V.Q....D..........V. 186

Human      QHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGS--SMV 233
Gorilla    .................................................. 233
Macaque    ..................................K............... 233
Elk        .................I...............Q...E......A...VI 234
Cattle     E................I...............Q..........A...VI 234
Sheep      .................I.I.............Q..........A...VI 234
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FIGURE 1 Amino acid comparison of human, gorilla, macaque, american elk, cattle and sheep PrP^C amino acid sequences. Only amino acids 88 to 233 (according to human PrP) are included in the comparison for clarity. Deletions are indicated by dashes. Points indicate identical residues. Amino acid numbering is indicated on the right. Species are named on the left. Amino acid changes in 166 and 168 positions (Met/Val and Glu/Gln respectively) are boxed

MATERIALS AND METHODS

Generation of transgenic mice expressing Val_{166}-Glu_{168} amino acid changes of human PrP^C

The pMo-huPrP129M.Xho plasmid previously used for the generation of HuPrP-Tg340 mouse line[49] has been used as template for directed mutagenesis reactions. pMo-huPrP129M.Xho plasmid contains the human PrP (Met_{129} variant) open reading frame inserted in the MoPrP.Xho expression vector.[50] The vector contains the murine wild-type *prnp* gene including its promoter, exon 1, exon 2 and 3′-untranslated sequences excepting intron 2 and the murine PrP ORF. pMo-huPrP129M.Xho was mutated to generate pMo-$hu_{166}VDQ_{168}$-PrP129M.Xho by using QuikChange II XL kit (Stratagene, CA) with specific oligonucleotides (5′-GTACTACAGGCCCGTGGATCAGTACAGCAACCAGAAC-3′ and 5′-GTTCTGGTTGCTGTACTGATCCACGGGCCTGTAGTAC-3′) following the procedures described by the manufacturer.

Transgenic mouse lines were generated using the same procedure and mouse colonies previously described for the generation of HuPrP-Tg340 mouse line.[49] Briefly, transgenes were excised from the expression vector (pMo-$hu_{166}VDQ_{168}$-PrP129M.Xho) by use of the restriction endonuclease NotI, leading to DNA fragments of ~12 kb. NotI digested products were fractionated on a 1% preparative low melting point agarose gel (TopVision™, Fermentas Inc.). The gel slice corresponding to 12 kb was excised and digested using β-agarase I (New England Biolabs) as described by the manufacturer. Purified DNA was resuspended in Tris-EDTA (10 mM Tris, pH 7.4, 0.1 mM Ethylenediaminetetraacetic acid) at a final concentration of 2–6 ng/mL. Finally, DNA was microinjected into pronuclear-stage embryos collected from superovulated B6CBAF1 females mated with 129/Ola males carrying a null mutation in their endogenous PrP gene. The homozygous PrP null mouse line used was generated by Manson et al.[51]

DNA was extracted from founders' tail by use of an Extract-N-Amp tissue PCR kit (Sigma-Aldrich) following the manufacturer's instructions. The presence of the human transgene was identified by PCR amplification using specific primers for mouse PrP exon and the human PrP ORF (5′-CATTCTGCCTTCCTAGTGGTACC-3′ and 5′-GTGTTCCATCCTCCAGGCTTC-3′). $muPrP^{+/-}$ $huPrP^{+/-}$ founders were backcrossed with homozygous PrP null animals ($muPrP^{-/-}$)[51] to obtain mice homozygosis for the null mutation ($muPrP^{-/-}$ $huPrP^{+/-}$). The absence of the murine PrP ORF in the transgenic mice generated was confirmed by PCR amplification using specific primers (5′-TAGATGTCAAGGACCTTCAGCC-3′ and 5′- GTTCCACTGATTATGGGTACC-3′). Later, $muPrP^{-/-}$ $huPrP^{+/-}$ mice (VDQ-HuTg372) were used for transmission experiments.

WB analysis of brain PrP^C expression in transgenic mice

Whole brains from mouse founders' lines were homogenised in extraction buffer (0.5% NP-40, 1% sodium deoxycholate in phosphate-buffered saline 10 mM pH 7.4, with Complete inhibitor cocktail [Roche]). Samples were precleared by centrifugation at 2,000*g* for 5 min. Supernatants were mixed with an equal volume of 2× SDS reducing sample loading buffer and boiled for 5 min before loading onto

a 12% Bis-Tris Gel (Criterion XT, BioRad). Protein concentration was measured with Pierce[TM] BCA Protein Assay kit (Thermo Scientific). For immunoblotting experiments, the monoclonal antibody (mAb) Pri308[52] which recognises the $_{111}$HMAGAAAA$_{118}$ epitope of the human PrP sequence was used at a concentration of 0.1 µg/mL. Monoclonal antibody recognising β-actin (Sigma-Aldrich) was used as loading control at a 1/20.000 dilution. Immunocomplexes were detected incubating the membranes for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare Amersham Biosciences). Immunoblots were developed with enhanced chemiluminescence ECL Select (GE Healthcare Amersham Biosciences). Images were captured using ChemiDoc XRS+System and Image Lab 6.0.1 Software was used for images processing and densitometry analysis. Values were normalised against β-actin levels.

Prion isolates and inocula preparation

In this work, a panel of field isolates from distinct origin and representing different TSE agents were used (Table 1). As negative control, TSE-free cattle brain was included on the panel. All inocula were prepared from brain tissues as 10% (w/v) homogenates in 5% glucose.

