Modelling Human Prion Replication

in Cell-free Systems

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DECLARATION

I declare that all work included in this thesis is the original work of the author, unless referenced to other sources. No part of this work has been or will be submitted for any other degree or professional qualification.

Marcelo Alejandro Barria Matus

Acknowledgments

I am because we are,
I am a human because I belong,
I participate because I am part

Ubuntu

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Thesis abstract

One of the key molecular events in the transmissible spongiform encephalopathies or prion diseases is the conformational conversion of the cellular prion protein PrP^C into the misfolded and pathogenic isoform, PrP^{Sc}.

Prion diseases are fatal neurodegenerative conditions affecting humans and other animal species, which present with diverse clinical and neuropathological phenotypes. In humans, prion diseases can occur as sporadic, familial or acquired forms. Sporadic Creutzfeldt–Jakob disease (sCJD) accounts for the majority of cases. The current classification system of human prion diseases recognizes several distinct clinico-pathological entities including sCJD, variant Creutzfeldt-Jakob disease (vCJD), Gerstmann–Straussler–Scheinker syndrome, fatal familial insomnia and variably protease-sensitive proteinopathy. Prion protein gene (*PRNP*) mutations and polymorphisms, and PrP^{Sc} types have a profound effect on these clinico-pathological phenotypes.

Prion diseases of sheep and goats, cattle, and cervids are all actual animal health problems and present potential risks to human health. Thus far the only known zoonotic prion disease is bovine spongiform encephalopathy, which has resulted in vCJD in humans. The recognition of new forms of prion diseases in animal and humans has generated increased awareness of the animal and public health risks associated with prion disease. However the mechanisms involved in prion replication, transmission, and neurodegeneration remain poorly understood.

This thesis uses *in vitro* PrP conversion assays (protein misfolding cyclic amplification and real time quaking-induced conversion) to model different aspects of human prion replication: Molecular susceptibility, genetic compatibility, spontaneous formation and the effect of molecules that might enhance or prevent conversion were each investigated in order to obtain a better understanding of the molecular mechanism of the prion replication. I have addressed the hypothesis that the major determinant factors in prion disease pathogenesis (*PRNP* genetics, PrP^{Sc} types and species barriers) are intrinsic to the prion protein conversion process and their effects can be faithfully recapitulated by *in vitro* conversion assays.

The results shows that *in vitro* conversion assays used in this thesis can model the combined effects of different PrP type and genotypes, can replicate aspects of cross-species transmission potential and provide information about molecular barrier to zoonotic transmission, can model *de novo* PrP^{Sc} formation, and can assess the potential impact of chaperones on conversion of the human prion protein. In summary, this work provides evidence that the origin, propagation and transmission of prions can be meaningfully investigated in cell-free systems.

Abbreviations

129 MM	Methionine homozygote at *PRNP* codon 129
129 VV	Valine homozygote at *PRNP* codon 129
129 MV	Methionine/Valine heterocygote at *PRNP* codon 129
ACDP	Advisory committee on dangerous pathogens
BSE or C-Type BSE	Bovine spongiform encephalopathy
Cb	Cerebellum
CJD	Creutzfeldt-Jakob disease
CJDE$^{200K-129M}$	Gentic CJD linked to a mutation of glutamic acid (E) with lysine (K) at *PRNP* codon 200, methionine at the codon 129
CJDV$^{180I-129M}$	Gentic CJD linked to a mutation of valine (V) to isoleucine (I) at *PRNP* codon 180, methionine at the codon 129
CNS	Central nervous system
CWD	Chronic wasting disease
D178N	Mutation at *PRNP* codon 178: Aspartic acid (D) to asparagine (N)
E200K	Mutation at *PRNP* codon 200: Glutamic acid (E) to lysine (K)
E219K	Mutation at *PRNP* codon 219: Glutamic acid (E) to lysine (K)
FC	Frontal cortex
fCJD	Familial Creutzfeldt-Jakob disease
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
GdnHCl	Guanidine hydrochloride
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann-Straussler-Scheinker Disease
H-type BSE	High type BSE
iCJD	Iatrogenic Creutzfeldt-Jakob disease
kDa	Kilo Dalton
mAb	monoclonal antibody
MM	Methionine homozygote at *PRNP* codon 129

MM1	Subtype MM1 of sporadic CJD
MM2	Subtype MM2 of sporadic CJD
MV	Methionine/Valine heterozygote at *PRNP* codon 129
MV1	Subtype MV1 of sporadic CJD
MV2	Subtype MV2 of sporadic CJD
L-type BSE	Low type BSE
LMWH	Low molecular weight heparin
NCJDRSU	National CJD Research and Surveillance Unit
Nor98	Atypical scrapie
ORF	Open reading frame
P101L	Mutation at *PRNP* codon 101: Proline (P) to leucine (L)
P102L	Mutation at *PRNP* codon 102: Proline (P) to leucine (L)
PBS	Phosphate buffered saline
pH	Measure of hydrogen ion concentration
PMCA	Protein misfolding cyclic amplification
PK	Proteinase K
PRNP	Human prion protein gene
prnp	Non-human prion protein gene
PrP	Prion protein, irrespective of conformations
PrP^c	Normal form of the prion protein
PrP^{res}	Protease-resistant prion protein
PrP^{Sc}	Disease-associated form of prion protein. Rich in β-sheet structure. Conformational alternated respective to the PrP^C
PrP^{sen}	Protease sensitive prion protein
PVDF	Polyvinylidene fluoride
recPrP	Bacterially expressed recombinant prion protein
$recPrP^{res}$	Protease resistant prion protein derived from recPrP
RT-QuIC	Real-time quaking induced conversion
sCJD	Sporadic Creutzfeldt-Jakob disease
sFI	Sporadic fatal insomnia
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% Tween 20

TME	Transmissible mink encephalopathy
TSE	Transmissible spongiform encephalopathy
UK	The United Kingdom
vCJD	Variant Creutzfeldt-Jakob disease
VPSPr	Variably protease sensitive prionopathy
V180I	Mutation at *PRNP* codon 180: Valine (P) to isoleucine (L)
V210I	Mutation at *PRNP* codon 210: Valine (P) to isoleucine (L)
VV	Valine homozygote at *PRNP* codon 129
VV2	Subtype VV2 of sporadic CJD
WB	Western blot

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Chapter One

General introduction

1.1 Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative conditions that affect humans and a diversity of animals. The diseases are accompanied by a change in the conformation of the normal prion protein (termed PrP^C) into an abnormal form of the native protein (termed PrP^{Sc}) (table 1.1) (Prusiner, 1998, Prusiner, 1982).

TSEs, particularly in humans, occur as sporadic, genetic and acquired diseases, the commonest of which is sporadic. TSE pathology is characterized by neurological dysfunction and death, gliosis and the deposition of PrP^{Sc} in the brain. TSE are (as the name indicates) transmissible, and the causal infectious agents are termed prions (Prusiner, 1982)

Term	Description
PRNP	Human prion protein gene
prnp	Non-human prion protein gene
PrP	Prion protein, irrespective of conformations
PrP^C	Normal form of the prion protein
PrP^{Sc}	Disease-associated form of prion protein. Rich in β-sheet structure. Conformational alternated respective to the PrP^C
PrP^{res}	Protease-resistant prion protein
PrP 27-30	PrP core fragment remaining after PK digestion
PrP^{sen}	Protease sensitive prion protein
recPrP	Bacterially expressed recombinant prion protein
$recPrP^{res}$	Protease resistant prion protein derived from recPrP

Table 1.1 Glossary of prion protein abbreviations used in this thesis

1.1.1 PrP^C, PrP^{Sc} and the "Prion hypothesis"

The gene coding for the prion protein (termed *PRNP*) is a conserved gene in mammals and avian species (Lee et al., 1998, Wopfner et al., 1999). In humans it is located on chromosome 20 but the location may vary depending of the carrier species (Sparkes et al., 1986, Kretzschmar et al., 1986). It encodes a 33-35 KDa protein which in its native state is sensitive to proteases, soluble in non-denaturing detergents, rich in alpha helix content (around 40%) with a small percentage of β-sheet (3%), and with a single disulfide bond between the residues 179 and 214 (Pan et al., 1993, Riek et al., 1996). In contrast, the protein in its abnormal folding state is rich in β-sheet structures, insoluble in detergents and partially resistant to proteases (Pan et al., 1993). The PrP can be found in non-glycosylated, mono-glycosylated or di-glycosylated forms (Haraguchi et al., 1989), predominantly inserted into the outer leaflet of the cell membrane through an glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1987). The GPI anchor can promote the association of PrP^C with cholesterol-rich lipid rafts involved in the trafficking of the protein and in the conversion to PrP^{Sc} (Hooper, 2005). The normal prion protein is present in different organs, with high expression in the brain and lymph nodes; moderate expression in spleen, heart, liver and lung; and lower expression in kidney (Ning et al., 2005).

A vigorous debate has developed in the scientific community regarding the nature of the infectious particles involved in TSEs. Some authors have argued that the infectious agent is a virion, composed of proteins plus nucleic acid molecules (Dickinson et al., 1968, Manuelidis, 2007). Another current claim is that the infectious agent is composed only of proteins, in particular the one coded by the *PRNP* gene. In 1982 Stanley Prusiner and colleagues suggested a concept to define

these new infectious agents responsible for TSEs. He coined the term "prion" for this small proteinaceous infectious particle resistant to procedures that attack nucleic acids and is partially resistant to proteases (Prusiner, 1982). This developed into the "protein only hypothesis" which recognizes as the "only" infectious agent the misfolded form of the prion protein. Two models have been proposed to explain the conformational conversion of prions. The "template directed refolding" model, in which an exogenous misfolded prion is required to convert endogenous cellular prion protein to PrP^{Sc}, and the "seeded nucleation" hypothesis, in which the two prion forms (PrP^{C} and PrP^{Sc}) are in thermodynamic equilibrium. According to the "seeded nucleation" hypothesis the infectious agent consists of a highly ordered arrangement of PrP^{Sc} molecules in the form of an aggregate, and the normal PrP^{C} protein is the precursor of further infectious PrP^{Sc}(Aguzzi and Calella, 2009, Griffith, 1967). This molecular interaction was confirmed when PrP^{C} knock-out animals were intracerebrally challenged with high doses of prions and they failed to develop any clinical signs (Bueler et al., 1993). The authors concluded that development of prion symptoms and pathology is strictly dependent on the presence of PrP^{C} and that incubation time and disease progression are inversely related to the level of PrP^{C} (Bueler et al., 1993). Furthermore, in order to evaluate the misfolding properties of the PrP^{C} in association with the GPI anchor, transgenic mice expressing prion protein lacking the GPI showed minimal clinical manifestations, although prion inoculation induced the formation and deposition as amyloid plaques. These results not only reinforce the relevance of PrP^{C} - crucial in the conversion process - they also suggest a fundamental link between the PrP^{C} and the cell membrane for the development of the disease (Chesebro et al., 2005).

1.1.2 Animal Prion Diseases

Wide ranges of animal species are known to be susceptible to prion disease, including several associated with human food consumption.

Animal species	Disease
Human consumption	
Sheep and Goats	Scrapie
	Atypical scrapie (Nor98)
Cattle	Bovine spongiform encephalopathy (BSE)
	High type BSE (H-type BSE)
	Low type BSE (L-type BSE)
Deer / Elk / White-tailed deer	Chronic wasting disease (CWD)
Non-human consumption:	
Mink	Transmissible mink encephalopathy (TME)
Cats	Feline spongiform encephalopathy (FSE)
Nyala and Oryx	Exotic ungulate encephalopathy (EUE)

Table 1.2 Non-human TSEs associated with human food consumption.

In general, all the above-mentioned TSEs share common features such as neurological signs, neuronal loss, astrocytosis, and amyloid formation. In the case of sheep and goats affected by scrapie, the animals exhibit changes in posture and movements (trembling, severe incoordination, stumbling and animals are unable to stand), skin irritation weight loss and finally death (Dickinson, 1976). The first described cases of scrapie can be traced back to 1773 in the UK (Reviewed by

Hunter N, 2007) (Hunter, 2007). Since then, the disease has been reported to be endemic throughout the world, except in Australia and New Zealand (Reviewed by Imram M, 2011) (Imran and Mahmood, 2011).

The route by which the infectious agent is transmitted between sheep and sheep-flocks is thought likely by the oral route (horizontal transmission) or contamination of the environment. Transmission studies in experimental animals have shown that scrapie-affected-animals can spread the disease to sheep and goats by contact of the offspring or other lambs with the placenta and placenta fluids (Dickinson et al., 1974). Additional information indicates that alternative transmission routes include skin scarification and blood transfusion (Taylor et al., 1996, Hunter et al., 2002). It bears noting that polymorphic variations of the ovine *PRNP* gene are associated with susceptibility to scrapie. Current research support that variations in the amino acid sequence, particularly associated at positions 136, 154 and 17, have a profound effect on the susceptibility to scrapie (Eiden et al., 2011, Bossers et al., 1996).

With the goal of supplementing the nutritional requirements of cattle, farmers in the UK started to feed them with recycled ruminant tissues (including sheep and goats tissues) or meat-and-bone-meal (MBM) as a source rich in proteins. The objective was to generate an economic impact increasing the milk and meat production. This "inoffensive" action brought an opposite and crushing effect, producing a financial catastrophe and a new disease in cattle called bovine spongiform encephalopathy or BSE. Although the original source of BSE is not known, one hypothesis is the transmission of scrapie prions (from sheep) to cattle. This hypothesis is supported by what is currently known about the species barrier phenomena. The species barrier can be defined as the difficulty in transmitting a prion disease from one species to

another in a primary transmission. In practice this can be recognized by an adaptation of the agent to the second species, producing a shorter incubation period and uniform transmission properties in a secondary transmission. The ability of a prion strain to be transmitted to a host is affected by the differences in primary sequence of the prion protein between donor and host species (Hill and Collinge, 2002). In this regard, a prion strain is defined as an infectious isolate which can cause a distinct prion-disease phenotype when it is inoculated to an identical host, maintaining the phenotypic hallmarks (incubation time, lesion profile, etc.) upon serial transmission. Considering the species barrier and the strain definition, Wilesmith and colleagues explored a possible connection between BSE and scrapie. This epidemiological study revealed that MBM containing ruminant-derived proteins were linked to the farms from which cases of BSE were reported (Smith and Bradley, 2003, Wilesmith et al., 1991). These results suggested a cattle-adapted scrapie like agent may have been responsible for BSE infection. An alternative hypothesis about the origin of BSE suggests that the bovine TSE may have originated spontaneously as a result of a genetic mutation, being later spread by the feeding of contaminated MBM to cattle.

Cattle affected by BSE usually present behavioural changes, tremors, ataxia, and hyperactivity to stimuli. The incubation time of BSE in cattle is between 2 to 8 years. Like sheep, several polymorphisms on the *PRNP* gene have been described to be associated with the susceptibility of cattle to BSE. Atypical forms of scrapie, and atypical forms of BSE have also been reported, e.g. Nor98 in sheep and low type BSE and high type BSE in cattle. Details of these prion strains will be discussed in Chapter 3.

Chronic wasting disease or CWD is another fatal TSE. CWD affect free-range and wild cervids, although it was first identified in captive mule deer in Colorado (Williams and Young, 1980, Williams and Young, 1992). The origin of CWD is still unknown and whether it has a relationship to scrapie or began as a sporadic disease will be difficult to know. Clinical signs in deer affected by CWD include behavioural changes, progressive loss of weight and death at the end-stage of the disease. Intracranial inoculations of experimental deer CWD have shown that the incubation time of CWD is between 17 to 21 months, and between 15 to 25 months by oral exposure (Sigurdson, 2008, Williams, 2005). Epidemiological and experimental data have provided substantial evidence of direct (animal to animal) and indirect (pasture contamination) transmission of CWD (Miller et al., 2004, Mathiason et al., 2009, Miller and Williams, 2003).

Transmissible mink encephalopathy (TME) and feline spongiform encephalopathy are very rare prion diseases. TME outbreaks have been reported in ranched mink in the United States of America (Wisconsin, Idaho and Minnesota); and FSE has been reported in domestic cats and captive wild members of the family *Felidae* in the UK, with a few cases identified in France, Australia, Ireland, and Germany. Experimental challenge of minks inoculated with TME showed spongiform changes in the cerebral cortex, with a severe vacillation in the gyri bordering, the cruciate sulcus and the anterior and posterior sigmoid gyrus (Liberski et al., 2009). The posterior part of the brain showed minimal lesions. Histopathological analysis of FSE cases revealed spongiform degeneration in the brain and spinal cords, with substantial variations in terms of vacuolation and PrP aggregation profiles depending on the affected species

(domestic cats, cheetahs, lions, ocelots, tigers and Asian golden cats). Coincidentally, for these two uncommon TSEs the death occurs after 2-8 weeks of the clinical onset.

1.1.3 Human Prion Diseases

Human prion diseases are a group of neurodegenerative disorder characterized by a rapidly progressive dementia, myoclonus, visual or cerebral impairment and pyramidal-extrapyramidal signs (Aguzzi et al., 2008, Ironside, 1998). Human prion diseases are classified according to their clinical, genetic and neuropathological features and fall into three aetiological groups:

- o **Sporadic or idiopathic** forms include sporadic Creutzfeldt–Jakob disease (sCJD), sporadic fatal insomnia (sFI) and variably protease sensitive prionopathy (VPSPr).
- o **Acquired** forms include iatrogenic (iCJD), variant Creutzfeldt–Jakob disease (vCJD) and kuru.
- o **Familial or genetic** forms include Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and familial Creutzfeldt-Jakob disease (fCJD).

The most common form of CJD is the sporadic form accounting for around 85% of human cases and with a relative incidence of 1-2 case / million population per annum worldwide (Ironside, 1998, Ironside et al., 2005). The main risk factors for genetic CJD are mutations in the *PRNP* gene.

As with animals affected by TSEs, *PRNP* polymorphisms appear to influence clinical and pathological phenotypes in the human prion diseases. The most critical polymorphism resides at codon 129, which may encode methionine or valine. The prevalence of the methionine / methionine (M/M) genotype at this codon is 39%, while methionine / valine (M/V) accounts for 50% and valine / valine (V/V) for only

11% in the normal Caucasian population. Nevertheless, 61% of sCJD cases are M/M at codon 129, 21% M/V and 18% V/V (Alperovitch et al., 1999, Ironside et al., 2005) In 1996 Will and colleagues reported 10 unusual cases of CJD in young individuals with a new neuropathological profile, suggesting the appearance of a new variant of Creutzfeldt–Jakob disease (vCJD) in the UK, with a possible link to the earlier bovine spongiform encephalopathy (BSE) epidemiology (Will et al., 1996). Animal transmission studies reinforced this view and strongly suggested that vCJD is: (i) caused by the same strain of agent that has caused BSE, FSE and TSEs in exotic ruminants; (ii) vCJD is distinguishable from sCJD; and (iii) CJD in two dairy farmers is of the sCJD type and is not linked to the causative agent of BSE (Bruce et al., 1997, Ironside, 1998).

TSEs in humans are diagnosed based on established diagnostic criteria which consider (i) clinical signs and symptoms, (ii) clinical tests, and (iii) post-mortem neuropathological confirmation. Diagnose of TSEs in animals is different since numerous high through-put "rapid tests" have been used. Today, considering the fundamental role played by the PrP in prion pathogenesis, several approaches have focused in detecting or amplifying the misfolded form of the prion protein PrP^{Sc}. Immunology-based techniques alongside the development of specific antibodies have led to immunoassays such western blotting, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC) or conformation-dependent immunoassay (CDI) being increasingly used for diagnose and screening of animal TSEs (Gavier-Widen et al., 2005). On the other hand, and based on the biochemical property of PrP^{Sc} to convert the normal prion conformation, researchers have also focused on investigating the *in vitro* conversion properties of the PrP as a potential tool for

developing a new test method and also to investigate the basis of the prion conversion. (Jones et al., 2011).

1.1.4 *In vitro* replication of prions

Caughey and colleagues developed an *in vitro* system in which recombinant hamster PrP^C, incubated with the misfolded form of the prion protein and a chaotropic agent, generated "new" protease-resistance material derived from the original PrP^C (Kocisko et al., 1994). Although this represented a major development in prion biology, a large excess of input material (PrP^{Sc} > 50 fold) was required to perform the conversion of the recombinant prion protein, placing constraints on its utility.

Subsequently and based on the nucleation and polymerization model, Soto and colleagues described an *in vitro* procedure that mimics the *in vivo* PrP^{Sc} conversion process. Using this approach it was possible to amplify minute quantities of PrP^{Sc} present in a sample.

This technology, called protein misfolding cyclic amplification (PMCA), consists of cycles of sonication followed by incubation periods of a mixture of a normal brain homogenate (rich in PrP^C) with a source of PrP^{Sc} in the presence of detergents (Figure 1.1) (Soto et al., 2002, Saborio et al., 2001)
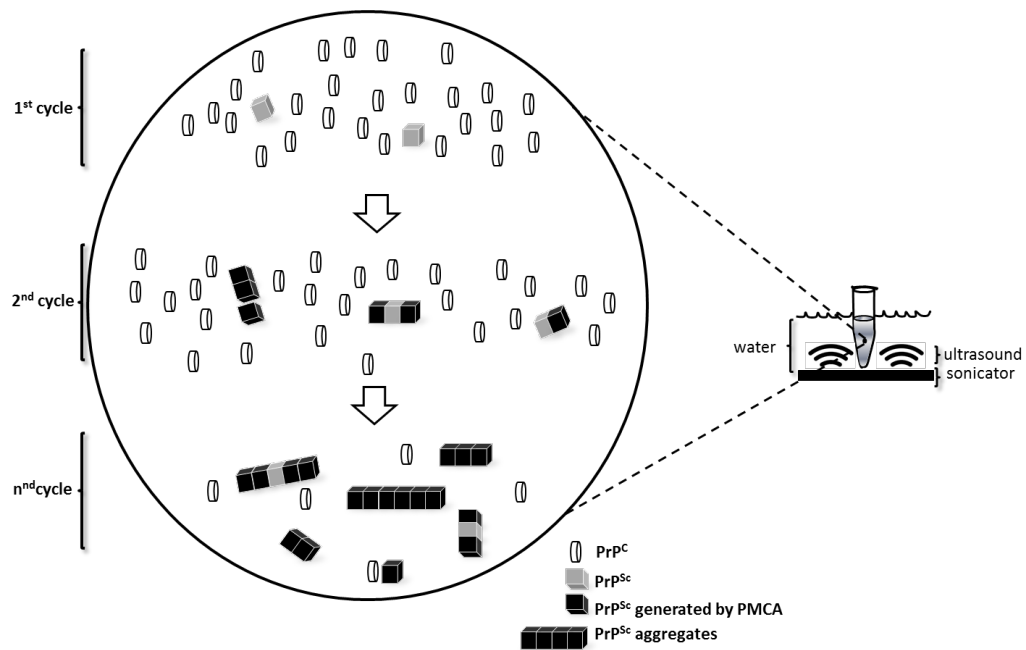
Figure 1.1 Schematic representation of protein misfolding cyclic amplification (PMCA) showing the presumed mechanism of molecular conversion. A large excess of PrP^C is incubated with minimal quantities of PrP^{Sc} followed by cycles of incubation and sonication. PrP^{Sc} aggregates are shown to grow, converting and incorporating PrP^C molecules. With each cycle of sonication and incubation (represented by arrows) PrP^{Sc} aggregates are broken, creating further seeds. After several cycles of sonication and incubation most of the PrP^C will have been converted into the PrP^{Sc} (Barria et al., 2014b).