Transmission studies

About 6–7 week old mice in groups of 5–9 MDE-HuTg340 mice (overexpressing human PrP^C fourfold the PrP expression level in human brain)[49] and VDQ-HuTg372 mice (expressing similar levels of PrP^C in the brain, the main target for prion propagation) newly generated were inoculated with the above-mentioned list of prion inocula by intra-cerebral route with 20 µL of 10% brain homogenate, as previously described.[49] After inoculation, mice were observed daily and their neurological status assessed twice a week. When the progression of the disease was evident or at the end of the lifespan (≈650 days), animals were euthanised for ethical reasons. Once euthanised, necropsy was performed and the brain was harvested for analysis. Half of the brain was fixed by immersion in neutral-buffered 10% formalin (4% 2-formaldehyde) and the rest of the tissue was frozen at −20°C and used for biochemical analysis.

Survival times were calculated as mean ±SD of the days post-inoculation (dpi) of all the mice scored positive for PrP^{res}. Attack rate was determined as the ratio of PrP^{res}-positive mice among all the inoculated mice.

WB analysis of brain PrP^{res} in transgenic mice

175 ± 20 mg of frozen brain tissue was homogenised in 5% glucose in distilled water in grinding tubes (Bio-Rad) adjusted to 10% (w/v) using a TeSeE[TM] Precess 48[TM] homogenizer (Bio-Rad) following the manufacturer's instructions. The presence of PrP^{res} in transgenic mice brains was determined by WB using the reagents of the ELISA commercial test (TeSeE, Bio-Rad). About 10–100 µL of 10% (w/v) brain homogenate was analysed as previously described[56] using 12% Bis-Tris Gel (Criterion XT, BioRad). For immunoblotting,

TABLE 1 Description of the isolates used in this study

Isolate	Description and references	Supplier
sCJD T1	Type 1 sCJD M129M-infected case (0.08.02523_001)[53]	BB[a]
sCJD T2/MDE-HuTg340	Terminally ill MDE-HuTg340 mice infected with Type 2 sCJD V129 V-infected case (BC1452) after two iterative passages	CISA[b]
vCJD	vCJD M129M-infected case (BC1458)[54]	BHUFA[c]
C-BSE	Classical BSE natural case from United Kingdom[55]	AHVLA[d]
H-BSE	Atypical H-BSE natural case from Poland. Po 45[56]	NVRI[e]
L-BSE	Atypical L-BSE natural case from Poland. Po 15[22]	NVRI
Scrapie 1	Naturally scrapie-infected goat from France (wt; S/P_{240}). Fr–2143[57]	INRA[f]
Scrapie 2	Naturally scrapie-infected sheep from France (ARQ/ARQ). Fr-PS21[24]	INRA
Scrapie 2/Tg110	Terminally ill Bo-Tg110 mice infected with Scrapie 2	CISA
Scrapie 3	Naturally scrapie-infected (ARQ/ARQ) sheep from Italy. It–198–9[58]	ISS[g]
Scrapie 3/Tg110	Terminally ill Bo-Tg110 mice infected with Scrapie 3	CISA
CWD	Naturally CWD infected Rocky Mountain elk. #3	CFIA[h]

[a]Basque Biobank. Bilbao. Spain.
[b]Centro de Investigación en Sanidad Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria. Valdeolmos, Madrid, Spain.
[c]CJD Reference Laboratory. Alcorcón. Spain.
[d]Animal Health and Veterinary Laboratories Agency. New Haw. Addlestone. Surrey, UK.
[e]National Veterinary Research Institute. Pulawy. Poland.
[f]French National Institute for Agricultural Research, Nouzilly, France
[g]Instituto Superiore di Sanitat. Rome. Italy.
[h]National and OIE Reference Laboratory for Scrapie and CWD. Canadian Food Inspection Agency. Ottawa. Ontario, Canada.

Sha31 mAb[52] was used at a concentration of 1 µg/mL. Sha31 recognises$_{145}$-YEDRYYRE-$_{152}$ epitope of the human PrP^C sequence. Immunocomplexes were detected as described above for brain PrP^C analysis. About 5–50 µL of 10% (w/v) brain homogenate equivalent was loaded per lane in the Western blot.

Paraffin-embedded tissue blot and histopathological analysis

Paraffin-embedded tissue blots (PET blot) were conducted as previously described[59] using the Sha31 mAb.[52] Lesion profiles of the brains were done using 4-µm thick tissue slices stained with haematoxylin and eosin according to the method described by Fraser and Dickinson.[60]

Modelling of Val$_{166}$-Gln$_{168}$ human PrP^C

In silico human PrP expressing Val$_{166}$-Gln$_{168}$ substitutions based on the prion protein model structures available were generated using Comparative Modelling with Rosetta (RosettaCM) at Robetta Web server on 23 April 2019. (http://robetta.bakerlab.org/).[61,62] Briefly, this protocol creates a homology model with given Protein Data Bank files corresponding to one or more template structures. It is used for comparative modelling of target proteins. Figures of the molecular models were obtained with PyMol software.[63]

RESULTS

Generation of transgenic mice expressing Val$_{166}$-Gln$_{168}$ human PrP^C

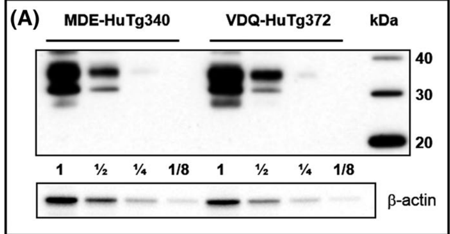
The same plasmid vector used to generate the MDE-HuTg340 mouse line[49] was used to generate the new transgenic mouse lines described in this work. Several mouse lines (founders) hemizygous for the transgene (Val$_{166}$-Gln$_{168}$HuPrP) were obtained. Founder animals also carrying the endogenous murine *prnp* gene (muPrP$^{+/-}$, huPrP$^{+/-}$) were crossed with *prnp* null mice (muPrP$^{-/-}$) to obtain transgenic hemizygous lines in a murine *prnp* null background (muPrP$^{-/-}$, huPrP$^{+/-}$). The absence of the murine *prnp* was determined by PCR using specific primers. Then, hemizygous mice were crossed to produce homozygous animals (muPrP$^{-/-}$, huPrP$^{+/+}$). At this point, PrP^C expression level was determined in brain homogenates of the founder mice by serial dilution in WB and compared to MDE-HuTg340 brain PrP^C expression levels. From the different founders, the VDQ-HuTg372 mouse line was selected as it showed comparable brain PrP^C levels and similar electrophoretic profile than MDE-HuTg340 mouse line (Figure 2). VDQ-HuTg372 mice reached the end of their lifespan with neither evidence of spontaneous prion disease nor behavioural defects such as alterations in reproduction rates or social deficits.

Prion resistance/susceptibility of VDQ-HuTg372 mice compared to MDE-HuTg340 mice

Once selected, VDQ-HuTg372 and its control counterpart MDE-HuTg340 mice were intracranially inoculated with a collection of isolates representative of different prion strains from human, cattle, goat, sheep and elk (Table 2). Since both transgenic lines share the same brain PrP^C expression levels, susceptibility/resistance differences among both lines will only be due to the two amino acid substitutions present on VDQ-HuTg372 mice.

Human prion transmission in VDQ-HuTg372

VDQ-HuTg372 mice and its control counterparts MDE-HuTg340 mice were inoculated with human prions sporadic CJD (sCJD) and vCJD. VDQ-HuTg372 mice were readily infected with sCJD T1



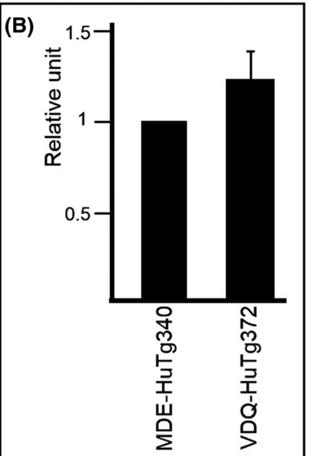


FIGURE 2 Brain PrP^C expression in transgenic mouse lines. (A) Immunoblots of brain PrP^C expression in MDE-HuTg340 in comparison to VDQ-HuTg372 transgenic mouse line detected with Pri308 mAb. Direct sample (60 micrograms of protein) and 1/2 dilutions were loaded on 12% Bis-Tris gels. Molecular mass in kDa (MagicMark^{TM} XP Western Protein Standard) are shown at the right side of the blot. β-actin was used as loading control. (B) Brain PrP^C expression was quantified and normalised against β-actin levels. Immunoblots illustrates a representative set of three independent experiments and the diagrams illustrates the mean densitometric values from these experiments. Data from MDE-HuTg340 brains was always standardised as 1 relative unit. Error bar represents the standard deviation of the mean value

prions showing even shorter survival times than MDE-HuTg340 mice. While VDQ-Tg372 mice had mean survival times shorter than 120 days, the MDE-HuTg340 mice had mean survival times around 60 days longer (Table 2). The second passage in VDQ-HuTg372 mice remained around 120 dpi. However, these extremely fast sCJD T1 prions passaged in VDQ-HuTg372 did not reduce the sCJD T1 typical mean survival time when inoculated into MDE-HuTg340 mice (Table 2). Attack rates reached 100% of the inoculated animals in all cases. The sCJD T2 isolate used in this study was previously adapted to MDE-HuTg340 mice to avoid the transmission barrier due to the codon 129 polymorphism in human PrP (Table 1). VDQ-HuTg372 showed similar survival times compared to MDE-HuTg340 mice inoculated with sCJD T2/MDE-HuTg340, 505 ± 34 and 469 ± 45 dpi respectively (Table 2). Attack rates reached 100% of the inoculated animals in both cases. This difference was found not significant as assessed by Mann-Whitney's *t*-test (p-value <0.05).