The aim of the sonication is to fragment the PrP^{Sc} aggregates to increase the amount of seeds present in the sample without affecting their ability to act as conversion nuclei (Saa et al., 2005). Castilla and colleagues reported that the PrP^{Sc} generated by PMCA and inoculated into wild-type animals retains the biochemical and pathological characteristics of the original source (Castilla et al., 2008b).

PMCA has also been used to address different questions in the field of prion research, such as the species barrier phenomenon. Hamster and mouse prions have shown major difficulties in the transmission of the infectious agent between these two species (Race et al., 2002). Nevertheless, Castilla and colleagues reported that using PrP^{Sc} from mice strains (as a seed) and PrP^{C} from hamster as a source of substrate it is possible to produce an adaptation on the seeds, generating "new" prions after serial rounds of PMCA. Similar results were obtained using hamster seeds and murine substrate (Castilla et al., 2008a)

Another example of the applicability of PMCA is the spontaneous generation of prions. Deleault and colleagues reported that using PMCA and minimal components such as native PrP^{C}, co-purified lipid molecules and polyanions, it is possible to generate and propagate prions *de novo* (Deleault et al., 2007).

In addition, Barria and colleagues generated prions spontaneously at a low and variable rate in the absence of pre-existing PrP^{Sc} using a modified version of PMCA. The PMCA product was infectious in wild-type hamsters, producing a new disease phenotype with unique clinical, neuropathological and biochemical features (Barria et al., 2009).

More recently, Wang and colleagues reported the spontaneous generation of infectious prion by PMCA using exclusively recombinant PrP (Wang et al., 2010). These findings provide an experimental approach for studying the origins, mechanism, and factors related to sporadic TSEs and their zoonotic potential.

1.1.5 Aims of the Thesis

This thesis aims to provide a better understanding of human prion replication using cell-free conversion systems. It will address the following four hypotheses:

1.   Homologous (human:human) amplification of PrP^{Sc} from different human prion diseases. (Chapter 3)

Hypothesis: PMCA can faithfully amplify PrP^{Sc} from a wide range of different human prion diseases conserving PrP^{res} types and respecting *PRNP* codon 129 barriers.

Human prion diseases occur as sporadic, familial or acquired forms with clinical, genetic and neuropathological variations in each group. Jones and colleagues reported that PrP^{Sc} in extracts of sCJD brain could be amplified by PMCA using human prion protein from transgenic mouse brains by PMCA (Jones et al., 2008). They further showed that the efficiency of PrP^{Sc} conversion appeared to depend on the sCJD subtype and the *PRNP* codon 129 genotype of the PrP^{C}. However, the above study was limited in scope and there is a need for a more detailed determination of the combined effects of substrate genotype and the human prion disease subtype of the seed on the efficiency of PrP^{Sc} conversion.

This thesis seeks to extend the investigation of the interaction of the *PRNP* codon 129 genotype (of the substrate) and the human prion disease type of the seed. PMCA was used as an *in vitro* model of the *in vivo* PrP^{C} to PrP^{Sc} conversion, hypothesised to be a central event in prion diseases.

To achieve the aim, brains from different lines of humanized transgenic mice that differ only in their *PRNP* codon 129 genotypes (M/M, M/V or V/V) of the human prion protein, non-CJD human brain (of each genotype), and human cell lines which

overexpress the human prion protein (codon 129 genotypes M and V) served as sources of PrP^C. Brain tissue from vCJD and sCJD patients served as source of PrP^{Sc} seed. The main objective was to determine, for each subtype of human prion disease, the substrate *PRNP* codon 129 genotypes that are most efficient for the amplification of PrP^{Sc} by PMCA. This thesis also explored the *in vitro* conversion properties of non-standard PrP^{Sc} types, such as those found in the brain in GSS, FFI and VPSPr cases.

2. Heterologous (animal:human) amplification of PrP^{Sc} from different animal prion diseases. (Chapter 4)

Hypothesis: Species barriers to prion transmission can be modelled using PMCA.

After the discovery of a link between BSE and vCJD, several investigators have examined the potential risk of transmission from animals with others TSEs to humans. For the purpose of this thesis, PMCA was used to model the susceptibility of humans to different animal prion diseases. A panel of seeds for PMCA amplification were prepared from cases of vCJD, cattle BSE, H-type BSE in cattle, L-type BSE in cattle, sheep scrapie, atypical scrapie in sheep and chronic wasting disease. The panel was assessed using human brain substrates from non-CJD individuals, transgenic mice brain, and cell lines, with genotypes of *PRNP* codon 129 being M/M and V/V. The aim was to use an *in vitro* method of assessing the propensity of human PrP^C to be converted by PrP^{Sc} of a potentially zoonotic strain and therefore obtain information relevant to zoonotic risk.

3.   Spontaneous PrP^{Sc} formation using *in vitro* conversion systems as a model for sporadic CJD. (Chapter 5)

Hypothesis: Unseeded *in vitro* conversion reactions can be used to explore the factors affecting spontaneous prion protein misfolding.

Sporadic CJD is the most common form of human TSEs, affecting approximately 1-2 persons per million people per year (Ironside and Head, 2008, Ironside et al., 2005). However, important basic questions remain about the aetiology of this disease and whether sporadic CJD arises in individuals as a result of a spontaneous conversion of PrP^C to PrP^{Sc}, which then self-propagates. Different approaches have been used to address these questions. For example, Deleaut and colleagues and Barria and colleagues both explored the possibility of spontaneous generation of PrP^{Sc} in PMCA using minimal components (Deleault et al., 2007, Barria et al., 2009). Recently an interesting approach was published by Edgeworth and colleagues in which they showed that the incubation of metal wires with susceptible cells was sufficient to generate PrP^{Sc} spontaneously and produce a new disease phenotype in recipient wild-type mice (Edgeworth et al., 2010). Using a transgenic mice model which expresses wild-type bank vole prion protein, Prusiner and colleagues generated the spontaneous conversion of the normal prion protein in a transgenic model, recapitulating several aspects of prion disease (Watts et al., 2012). Finally, Wang and colleagues reported spontaneous generation of infectious prion by PMCA using exclusively recombinant PrP (expressed in bacteria), purified RNA from mice liver and synthetic lipids (Wang et al., 2010). As an alternative approach, in this thesis, two *in vitro* PrP conversion assays – protein misfolding cyclic amplification (PMCA) and real time quaking-induced conversion (RT-QuIC) – were used to try to identify conditions that promote

the spontaneous generation of human PrP^{Sc}. PMCA is a complex cell free system which typically utilizes brain homogenate, glycosylated and glipiated PrP, whereas RT-QuIC is much more simple and assesses amyloid formation using a naked protein backbone. Both cell free systems can used to model fundamental aspects of prion replication. The aims were (i) to establish a protocol for the spontaneous generation of the abnormal form of the human PrP *in vitro*, (ii) to determine whether the spontaneous *in vitro* generated human PrP^{res} can propagate *in vitro* and (iii) to determine whether PrP^{C} methionine or valine at the position 129 conferred greater susceptibility to the spontaneous conversion.

4.   A possible role for molecular chaperones in prion protein conversion. (Chapter 6)

Hypothesis: Members of the α-crystallin / small heat shock protein gene family affect prion protein conversion efficiency *in vitro*.

The α-crystallin / small heat shock proteins are a family of proteins with differential constitutive tissue expression patterns and stress-inducible, chaperone-related functions that have been implicated in responses to neurodegeneration among other pathologies. The cellular prion protein (PrP^{C}) is also thought to have a stress-related function which has also been associated with the autocatalytic propagation of the misfolding disease-associate PrP^{Sc}. In order to test for a possible role for α- crystallin in the conversion and propagation of the human prions, two prion conversion assays: PMCA and RT-QuIC were used to evaluate the activity of α-crystallin in the generation, conversion and propagation of human prions *in vitro*. The aims of this chapter were to (i) determine the effect of α-crystallin in a seeded vCJD PMCA

reaction, and (ii) evaluate if there is any effect on the formation of spontaneous amyloids by RT-QuIC.

Chapter Two

General Materials and Methods

2.1 Materials and methods

2.1.1 Laboratory requirements in handling TSE samples

TSE-tissue handling was carried out following the biosafety procedures described by the Laboratory Code of Practice of the National CJD Research & Surveillance Unit (NCJDRSU), Edinburg, UK. The Advisory Committee on Dangerous Pathogen (ACDP) classifies TSE agents in various species as hazard group 3. Following the recommendations suggested by the ACDP, the inactivation of the infectious agent was conducted by incubation of contaminated material to a solution of sodium hydroxide at a concentration of 2N for minimum 60 minutes.

All tissue sampling from cases of human prion disease was performed in a Class 1 microbiological safety cabinet. Handling of infected tissue homogenate (usually 10%) was performed under the same conditions stated above. The preparation and handling of uninfected tissue (10% uninfected brain homogenate) was carried out in a Class 2 microbiological safety cabinet. All the experimental work conducted for the accomplishment of this thesis was performed in a category 3 laboratory (with derogations) at the NCJDRSU.

2.1.2 Biological Samples

2.1.2.1 Human and non-human brain tissue

Human brain specimens were obtained at autopsy form UK cases referred to National CJD Research and Surveillance Unit with consent for research. The brain material was obtained by the submission of a tissue request to the NCJDRSU Brain and Tissue Bank committee for approval. Human brain specimens were selected based on the following criteria: (i) availability of a frozen half brain , (ii) consent for

research, (iii) neuropathological confirmation of diagnosis, (iv) samples selected previously for published scientific research were considered in preference to samples that had not previously been used, (v) CJD brain specimens that had mixtures of PrP^{Sc} types were excluded. Age, gender, post mortem delay, were not considered as a part of the selection criteria. Ethical approval for the use of these tissues was covered by LREC 2000/4/157 (Professor James Ironside).

Non-human brain tissues (non-human TSE samples) were obtained by request form the Animal Health Veterinary Laboratory Agency TSE Archive (AHVLA), Weybridge, UK. A detailed list of the analysed specimens is described in Chapter 4, Materials and method section.

2.1.2.1.1 Normal brain homogenate

Humanized MM, MV and VV transgenic mouse and frontal cortex form non–CJD patient were used as a substrate for the PMCA experiments. A detailed list of the utilized specimens is described in each specific Chapter in the Materials and method section. Transgenic mouse lines were kindly provided by Professor Jean Manson and Dr. Rona Barron, Roslin Institute, The University of Edinburgh, UK.

2.1.2.1.2 Human prion disease brain homogenate

Brain homogenates used in this study were derived from patients with a vCJD, sCJD, GSS, GSS with an 8kDa PrP^{res} fragment, FFI, VPSPr and pateints who were considered from a diagnosis of CJD but who were found to be suffering from a condition unrelated to human prion diseases such as CJD, GSS, FFI or VPSPr. A

detailed list of the analysed specimens is described in each specific Chapter in the

Materials and method section.

2.1.3 Solutions, reagents and buffers

The following reagents were used for the preparation and performance of PMCA and

Western blotting experiments.

	Reagents /Solution/Antibodies	Brand
PMCA Conversion Buffer	PBS 1X, 150mM NaCl, 1% Triton X-100, 1X Protease Inhibitor	
	PBS 1X	Sigma, Cat No. D8537
	NaCl	Sigma S3014-500G
	Triton X-100	Cat No T9284-100ML
	Complete Protease Inhibitor Cocktail Tablets (PI)	Roche, Cat No 11836170001
	EDTA 0.5M	Fluka, Cat No 100967520
	Low molecular weight heparin (final concentration 100μg/ml)	
Western blotting	Gels Nu-PAGE 10%	Invitrogen, Cat No NPO 301BOX
	Sampler buffer	Invitrogen
	Phenylmethanesulfonyl fluoride (PMSF) 50 mM	Sigma, Cat No P7626
	PVDF membrane	Bio-Rad Cat No 132-0177
	Anti-Prion Protein, a.a. 109-112, clone 3F4	Cat No mAb 1562
	Monoclonal anti-PrP antibody 6H4 (mAb 6H4)	Prionics, Cat No 01-010
	Proteinase K (Solution PK 1μg/μl in water)	Novagen Cat No 71049-3
	ECL Plus Western blotting detection reagents GE Healthcare	GE Healthcare Cat No RPN2132

Table 2.1 Reagents, solution and antibodies utilized to perform PMCA and Western blotting.

2.1.4 Methods

2.1.4.1 Preparation of PMCA Substrate (PMCA-Substrate)

2.1.4.1.1 Preparation of normal brain homogenate

Normal brain homogenates (humanized MM, MV and VV transgenic mice and frontal cortex from non–CJD patient) were prepared using the tissue grinder homogenate and chilled conversion buffer to obtain a final 10% weight/volume (w/v) solution. The homogenized tissue was centrifuged at 2000 rpm for 40 seconds in a refrigerated centrifuge (4ºC). After centrifugation the samples were removed carefully form the centrifuge and the supernatant retained. The normal brain homogenates or substrate was aliquoted in 1.5 ml micro-centrifuge tube frozen and stored at -80 until to be used.

For the preparation of PMCA-substrate, independent of its origin, low molecular weight heparin was added at 100μg/ml. EDTA was included at 500μM in all PMCA reactions. Both reagents (heparin and EDTA) were incorporated to the PMCA-substrate just before use.

2.1.4.1.2 Preparation of cell PMCA substrates

Cell PMCA substrate was prepared as described by Yokoyama and colleagues (Yokoyama et al., 2011). 293F cell extract (Codon 129M and V) was kindly provided by Professor Testsuyuki Kitamoto (Tohoku University Graduate School of Medicine, Sendai, Japan) and Dr. Masanori Morita (Japan Blood Products Organization, Kobe,

Japan).

2.1.4.2 Preparation of human and non-human prion disease brain homogenate (PMCA-seeds)

For the preparation of human and non-human prion disease brain material the same protocol was used as for the preparation of normal brain homogenate, except that quick freezing was not judged to be critical.

2.1.4.2.1 Determination of the PMCA-seed concentration prior to the amplification

All human and non-human prion disease brain material (10% brain homogenate) were evaluated for the presence of PrP^{res}. Serial dilutions of the TSE seed brain homogenate were used to determine the PrP^{res} levels by Western blotting. Likewise to determine the PrP^{res} signal which were faintly detected by Western blotting and densitometry. The final aim was to calculate the seed-dilution (PrP^{res}) (regarding the 10% brain homogenate) which was faintly detectable before amplification in order to see clearly the increased signal after PMCA. For this purpose, variations of the seed concentration among samples were utilized for the *in vitro* amplification.

2.1.4.3 Preparations of serially diluted samples for PMCA

A set of serially diluted PMCA reactions were prepared. Aliquots of normal brain homogenate and human and non-human prion disease brain homogenate were thawed by incubating in water at room temperature. PrP^{Sc} seeds (1.3μl) were carefully mixed with near 130.7μl PMCA substrate in 0.2ml PCR tubes to prepare a standard PMCA dilution of 1/100 (Figure 2.1). Serial dilutions 1/10 and 1/5 and 1/2 were used for some reactions. For serial dilution based on 1/10: 12 μl from the first

PMCA dilution was taken and transferred to the second tube (d-10^{-2}) followed by careful up-and-down mixture by pipetting. This process was repeated until dilution of interest d-10^{-n} (Figure 2.1).

For the non-sonicated sample (termed "Frozen" samples) 19 µl from the serially diluted samples was taken and frozen immediately. These samples served as non-amplified controls. All the samples were carefully mixed and then placed in the sonicator (incubation / sonication process).
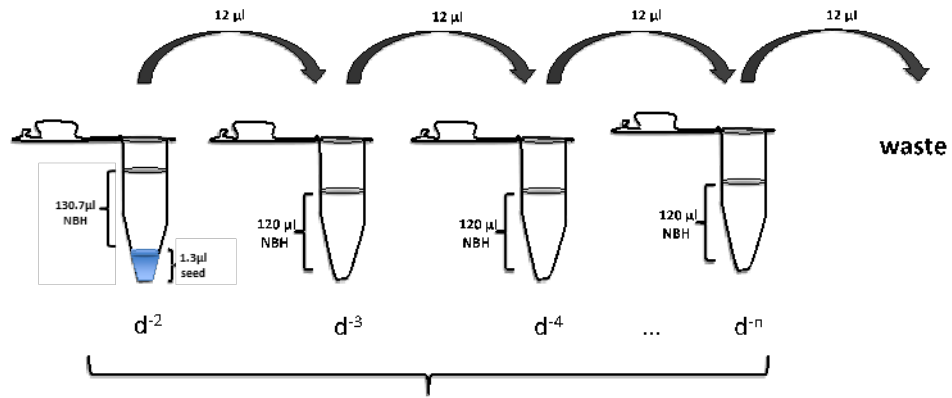
Once prepared the diluted samples, 19 µl of each tube were taken and freeze immediately ("Frozen" samples).

Figure 2.1 Diagrams represent serial dilution of infected brain in normal brain homogenate. The first dilution (1/100) contains 1 volume of 10% w/v infected brain homogenate (source of PrP^{Sc}, PMCA-seeds) and 99 volumes of normal brain (source of PrP^C, PMCA-substrate); the second tube is a 10x dilution of the previous mix; the third tube is a 100x dilution of the first tube; etc. All tubes end up with the same final volume (120 µl). From each tube 19 µl are removed and kept frozen for the non-amplified control. The final reaction volume is around 100µl (Barria et al., 2012).

2.1.4.4 Automatic Cyclic Amplification

The sonication step in the PMCA technique is the key element used to increase the efficiency and decrease the time used in the conversion of the PrP^C into the abnormal form of the prion protein. The following methodological parameters were used during the PMCA protocol:

Machine: Sonicator S4000

- Sonication time (Pulse ON): 20 seconds.

- Incubation time (Pulse OFF): 29 minutes and 40 seconds.

- Duration of PMCA reaction: 48 hours.

- Amplitude of sonication: 85-91

- Energy (in watts) : 280-290

- Temperature: 37°C

Clear nano-pure water (200ml) was added to the microplate horn to perform the reaction. All samples were placed in the PMCA tube holder and dipped into the water checking carefully that the levels of water did not reach the tube holder.

2.1.4.5 PrP Digestion

To detect the PrP^{res} product, proteinase K (PK) digestion was performed to remove the remaining PrP^C from the samples followed by Western blotting. Nineteen microliters of each sample were incubated with 1µl of 1µg/µl Proteinase K (final concentration 50µg/ml) for 1h at 37°C in a standard thermoblock.

One microliter of 50 mM PMSF was added to each reaction to inhibit PK activity. Samples were mixed with appropriate volumes of 4X loading buffer and centrifuged at 2000 rpm for 20 sec.

2.1.4.6 Western blotting analysis

Samples were boiled at 100°C for 10min and centrifuged at 2000 rpm for 20 sec to bring down the condensate. The samples were then loaded into the wells of a NuPAGE Novex 10% Bis-Tris Gel (1.0 mm, 10 well) and electrophoresed at 200V for 50 minutes (in NuPAGE® MES SDS Running Buffer).

Proteins were electro-transfered to a PVDF membrane (Bio-Rad) using 800 mA (constant) for 1 h. Membranes were blocked with 2% milk for 1hr followed by incubation with specific antibodies diluted in 1X PBS with 0.05% Tween 20 (PBST) (3F4, 6H4 mAbs) depending on the experiment. Blots were rinsed 3 times (10 min each time) with 1X PBST (washing buffer). ECL Anti-mouse IgG, peroxidase-linked species specific F(ab')2 fragment (from sheep) was used as a secondary antibody diluted 1/25,000 in washing buffer and incubated for 1 h. Again blots were rinsed 3 times for 10 min each with washing buffer to eliminate excess of secondary antibody.

Membranes were developed by a luminescent peroxidase substrate ECL-Plus from GE. Finally image of blots were acquired using the XRS digital CCD camera system.

2.1.4.7 Densitometric Analysis

Densitometric analyses were performed using Image Lab software version 2.01 (Bio Rad). All images were captured using a CCD camera and saved in TIFF format for further analysis. Densitometric Analysis of relative bands intensity were performed considering lineal range of PrP immunodetection signal by Western blotting.

In order to evaluate the potential variability in densitometric assays and western blotting, serial dilutions of 10% brain homogenate (from transgenic mice expressing the human form of the prion protein) were performed and subjected to immunoblot analysis using 3F4 antibody. Densitometric analysis in three independent immunoblots showed minimal variability across experiments.

2.1.4.7.1 PMCA normalization and quantification

To estimate the PMCA amplification efficiency, PrP^{res} densitometric signals (after PK treatment and Western blotting) were calculated using the Image Lab software.2.0.1. The densitometric value of the intensity of PrP immunoreactive bands was calculated for the samples before and after PMCA measuring an equal designated area (number of pixels). To obtain a better comparison among the samples, background subtraction was performed. The densitometric values of "sonicated" samples were divided by the "frozen" measures. The increase in signal (ratio between the detectable levels of PrP^{res} before and after PMCA) was designated as the amplification efficiency of *in vitro* conversion.