In the case of vCJD prions, VDQ-HuTg372 mice died at 357 ± 28 days after inoculation showing 100% attack rates, while MDE-HuTg340 mice showed a much longer survival time of 545 ± 146 dpi and reached 100% attack rates. After the second passage, survival time was maintained for MDE-HuTg340 mice while it was reduced to 236 ± 10 dpi for VDQ-HuTg372. Identical brain PrP^{res} electrophoretic signatures were observed in both mouse lines after inoculation with the different human prion strains used in this work (Figure 3A). The sCJD T1 PrP^{res} phenotype is observed for sCJD T2 prion strain in the context of human Met129 genotype as was previously reported.[24,64,65]

Animal prion transmission in VDQ-HuTg372 mice

Cattle prion strain C-BSE, as well as atypical BSE strains L-BSE and H-BSE, were inoculated in VDQ-HuTg372 and MDE-Tg340 mice. Remarkably for C-BSE inoculation, only one out of eight MDE-HuTg340 mice were infected beyond 650 dpi while all VDQ-HuTg372 mice were positive for brain PrP^{res} after survival times of 592 ± 85 dpi (Table 2). As expected, the second passage showed a 100% attack rate in both models, but in VDQ-HuTg372 mice the survival time was reduced to 328 ± 32 dpi while MDE-HuTg340 mice remained at 633 ± 32 dpi (Table 2). Neither VDQ-HuTg372 nor MDE-HuTg340 mice were infected with H-BSE prions. By contrast, L-BSE was efficiently transmitted in both mouse lines showing 100% attack rates in both cases (Table 2). Again, VDQ-HuTg372 showed shorter survival times than MDE-HuTg340 mice, 210 ± 13 (6/6) and 607 ± 13 (7/7) dpi respectively. Second passage in both models resulted in a survival time reduction of around 20%. Identical brain

TABLE 2 Effect of the presence of the $_{166}$VDQ$_{168}$ amino acid residues in the human PrP sequence in the replication of several prion strains from human, bovine, goat, sheep and elk as assayed in MDE-HuTg340 and VDQ-HuTg372 mouse models

			Mean survival time in days ± SD, (n/n_0)	
Prion origin	**Inocula**	**Passage**	**MDE-HuTg340**	**VDQ-HuTg372**
Human	sCJD T1	1st	185 ± 7 (7/7)	112 ± 10 (6/6)
		2[a]	190 ± 8 (5/5)	113 ± 2 (6/6)
	sCJD T1/VDQ-HuTg372	1st	196 ± 16 (6/6)	113 ± 2 (6/6)
	sCJD T2/MDE-HuTg340	1st	469 ± 45 (5/5)	505 ± 34 (7/7)
	vCJD	1st	545 ± 146 (5/5)[a]	357 ± 28 (7/7)
		2[a]	564 ± 39 (4/4)[a]	236 ± 10 (6/6)
Cattle	H-BSE	1st	>650 (0/6)	>650 (0/6)
	L-BSE	1st	607 ± 13 (7/7)[c]	210 ± 13 (6/6)
		2[c]	487 ± 16 (4/4)[c]	174 ± 5 (5/5)
	C-BSE	1st	>650 (1/8)[b]	592 ± 85 (5/5)
		2[b]	633 ± 32 (4/4)[b]	328 ± 32 (6/6)
Goat	Scrapie 1	1st	>650 (0/6)	>650 (0/6)
Sheep	Scrapie 2	1st	>650 (0/6)[d]	403 ± 20 (7/7)
	Scrapie 2/VDQ-HuTg372	1st	>650 (0/6)	378 ± 53 (6/6)
	Scrapie 3	1st	>650 (0/6)	>650 (0/6)
Elk	CWD	1st	>650 (0/6)	427 (1/7)
	CWD/HuVDQ-Tg372	1st	509–594 (2/5)	236 ± 10 (6/6)

n/n_0: diseased, PrP^{res} positive/inoculated animals. Mean survival time is indicated for all mice scored positive for PrP^{res}.
[a]Published in reference.[54]
[b]Published in reference.[56]
[c]Published in reference.[22]
[d]Published in reference.[24]

PrP^{res} electrophoretic signatures were observed in both mouse lines after inoculation with the different cattle prion strains used in this work (Figure 3A). Interestingly, the western blot conditions used in this work detected a slightly higher relative mobility in brain PrP^{res} from both mouse lines inoculated with L-BSE than in the original cattle L-BSE inoculum. This difference in the L-BSE migration of PrP^{res} bands in the humanised mice when compared to L-BSE from cattle could be explained by changes in the electrophoretic mobility due to differences in the amino acid sequence, as was previously described with classical-BSE after passage in sheep, which also shows a slightly higher mobility than classical-BSE from cattle.[66]

In accordance with previous studies,[24] none of the MDE-HuTg340 mice inoculated with sheep or goat classical scrapie was scored positive for the disease in the first passage. The same outcome was obtained for VDQ-HuTg372 mice with isolates Scrapie 1 and 3. Whereas Scrapie 2 infected VDQ-HuTg372 mice with 100% attack rate and a survival time of 403 ± 20 dpi (Table 2). These prions were then inoculated in MDE-HuTg340 mice and none of the animals was scored positive for the disease (Table 2). By contrast, second passage in VDQ-HuTg372 mice resulted in a survival time reduction to 378 ± 53 dpi. Brain-PrP^{res} from VDQ-HuTg372 mice inoculated with Scrapie 2 showed an unglycosylated band of 19 kD resembling the human sCJD type 2 PrP^{res} electrophoretic signature (Figure 3B).

Classical scrapie isolates adapted to the bovine PrP^{C} sequence were also inoculated in both models to assess how passage through the bovine species barrier affected classical scrapie zoonotic abilities. Differences were not observed for Scrapie 2, as low attack rates and long survival times were observed in both MDE-HuTg340 and VDQ-HuTg372 mice (Table 3). By contrast, bovine-adapted Scrapie 3 infected just VDQ-HuTg372 mice showing 100% attack rates and long survival time of 576 ± 37 dpi (Table 3).

In this case, differences in the brain PrP^{res} signature were observed in MDE-HuTg340 and VDQ-HuTg372 mice inoculated with the bovinised scrapie prions (Figure 3B). For Scrapie 2, MDE-HuTg340 mice showed a PrP^{res} electrophoretic signature resembling

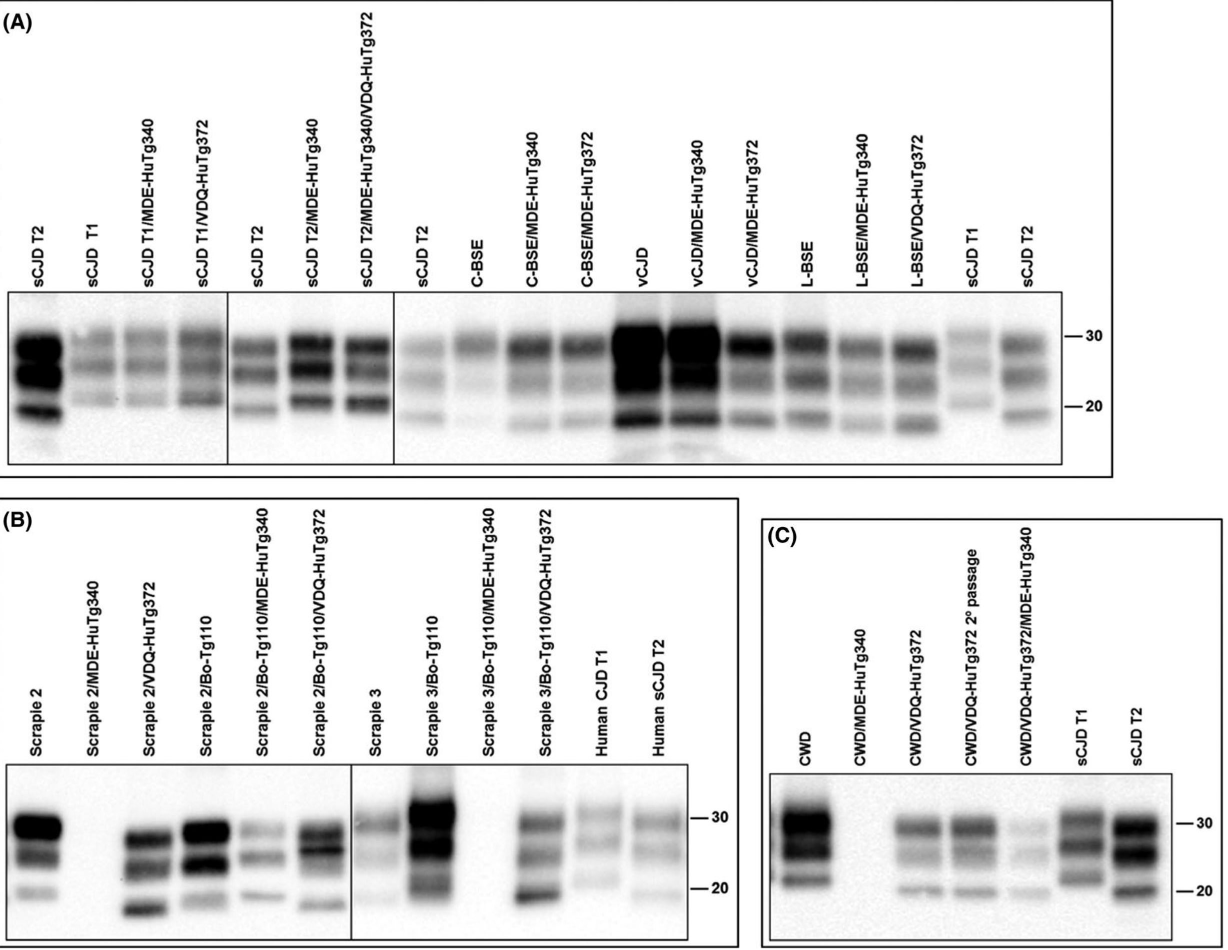


FIGURE 3 Electrophoretic profiles of PrP^{res} as detected by mAb Sha31 in brain extracts from mice infected with the prion agents indicated in the top. (A) Human and cattle inocula compared with the positive transmissions in MDE-HuTg340 and VDQ-HuTg372 transgenic mice. (B) Sheep and sheep-derived inocula compared with the transmissions in MDE-HuTg340 and VDQ-HuTg372 transgenic mice. (C) Elk inoculum compared with the transmissions in MDE-HuTg340 and VDQ-HuTg372 transgenic mice. T1 and T2 sCJD from human brain have been included as controls for better comparison. Similar quantities of PrP^{res} were loaded in each lane for better comparison. Molecular mass (in kD) is shown at the right side of the blots

type 1 PrP^{res} with a 21 kD unglycosylated band. By contrast, VDQ-HuTg372 mice showed a PrP^{res} electrophoretic signature characterised by a 19 kD unglycosylated band. Finally, VDQ-HuTg372 mice inoculated with Scrapie 3 showed an unusual PrP^{res} electrophoretic signature with a 19-kD predominant unglycosylated band.