2.1.4.7.2 Selection criteria for PMCA seed-range dilution and densitometric analysis

All PMCA-seeds were evaluated for the levels of PrP^{res} after protease K digestion and Western blotting. The selection criteria used to determine the dilution utilized for prion amplification (and further densitometry) was based on the detection of faint levels of PrP^{res} before PMCA in order to visualize a measurable increase of signal post amplification. For this purpose variations of the seed concentration were utilized for the PMCA reactions.

	Equipment	Brand
PMCA		
	Sonicator Misonix S4000	Part S4000 Misonix
	Tube holder and cover for PMCA Incubator Leec compact incubator	Part # 444 Misonix
To substrate preparation	Potter-Elvehjem Tissue Grinders 15 or 30 ml	14231-368
Western blotting		
	Trans-Blot Cell With Plate Electrodes Thermoblock	170-3939 QBD2
	Trans-Blot Electrophoretic Transfer Cell	170-3954
	FisherBiotech™ Electrophoresis Power Supplies	FB300Q
	Densitometric Analysis, Image Lab software version 2.01, Bio Rad	
Others		
	Refrigerated centrifuge	Sigma 3-16K
	Ultra low freezer So-Low	Nuaire -86 ultra

Table 2.2 Equipment utilized to perform PMCA and Western blotting.

Homologous (human:human) amplification of PrP^{Sc} from different human prion diseases.

3.1 Introduction

3.1.1 Overview

Human prion diseases are a group of neurodegenerative conditions associated with misfolding and aggregation of the prion protein. They are classified as idiopathic, familial or acquired. They comprise a wide range of clinical and neuropathological phenotypes including Creutzfeldt–Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia, among others (Table 3.1).

The conformational change of the PrP^C to PrP^{Sc} is considered a key element to understand the molecular mechanism of the disease, and also their current classification. The current classification of human prion diseases relies mainly on international well accepted protocols, wherein the clinical signs, life-test analysis (magnetic resonance imaging, electroencephalography, 14-3-3 protein test), and post mortem confirmation, are critical parameters (http://www.cjd.ed.ac.uk/documents/criteria.pdf). For the familial human prion diseases group, the presences of mutations on the *PRNP* are also essential.

For the sCJD cases, a molecular typing subclassification was proposed by Parchi and colleagues which relies mainly on: (i) the polymorphic variation associated with the *PRNP* gene and (ii) the mobility and glycoform ratio of the protease-resistant core fraction of the PrP after proteinase K cleavage, visualized by Western blotting (Cali et al., 2006, Cali et al., 2009, Parchi et al., 1997, Ironside, 1998, Parchi et al., 1999b).

To gain a deeper understanding, the present introduction will be divided into the following two sections:

1. **Human prion diseases: mutations and polymorphic variations of the** *PRNP* **gene.**

2. **PrP glycosylation and conformation.**

	Disease	*Epidemiology*
Sporadic		
	Sporadic Creutzfeldt-Jakob Disease (sCJD)	1-2 /million/annum
	Sporadic fatal insomnia (sFI)	~2 /100million/annum
	Variably protease-sensitive prionopathy(VPSPr)	~2/100million/annum
Familial		
	Familial CJD (fCJD)	~200 worldwide [*]
	Gerstmann-Strausler-Scheinker (GSS)	1/100million/annum
	Fatal familial insomnia (FFI)	~100 worldwide [*]
Acquired		
	Kuru	Historically prevalent, fore tribe, New Guinea
	Iatrogenic CJD (iCJD)	~400 worldwide [*]
	Variant CJD (vCJD)	~200 mostly UK [*]

Table 3.1 Incidences and worldwide cases of human prion diseases. Epidemiology data is presented in number of cases/million/annum and total number of cases. ([*]) Incidence is not available.

3.1.2 Human prion diseases: mutations and polymorphic variations of the *PRNP* gene

The human prion protein gene (*PRNP*) is located on the short arm of chromosome 20 and comprises two exons. The exon two contains the open reading frame which encodes the 253 amino acid polypeptide, which after cellular processing results in a protein with a molecular mass of the 35-36 kDa termed the PrP^C (Oesch et al., 1985, Liao et al., 1986, Puckett et al., 1991).

It has been proposed that genetic variations of the *PRNP* gene appears not only to determine the susceptibility to certain prion diseases but also determines the disease phenotype (Reviewed by Parch P, 2012 and Head M, 2012) (Head and Ironside, 2012b, Head and Ironside, 2012a, Parchi and Saverioni, 2012). In this sense, the status of codon 129 – which has been described to encode either methionine or valine – has been shown to have a profound effect on the susceptibility to acquire / develop human prion diseases and their classification.

In the general Caucasian population, the estimated genotype frequencies at codon 129 are 39% methionine homozygotes (MM) versus 11% of the alternative valine homozygotes (VV). The remaining 50% of the population harbour the heterozygous methionine / valine genotype (MV). However, the genotype frequencies among individuals affected by prion diseases follow a different distribution, suggesting a correlation between codon 129 genotypes and diseases susceptibility. Specifically, 61% of sCJD cases are M/M at codon 129, 21% M/V and 18% V/V (Alperovitch et al., 1999, Ironside et al., 2005).

3.1.2.1 Sporadic human prion diseases

3.1.2.1.1 Sporadic Creutzfeldt-Jakob disease

With a worldwide incidence of one to two cases per million individuals per year, sCJD is considered the most common form of human prion diseases, accounting for around 85% of all CJD cases (Ironside et al., 2005, Will et al., 1996). Typically, sCJD occurs in middle-aged and elderly individuals, with a peak age of onset between 55 to 75 years. sCJD comprises a number of diverse clinical and neuropathological phenotypes. In general, most of the cases exhibit a progressive dementia, cerebellar dysfunction including muscle incoordination, visual disturbances and myoclonus. At the late stages of the disease, most of the patients develop akinetic mutism, and are unable to respond to external stimuli (Sikorska et al., 2012, Sikorska and Liberski, 2012, Ironside, 1998). The majority of sCJD cases die within six months of onset; however, in Japan a longer median survival time has been reported (Kitamoto and Tateishi, 1988).

The considerable heterogeneity of the clinical and pathological phenotypes of sCJD has been attributed to polymorphisms on the *PRNP* gene. The current molecular / genetic subclassification of sCJD was defined in 1999 by Parchi and colleagues (Parchi et al., 1999b). This classification relies on the homozygosis or heterozygosis at codon 129, the phenotypic heterogeneity of the pathology, and the physicochemical properties of PrP^{Sc}. Six subgroups were proposed: The first subgroup correspond to the MM1/MV1 cases, which accounted for 70% of the total number of cases and were previously classified as typical or classical CJD. The second subgroup corresponded to the VV2 cases and comprised the previous cases described as ataxic variants of sCJD. They account for 16% of the cases. The third

subgroup corresponded to the MV2 cases, formerly classified as Kuru plaque variant of sCJD. They accounted for 9% of cases. Representing 1% cases of the cases, the subgroup VV1 incorporated the cases described as atypical CJD. Finally, the last two subgroups correspond to the MM2-thalamic (MM2t) and cortical (MM2c) that represented 1% of the cases each. The MM2t subgroup incorporated the cases previously classified as the thalamic variant, which shares a similar disease phenotype with the genetic condition known as fatal familial insomnia. This disease entity is characterized pathologically by thalamic atrophy, and clinically by insomnia. The disease is known as sporadic fatal insomnia or sFI (Parchi et al., 1999a, Kawasaki et al., 1997, Mastrianni et al., 1999). The MM2c subgroup, which is denoted mainly by a cortical pathology, did not present a prior classification (Table 3.2). (Parchi et al., 1999b).

An important factor not considered in detail by Parchi's proposed classification refers to the possibility of mixtures of types (type 1 and type 2) in the same individual. This interesting fact, although reported in 5% of the cases, was not considered in this classification system (Parchi et al., 1999b). Several studies have confirmed the existence of mixtures, particularly in sCJD in which estimates of incidence range between 5 to 36% of cases across all three 129 codon genotypes (Parchi et al., 2009, Cali et al., 2009, Head et al., 2004, Puoti et al., 1999). Western blotting analysis using a monoclonal antibody that specifically recognizes PrP^{res} type 1 identified the occurrence of PrP^{res} type 1 in all the cases of a cohort of sCJD type 2 (Polymenidou et al., 2005). Using a similar methodology, but an alternative monoclonal antibody (12B2), Yull and colleagues reported for the first time the detection of PrP^{res} type 1 in the brain of 21 vCJD patients; PrP^{res} type 1 was also

detected in peripheral tissue (Yull et al., 2006). A recently revised subclassification of sCJD proposed the active incorporation of the co-occurrence cases (type 1 + type 2), integrating six additional subgroups to those already described (Parchi et al., 2009).

An alternative human prion classification has been proposed by Collinge and colleagues (Collinge and Clarke, 2007, Collinge et al., 1996, Wadsworth et al., 2003). This classification recognize three PrP^{res} types (contrary to the two presented by Parchi), and also distinguish more PrP^{res} glycoform of the three recognize by Parchi and colleagues. This thesis employs the subclassification proposed Parchi and colleagues (Parchi et al., 1999b, Parchi and Saverioni, 2012)

sCJD subtype codon129/PrP^{Sc} type	Previous classification	% of cases	Duration (months)	Clinical Features	Neuropathological Features
MM1 or MV1	Classical CJD	70	3.9	Rapidly progressive dementia, early and prominent myoclonus, typical EEG; visual impairment or unilateral signs at onset in 40% of cases	"Classic CJD" distribution of pathology; often prominent involvement of occipital cortex; "synaptic type" PrP staining; in addition, one-third of cases shows confluent
VV2	Ataxic variant	16	6.5	Ataxia at onset, late dementia, no typical EEG in most cases	Prominent involvement of subcortical, including brain stem nuclei; in neocortex, spongiosis is often limited to deep layers; PrP staining shows plaque-like, focal deposits, as well as prominent perineuronal staining
MV2	Kuru-plaques variant	9	17.1	Ataxia in addition to progressive dementia, no typical EEG, long duration (more than 2 years) in some cases	Similar to VV2 but with presence of amyloid-Kuru plaques in the cerebellum, and more consistent plaque-like, focal PrP deposits
MM2-thalamic	Thalamic variant	2	15.6	Insomnia and psychomotor hyperactivity in most cases, in addition to ataxia and cognitive impairment, no typical EEG	Prominent atrophy of the thalamus and inferior olive (no spongiosis) with little pathology in other areas; spongiosis may be absent or focal, and PrP^{Sc} is detected in lower amount than in the other variants
MM2-cortical	Not established	2	15.7	Progressive dementia, no typical EEG	Large confluent vacuoles with perivacuolar PrP staining in all cortical layers; cerebellum is relatively spared
VV1	Not established	1	15.3	Progressive dementia, no typical EEG	Severe pathology in the cerebral cortex and striatum with sparing of brain stem nuclei and cerebellum; no large confluent vacuoles, and very faint synaptic PrP staining

Table 3.2 Classification of sCJD proposed by Parchi and colleagues (1999) based on the phenotypic and molecular variability of 300 sCJD patients (Parchi et al., 1999b)

3.1.2.1.2 Variably protease-sensitive proteinopathy (VPSPr)

Variably protease-sensitive proteinopathy is a novel human prion disease characterized (like other forms of prion disease) by the presence of PrP^{Sc}. Nevertheless the PrP^{Sc} present in these cases show a distinctive sensitivity to treatment with protease. Although first described only for cases having homozygosity for valine at codon 129 (Gambetti et al., 2008), VPSPr has now been identified in all three genotypes (Rodriguez-Martinez et al., 2010, Head et al., 2010, Giaccone et al., 2007). Western blot analysis of VPSPr brain homogenates after PK treatment exhibit a distinctive electrophoretic mobility of the PrP resistant fragments, resulting in a ladder of bands with a prominent band at 8kDa, similar to those detected in Gerstmann–Straussler–Scheinker syndrome. So far very little is known about the mechanisms involved in this disease. Further work is required to establish the epidemiology, transmission and molecular characteristics of VPSPr (Head et al 2013).

3.1.2.2 Familial human prion diseases

Familial prion diseases, also known as genetic prion diseases, are a group of inherited neurodegenerative disorders associated with the presence of mutations in the *PRNP* gene. The pattern of inheritance of genetic prion diseases is autosomal dominant; only one copy of the mutated gene is required to develop the disease (Lloyd et al., 2011). A large number of mutations and polymorphisms have been identified underlying multiple prion disease phenotypes (Mead, 2006). Typically these mutations are of three types: point mutations, which lead to (i) an amino acid change or (ii) a premature stop codon, and (iii) insertions that alter the total number of octarepeats (OPRI) (Lloyd et al., 2011, Mead, 2006). Although it is not entirely clear how these mutations result in the production of misfolded prion protein, it has been proposed that they can lead to structural (i.e. conformational) changes of PrP^C, thus predisposing the normal isoform to be more susceptible to acquire the PrP^{Sc} conformation. Moreover, the occurrence of mutations within critical regions in the PrP^C greatly increases the propensity to undergo a pathogenic conversion (Legname, 2012, Jackson et al., 2009).

The genetic forms of human prion diseases account for 10–15% of all human prion incidences (Mead, 2006, Windl et al., 1999, Kovacs et al., 2005). Patients affected with familial prion diseases exhibit a wide spectrum of clinical phenotypes, even among individuals carrying the same gene mutations (Barbanti et al., 1996, Collinge and Palmer, 1992, Zarranz et al., 2005, Zerr et al., 1998). The heterogeneity of prion diseases can be attributed to several factors, although the PrP polymorphic genotype and the PrP^{Sc} type are considered to be the major influence (Gambetti et al., 2003). Genetic prion diseases are classified according to the clinico-pathological phenotypes

of the patients as either CJD, Gerstmann–Straussler–Scheinker syndrome or fatal familial insomnia.

3.1.2 2.1 Genetic or familial Creutzfeldt-Jakob disease (gCJD)

Genetic CJD (gCJD) is an inherited form of the Creutzfeldt-Jakob disease that was first recorded in 1924 by Kirchbaum (Reviewed by Gambetti P, 2003) (Gambetti et al., 2003). The affected family was later shown to carry an inherited form of CJD (Kretzschmar et al., 1995). gCJD is classified into several haplotypes based on pathogenic mutations and the polymorphism at the codon 129 of the *PRNP* gene. Among gCJDs, CJD with the E200K 129M haplotype (CJD$^{E200K–129M}$) is the most common form. The mean age of onset is 58 years and the mean duration is 6 months. The clinical presentation of CJD$^{E200K–129M}$ includes cognitive and mental abnormalities (~80% of patients), cerebellar signs (~43%), and visual signs and myoclonus (both <20%) (Hsiao et al., 1991, Brown et al., 1991). During the course of the disease all patients experience dementia. Another example of gCJD, but with rare mutations is the V180I–129M haplotype (CJDV$^{180I–129M}$). A total of seven cases have been reported worldwide, six Japanese and one American. Among the affected individuals four carried 129M on the normal allele and one had a second mutation on the other allele, M232R. The distinguishing clinical features of CJD$^{V180I–129M}$ are the long duration (1-2 years) and possible presence of Kuru plaques and plaque-like formations in subjects with 129M genotypes (Hitoshi et al., 1993, Kitamoto, 1994, Iwaski et al., 1999). As the majority of gCJD cases (>50%) have been diagnosed in individuals without a positive family history of the disease, the term gCJD is now being used more frequently instead of fCJD. Indeed, disease penetrance is highly

variable, as shown by considerable intra-familial and intra-generational phenotypic variability (Imran and Mahmood, 2011).

3.1.2.2.2 Gerstmann–Straussler–Scheinker syndrome

Gerstmann–Straussler–Scheinker syndrome (GSS) is a rare human prion disease, with an incidence of 1 in 100 million per population per year (Belay, 1999). The syndrome was first described in 1936 in a large Austrian family, in which members manifested a slow and progressive cerebellar ataxia admixed with cognitive decline at some time in the course of the disease (Reviewed by Liberski P, 2012) (Liberski, 2012)). A number of descriptive and eponymous titles have been described to analogous prion disorders; and only recently they have been recognized as a cohesive clinicopathological entity with a nosological classification as a TSE (Masters et al., 1981). The clinical features of GSS are early age onset (30-40 years) and slow progression extended over an average period of 5 years (Liberski, 2012). The variation in age of the onset is associated with the P102L mutation (Piccardo et al., 1998). Clinical symptoms include cerebellar ataxia, gait abnormalities, dementia, dysarthria, ocular dysmetria, infrequent myoclonus, spastic paraperesis, Parkinsonian signs and hyporeflexia or areflexia in the lower extremities (Belay, 1999, Liberski, 2012, Liberski and Budka, 2004). Although the main clinical features of dementia associated with progressive cerebellar ataxia and / or spastic paraparesis are non-specific, GSS patients share the characteristic neuropathological feature of multicentric amyloid plaques (Kovacs and Budka, 2009).

The genetic basis of GSS was first confirmed by the association of the P102L mutation with the disease phenotype (Hsiao et al., 1990). Further, the most common

mutation in GSS is the P102L substitution. This mutation is usually associated with the classical 'ataxic' GSS clinopathological profile. Other mutations in the *PRNP* gene have been described; however, they are often associated with less typical phenotypes (Kitamoto et al., 1993b, Hsiao et al., 1992, Kitamoto et al., 1993a). Some missense mutations that have been reported to co-segregate with the syndrome are: P102L-129M; P105L–129V; A117V–129V; Y145STOP–129M; F198S–129V; D202N–129V; Q212P; and Q217R–129V (Belay, 1999, Jansen et al., 2010, Kovacs et al., 2005).

3.1.2.2.3 Fatal familial insomnia (FFI)

Fatal familial insomnia was originally considered a thalamic dementia, and it was not until 1986 that it was reported as a disease afflicting five members of a large Italian family (Lugaresi et al., 1986). Subsequently, in 1992, FFI was proposed as a novel, genetically determined prion disease (Medori et al., 1992). FFI is caused by the effect of a mutation combined in *ci*s: an amino acidic change from aspragine to aspartic acid at codon 178 (D178N); and methionine at codon 129 (129 MM) (Goldfarb et al., 1992). Patients with D178N and valine at codon 129 exhibit a gCJD phenotype (Capellari et al., 2011, Lugaresi et al., 1986, Montagna et al., 2003). Further analysis revealed subtle differences in the gene products of D178N–129M and D178N–129V, as well as more discernable differences in the glycoform ratios and electrophoretic mobility's of their protease-resistant derivatives (Monari et al., 1994). The brains of FFI patients show a characteristic degeneration largely confined to the part of the brain involved in the sensory perception and prioritization of peripheral sensory information, the thalami (Lugaresi et al., 1986, Medori et al.,

1992, Parchi et al., 1998). The core clinical features of FFI consist of a profound disruption of the normal sleep-wake cycle (the main clinical hallmark), sympathetic over-reactivity, diverse endocrine abnormalities and markedly impaired attention (Montagna et al., 2003). An overview of the global occurrence of FFI indicates that 100 cases have been reported in almost 40 families from Italy, Germany, Austria, Spain and the UK, among other countries (Baldin et al., 2009). Although the incidence among men and women was similar, the clinical phenotype of FFI is apparently dependant on the genotype at codon 129. Myoclonus, spatial disorientation and hallucinations are more frequent in patients homozygote for the amino acid methionine (129 MM) (Zarranz et al., 2005, Krasnianski et al., 2008). Whereas symptoms such as bulbar disturbances and vegetative dysfunction were more common in heterozygous methionine / valine FFI patients (129 MV). These phenotypic differences between MV and MM FFI patients may be caused by different rates of prion conversion (Gambetti et al., 1995). Likewise, the duration of the disease has also been shown to vary between genotypes.

3.1.2.3 Acquired human prion diseases

Acquired prion disease results from the exposure to exogenous prions. The transmission of infection can occur from human to human or as a zoonosis, from an animal to humans. Acquired forms of human prion diseases are of importance because of their consequences for public health. Three different clinical phenotypes have been described so far: Variant Creutzfeldt–Jakob disease, Iatrogenic Creutzfeldt–Jakob disease and Kuru.

3.1.2.3.1 Variant Creutzfeldt–Jakob disease (vCJD)

In 1995 and early 1996 a small number of cases of CJD in unusually young individuals were reported. The average age at death was 29 and the clinical and pathological features of the patients differed from those of CJD in several ways (e.g. the presence of florid plaques in the brain). In April 1996 the disease was defined as a new variant of CJD (vCJD), and a link to the epidemic of BSE in the UK cattle was suggested (Will et al., 1996). Several laboratory and biological studies confirmed that the infectious agent responsible for vCJD was similar to the BSE agent (Bruce et al., 1997). Scott and colleagues performed transmission studies in wild-type human transgenic and bovine transgenic mice and provided compelling evidence of a causal link between BSE and vCJD (Scott et al., 1999). Since first reported by Will and colleagues in 10 patients, the majority of additional cases have been identified in the UK (total 177 cases) and France (~25 cases). Although the incidence of vCJD is declining, a small number of cases are still being identified.

In comparison to sCJD, vCJD usually affects younger age groups (onset around 27 years) and presents a longer duration (~14.5 months). Also, the symptoms of vCJD

are remarkably stereotyped compared to the varied clinical presentations of sCJD (Will et al., 1996, Ironside et al., 1996). The prominent presenting symptoms of vCJD are mainly psychiatric and behavioural. Neurological features can appear more than 6 months after the initial symptoms. As the disease progresses, signs of cerebellar ataxia with cognitive impairment and involuntary movement arise. The characteristic pathological changes observed in brains affected by vCJD are the formation of florid plaques, non-CNS tissue deposition of PrP^{Sc}, type 2B pattern of protease treated PrP^{Sc} by Western blotting, and hyperintensity of the pulvinar of the thalamus region seen on MRI scans (Ironside, 1998, Ironside and Head, 2004, Ironside et al., 1996, Will et al., 1996).

Nearly all cases of vCJD have been *PRNP* 129MM individuals (http://www.cjd.ed.ac.uk/data.html) apart from one possible MV case (Kaski et al., 2009). However, the accumulation of abnormal PrP has been identified in the appendix of three asymptomatic individuals, two of which were VV at the codon 129 of the *PRNP* gene (Hilton et al., 2004a, Hilton et al., 2004b, Ironside et al., 2006). Animal studies have shown that different clinical phenotypes could occur in people (exposed to the infectious agent) with polymorphic variations at the codon 129 of the prion protein gene (Asante et al., 2006, Wadsworth et al., 2004).