Elk CWD prions were unable to infect MDE-HuTg340 mice (Table 2). By contrast, one out of seven VDQ-HuTg372 mice was infected showing a 427 dpi survival time. Second passage in VDQ-HuTg372 mice achieved 100% attack rate and 236 ± 10 dpi survival time. However, these CWD prions adapted to VDQ-HuTg372 mice were poorly transmitted when inoculated in MDE-HuTg340 mice as only two out of five animals were scored positive at 509 and 594 dpi respectively.

The brain PrP^{res} electrophoretic signature in VDQ-HuTg372 or MDE-HuTg340 mice infected with CWD showed a 19-kD unglycosylated band resembling type 2 sCJD electrophoretic signature (Figure 4C).

Comparison of the neuropathological assessment of VDQ-HuTg372 and MDE-HuTg340-infected mice

Vacuolar lesions (Figure S1) and PrP^{res} distribution patterns were compared in VDQ-HuTg372 and MDE-HuTg340 mice inoculated with sCJD T1, sCJD T2/MDE-HuTg340, vCJD and L-BSE. The same lesion profiles and PrP^{res} distribution patterns were obtained for all strains when compared among the two different mouse models. sCJD prions were characterised by strong PrP^{res} distribution in thalamus and cerebral cortex as well as vacuole accumulation in the superior colliculus, medial thalamus, hippocampus, cerebral cortex and pyramid tract areas in both mouse models as previously described.[22,24] vCJD prion transmission showed the histopathological features already reported for C-BSE-derived prion transmission, such as granular and strong PrP^{res} distribution, in both mouse lines.[54] Finally, L-BSE prions were transmitted in MDE-HuTg340 and VDQ-Tg372 mice showing unique strain features mainly characterised by a fine non-granular PrP^{res} distribution which was more intense in the habenular, geniculate and dorsal nuclei of the thalamus and weak vacuole accumulation, more prominent in the cerebellar cortex and cerebellum and mesencephalic tegmentum white matter areas.[22] The only remarkable difference among the two mouse lines was the consistent weak signal intensity in sCJD T1 PrP^{res} pattern in VDQ-Tg372 mice (data not shown). This could be explained by the extremely short incubation times of around 120 dpi that may not produce big enough PrP^{Sc} deposits able to be fully detected by the Pet Blotting technique.

In silico comparative structural analysis

In order to assess if Val_{166}-Gln_{168} amino acid changes introduced in VDQ-Tg372 mice affect the structure of human PrP^C, an in silico model was generated and compared with the one of wild-type human PrP^C (Figure 4). Minor changes are observed in the local region of the amino acid changes, which is more structured in Val_{166}-Gln_{168} human PrP than in the wild-type counterpart. Additionally, Val_{166}-Gln_{168} human PrP shows a slight deviation from the straight helix axis after residue 220 and enhanced definition of the carboxy-terminal amino acids of α-helix 3 when compared to wild-type human PrP (Figure 4).

DISCUSSION

vCJD cases due to the dietary exposure to the epidemic C-BSE agent in UK cattle raised concerns about the transmissibility of other animal prions to humans. To date, C-BSE is the only recognised zoonotic prion. Nevertheless, L-type BSE seems to propagate with no obvious transmission barrier in transgenic mice expressing Met_{129} human PrP^C[19–22] but not in mice expressing either Val_{129} or Met/Val_{129} PrP variants.[22] In addition, some scrapie isolates were successfully transmitted to humanised mice showing a transmission barrier comparable to that of C-BSE.[24] Considering this limited susceptibility of human species to prions, we have assessed the transmission features of a collection of prion isolates representative of diverse prion strains in transgenic mice VDQ-Tg372 expressing a mutant human PrP^C containing Val_{166}-Gln_{168} amino acid changes. Comparison with MDE-Tg340 mice that express the same levels of wild-type human PrP^C in brain would elucidate to what extent wild-type Met_{166} and Glu_{168} amino acid residues define the human transmission barrier to prions. Intracranial inoculation was used as the brain is the main target for prion propagation. MDE-HuTg340 transgenic mice previously described[49] are relevant animal models to assess prion transmission across the human transmission barrier.[22,49,54] Both transgenic mouse lines originated in such a way that the differences in both models are limited to the amino acid changes at 166 and 168 positions, so the alterations observed in prion propagation are directly related to the referred mutations. Potential differences due to the mixed genetic background of the mouse lines were minimised by inoculating a number of animals (5 to 9) on each experiment. Both amino acid substitutions are included in the β2-α2 loop, a region previously reported to be of special importance for prion propagation.[38,41–44,48] Met_{166}-Glu_{168} residues were chosen for mutation because they are together in human PrP while Val_{166}-Gln_{168} variants are present in most mammals species like sheep, cattle and elk.[67] The molecular modelling of the human PrP including Val_{166}-Gln_{168} amino acid changes do not present substantial changes in the overall PrP^C structure although slight differences between both PrP models were found. However, it must be taken into account that the β2-α2 loop region is generally not well defined in PrP molecular models thus the structural changes produced by mutations on this area may remain underestimated.[40,68,69] The model shown in this study at least suggests that Val_{166}-Gln_{168} substitutions in human PrP^C alter α-helix 3. The alterations in these residues are probably involved in the anchoring of the α-helix 3 against the β2-α2 loop and the residues following the first β-sheet strands previously proposed.[48,69] The Y_{169},

TABLE 3 Comparative transmission of sheep scrapie in MDE-HuTg340 and VDQ-HuTg372 mouse models before and after adaptation to cattle-PrP amino acid sequence