3.1.2.3.2 Iatrogenic Creutzfeldt–Jakob disease (iCJD)

The iatrogenic form of the Creutzfeldt–Jakob disease (iCJD) is a result of accidental exposure of patients to prions during medical procedures. The first case iCJD was reported in 1974 in the recipient of a corneal graft from a donor who had died of pathologically confirmed CJD (Duffy et al., 1974). To date, more than 400 cases of

iatrogenic iCJD have been reported worldwide (Hamaguchi et al., 2009). The mechanisms of transmission are variable and include neurosurgical instruments, depth electrodes, human pituitary hormones and dura mater grafts, all of which have involved cross-contamination with prion contaminated material (Will, 2003). When transmission is intracerebral the symptoms resemble those of sCJD. As for other prion diseases, genetic factors play an important role in the disease phenotype of the disease. The number of iCJD cases has decreased over time probably due to increased surveillance and use of effective decontamination measures (Hamaguchi et al., 2009).

3.1.2.3.3 Kuru

Kuru ("to shake" or "tremble" in the Fore language) was first reported in 1959 (Gajdusek and Zigas, 1959). Kuru was recognized to be endemic amongst the Fore tribe resident in the Eastern Highlands of New Guinea, although it was also seen in neighbouring tribes with whom the Fore often inter-married (Gajdusek and Zigas, 1957, Gajdusek, 1977). The clinical presentation of Kuru is characterised by cerebellar ataxia with relatively preserved cognition, preceded by headaches, joint pains and shaking of the limbs. The mechanism of transmission was identified as ritualistic cannibalism practices. No children born after 1959 have been affected. There is no evidence for vertical transmission of the disease, despite breast feeding of infants by clinically affected mothers (Liberski and Brown, 2009, Liberski et al., 2012). Since endocannibalism ceased in the late 1950s, the incidence of Kuru decreased dramatically. The last cases, mostly MV, appeared over 40 years after cessation of cannibalism, suggesting a very long incubation period (Collinge et al.,

2006). In contraposition, given that MM genotypes at codon 129 were initially diagnosed in the Fore population but then suffered a reduction on its frequency over time, a genetic susceptibility and a shorter incubation period for the MM genotype was suggested (Cervenakova et al., 1998, Collinge et al., 2006, Lee et al., 2001). Notably, a Kuru resistant factor has been reported in people who survived the epidemic in Papua New Guinea - those individuals harbouring the genetic variant G127V showed a lower predisposition to the disease (Mead et al., 2009).

3.1.3 PrP glycosylation and conformation

The presence of two potential N-glycosylation sites at the residues (Asn-181 and Asn-197 in humans and Asn-180 and Asn-196 in mice) allows PrP^C to be expressed as three different glycoform: non-glycosylated, mono-glycosylated or di-glycosylated (Haraguchi et al., 1989, Rudd et al., 2002, Rudd et al., 2001). These two glycosylation sites can be occupied by high-mannose type oligosaccharides attached to PrP in the endoplasmic reticulum before secretion.

In order to determine whether or not heterogeneity of the PrP^C glycoforms exist as a function of the brain region, DeArmon and colleagues used a two dimensional PAGE immunoblot and a panel of antibodies, showing differences in the relative concentration and isoelectric point of the PrP^C as a function of the hamster brain region. They hypothesised that the selective targeting of neuronal cell is relative to the affinity PrP^C / PrP^{Sc} modulated by differences in the PrP^C glycosylation (DeArmond et al., 1999). Differences in the proportion of glycans between PrP^C and the PrP^{Sc} have also been reported. Purification of PrP^C and PrP^{Sc} from Syrian hamster brain revealed a broad heterogeneity in their glycan populations between these two isoforms, with more than 50 different sugar chains attached. Presumably, this difference can be linked with a perturbation of the glycosylation machinery specifically associated with the galactosyl trans-ferase activity, and also glycoform selection (Rudd et al., 1999, Endo et al., 1989). Variation in the PrP^{Sc} glycoform ratio can also be observed when transmitting prions from one species to another. Analysis of brain stem tissue from sheep infected with scrapie and BSE showed variations in the relative amount of PrP^{res} glycoform, with a higher level of diglycosylated PrP^{res} and lower levels of the mono and unglycosylated form in the

samples derived from BSE-infected sheep compared with those derived from sheep with-scrapie (Thuring et al., 2004).

A clear example where the conformation of PrP^{Sc} has been employed as potential markers to distinguish between different strains has come from the studies of two hamster-adapted-TME strains termed hyper (HY) and drowsy (DY). Experimental transmission of TME to Syrian golden hamsters resulted in the divergence of two disease phenotype characterized by hyperexcitability and cerebellar ataxia (HY), and progressive lethargy (DY), respectively (Bessen and Marsh, 1992b). Biochemically these two strains can also be distinguished. Western blot analysis of PK resistant core fragment confirmed a 1 to 2kDa difference in the migration of the non-glycosylated band, showing a lower molecular weight band associated to DY PrP^{Sc}. Protein sequencing experiments (after proteinase K digestion) revealed that the major N-terminal cleave sites of DY PrP^{Sc} was positioned at least 10 amino acids residues further towards the C terminus prior to HY, suggesting potential conformational differences between these two strains (Bessen and Marsh, 1992a). In order to evaluate more in detail the possibility that these two isolates possess variations in the structure, Caughey and colleagues evaluated the conformation variability over three hamster adapted prion strains: HY, DY (TME-derived) and 263K (scrapie-derived). Fourier transform infrared spectroscopy analysis revealed post-translational modifications associated to DY and HY conformers which could explain the biochemical differences between these two isolates. Perhaps more importantly, the studies of HY and DY strains demonstrate that different conformers of the PrP^{Sc} are associated with distinct prion disease-phenotypes, providing an important

contribution to the understating of the current classification of the human prion disease.

3.1.3.1 Molecular typing of human prion diseases

The proteolytic degradation of the prion protein by proteinase K has been used as a fundamental tool to discriminate between the normal prion protein and the misfolded isoform. Likewise it has been used to distinguish between diverse prion strains and types. In the current classification of the human prion diseases, Western blotting analysis of the PK resistant core fragments of the PrP has allowed the distinction of two major prion types (type 1 and type 2), which differ in the relative molecular mass. (Cali et al., 2006, Gambetti et al., 2003, Parchi et al., 2012, Parchi et al., 1999b) The results of the combined analysis (Western blotting and proteinase cleavage) indicated that type 1 has a main cleavage site at glycine 82 thereby producing a N-terminal truncated fragment of about 21kDa, whereas type 2 is predominantly truncated at serine 97, generating a N-terminal truncated fragment of about 19kDa (Parchi et al., 2000b, Parchi et al., 2000a). Types 1 and 2 have intact C-termini in their protease-resistant core; however, in certain prion pathologies the presence of small fragments of about 8kDa fragments have been described. These fragments correspond to the central region of PrP[res] post-truncation of the N and C-termini by proteinase K. These PrP[res] fragments have been mainly described in some forms of GSS and in VPSPr (Zou et al., 2010, Parchi et al., 1997, Piccardo et al., 1998, Piccardo et al., 2001, Head et al., 2010, Head et al., 2013). As well as the electrophoretic mobility, the glycosylation ratios of the di, mono and non-glycosylated PrP[res] play a role structuring the actual classification of PrP[res] types. A glycoform ratio characterised by diglycosylated PrP[res] is designated by the suffix B. In contrast, the suffix A denotes a higher proportion of the monoglycosylated form. In addition, where the glycosylated bands (di and mono) are similarly abundant at the

expense of the non-glycosylated band they are designated with the suffix A/B (Parchi et al., 1997, Head, 2013, Head and Ironside, 2012b, Head and Ironside, 2012a). Figure 3.1 shows a schematic representation of the PrP^{res} profile of several human prion diseases reported so far.
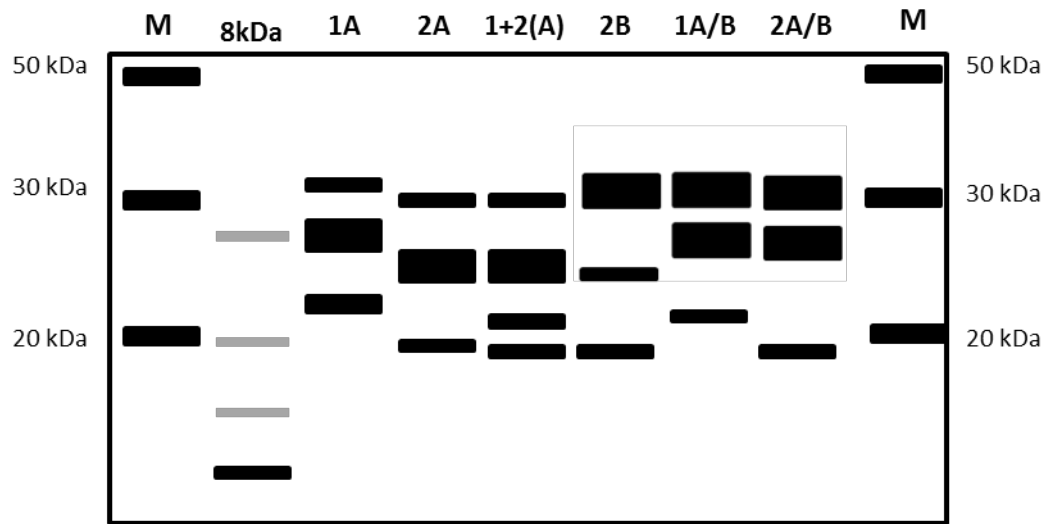
Figure 3.1 Schematic diagram of the spectrum of PrP^{res} fragments observed in human prion diseases.

3.1.4 PMCA and human prion replication

PMCA has been proven to be a very useful technique to investigate several aspect of the human prion replication. Jones and colleagues reported that PrP^{Sc} in extracts of sCJD could be amplified by PMCA using PrP^C from transgenic mouse brains by PMCA (Jones et al., 2008). They further showed that the efficiency of PrP^{Sc} conversion appeared to depend on the sCJD subtype and the *PRNP* codon 129 genotype of the PrP^C. However, the study was limited in scope and there is a need for a more accurate and detailed determination of the combined effects of substrate genotype and the human prion disease subtype of the seed on the efficiency of PrP^{Sc} conversion.

This chapter seeks to extend the investigation of the interaction of the *PRNP* codon 129 genotype (of the substrate) and the human prion disease type of the seed. PMCA was used as an *in vitro* model of the *in vivo* PrP^C to PrP^{Sc} conversion, hypothesised to be a central event in prion diseases. To achieve the aim, brains from three lines of humanized transgenic mice that differ only in the *PRNP* codon 129 genotypes (M/M, M/V or V/V) of the human prion protein, non-CJD human brain (of each genotype) and transfected 293F cells, served as sources of PrP^C. Brain tissue from vCJD, sCJD and atypical forms of human prion diseases served as source of PrP^{Sc} seed. The main objective was to determine for each subtype of human prion disease, substrate *PRNP* codon 129 genotypes that are compatibles and most efficient for the amplification of PrP^{Sc} by PMCA. Also, this chapter explores the *in vitro* conversion properties of non-standard PrP^{Sc} types, such as those found in GSS, FFI and VPSPr cases.

3.2 Material and methods:
3.2.1 Biological Samples

3.2.1.1 Human brain tissue

Human brain specimens were obtained at autopsy form UK cases referred to the National CJD Research and Surveillance Unit with consent for research. Selection criteria and ethical approval are described in General Material and Methods, section 2.1.2.1.

3.2.1.1.1 Human normal brain homogenate

Frontal cortex form non–CJD patient were used as a substrate for the PMCA experiments. A detailed list of the utilized non-CJD specimens is described in table 3.7.

3.2.1.1.2 Human prion disease brain homogenate

Brain homogenates used in this study were derived from patients with: sCJD, VPSPr, FFI, GSS, GSS with 8kDa fragment and vCJD. Detailed lists of the analysed PrP^{Sc} samples are shown in the following tables (Tables 3.3, 3.4, 3.5 and 3.6).

3.2.1.2 Humanized transgenic mouse brain tissue

The entire brain form inbred humanized transgenic mouse lines expressing the human from of the prion protein MM, MV and VV at the *PRNP* codon 129 (Bishop et al., 2006), were used for substrate preparation and PMCA reactions.

3.2.1.3 Human cells lines

Transfected human 293F cells, overexpressing a chimeric mouse-human prion protein (methionine or valine at the position codon 129 of the prion protein gene) were obtained by supressing the endogenous PrP protein expression with RNAi Subsequently, 293F cell were transfected with PIRESneo3-cDNA-quemier-*PNRP* expression vector. Cells were transfected and were grown by cell culture (Yokoyama et al., 2011).

3.2.2 Methods

3.2.2.1 Preparation of brain PMCA substrates

Human Brain and transgenic mouse brain PMCA substrates were prepared exactly as described in General Material and Methods (Chapter 2)

3.2.2.2 Preparation of cell PMCA substrates

Cell PMCA substrates was prepared as described in General Material and Methods (Chapter 2)

3.2.2.3 PMCA procedure

PMCA reactions were prepared exactly as described in General Material and Methods (Chapter 2)

3.2.2.4 Protease digestion and Western blot analysis

Western blotting experiments were prepared exactly as described in General Material and Methods (Chapter 2)

3.2.2.5 Densitometric Analysis

Densitometric analyses were performed using Image Lab software version 2.01, Bio Rad.

3.2.2.6 Data analysis

All statistical analysis, graphs and further analysis were completed using GraphPad Prisms 6.

Diagnosis (subtype)	Thesis Samples ID	PRNP codon 129 genotype	Brain region	PrP^{res} type
sCJD (MM1)	sCJD-1	MM	FC	1A
sCJD (MM1)	sCJD-2	MM	FC	1A
sCJD (MV1)	sCJD-3	MV	FC	1A
sCJD (MV1)	sCJD-4	MV	FC	1A
sCJD (MV1)	sCJD-5	MV	FC	1A
sCJD (MV1)	sCJD-6	MV	FC	1A
sCJD (MV1)	sCJD-7	MV	FC	1A
sCJD (VV1)	sCJD-8	VV	FC	1A
sCJD (VV2)	sCJD-9	VV	FC	2A
sCJD (MM2c)	sCJD-10	MM	FC	2A
sCJD MM2c)	sCJD-11	MM	FC	2A
sCJD (MM2t)	sCJD-12	MM	FC	2A
sCJD (MV2)	sCJD-13	MV	FC	2A
sCJD (MV2)	sCJD-14	MV	FC	2A
sCJD (MV2)	sCJD-15	MV	FC	2A
sCJD (MV2)	sCJD-16	MV	FC	2A
sCJD (MV2)	sCJD-17	MV	FC	2A
sCJD (VV2)	sCJD-18	VV	FC	2A
sCJD (VV2)	sCJD-19	VV	FC	2A

Table 3.3 Genetic and PrP^{Sc} biochemical information for the sporadic CJD cases and specimens selected for *in vitro* prion conversion (PrP^{Sc} source).

Diagnosis	Thesis Samples ID	PRNP codon 129 genotype	Brain region	PrP^{res} type
VPSPr	VPSPr-1	VV	FC	2A+8kDa
	VPSPr-1	VV	Cb	2A+8kDa
	VPSPr-2	VV	FC	2A+8kDa
	VPSPr-2	VV	Cb	2A

Table 3.4 Genetic and PrP^{Sc} biochemical information for the variably protease-sensitive prionopathy cases and specimens selected for *in vitro* prion conversion (PrP^{Sc} source).

Diagnosis	Thesis Samples ID	Brain region	PRNP Mutation	PRNP codon 129 genotype	PrP^{res} type
FFI	FFI-1	FC	D178N	MM	0
FFI	FFI-2	FC	D178N	MM	2
GSS	GSS-1	FC	P102L	MM	1A
GSS	GSS-2	FC	P102L	MM	1A+8kDa

3.5 Genetic and PrP^{Sc} biochemical information for the familial cases and specimens selected for *in vitro* prion conversion (PrP^{Sc} source). PrP^{res} type (0) corresponds to undetectable PrP^{res} using conventional means.

Diagnosis	Thesis Samples ID	PRNP codon 129 genotype	Brain region	PrP^{res} type
vCJD	vCJD-1	MM	FC	2B
vCJD	vCJD-2	MM	FC	2B

Table 3.6 Genetic and PrP^{Sc} biochemical information for the vCJD cases and specimens selected for *in vitro* prion conversion (PrP^{Sc} source).

Diagnosis	Thesis Samples ID	PRNP codon 129 genotype	Brain region	PrP^{res} type
Guillan-Barre syndrome	Substrate-1	MM	FC	0
Lewy body dementia	Substrate-2	VV	FC	0

Table 3.7 Diagnostic and genetic information of non-CJD cases and specimens selected for substrate (PrP^{C} source) preparation. PrP^{res} type (0) corresponds to undetectable PrP^{res} using conventional means.

3.3 Results

As suggested above, PMCA is an *in vitro* conversion technique that can be used to model aspects of prion replication. Modifications have been incorporated into this technique to analyse, for example, the convertibility of different substrates by various seeds; to increase the efficiency of the technique; to develop a diagnostic test or to explore the factors involved in prion conversion (Fujihara et al., 2009, Jones et al., 2007, Wang et al., 2010, Yokoyama et al., 2011).

The in *vitro* conversion process of PrP^C to PrP^{Sc} most likely depends on the molecular compatibility of the two isoforms, the competence of the substrate to support amplification (PrP^C source), the amount of seed ($PrPS^{Sc}$ source), and the chemical or physical conditions employed. First, in order to determine efficiency and reproducibility of PMCA for human prion seeds, humanized transgenic animals and post mortem brain homogenate substrates were used to evaluate the *in vitro* conversion of vCJD PrP^{Sc}.

3.3.1 *PRNP* codon 129 MM post mortem human brain homogenate and humanized transgenic mouse brain homogenate as a substrate for *in vitro* conversion using variant CJD brain homogenate as seed

Conversion of human PrP^C was analysed using substrate derived from either 1) post mortem brain tissue from non-CJD patients homozygous for methionine (MM) at codon 129 or 2) brain tissue from transgenic mice (Tg) expressing the human form of the prion protein (MM at codon 129 of *PRNP*). In both cases normal brain homogenate was prepared following the protocol described in the materials and methods section 2.1.4. Ten percent brain homogenate from human post mortem tissue or transgenic animals was mixed with different volumes of a 10% brain homogenate made from vCJD brain specimen (source of seed) (Figure 3.2). Serial dilutions of the seed: 1/5, 1/10, 1/20, and 1/40 (in 10% normal brain homogenate) were made. Seeded, non-sonicated samples were used as a "before" PMCA control (termed "Frozen" sample or "F"). After PMCA ("S" samples) and the "F" samples were treated with proteinase K (PK) and analysed by Western blotting (General Material and Methods, Chapter 2). Sonicated samples ("S" samples) showed higher levels of PrP^{res} compared to frozen samples incubated with post mortem tissue (Figure 3.2A) or Tg mouse brain homogenate (Figure 3.2B). Densitometric analysis of PrP^{res} of Western blots showed 10.2 fold amplification using non-CJD human brain homogenate and a 17.5 fold amplification using Tg mouse brain homogenate substrate (Figure 3.2A, lanes 7 and 8, Figure 3.2B, lanes 7 and 8). PrP^{res} formation was consistently more efficient when vCJD PrP^{Sc} seed sample were amplified with brain homogenate from transgenic mice compared with post mortem human brain (Figure 3.2B).
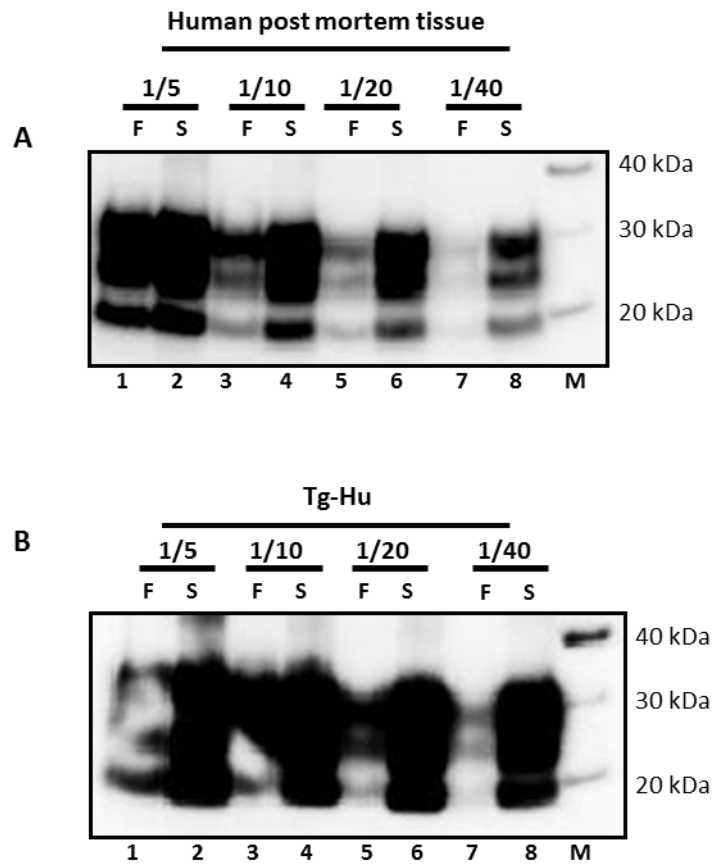
Figure 3.2 Serial dilutions of vCJD brain homogenate seed mixed with two different PMCA substrates: A: Post mortem tissue from non-CJD patients (MM at codon 129 of *PRNP*). **B:** Transgenic mice expressing the human form of the prion protein, homozygous for methionine (MM) at codon 129. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.2 Reproducibility of PMCA for vCJD seeds

To analysis the reproducibility of the PMCA technique, two independent sets of experiments were carried out (Figure 3.3A and B):

1. To evaluate the efficiency of amplification over two times points (called experiment 1 and 2) serial dilutions of vCJD seeds (1/20, 1/100, 1/500, 1/2500 and 1/100, 1/500, 1/2500, 1/12500) were mixed with 10% Tg mouse (MM) brain homogenate and subjected to rounds of incubation and sonication for 96 PMCA cycles (following the protocol described in General Material and Methods, Chapter 2). Densitometric analysis showed an amplification rate around 15.5 to 16.5 fold (Figure 3.3A, Experiment 1) compared with around 15.7 to 16.2 fold calculated for the samples in the experiment 2. The determination of the PMCA amplification rates were following the procedures described in the section: PMCA normalization and quantification in General Material and Methods, Chapter 2. (Figure 3.3A).

2. To analyse the reproducibility between different machines, a second S-4000 sonicator was used in parallel with similar settings applied for the experiment 2. Sonicator B showed an amplification rate around 17 to 18 fold higher intensity in the sonicated samples compared with the non-sonicated samples (Figure 3.3B, lanes 2, 4, 6 and 8).

These results therefore demonstrated good reproducibility for PMCA using two different machines between two independent experiments.
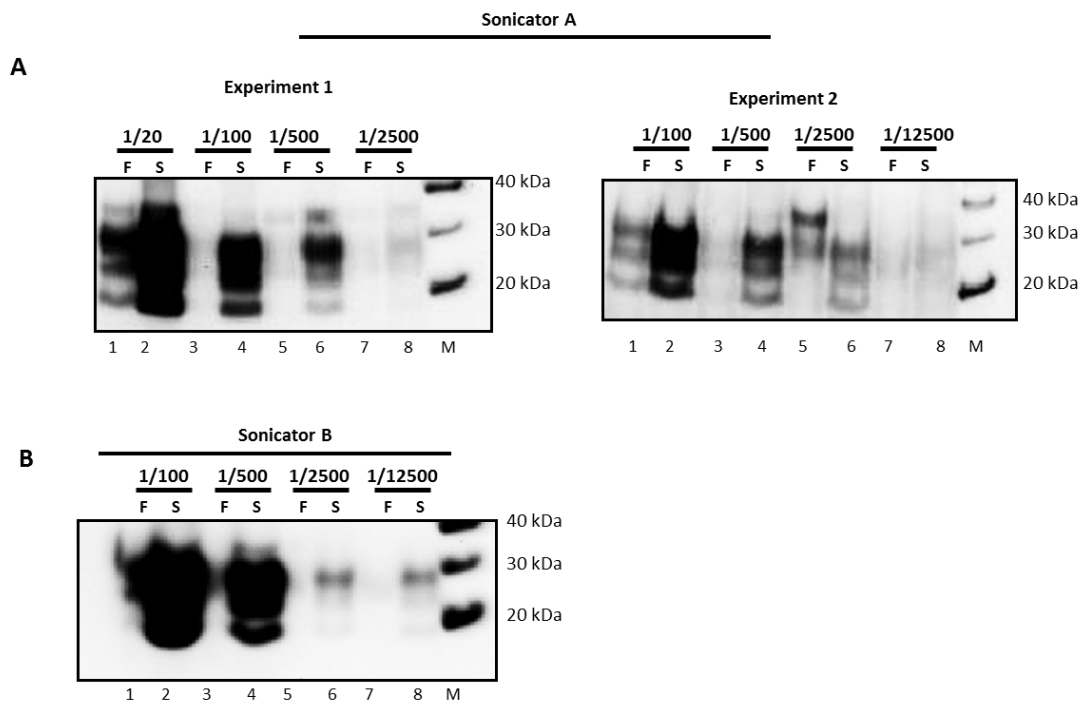
Figure 3.3 Reproducibility of PMCA for vCJD seed. A: amplification carried out over two times points (Experiment 1 and 2). **B**: To analyse reproducibility between independent machines, a different S-4000 sonicator was used in parallel to the experiment 2. The dilution 1/500 (for each experiment) was used to calculate the PMCA efficiency. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.3 *In vitro* conversion of vCJD PrP^{Sc} using human brain homogenate, humanized transgenic mouse and 293F cells extract substrates

To analyse the *in vitro* conversion capacities of vCJD using three different sources of PrP^C, 10% human brain homogenate, 10% humanized transgenic, and 20% 293F cells extract, were seeded, with vCJD and 96 cycles of PMCA were conducted. vCJD seed (10% brain homogenate) was diluted 1/50 and 1/250 in all three substrates. After 96 cycles of PMCA, the human post mortem substrate showed increased levels of PrP^{res} present in the sonicated samples compared with the equivalent non-sonicated samples. Densitometric analysis of PrP^{res} on Western blots showed an *in vitro* amplification of around 10 fold for the human post mortem substrate (Figure 3.4, lanes 2 and 4). The humanized transgenic mice substrate showed higher levels of amplification compared with the human post mortem tissue with an amplification factor of around 21 fold (Figure 3.4, lanes 6 and 8). The 293F cells extract showed an intermediate amplification rate with around 16 fold (Figure 3.4, lanes 10 and 12). Irrespective to the origin, all substrates showed an increase of signal across the serial dilution preformed for the seed. The transgenic mice substrate gave the highest levels of amplification, followed by the 293F cell substrate, in turn followed by the human post mortem tissue substrate. The determination of the PMCA amplification efficiency followed the procedures described in the section: PMCA normalization and quantification in General Material and Methods, Chapter 2.
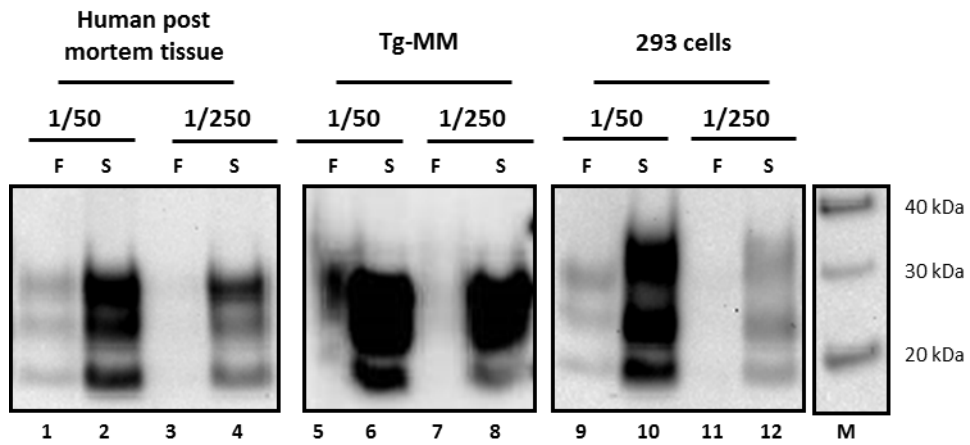
Figure 3.4 Amplification of vCJD PrP^{Sc} using human brain homogenate, humanized transgenic mice and 293F cells extract. A: PMCA seeded with serial dilutions of vCJD PrP^{Sc} (1/50 and 1/250 regarding the 10% brain homogenate). The seed was mixed with three different substrates and amplified for 96 cycles of PMCA. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.4 *In vitro* conversion of sCJD PrP^{Sc} subtypes in human brain homogenate, humanized transgenic mice and 293F cells extract

Six clinic-pathological subtypes of sCJD have been identified (MM1 and MV1, MM2-thalamic, MM2-cortical, MV2, VV1 and VV2) that correlate with the PrP^{res} electrophoretic mobility / glycosylation ratios, and the polymorphic status at codon 129 of the *PRNP* gene.

In order to evaluate the ability to support *in vitro* amplification, brain homogenates from the six different subtypes of sCJD were mixed with different sources of PrP^{C}. The seed and substrate compatibility (in terms polymorphic variation associated with codon 129: MM MV and VV) were tested for their *in vitro* conversion capacities.

3.3.4.1 *In vitro* conversion of sCJD MM1, MM2c and MM2t PrP^{Sc} subtypes in human brain homogenate, humanized transgenic mice and 293F cells extract

In order to determine the potential *in vitro* conversion capacities of sCJD MM1, MM2c and MM2t, three distinct sources of PrP^{C} (all carriers of the amino acid methionine at codon 129 of *PRNP* gene) were mixed with PrP^{Sc} derived from sCJDMM1, MM2c and MM2t brain specimens. To evaluate the PrP^{Sc} amplification, serial dilutions of the seeds (10% brain homogenate) were performed. For sCJD MM1 and MM2c dilutions 1/10 - 1/50 were used, for sCJD MM2t, 1/3 - 1/6. Substrate prepared form human post mortem brain, transgenic mouse brain and 293F cell were utilized. After 96 PMCA cycles, sCJDMM1 amplification reactions showed PrP^{res} formation on both dilutions (Figure 3.5A, lanes 2, 4, 6, 8, 10 and 12 compared with lanes 1, 3, 5, 7, 9 and 11). Independent of the origin of the PrP^{c}, all

substrates supported the amplification of the sCJD MM1 PrP^{Sc} seeds. Densitometric analysis showed an amplification factor of around 4 fold using the human brain substrate. The transgenic mice derived substrate show an amplification factor of around 5 fold; follow by the 4 fold for the 293F cells extract.

sCJD MM2t did not show an increase of signal at any of the seeding dilutions when incubated with human brain homogenate or humanized transgenic substrate (Figure 3.5B, lanes 2, 4 and 6). However a small increase of signal was detected on the seed dilution 1/3 (Figure 3.5B, lane 8) in the cells extract substrate. This observation was not seen in the following dilution (Figure 3.5B, lanes 9 and 10), or in a duplicate experiment. These results confirmed the lack of amplification presented by sCJD MM2c.

In summary, among the three homozygous methionine PrP^{Sc} subtypes analysed, only sCJD MM1 showed increased levels of PrP^{res} formation by PMCA. The other two PrP^{Sc} subtypes did not result in detectable *in vitro* conversion of human PrP^{C}.
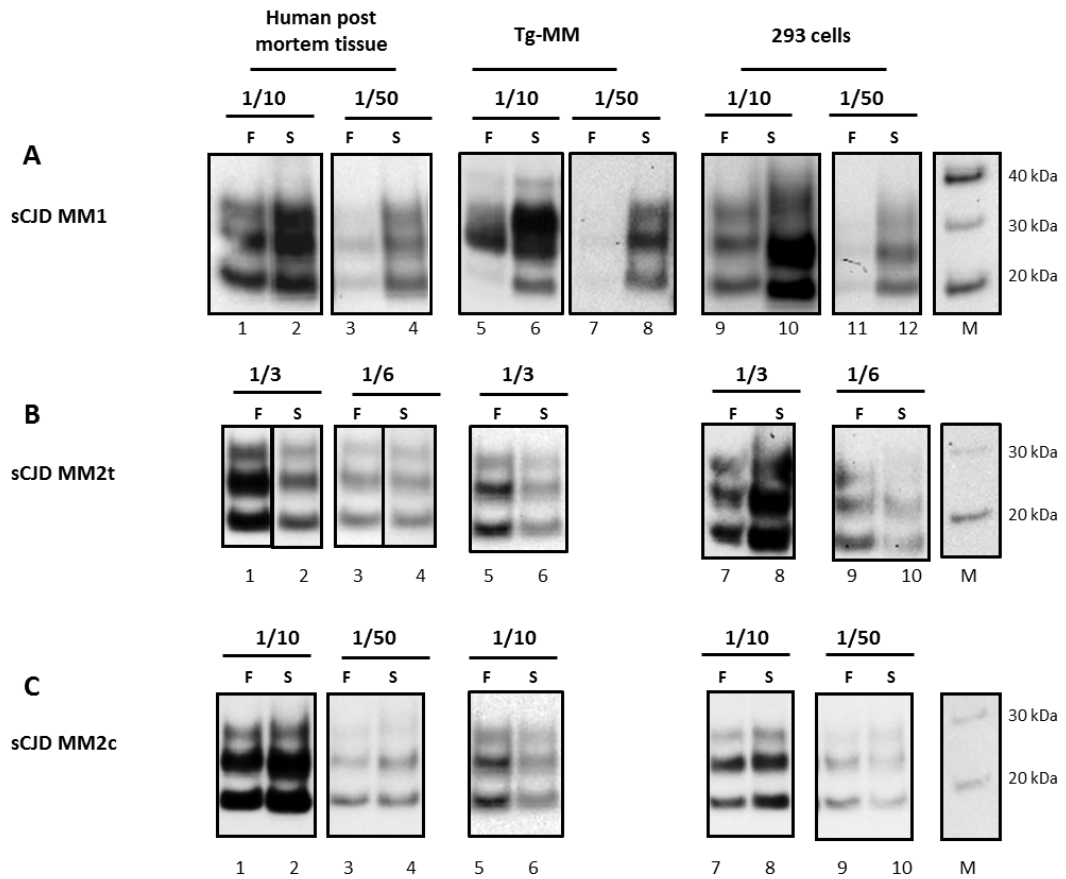
Figure 3.5 PMCA reaction seeded with sCJD MM1, MM2c and MM2t and supplemented with human brain homogenate, humanized transgenic mice and 293F cell extract. Serial dilutions of **(A)** sCJD MM1 (1/10 and 1/50), **(B)** MM2t (1/3 and 1/6) and **(C)** MM2tc (1/10 and 1/50) were incubated with three independent sources of PrP^C: 10% human brain homogenate, 10% humanized transgenic mice and 20% 293F extract, all codifying methionine at the codon 129 of the *PRNP* gene. PMCA reactions seeded with sCJD MM2t 1/3 and 1/6 (corresponding to the Human post mortem tissue substrate) were blot from the same gel and developed under the same exposure settings, however they do not correspond to consecutive lanes. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.4.2 *In vitro* **conversion of sCJD VV1 and VV2 PrP^{Sc} subtypes in human brain homogenate, humanized transgenic mice and 293F cells extract**

To investigate the potential *in vitro* conversion of sCJD VV1 or sCJDVV2 PrP^{Sc} *in vitro*, similar experiments to those previous described with MM subtypes were performed. Brain homogenate from patients with sCJD VV1 and sCJDVV2 were serially diluted (1/3 - 1/6 or 1/20 - 1/40 respectively) in the three different sources of the human PrP^{C}. Substrate prepared from human brain, transgenic mouse brain or 293F cell extract were used. Considering the polymorphic compatibility of seed / substrate, all substrates were carriers of valine at codon 129 of *PRNP* gene. Reactions seeded with sCJD VV1, incubated with human brain homogenate (Figure 3.6A, lanes 2 and 4), humanized transgenic mice (Figure 3.6A lanes 6 and 8), or the transfected cells (Figure 3.6A, lanes 10 and 12) did not show any increase PrP^{res} over the unamplified samples. PMCA reactions seeded with sCJD VV2 in human brain post mortem tissue, showed increased levels of PrP^{res} on both serial dilutions in the sonicated samples (Figure 3.6B, lanes 2 and 4). Densitometric analysis showed a 7 fold increase for human post mortem brain substrate. The remaining two substrates also supported *in vitro* amplification, with amplification rates of around 11 fold for the transgenic mouse derived substrate (Figure 3.6B, lanes 6 and 8) and around 8 fold for the cell cultures substrate (Figure 3.6B, lanes 10 and 12).

Contrary to what was observed for to the *in vitro* conversion reactions seeded with sCJD MM subtypes, which amplified type 1 but not type 2, the homozygous valine sCJD subtypes supported amplification on the sCJD VV2 seeds, and failed to support *in vitro* conversion of the sCJD VV1 PrP^{Sc}.
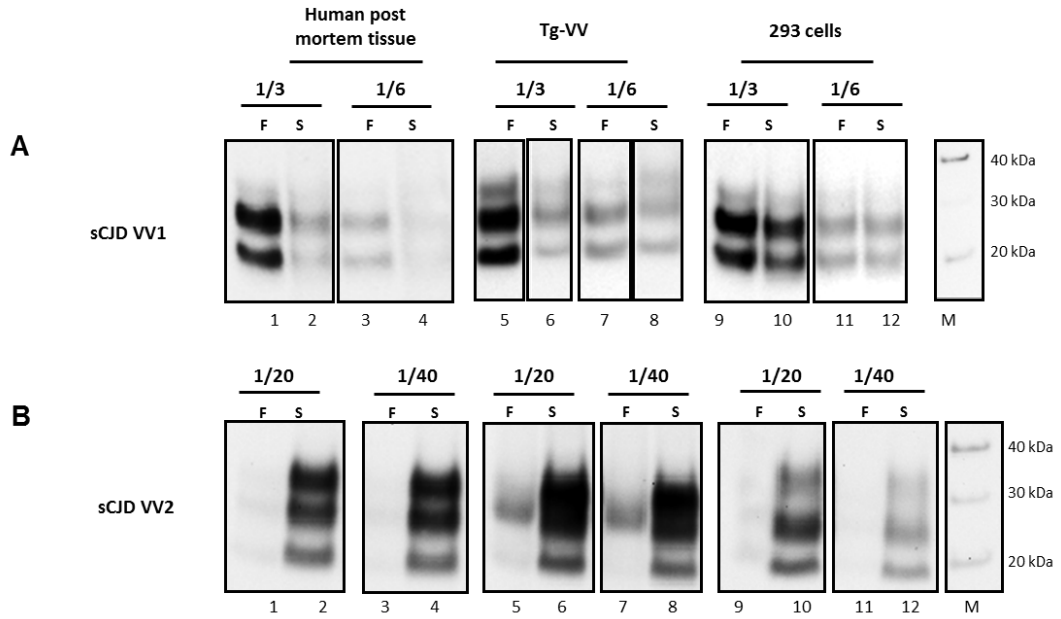
Figure 3.6 PMCA reactions seeded with sCJD VV1, VV2 and supplemented with human brain homogenate, humanized transgenic mice and 293F cell extract. Serial dilutions of **(A)** sCJD VV1 (1/3 and 1/6), **(B)** VV2 (1/20 and 1/40) were incubated with three independent sources of PrP^C: 10% human brain homogenate, 10% humanized transgenic mice and 20% 293F cell extract. All substrates coded for the amino acid valine at the codon 129 of the *PRNP* gene. PMCA reactions seeded with sCJD VV1 1/3 and 1/6 (corresponding to the Tg-VV substrate) were blot from the same gel and developed under the same exposure settings, however they has not correspond to consecutive lanes. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.4.3 *In vitro* conversion of sCJD MV1 and MV2 PrP^{Sc} subtypes in human brain homogenate and humanized transgenic mice

To evaluate the capacities of heterozygous sCJD MV seeds to propagate in PMCA, five sCJD MV1 and five MV2 cases were used (See details in Table 3.3 Material and methods, Chapter 3). Normal brain homogenate derived from human post mortem tissue and transgenic mice brain, both expressing the heterozygous (MV) human PrP^C, were incubated and sonicated for 96 cycles of PMCA. sCJD MV1 and MV2 seeds were dilution 1/12. Different sCJD MV1 seeds incubated with 10% post mortem human brain homogenate showed variable amplification rates. In one case amplification was undetectable. Low to moderate levels of amplification were detected to the other four cases, with an amplification factor of around 2 to 4 fold (Figure 3.7A, lane 2). The transgenic mice brain substrate gave similar results (Figure 3.7B, lane 4).

The PMCA reactions seeded with MV2 PrP^{Sc} gave similar results to those observed for sCJD MV1. Low levels of amplification were detected, with variable amplification rate between 2 to nearly 4 fold, independent of the source of PrP^C (human post mortem or transgenic mice brain substrate) (Figure 3.7B, lane 2 and 4). Representative blots are shown in Figure 3.7.

Figure 3.7 *In vitro* conversion reaction seeded with sCJD MV1 and MV2 PrP^{Sc} — rendered as:
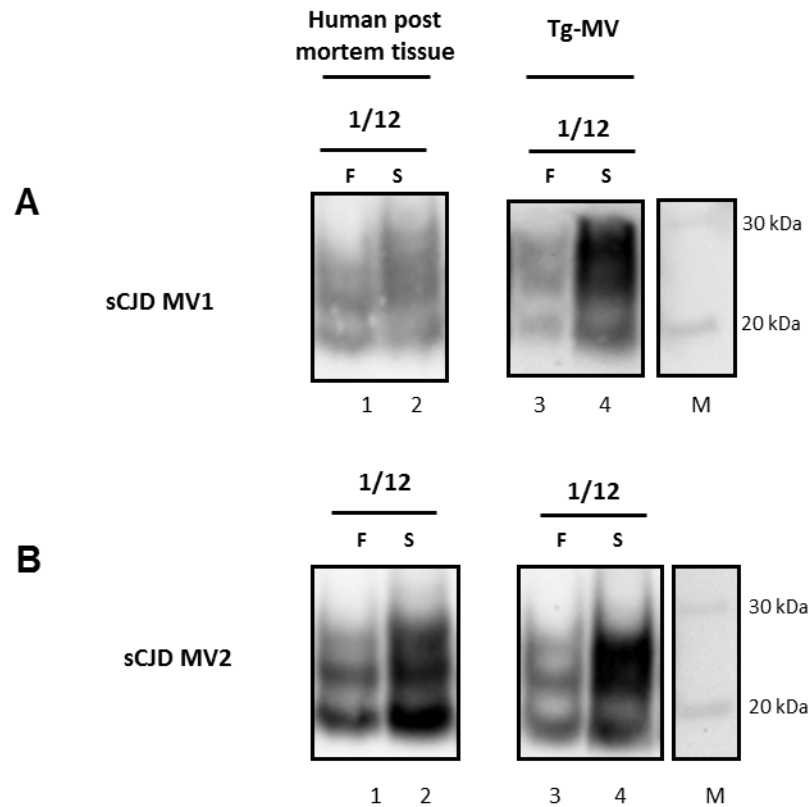
Figure 3.7 *In vitro* conversion reaction seeded with sCJD MV1 and MV2 PrP^{Sc} in heterozygous *PRNP* codon 129 human brain homogenate and humanized transgenic mice substrates. Dilution of the 10% brain homogenate (1/12) from **(A)** sCJD MV1, **(B)** sCJD MV2 were incubated with two sources of PrP^{C}: 10% human brain homogenate, 10% humanized transgenic mice. The two sources of PrP^{C} were heterozygous (MV) at the codon 129 of the *PRNP* gene. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.5 *In vitro* amplification properties of vCJD and sCJD: MM1, MV1, MV2, VV2 conversion of human PrP^C of different codon 129 genotypes

The amplification of vCJD, sCJD MM1 and VV2 will be discussed in the following section 3.3.5.1. The amplification of sCJD MV1 and MV2 will be discussed in the section 3.3.5.2.