Prion origin	Inocula	Passage	Mean survival time in days ± SD, (n/n_0)	
			MDE-HuTg340	VDQ-HuTg372
Sheep	Scrapie 2	1st	>650 (0/6)[a]	403 ± 20 (7/7)
		2nd	369,579 (2/6)[a]	378 ± 53 (6/6)
	Scrapie 2/Bo-Tg110	1st	534 (1/5)	555 (1/5)
	Scrapie 3	1st	>650 (0/6)	>650 (0/6)
	Scrapie 3/Bo-Tg110	1st	>650 (0/6)	576 ± 37 (6/6)

n/n_0: diseased, PrP^{res} positive/inoculated animals. Mean survival time is indicated for all mice scored positive for PrP^{res}.
[a]Published in reference.[24]

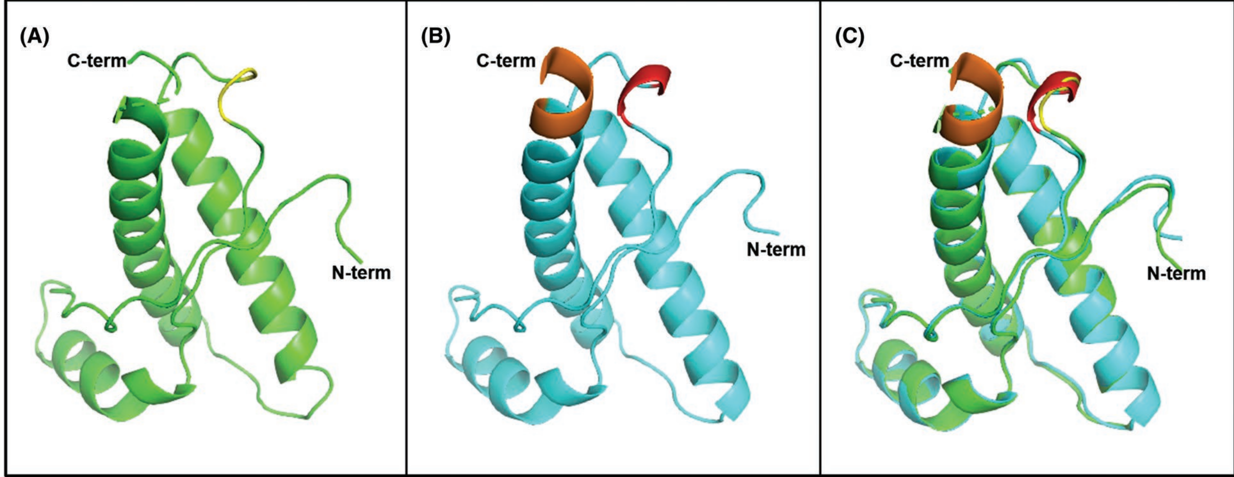


FIGURE 4 Structural models. (A) Structural Cα backbone of human wt-PrP^C from amino acids 124 to 227 (green). 167–171 positions shown in yellow. (B) Structural Cα backbone of Val_{166}-Gln_{168} human PrP^C (blue) from amino acids 124 to 227; 167–171 positions shown in red. (C) Structural Cα backbone superposition of 124–227 amino acids of human wt-PrP^C (green) and Val_{166}-Gln_{168} human PrP^C (blue). Overall folding of the models showing only differences in the carboxy-terminal region and the β2-α2 loop. 223–227 amino acid residues in Val_{166}-Gln_{168} human PrP^C (brown) fold as α-helix that is not structured in human wt-PrP^C and causing a slight deviation from the straight helix in the α-helix 3

F_{175} and Y_{218} aromatic cluster present in the wt human PrP could be altered in the Val_{166}-Gln_{168} mutant of human PrP^C due to modifications in the solvent exposure of Y_{169}. This alteration could allow new interactions with α-helix 3 as previously proposed for other human PrP mutants associated with disease.[45,46]

Accelerated propagation of the disease in Val_{166}-Gln_{168} human PrP^C context

In general terms, the presence of Val_{166}-Gln_{168} amino acid changes never delayed the survival time or reduced the attack rates for the prion strains used in this work. Indeed, the prion disease progression was faster in Val_{166}-Gln_{168} human PrP^C context than in the wild-type human PrP^C one. The only exception was Val_{166}-Gln_{168} mice inoculated with human type 2 sCJD. In this case, the disease progression remained unchanged in mice expressing either Val_{166}-Gln_{168} human PrP^C or wild-type human PrP^C. This effect observed with type 2 sCJD strain suggests that Met_{166}-Glu_{168} amino acids are not relevant for the propagation of the type 2 sCJD PrP^{Sc} conformers since its mutation does not affect prion propagation. Human strains sCJD type 1 and vCJD and animal strain L-BSE showed shorter survival times in mutated mice suggesting that Met_{166}-Glu_{168} residues somehow increased the resistance to these strains. A mouse model expressing mutant human PrP^C Val_{166}-Gln_{168}-Asn_{170}-Thr_{174} was infected with type 1 sCJD prions showing a 60% increase in the survival time.[38] Thus, evidencing that further modification of the human PrP^C β2-α2 loop region in Ser_{170}-Asn_{174} positions creates a significant transmission barrier at least for this prion strain.