3.3.5.1 *In vitro* amplification properties of vCJD, sCJD MM1 and sCJD VV2 to convert the human PrP^C of different codon 129 genotypes

All of the PMCA reactions described above were performed using seeds and substrates matched for *PRNP* codon 129. In order to evaluate the *in vitro* conversion capacities of the PrP^{Sc} that previously supported the human PrP^C conversion, but using different codon 129 genotype substrates, homogenate from vCJD, sCJD MM1 and sCJD VV2 brain specimens were selected, diluted and amplification attempted. Normal human post mortem tissue and transgenic mice brain, expressing the human prion protein (in the three possible polymorphic forms: MM, MV, VV) were used as a source of PrP^C. PrP^{Sc} seeds were diluted as follows: vCJD 1/100, sCJD MM1 and VV2 were diluted 1/40 into human post mortem brain substrate. Considering the highest efficiency observed for PMCA reactions (incubated with transgenic mice brain substrate), a higher dilution of the vCJD PrP^{Sc} seed was performed (1/1000). For the sCJD seeds (MM1 and VV2), the dilution 1/40 was maintained.

After PMCA, samples incubated with homozygous (129 MM) post mortem substrate and seeded with vCJD, showed high levels of PrP^{res} formation (Figure 3.8A, lane 2). Densitometric analysis of PrP^{res} on Western blot showed an amplification rate over 10 times fold with the compatible seed / substrate allele (129 MM). A dramatic

decrease in the PrP^{res} formation was observed when the heterozygous MV or the homozygous VV substrates were similarly seeded (Figure 3.8A, lanes 4 and 6). Amplification efficiency of around 2.5 to 3.5 fold were observed for the MV and VV substrates respectively (Figure 3.8A). Using transgenic mice substrate, similar results were observed. vCJD PrP^{Sc} seeds showed *in vitro* conversion when incubated with MM substrate (Figure 3.8D, lane 2), with a decrease of conversion efficiency in MV and VV PMCA substrates (Figure 3.8D, lanes 4 and 6). Densitometric analyse demonstrated around 3 to 4 times lower PrP^{res} formation on the samples incubated with the heterozygous MV substrate, with a further decrease of intensity in the VV PrP^{C} substrate (Figure3.8D).

As expected, sCJD MM1 seeds showed lower levels of *in vitro* conversion compared with vCJD seeds. The homologous (MM) seed / substrate PMCA reactions showed a similar amplification factor between 3 to 4 fold using the human brain (Figure 3.8B, lane 2), and the transgenic mice substrate (Figure 3.8E, lane 2). Amplification rate around 2 to 3 fold were observed for the MV substrate on both, the transgenic and human post mortem homogenate (Figure 3.8 and E, lane 4). The VV substrates showed amplification rate around 1.5 to 2.5 fold for the post mortem substrate, and the transgenic mice substrate respectively (Figure 3.8B and E, lane 6).

The sCJD VV2 seeds resulted in PrP^{res} formation into all three allelic substrates (Figure 3.8C and F, lanes 2 4 and 6), however a preference for the codon 129 (VV) substrate was observed (Figure 3.8C and F, lane 6). After PMCA, higher levels of PrP^{res} formation were detected for the samples incubated with the VV human or transgenic brain substrate, with amplification rate around 6 to 10 fold respectively (Figure 3.8C and F).

In summary, the amplification efficiency of vCJD PrP^{Sc} appears to depend upon the allelic compatibility at the codon 129. This was also observed for the PMCA reactions seeded with sCJD MM1 in which the favoured substrate was homozygous for methionine at position 129 in PrP^{C} and in samples seeded with sCJD VV2, in which the favoured substrates was homozygous for valine at position 129 in PrP^{C}.

Human post mortem tissue

A vCJD

B sCJD MM1

C sCJD VV2

Tg-Hu

D vCJD
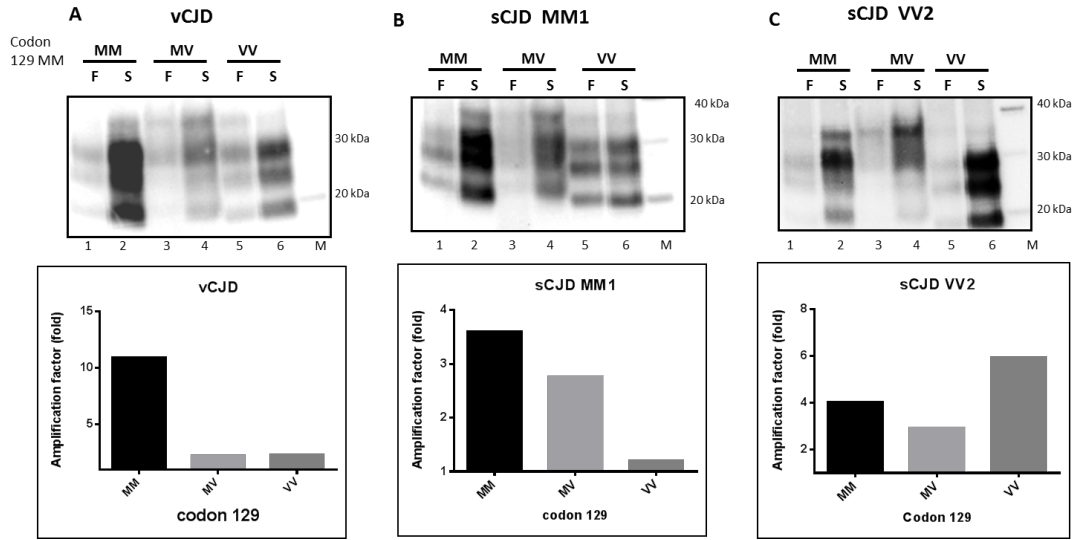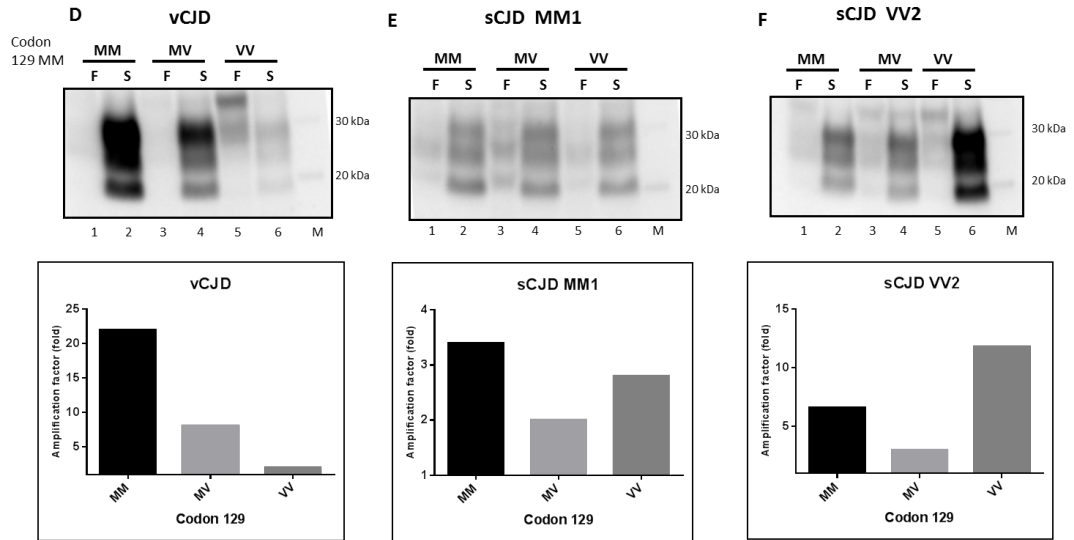
E sCJD MM1

F sCJD VV2

Figure 3.8 PMCA using vCJD, sCJD MM1 and sCJD VV2 subtypes to convert the human PrP^C carrying the three different codon 129 genotypes. Dilutions of **(A)** vCJD 1/100, **(B)** sCJD MM1 1/40, **(C)** sCJD VV2 1/40 were incubated with two different sources of PrP^C: 10% human brain homogenate, 10% humanized transgenic brain homogenate. The two different substrates expressed the human prion protein in the three possible polymorphic genotypes: MM, MV, VV. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb. Densitometric analyses of PrP^{res} on Western blot were performed following the procedure described in General Material and Methods (Chapter 2). The graphics representations of the PMCA amplification efficiency were performed using GraphPad Prisms 6. The (Y) axis represents the amplification factor (in fold of amplification), the (X) axis shown the three human polymorphic substrates (codon 129): MM, MV, VV, utilized for the PMCA reactions.

3.3.5.2 *In vitro* amplification properties of sCJD MV1 and sCJD MV2 to convert the human PrP^C of different codon 129 genotypes

Previous experiments indicated that the *in vitro* conversion efficiency of human PrP^C was affected by compatibility between the codon 129 status of seed and substrate. In order to evaluate the *in vitro* conversion capabilities of heterozygous (codon 129 MV) sCJD PrP^{Sc} seeds to convert the human prion protein, sCJD MV1 and MV2 subtype brain homogenates were used to seed PMCA reactions. Specimens from five sporadic CJD MV1 and five MV2 subtype cases were homogenized and diluted in each humanized transgenic (MM, MV, VV) substrate. After 96 cycles of PMCA, sCJD MV1 brain homogenate incubated with MM 129 codon substrate, showed conversion, with an amplification factor of around 2 to nearly 5 fold and with an average of 3.2 fold (Figure 3.9A). The heterozygous MV codon 129 substrate showed a lower amplification; with one fold less efficacy compared to the homozygous MM substrate. Even lower efficiency was seen in the reaction performed using the homozygous VV substrate (Figure 3.9A). An opposite tendency was observed in the samples seeded with sCJD MV2, in which the average amplification for the reactions incubated with 129 codon MM substrate was 2 fold (Figure 3.9B). Finally, the PMCA reactions incubated with substrate homozygous for valine (VV) at the codon 129 showed the highest levels of amplification (between 1.7 to 8.5 fold), with an average of 4 fold, which is twice as efficient as that of the comparable MM substrate (Figure 3.9B).

The results therefore indicate that at least two major effects influence the conversion of the human PrP^C in sCJD cases: the PrP^C / PrP^{Sc} polymorphic variability presented by the codon 129, and the PrP^{Sc} type. Example blots are shown in Figure 3.9.
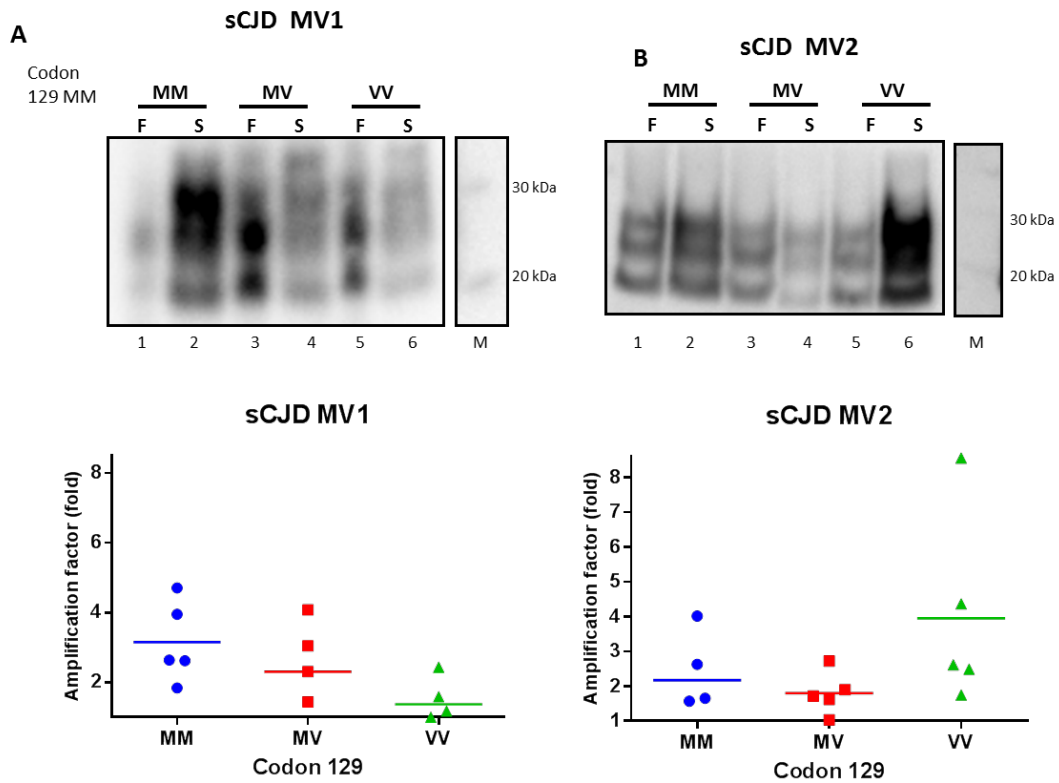
Figure 3.9 PMCA using sCJD MV1 and MV2 seeds to convert the human PrP^C carrying distinct codon 129 genotypes Five **(A)** sCJD MV1, and five **(B) s**CJD MV2 cases were homogenized, mixed and incubated in each polymorphic substrate (MM, MV, VV) for 96 cycles of PMCA. Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb. Densitometric analyses of PrP^{res} on Western blot were performed following the procedure described in General Material and Methods (Chapter 2). The graphics are representations of the PMCA amplification efficiency and were performed using GraphPad Prisms 6. The (Y) axis represents the amplification factor (in fold amplification), the (X) axis shown the three polymorphic codon 129 substrates utilized for *in vitro* amplification: Codon 129 MM (blue), MV (red), VV (green). The horizontal lane (-) for each group represents the means of value. Immunoblots presented in the figure A and B are examples of the western blot analysis.

3.3.6 In vitro conversion properties of other PrP^{Sc} types

In order to test whether PrP^{Sc} from different human prion diseases are able to convert the normal human prion protein, genetic and idiopathic prion disease cases were selected to perform *in vitro* conversion assay. Samples form brain specimens of Gerstmann-Straussler-Scheinker syndrome, and its variant with ~8kDa fragment PrP^{res} fragment, fatal familial insomnia and variably protease sensitive prionopathy were homogenised and used as seeds in PMCA. Prior to amplification, all disease brain homogenates (seeds) were characterized for PrP^{res} abundance and type.

3.3.6.1 Characterization of human prion diseases brain homogenate

To characterize total PrP and PrP^{res} in the seeds used for the *in vitro* conversion reactions, tissue from: one case of GSS, one case of GSS with 8kDa fragment, two cases of FFI and two cases of VPSPr, were homogenized in conversion buffer, loaded on SDS gels, and analysed by Western blot. To confirm previously reported differences in PrP^{res} type from different brain regions, tissue from frontal cortex and from the cerebellum in the VPSPr cases were selected (Figure 3.10A). The total PrP levels were evaluated in the form of bands located in the molecular mass range of 20-40 kDa, probably corresponding to the diglycosylated full length PrP and the monoglycosylated PrP or N-terminally truncated diglycosylated form of the human prion protein. Differences in the total PrP levels were quantified by densitometric analysis. When the seed samples were compared with the unaffected prion diseases control, around 60-80% less total PrP was observed in the FFI samples (Figure 3.10A, lanes 1 and 2). Similar levels of total PrP were observed in frontal cortex and cerebellum tissue of VPSPr samples derived from the same individual (Figure 3.10A,

lanes 5-6 and 7-8). However, clear differences were noted after to treat the samples with proteinase K. Near to undetectable levels of PrP^{res} were seen in the FFI samples (Figure 3.10B, lanes 1 and 2), and the VPSPr frontal cortex samples (Figure 3.10B, lanes 6 and 8). In contrast, PrP^{res} was more abundant in VPSPr cerebellum tissue (Figure 3.10B, lanes 7 and 9) and the GSS tissue (Figure 3.10B, lanes 3 and 4). GSS with 8kDa fragment sample, and the VPSPr frontal cortex brain homogenate showed the appearance of a low molecular weight band (~8kDa) characteristic of these two prion pathologies (Figure 3.10B).
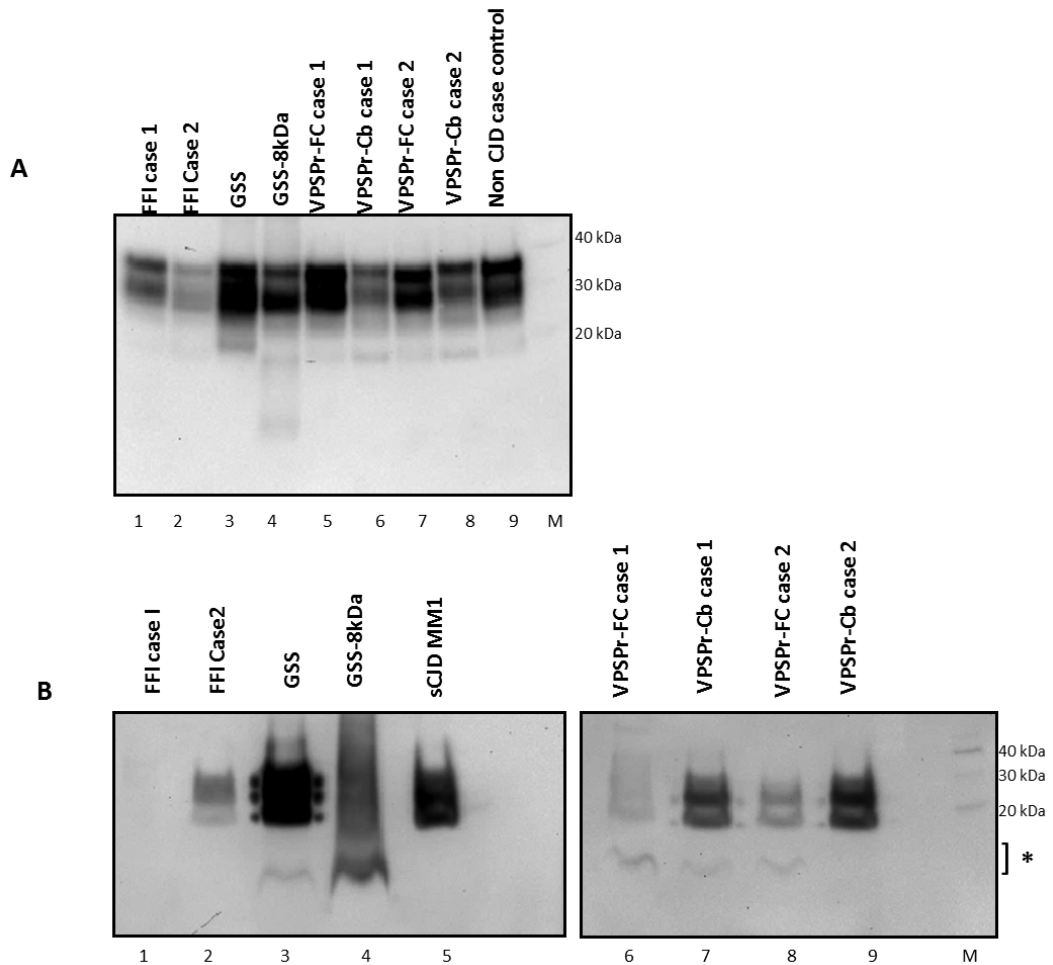
Figure 3.10 Characterization of total PrP and PrP^{res} in the FFI, GSS, VPSPr samples. (A) One µl of 10% brain homogenate for each human prion disease related samples (lanes 1 to 8) were loaded in parallel to an unaffected prion disease control (lane 9) for comparative purposes. **(B)** A total of 25µl of 10% brain homogenate for each human prion disease related sample were treated with 50µg/ml PK for one hour at 37ºC and analysed by Western blot. Fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker syndrome (GSS), Gerstmann-Straussler-Scheinker syndrome variant with 8kDa fragment (GSS-8kDa), variably protease sensitive prionopathy (VPSPr), forntal cortex (FC) and Cerebellum (Cb). Low molecular weight band around 8kDa (*). M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.6.2 Amplification of GSS, GSS with 8kDa fragment and FFI PrP^{Sc}

Little is known about the abilities of these non-standard PrP^{Sc} types to convert human PrP^{C} *in vitro*. Therefore, two FFI and two GSS cases (one with 8kDa fragment) were used to evaluate the *in vitro* behaviour of PrP^{Sc} to convert human prion protein substrate, derived from human post mortem tissue and transgenic mouse brain, both MM at codon 129 (and without mutations in the *PRNP* coding sequence). The seeds were diluted 1/10 in the substrates. After 96 cycles of PMCA, the human post mortem tissue substrate seeded with GSS type 1 PrP^{res} showed an increase in PrP^{res} whereas the reaction seeded with GSS with 8kDa fragment did not show an increase in signal (Figure 3.11A, lanes 7 and 9). Repeating the same experiment, but using transgenic mouse substrate, gave similar results. The transgenic substrate confirmed the amplification of the GSS sample; however, GSS with 8kDa fragment did not show increased levels of PrP^{res} (Figure 3.11B, lanes 7 and 9). Three independent experiments confirmed the low levels and the lack of PrP^{res} formation detected after PMCA and Western blotting by GSS and GSS with 8kDa fragment seeds respectively.

Unexpectedly, one of the FFI seeded samples (case 2) showed higher levels of PrP^{res} formation with a clear PrP^{res} fragments after PK treatment in reactions incubated with human post mortem substrate (*PRNP* codon 129 MM) (Figure 3.11A, lane 5). The amplification reaction seeded with the FFI case 1 brain homogenate did not show increased levels of PrP^{res} (Figure 3.11A, lane 3). PMCA using transgenic mice substrate (*PRNP* codon 129 MM) showed a clear PrP^{res} amplification in the reaction seeded FFI case 1 (Figure 3.11B, lane 3), however, an apparent lack of amplification

for the case 2 (Figure 3.11B, lane 5). This difference between the two FFI cases was

seen in three independent PMCA experiments.
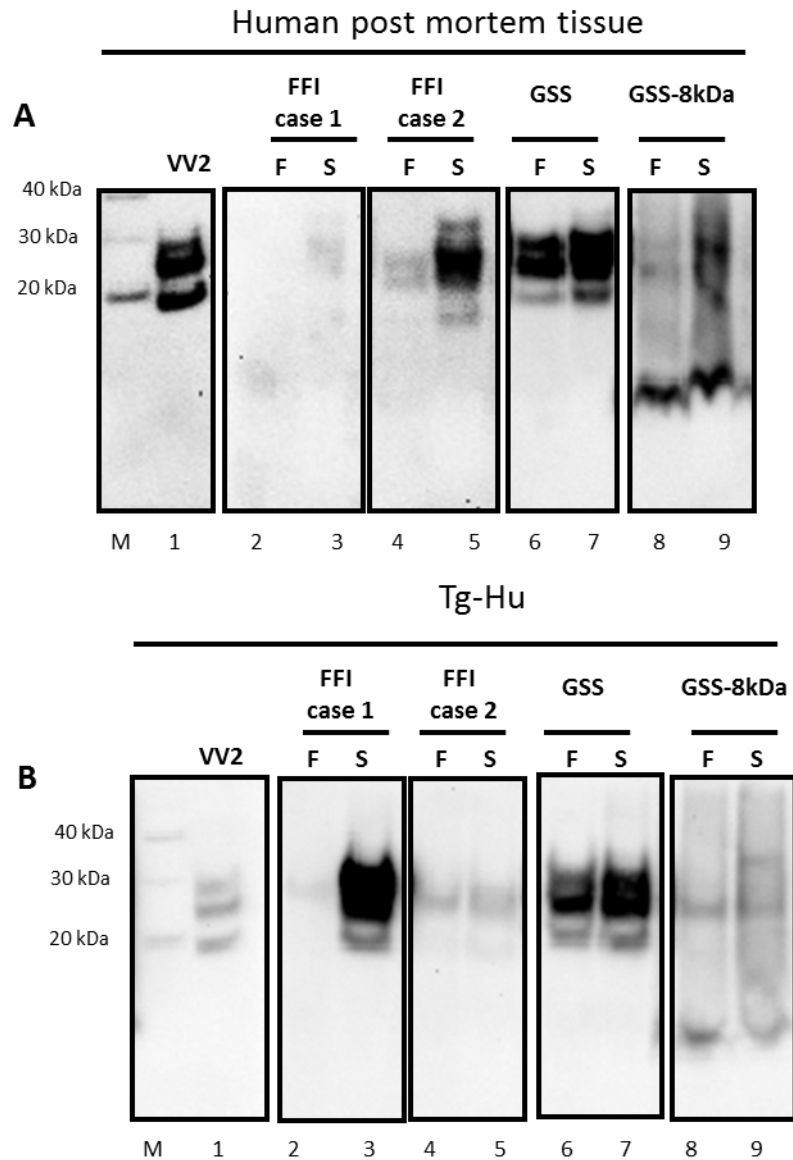
Figure 3.11 PMCA reactions seeded with homogenates of FFI, GSS and GSS with 8kDa fragment brain tissue. PMCA reactions seeded with brain homogenate diluted 1/10 of FFI (2 cases), GSS and GSS with 8kDa fragment were incubated with human brain substrate and transgenic mouse brain substrate, both *PRNP* codon 129 MM genotype and subjected to 96 cycles of amplification. VV2 brain homogenate (2μl) was used as a PrP[res] type standard reference. Fatal familial insomnia (FFI), Gerstmann-Straussler-Scheinker syndrome (GSS), Gerstmann-Straussler-Scheinker syndrome variant with 8kDa fragment (GSS-8kDa) F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.6.3 Amplification of FFI-PMCA products in a second round of PMCA

In order to determine whether the FFI-PMCA material retained or lost the ability to convert human PrP^C, the two FFI-PMCA-products (Figure 3.11B, lanes 3 and 5), were diluted 1/10 in fresh transgenic mice substrate (codon 129 MM) and subjected to a second round of PMCA. After 96 PMCA cycles, case 1 retained the ability to convert fresh PrP^C (Figure 3.12, lane 2). Interestingly, FFI case 2 showed clear PrP^{res} formation in the sonicated sample compared to the frozen one, in contrast to what was observed in the first PMCA round using the same substrate (Figure 3.12, lane 6 compared to lane 7) and (Figure 3.11B, lane 5). Several technical and biochemical factors might have contributed to this blocking effect. The presence of an inhibitory component present in the original homogenate might have been diluted in the second round (1/10), causing the release of the inhibition. Additional experiments are needed to clarify this point. More important is the observation that both FFI-PMCA products propagate, and have the ability to convert PrP^C *in vitro*, producing PrP^{res}.
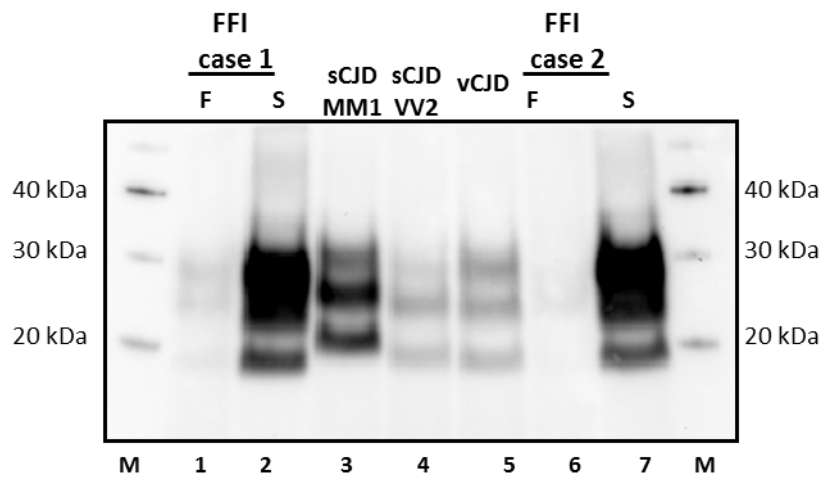
Figure 3.12 Conversion properties of FFI amplification products in a second round of PMCA. The two FFI-PMCA products (cases 1 and 2), generated from a previous round of amplification, were incubated with fresh transgenic mouse substrate expressing the human form of the prion protein homozygous for methionine (MM) at codon 129 and subjected to a second round of PMCA. sCJD MM1, sCJD VV2 and vCJD were used as a PrP^{res} type standard reference. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.6.4 Molecular typing of the PMCA- generated FFI product

Western blots analysis of the PrP^{res} fragments derived from FFI specimens, have previously shown the presence of two major bands that correspond to the diglycosylated and monoglycosylated protease resistant fragment to predominate. The non-glycosylated band is under-represented, but has a molecular mass of 19 kDa (ie type 2). Two FFI cases were used to test the ability of FFI PrP^{Sc} to propagate *in vitro,* both with positive results. In order to determine the molecular type of the FFI-PMCA generated material, three of the most common forms of human prion diseases were used as reference standards. sCJD MM1, sCJD VV2 and vCJD brain homogenate were analysed by Western blot in parallel with the FFI-PMCA products (Figure 3.13) . In term of electrophoretic mobility, both FFI-PMCA samples showed three major PrP^{res} fragments, with an apparent molecular mass of the non-glycosylated PrP^{res} fragment of around 19kDa. In term of glycosylation profile, the diglycosylated PrP^{res} predominated over the mono- and non-glycosylated fragments. In summary, the FFI-PMCA generated material showed an electrophoretic mobility and glycosylation pattern that resembles type 2B.
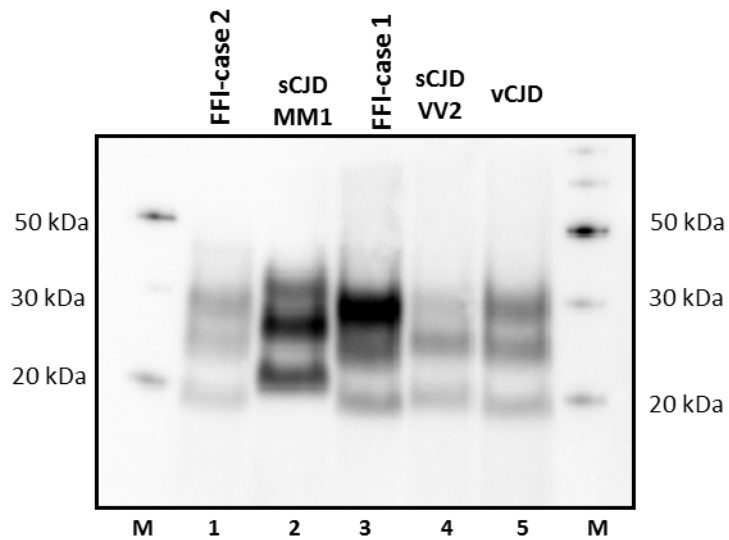
Figure 3.13 Molecular typing of FFI-PMCA products: comparison with sCJD MM1, sCJDVV2 and vCJD standards. The two FFI-PMCA products (cases 1 and 2) were treated with proteinase K (50μg/ml) and analysed by Western blotting. sCJD MM1 (lane 2), sCJD VV2 (lane 4) and vCJD (lane 5) were used as a PrP^{res} type standard reference. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.6.5 Comparison of the susceptibility of the FFI PrP^{Sc} and FFI-PMCA products to PK degradation

A PK titration assay was performed to compare the sensitivity of PrP^{Sc} from the FFI (brain tissue) and that found in the PMCA products to proteolytic digestion. Similar quantities (19µl) of 10% brain homogenate from brain tissue of both FFI cases and their FFI-PMCA generated products were treated for 60 min with 50, 10, 2.5 and 1µg/ml of PK. The protease sensitivity of PrP in brain tissue and the resultant PMCA product appeared to differ. PrP digestion was complete when 50 µg/ml of PK was used with the human tissue brain homogenate. Whereas when the same PK concentration was used to digest the PMCA product detectable PrP^{res} remained (Figure 3.14A and B, lane 1 compared to lane 6). Whether this increase in PK resistance is the result of misfolding of wild-type human PrP^{C} (as opposed to mutant PrP) is not clear, and requires further investigation.
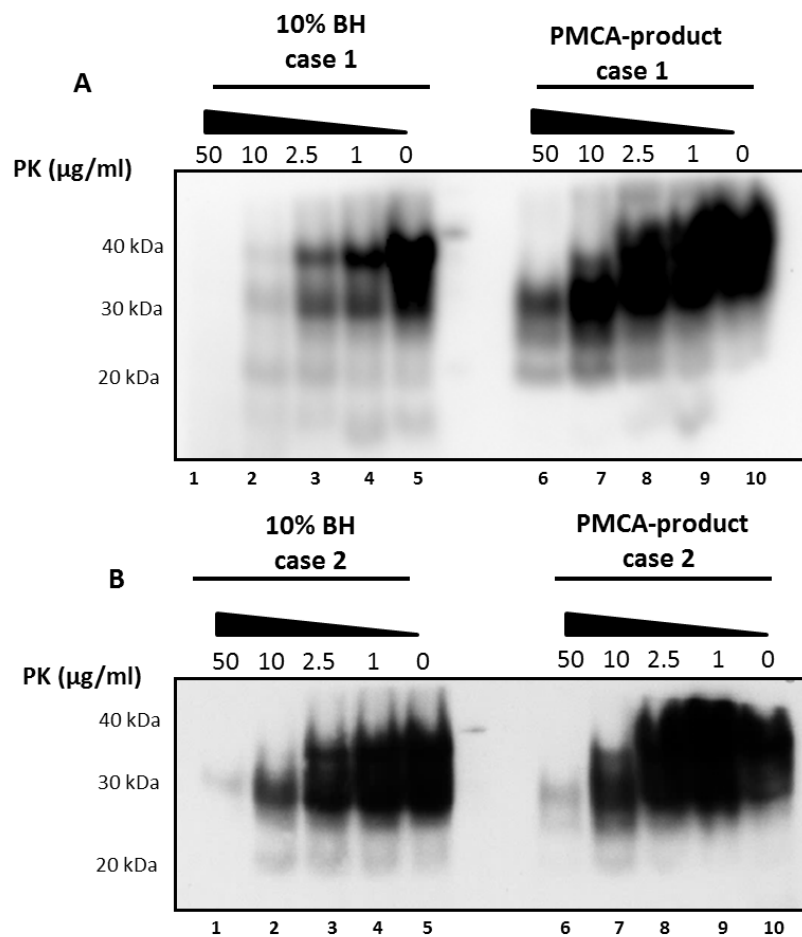
Figure 3.14 Protease resistance profile of PrP^res associated with FFI brain homogenate and FFI-PMCA products. Aliquots of brain homogenate 10% brain homogenate derived from FFI cases or the amplification products seeded with the same material (19µl) were incubated for 60 min at 37ºC with different concentrations of PK (50, 10, 2.5, 1µg/ml and non PK). All samples products were detected by Western blotting analysis **(A)** Comparative protease resistance profile observed for the FFI case 1 and **(B)** for the FFI case 2. BH: brain homogenate. M: Molecular Marker. Immunodetection by 3F4 mAb.

3.3.6.6 *In vitro* conversion of VPSPr PrP^{Sc}

Variably protease-sensitive proteinopathy (VPSPr) is a rare human prion disease characterized by the presence of a very particular form of PrP^{Sc} that is poorly resistant to proteolytic digestion. After PK treatment and Western blot analysis, VPSPr samples show a faint ladder of bands into the range of 18-30kDa, with a prominent and characteristic band of 7-8kDa. Thus far very little is known about the molecular mechanisms associated with the conversion of the PrP^C to PrP^{Sc} in VPSPr patients. In order to evaluate the amplification properties of VPSPr PrP^{Sc}, two VPSPr cases (from which two different brain region were tested: frontal cortex and cerebellum) were homogenized, and incubated in human post mortem brain or transgenic mice substrate and subjected to 96 cycles of PMCA. Both substrates contained valine at codon 129 of the prion protein gene (*PRNP*) to match that of the VPSPr seeds.

Independent of the origin of the substrate, PrP^{res} amplification was observed in the samples seeded with cerebellar (Figure 3.15A and B, lanes 4 and 8), and showing a clear diglycosylated PrP^{res} that was largely absent in the non-amplified samples (Figure 3.15A and B, lanes 3 and 7). Frontal cortex seeds did not result in obvious amplification (Figure 3.15A and B, lanes 2 and 6). The characteristic 8kDa band, present in the frontal cortex and cerebellum of case 1, and in the frontal cortex of case 2, did not show evident increase of PrP^{res} signal, suggesting a lack of amplification for that particular form of PrP^{Sc}.
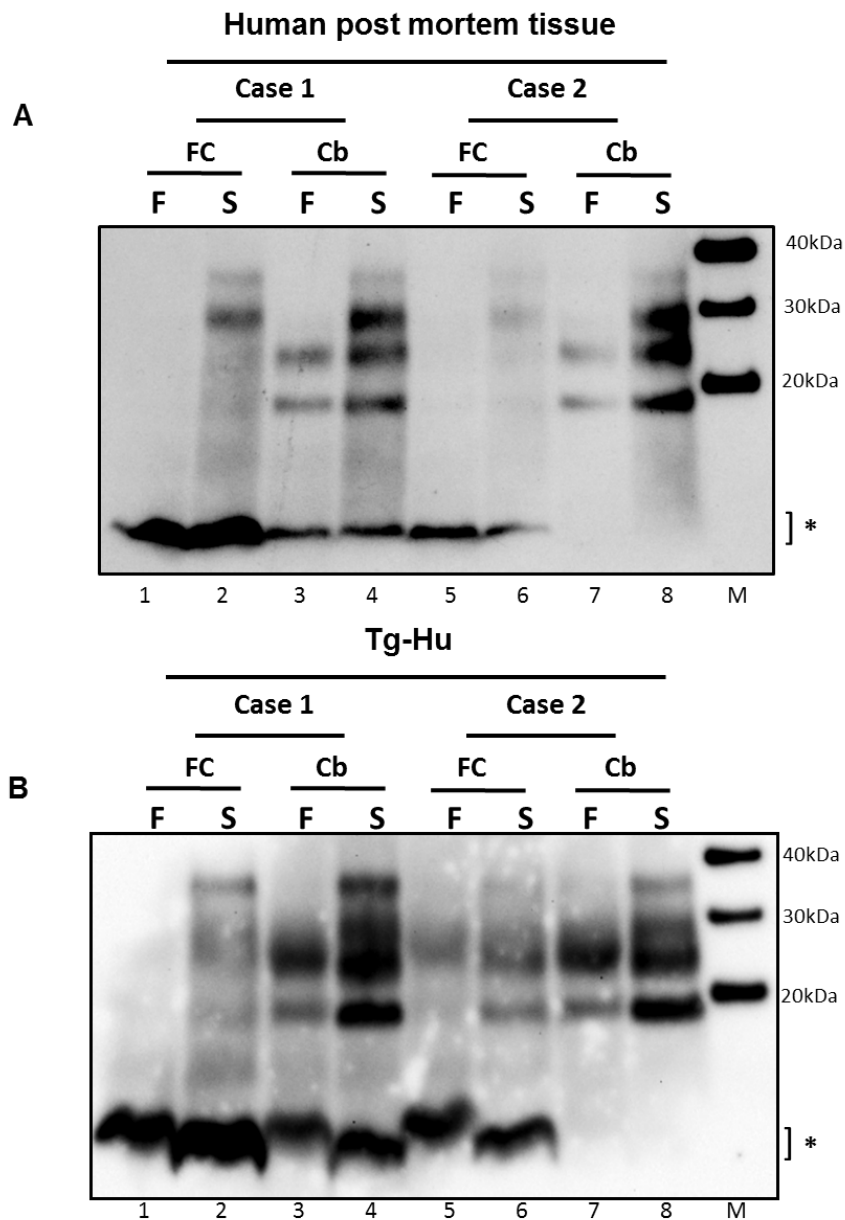
Figure 3.15 Amplification reactions using VPSPr samples. Brain homogenate prepared from two VPSPr cases (Cases 1 and 2) diluted 1/5 and incubated with 10% brain homogenate from: **(A)** post mortem tissue and **(B)** transgenic mouse homogenate, both expressing the human form of the prion protein homozygous for valine at codon 129 (VV), were subjected to 96 cycles of PMCA. The VPSPr seeds were derived from two regions of the brain, frontal cortex (FC) and cerebellum (Cb). Amplified samples are represented by (S for sonicated) and non-amplified by (F for frozen). Low molecular weight band (*). M: Molecular Marker. Immunodetection by 3F4 mAb.

3.4 Discussion

3.4.1 Overview

The present chapter addresses of the complexity and heterogeneity of human prion diseases. Mutations, polymorphic variation of the human prion protein gene, types or subtypes are just some aspects of the extensive variability of this multifaceted disease.

The present chapter aimed to assess the ability of PrP^{Sc} of different human prion diseases to convert the native human PrP^{C} *in vitro* to its abnormal, disease-associated isoform PrP^{Sc}. The main objective was to determine, for each subtype of sCJD, the substrate *PRNP* codon 129 genotypes, that are most efficient for the amplification of PrP^{res}. Also this chapter explored the *in vitro* conversion properties of non-standard PrP^{Sc} types, such as those found in GSS, FFI and VPSPr cases, to propagate *in vitro*. First, the susceptibility of the human PrP^{C}, along the efficacy and reproducibility of vCJD PrP^{Sc} *in vitro* conversion was considered.

3.4.2 Susceptibility of human PrP^{C} to be converted *in vitro* by vCJD, sCJD, GSS, FFI and VPSPr PrP^{Sc}

3.4.2.1 *In vitro* conversion activity of vCJD PrP^{Sc} in PMCA

Diverse sources of the human PrP protein can be used to mimic the *in vitro* conversion process of PrP^{C} into PrP^{Sc}. Previously, Jones and colleagues reported the competence of humanized transgenic mouse brain and human post mortem brain tissue homogenates to act as substrates for the amplification of vCJD and sCJD PrP^{Sc} seeds in a cell-free conversion system (Jones et al., 2007, Jones et al., 2008). A ten

fold increase in vCJD PrP^{res} was achieved by Jones and colleagues after each round of PMCA (Jones et al., 2009).

For the purpose of this thesis, the evaluation of substrates (MM at position 129) derived from non-CJD post mortem tissue and humanized transgenic mice, showed around 10 and 17 fold amplification respectively after 96 cycles of PMCA. Several factors may explain the difference in the amplification efficiency, including the delay between deaths and post mortem, cell lysis and proteinase activity, which could be expected to diminish the efficiency of the human brain substrate.

Several groups have used PMCA to address a variety of questions concerning prion conversion (Jones et al., 2007, Saborio et al., 2001, Wang et al., 2010, Yokoyama et al., 2011). To evaluate the reproducibility of PMCA technique used in this thesis, two sets of experiment were performed to examine two variables, 1) experiment-to-experiment variation (two independent experiments) and 2) equipment effects (two different Misonix S-4000 sonicators). Similar amplification levels were observed for two independent experiment seeded with vCJD showing around 15 and 16 fold amplification respectively. Variations of between 1 and 2 fold amplification were observed when comparing two different sonicators (Figure 3.3A and B). It should be noted that one of the machines had been used more than the other and anecdotal evidence implicates both a "running in" effect and a later time-dependent loss of potency applying to this equipment. Considering the relative difference in amplification rates, this data suggests an acceptable reproducibility of PMCA conversion between independent experiments and the use of different machines.

After evaluating the amplification of vCJD, sCJD seeds were used to assess the ability, compatibility and susceptibility of human PrP^C to convert *in vitro* by different sCJD PrP^{Sc} subtypes.

3.4.2.2 *In vitro* conversion activity of sCJD PrP^{Sc} subtypes in PMCA

The most common form of CJD is the sporadic, representing around 85% of the disease in humans (Ironside, 1998). In order to evaluate the ability to support amplification, all sCJD subtypes (MM, MV1, MM2-thalamic, MM2-cortical, MV2, VV1 and VV2) were used as a seeds. These seeds were diluted in substrate from three different sources (human brain homogenate, humanized transgenic mice and 293F cells) prior to PMCA. All three subtypes supported in *vitro* conversion, however not all seed types were found to amplify in each substrate. The reactions seeded with two sCJD MM1 cases showed a moderate amplification with around 4 to 5 fold amplification after 96 cycles of PMCA. No amplifications were detected for the samples seeded with brain homogenate derived from one MM2t and two MM2c sCJD cases. Jones and colleagues reported the *in vitro* conversion of sCJD MM1, sCJD MM2c and MM2t seeds, and showed its dependence on a compatible *PRNP* codon 129 genotype of the substrate source of PrP^C (Jones et al., 2008). The results presented in this chapter have confirmed the observations of Jones and colleagues for the amplification of sCJD MM1 seed, but presented some differences over the conversion of sCJD MM2t / MM2c seeds.