Enhanced transmissibility of the infection in Val_{166}-Gln_{168} human PrP^C context

The reduction of prion transmission barriers in mice expressing the Val_{166}-Gln_{168} human PrP^C is also observed in terms of attack rates. Partial attack rates observed for C-BSE prions in mice expressing wild-type human PrP^C were improved to full attack rates in mice

expressing Val_{166}-Gln_{168} human PrP^C. Furthermore, prion strains that were unable to propagate in the wild-type human PrP^C context, like one classical scrapie strain or elk CWD, can propagate in the Val_{166}-Gln_{168} human PrP^C context. This shows that Met_{166}-Glu_{168} residues in wild-type human PrP^C were somehow preventing those strains crossing the human transmission barrier. However, this situation seems again to be strain specific since H-BSE and other classical scrapie strains were unable to amplify in both animal models despite the amino acid substitutions. As it applies to type 2 sCJD human prions, Met_{166}-Glu_{168} residues are not relevant for H-BSE and certain classical scrapie strains prion propagation. These findings support the idea that different prion PrP^{Sc} conformers initially interact and/or convert host PrP^C through different regions.

To date, CWD cannot be transmitted to transgenic mice expressing wild-type human PrP^C mice [37–39,70,71 and this work] The inability of CWD to propagate in the wild-type human PrP^C context is abolished in the mouse model expressing Val_{166}-Gln_{168} human PrP^C even though with a high transmission barrier. This is in agreement with the transmission of CWD in mice expressing mutant human PrP^C with Val_{166}-Gln_{168}-Asn_{170}-Thr_{174} amino acid changes[38] supporting the key role of the β2-α2 loop in the transmission barrier of CWD. The comparison of Val_{166}-Gln_{168} and Val_{166}-Gln_{168}-Asn_{170}-Thr_{174} mouse models[38] suggest that just Met_{166}-Glu_{168} amino acids account for wild type human PrP^C resistance against the efficient transmission of CWD. While CWD or certain classical scrapie prions transmitted very efficiently after iterative passage in mice expressing Val_{166}-Gln_{168} human PrP^C, further transmission of the adapted prions in the wild-type human PrP^C context was inefficient, highlighting the relevance of Met_{166}-Glu_{168} amino acid residues as key molecular elements involved in the resistance to propagation of certain classical scrapie or CWD strains.

Biological influence of Met_{166}-Glu_{168} amino acid changes

The alterations observed in the propagation of prion strains in Val_{166}-Gln_{168} human PrP^C context when compared to the wild-type human PrP^C suggest a general but strain-specific key role of these modifications in the β2-α2 loop either in the PrP^C-PrP^{Sc} heterologous interactions and/or in the PrP^{Sc} propagation rates. The higher transmission barrier associated with the wild-type human PrP^C suggests that Met_{166}-Glu_{168} amino acid residues could have been selected through evolution as molecular elements enhancing the resistance of human ancestors to circulating prion strains. These prions could affect human ancestors through dietary exposure to prion-infected tissues either from animals or human cadavers in cannibal rituals. In those cases of dietary exposure to prion-infected tissues, individuals harbouring Met_{166}-Glu_{168} residues in human PrP^C putatively would experiment delayed onset of disease and/or poor transmissibility both for intra and interspecies prion transmissions rendering a clear evolutionary advantage over individuals harbouring Val_{166}-Gln_{168} amino acid residues in human PrP^C.

Prion intraspecies transmission among human ancestors should not be underestimated since ritual cannibalism, besides other kinds of cannibalism, may have formed a driving force in the selection of advantageous human PrP variants, as was previously described for kuru disease[72] and for some prion diseases in the case of $Met_{129}Val$ dimorphism.[22,54,73,74] Such beneficial human PrP^C variants may have directed the evolution of modern humans in situations of ancestral prion disease epidemics.[23] Several pieces of evidence support widespread cannibalistic practices in many ancient human populations, for example, anthropic marks on Neanderthal bones[75] and biochemical analysis of fossilised human stools,[76] independently of the funerary or aggressive cause of the cannibalism. Furthermore, non-human primates evolutionarily related to humans, such as chimpanzees, eat head tissue from hunted prey, due to the high nutritional value of brain.[77] We can speculate that the ancient hunting habits, independent of whether the prey was human or not, exerted pressure for the selection of PrP variants that reduced the susceptibility to circulating prions thus promoting the selection of the Met_{166}-Glu_{168} amino acid residues in human PrP sequence. It should be noted that human PrP is the only primate PrP sequence harbouring Met_{166}-Glu_{168} amino acid residues. A few highly related primate species such as gorilla, chimpanzee and gibbon harbour only the Met_{166} residue,[78] suggesting that the Met_{166} variant was probably obtained earlier than the Glu_{168} amino acid change during primate evolution.

We can conclude that Met_{166}-Glu_{168} amino acids in the human PrP sequence are molecular elements highly involved in the human reduced susceptibility to prion infection although certain prion strains may not be affected by their presence.

ETHICS APPROVAL

Animal experiments were carried out in strict accordance with the recommendations stated in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 2010/63/EU). All efforts were made to minimise suffering. Experiments were approved by the Committee on the Ethics of Animal Experiments of the author's institution (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria); Permit Number CEEA 2012/002.

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CONFLICT OF INTEREST

There are no conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

The study was conceived by JCE and JMT. JCE, AMM, PAC and JMT performed research. JCE, AMM and JMT analysed data; and JCE, AMM and JMT wrote the paper. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are in the article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.Fig S1

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