Amplification of PrP^{res} was detected in PMCA reactions seeded with two confirmed sCJD VV2 cases, with an amplification rate in the range of 6 to 11 fold. In contrast to what was observed for the amplification of sCJD MM1 seeds, a single case of sCJD

VV1 failed to amplify in any of the substrates used. PMCA reactions seeded with five sCJD MV1 and MV2 cases showed low levels of amplification when compared to the MM1 or VV2 seeds.

Although the amplification of sCJD MM1, VV2, MV1 and MV2 is in agreement with the results reported by Jones and colleague, the lack of amplification of sCJD MM2t / MM2c and VV1 PrP^{Sc} suggests certain degree of contradictions. The use of different amplification protocols might be part of the explanation of these differences. It is now well recognized that minor differences in experimental protocols can have dramatic effects on amplification efficiency, for example the use of heparin, inclusion of beads, and the power delivered by different sonicators (Barria et al., 2011, Deleault et al., 2007, Jones et al., 2007, Makarava et al., 2013, Wang et al., 2010, Yokoyama et al., 2011). The protocol used by Jones and colleagues (Jones et al., 2009), exhibited certain degree of differences to the PMCA procedure used here. The use of different PrP^{Sc} samples could account for part of this discrepancy. An important point to note is that the transgenic substrate (PrP^{C}) used in both protocols were derived from the same strain of transgenic animals. The apparent lack of conversion seed in human PrP^{C} when seeded with sCJD MM2t / MM2c and VV1 PrP^{Sc} were consistent using three different sources of substrates.

According to the current subclassification of sCJD (proposed by Parchi and colleagues), the majority of the sCJD cases correspond to the subtypes MM1/MV1 and VV2, accounting for 70 and 16% of the cases respectively. The remaining subtypes, the MV2, MM2 (thalamic and cortical) and VV1 account for 14% of the cases.

(Parchi et al., 1999b). Attempting to draw a parallel with the sCJD incidence reported by Parchi and colleagues, the results presented here suggest that the PrP^{Sc} types present in the most common sCJD subtypes have a better ability to convert *in vitro*, whereas the less common subtype has a poor amplification potential. sCJD MM1 presented a moderate amplification potential with around 4 to 5 fold of conversion. sCJD MV1 seeds instead showed low level of amplification (2 to 4 fold). The VV2 PrP^{Sc} seeds showed the highest level of *in vitro* amplification with around 10 fold. sCJD MV2 PrP^{Sc} seeds showed little levels of PrP^{C} conversion (2 to 4 fold), whereas the other three seeds (sCJD MMt, sCJD MMc and sCJD VV1) did not show any signs of conversion. This might be taken to suggest that the amplification procedure utilized over the course of this thesis reflects the distribution of sCJD subtypes, and also suggests that PrP^{Sc} *in vitro* conversion properties may correlate with the *in vivo* conversion and account in part for the aforementioned distribution.

sCJD MM1 PrP^{Sc} seeds amplified efficiently in MM substrate, but less so in MV or VV PrP^{C} containing substrates, which confirms the observation of Jones and colleagues. A similar result was observed for reactions seeded with sCJD VV2, which showed greater compatibility with the VV substrate, some amplification with MV, and less with MM. More interestingly, MV1 PrP^{Sc} seeds showed a trend towards more efficient amplification in MM substrates compared to VV substrates, whereas MV2 PrP^{Sc} displayed a preference for VV, rather than MM at the codon 129. The factors that govern this molecular inclination remain unclear but could include the structural conformation of the PrP^{C} / PrP^{Sc} sCJD subtypes. In terms of PrP^{C}, a close similarity was found by Hosszu and colleagues on the stability and conformation of the two allelic forms of PrP^{C}, suggesting that the difference of the

molecular behaviour of the two conformations is related more to later states of $PrP^C \rightarrow PrP^{Sc}$ conversion rather than to the native state of the protein (Hosszu et al., 2004). In terms of PrP^{Sc}, differences in the dynamic of prion propagation and susceptibility of conversion have been described *in vivo* and *in vitro* for all three codon 129 alleles (Tahiri-Alaoui et al., 2004, Lewis et al., 2006, Baskakov et al., 2005, Head and Ironside, 2012a, Bishop et al., 2010).

Inoculation of examples of each of the six sCJD subgroups (MM1, MM2, MV1, MV2, VV1 and VV2) to a panel of transgenic mice which express the three polymorphic form of the prion proteins (MM, MV and VV) revealed that all sCJD subtypes were transmissible to at least in one transgenic mice genotype, and according to the animal transmission properties, it was possible to discriminate four sCJD strains: M1 (which together correspond to the subtypes MM1 and MV1), V2 (that corresponded to the subtypes VV2 and MV2), M2 (corresponded to MM2 subtypes), and V1 (that corresponded to the VV1 subtypes) (Bishop et al., 2010). Using brain homogenate (a source of PrP^C) from the same transgenic mouse strains employed in the above mention study, *in vitro* propagation experiments exhibit a certain degree of resemblance with this classification. The PMCA reactions seeded with MM1 and MV1 sCJD seeds presented similar in *vitro* amplification efficiency, within the range of 3 to 5 fold amplification (low to moderate), both seeds grouped as M1 in the transmission study. The M2 and V1 strains, which correspond to the sCJD MM2 and VV1 showed no clinical disease and very low death rate across the three mice lines. *In vitro*, these two subgroups showed a lack of *in vitro* conversion. The strain V2 showed a short incubation period over the mice line homozygous for valine, with a much longer incubation in the other two transgenic models (MM and

MV). Similarly, PMCA reactions seeded with MV2 and VV2 exhibited moderate to robust amplification efficiency when the reactions possessed substrate valine homozygous at the codon 129, presenting also a reduction of the amplification potential by being incubated into the other two polymorphic substrates. These results support the association between codon 129 and the two forms of PrP^{res} founded in the CJD cases, the type 1 and 2. Data also reinforce the observation in which type 1 sCJD has an intrinsic bias to convert MM substrates, contrasting to type 2 which displays the trend toward its counterpart valine. Biochemical analysis of 41 patients with sCJD showed discrimination of four distinct subgroups, that fit as well with the strain classification previously mentioned (Uro-Coste et al., 2008).

From a molecular perspective, the discrepancy of the in *vitro* conversion capacities observed for sCJD MM1 and MM2 to propagate *in vitro* could be explained by mechanistic and structural properties of each PrP^{Sc} subtype (Haldiman et al., 2013, Kabir and Safar, 2014, Kim et al., 2011, Kim et al., 2012, Safar, 2012). *In vitro* conversion studies using sCJD MM1 and MM2 revealed an inverse correlation between *in vitro* conversion efficiency and the presence of small aggregates of PrP^{Sc} (protease sensitive oligomers), which potentially correlate with the duration of the disease (Kim et al., 2012). These results could be considered as a potential explanation for the molecular behaviour of sCJD MM1 and MM2 in PMCA. However it remains to be explained why the reverse effect is observed for sCJD VV1 and VV2 seed, which presented the highest level of amplification by the sCJD type 2. If the presence of valine at the codon 129 has an impact on this molecular trend, it needs to be further investigated.

Overall, the results presented in this chapter confirm the ability of this *in vitro* conversion system to recapitulate aspects of prion strain replication, particularly those associated to the molecular interaction between the prion agent and the host genotype.

3.4.2.3 *In vitro* conversion activity of GSS, FFI and VPSPr PrP^{Sc} in PMCA

Soto and colleagues reported that PrP^{Sc} generated *in vitro* by protein misfolding cyclic amplification, maintains the biochemical and the biological properties of the original seeds. Comparison of the glycoform ratio and the electrophoretic mobility after PK digestion and deglycosylation between brain-derived and PMCA-generated PrP^{Sc} for each strains showed that the biochemical features were maintained upon *in vitro* propagation (Castilla et al., 2008b).

To evaluate for the first time the *in vitro* conversion properties of non-standard PrP^{Sc} types such as those found in GSS, FFI and VPSPr cases, PMCA experiments were performed with samples from these rare human prion diseases. The GSS PrP^{Sc} did show an increase of PrP^{res} formation using either normal human post mortem brain tissue or using humanized transgenic substrate (both methionine homozygous at codon 129 of *PRNP*). However, under the same experimental conditions GSS with 8 kDa fragment seed did not convert. The FFI samples showed PrP^{res} formation after PMCA, with a predominantly diglycosylated profile, characteristic of type 2B and similar to that of the seed material. PMCA products derived from FFI amplification exhibit PrP^{res} formation after a second round of PMCA, thereby confirming their capability to propagate *in vitro*. Previously Soto and colleagues reported that even following multiple rounds of PMCA the *in vitro* generated PrP^{res} has identical biochemical and structural properties to the original seed (Castilla et al., 2008b, Castilla et al., 2005). However, during the *in vitro* propagation of FFI samples, the PrP^{res} type produced by the *in vitro* propagation was maintained, but the product acquired a higher degree of protease resistance. This may be because the amplification product was now wild-type human prion protein rather than the mutant

form. Genetic prion diseases are usually heterozygous for the causative mutation and PrP^{Sc} contributions from both the wild-type and mutated forms of the prion protein are possible. Chen and colleagues examined the allelic origin of the misfolded form of the human prion protein in the FFI brain. They observed that the majority of PrP^{res} produced in FFI cases derives from the mutated PrP isoform, with only a small contribution from the wild-type protein. In contrast, the genetic condition familial Creutzfeldt-Jakob disease, which present the same mutation (D178N) but carrying valine at the codon 129, seems to be constituted of both the mutated and the wild-type misfolded isoforms (Chen et al., 1997).

The observation presented in this chapter opens the possibility of an interesting interaction among the mutated FFI PrP^{Sc} recruiting wild-type PrP^{C} to finally produce PrP^{res}. To have a better understanding, PMCA and systematic evaluation of mutated and wild-types seed and substrates - with their respective polymorphic variations - (D178N mutations / M129V polymorphisms) need to be performed.

To assess whether VPSPr PrP^{Sc} has *in vitro* conversion potential, 96 cycles of PMCA were performed. Samples prepared form frontal cortex and cerebellum form two VPSPr cases served as the seed. After PMCA, VPSPr PrP^{Sc} samples showed an increase in PrP^{res}, but only of the higher molecular mass forms found in cerebellum rather than the 7-8kDa PrP^{res} fragments found in frontal cortex and cerebellum. An unexpected change in the PrP^{res} glycosylation profile was also found (when seed was compared with PMCA product) with the appearance of the diglycosylated form in cerebellar samples. There was no evident amplification for the small fragment (8kDa) presented in the majority of the VPSPr cases.

It is noteworthy that the form of VPSPr PrP^{Sc} that is amplified in PMCA resembles that of sCJD (PrP^{res} type 2) whereas the form of PrP^{Sc} that does not amplify in PMCA resembles that of GSS (PrP^{res} with 8kDa). VPSPr has been proposed (rightly or wrongly) to be the sporadic form of GSS (Zou et al., 2010). Whether the different PrP^{Sc} forms present in VPSPr share similar capacities with PrP^{Sc} fragments founded in sCJD type 2 or GSS to propagate *in vitro* is unknown and needs to be explored. The results presented in this chapter showed for the first time, an experimental *in vitro* approach to trying to understand the replication of VPSPr PrP^{Sc}.

Finally, the results in this chapter show that: (i) PrP^{Sc} from different human prion diseases have different amplification potentials *in vitro*, (ii) the appropriate matching of codon 129 seed / substrate, influence the abilities of PrP^{Sc} to convert efficiently *in vitro* (iii) mutations in the *PRNP* gene have an effect on the structural conformation of PrP^{Sc} and seems to give them differential propagation properties, (iv) in situations where different type are present such as VPSPr and GSS these have altered *in vitro* amplification in which the forms that are protease resistant over a greater extent of the protein (PrP^{res} types 1 and 2) replicate better *in vitro* than those in which protease resistance is lower overall or is confined to the central region of the protein (8kDa PrPres). This implies that different conformers and glycotypes present in the brain might compete during replication with some environments favouring one over the other.

Macro - and micro - heterogeneity of PrP^{Sc} conformers is well accepted and has been reported in the majority of human prion pathologies (Head et al., 2013). However,

the impact and consequences of this phenomenon are only beginning to be understood.

Whether the *in vitro* conversion of the different PrP^{Sc} conformations have distinctive amplification properties or competence is unclear and remains to be addressed. The kinetics of prion propagation by *in vitro* conversion systems could be used as a model in which to evaluate candidate treatment or to develop sensitive diagnostic tests for these fatal neurologic disorders.

Heterologous (animal:human) amplification of PrP^{Sc} from different

animal prion diseases

4.1 Introduction

4.1.1 Overview

Transmissible spongiform encephalopathies have been described in humans and in a range of farmed, captive and free-ranging animal species, including bovine spongiform encephalopathy in cattle, scrapie in sheep and goats and chronic wasting disease in deer and elk. In humans, Creutzfeldt–Jakob disease.

Especially following the BSE epidemic in the UK and its link to a new form of CJD in humans, the potential for animal prion diseases to affect humans has been recognized as a subject of importance. Efforts to understand zoonotic potential and evaluate risk have gone hand-in-hand with the development of new *in vivo* and *in vitro* biological models and a better understanding of prion biology.

The increasing evidence in favor of the prion hypothesis has involved epidemiological, clinical and pathological research on TSEs in humans and animals. Increasingly sophisticated experimental models, including the use of non-human primates, wild-type, humanized transgenic mice and cell-free molecular conversion systems, have been proven very useful in the study of prions.

4.1.1.1 Transgenic mice models

Ten years after the initial development of trangenesis in animal models, a transgenic rodent with a single amino acid substitution (P101L) in the murine prion protein gene was developed. This transgenic model showed a neurodegenerative process, similar to that in humans affected by a corresponding P102L mutation in the *PRNP* gene. This achievement revealed that experimental animals (genetically modified) can

model certain aspects of prion diseases (Hsiao et al., 1990). In order to evaluate the association between the *PRNP* gene expression and the prion transmission, Bueler and colleagues intracerebrally inoculated *PRNP* knock-out mice with high doses of scrapie prions. The animals failed to develop any clinical signs of TSE disease, and showed no TSE pathology in the brain. The authors concluded that development of prion symptoms and pathology is strictly dependent on the presence of PrP^C, and that incubation time and disease progression are inversely related to the levels of PrP^C (Bueler et al., 1993).

Transgenic models have not only been relevant to understand basic questions of prion replication, they have also contributed to our understanding of their transmission between species.

4.1.1.2 *In vitro* **Prion conversion systems**

In vitro conversion systems attempt to model the conversion mechanism of PrP^C to the misfolded form of the prion protein. One of the first attempts was developed by Coughey and colleagues, in which recombinant hamster PrP^C incubated with PrP^{Sc} and chaotropic agents generated PrP^{res} at the expense of the normal PrP isoform after several hours of incubation. Based on this finding a number of *in vitro* conversion models have been developed. Protein Misfolding Cyclic Amplification (PMCA), quaking-induced conversion (QuIC) - and their respective variations - have been utilized to model several aspects of prion phenomenon, such the potential zoonotic risk for animal prions diseases to affect humans.

4.1.2 vCJD and BSE: The first example of a zoonotic prion disease

In 1985 the emergence of a new TSE affecting cattle was reported in the United Kingdom. The disease, called bovine spongiform encephalopathy or BSE eventually affected more than 180,000 animals in the United Kingdom with more than 70,000 confirmed clinical cases between 1992 and 1993 (Smith and Bradley, 2003). The animals affected showed a slowly progressive neurological disorder affecting sensation, posture and movement, in which weight loss and reduced milk yield were observed. Affected cattle were pathologically characterized by brain stem grey mater vacuolisation (Ducrot et al., 2008).

At the time it was unclear whether BSE would prove a threat to human health.

BSE is now known to be the pathogen that causes variant CJD (vCJD) in humans, whereas scrapie in sheep is thought to pose little or no risk to human health. In retrospect, the association between these two pathologies is obvious, but at the time it required careful surveillance and research for the connection to be established.

In 1996 Will and colleagues reported the identification of 10 unusual cases of CJD in young individuals in the UK. Active surveillance identified no obvious increase in the incidence of CJD in young patients in other European countries. Taking into account the young age at onset of the reported cases, a distinctive neuropathological profile, the presence of "florid" PrP plaques and the apparent absence of cases with a similar profile in Europe, the authors suggested that the appearance of vCJD in the UK was linked to the earlier BSE epidemic (Will et al., 1996).

In order to further test the link between BSE and vCJD, non-human primates were intracerebral inoculated with BSE brain homogenate. All three BSE inoculated *Cynomolgus macaques* developed abnormal behaviour similar to that observed in the

vCJD cases and had a neuropathology including PrP^{Sc} deposition reminiscent of that seen in vCJD patients. This further supported the hypothesis that BSE is the responsible agent for vCJD. Transmission studies in inbred mouse strains reinforced this view, and strongly suggested that vCJD is caused by the same strain of agent that caused BSE, feline spongiform encephalopathy (FSE) and TSEs in exotic ruminants (Ironside, 1998, Bruce et al., 1997). More compiling evidence of a link between BSE and vCJD was presented by Scott and colleagues who inoculated bovinized transgenic mice with BSE, scrapie, and vCJD. All three inocula propagated efficiently; reinforcing the hypothesis that vCJD is caused by the same strain of agent that has caused BSE (Scott et al., 1999).

In order to study the transmissibility of vCJD, sCJD and BSE to humans, transgenic animals that express human *PRNP* valine (V) or methionine (M) at codon 129 were inoculated with the aforementioned prion diseases (Asante et al., 2002, Hill et al., 1997). The transgenic mice presented evidence of transmission for all the inocula. However, the molecular signature of the vCJD PrP^{res} generated by the infected animals carrying valine at the position 129 did not share the same electrophoretic mobility as the original inoculate (Hill et al., 1997) In contrast, the animals carrying the polymorphism methionine and inoculated with the same inoculum, conserved the molecular pattern as the original inoculum (Asante et al., 2002). To further evaluate prion transmissibility of BSE and vCJD and assess effect of codon 129 polymorphism on human susceptibility, Bishop and colleagues produced humanized transgenic mice carrying three distinct genotypes at codon 129: methionine and valine homozygous and methionine/valine heterozygous. After inoculation with BSE and vCJD prions, different pathological characteristics and transmission efficiency

was detected among genotypes. In terms of vCJD propagation, the MM genotype at codon 129 was more susceptible than MV, which in turn was more susceptible than VV (Bishop et al., 2006). This genotype dependant gradient of susceptibility to transmission was suggested by the authors to be explained by a previous study in which recombinant human PrP with M at position 129 was shown to be more efficiently converted than with 129 V by using a cell-free conversion assay (Raymond et al., 1997).

In order to test whether inter-species transmission barriers could be modelled using PMCA, Jones and colleagues tested the ability of BSE and scrapie to convert human prion protein of the three major human prion protein polymorphic variants (*PRNP* codon 129 MM, MV and VV) expressed in humanized transgenic mouse brain. The results showed that cattle BSE effected efficient conversion of human PrP with a human *PRNP* genotypic preference similar to that of human variant CJD (MM>MV>VV) and that scrapie failed to convert the human substrate (Jones et al., 2009). These results mean that PMCA can faithfully replicate aspects of cross-species transmission potential and might provide useful additional information concerning the molecular barrier to zoonotic transmission.

The establishment of a causative link between BSE and vCJD clearly involved epidemiology, clinical and neuropathological investigation, but as shown above, it also involved the use of carefully selected *in vivo* and *in vitro* model systems. The history of BSE / vCJD can be used as a paradigmatic example of how to consider possible links between other animal prion diseases and how to investigate zoonotic potential.

4.1.3 Atypical TSEs

Following the BSE epidemic in the UK, active surveillance programs for animal prion diseases in Europe and elsewhere led to the identification of rare atypical forms of prion diseases in sheep and cattle. These included Nor98 or atypical scrapie in sheep (Benestad et al., 2003) and two prion diseases of cattle: bovine amyloidotic spongiform encephalopathy or L-type BSE (Casalone et al., 2004) and H-type BSE (Biacabe et al., 2004) both of which have a pathology and epidemiology distinct from classical or (C-type) BSE .

4.1.3.1 Atypical scrapie: **Nor98**

First diagnosed in Norway in 1998, Nor98 affected animals presented with progressive ataxia and showed vacuolar lesions and PrP^{Sc} accumulation in the cerebral and cerebellar distinct from classical scrapie. Nor98 affected sheep are of *prnp* genotypes rarely affected by the classical scrapie. Molecular analysis of protease-resistant prion protein in the brain revealed a low Proteinase K resistance and the presence of a low molecular weight PrP^{res} band hitherto not described in animal TSE. These features suggested the identification of a novel scrapie phenotype or strain (Benestad et al., 2003, Tranulis et al., 2011)

Transmission of scrapie and atypical scrapie to a transgenic mice model that overexpresses the ovine prion protein of the ARQ/ARQ *prnp* genotype revealed differences in the transmission rate between these two sheep TSEs. These results suggested that the two forms of scrapie correspond to different strains and that the risk of transmission of atypical scrapie (between sheep) was lower than classical

scrapie (Arsac et al., 2009). Wilson and colleagues reported a failure of transmission of Nor98 in humanized transgenic model mice, arguing a substantial transmission barrier between atypical scrapie and humans (Wilson et al., 2012). In agreement with Wilson and colleagues, recently, Wadsworth and colleagues showed that transgenic mouse that overexpress the human protein presented a lack of infectivity after challenge with ovine prions (scrapie and atypical scrapie) (Wadsworth et al., 2013).

4.1.3.2 L-type BSE / H-type BSE: *in vivo* models

Intracranial inoculation of cynomolgus monkeys (*Macacca fasicularies*) with three bovine strains, including C-type BSE, L-type BSE and vCJD revealed a distinctive pathological profile for L-type BSE.

These animals had a shorter survival time compared with those challenged with C-type BSE inoculated macaques, although the dose was 4 times less in terms of the amount of tissue compared to C-type BSE inoculum. Furthermore, PrP^{res} was 10 fold more concentrated in the C-type BSE inoculum than L-type BSE material. These results show the susceptibility of non-human primates to L-type BSE and suggest a higher pathogenicity of L-type BSE compared to the C-type BSE (Comoy et al., 2008).

Active animal TSE surveillance programs worldwide have identified H-type BSE and L-type BSE in countries outside the European Union (e.g. Japan). The evaluation of the infectivity and the prion disease phenotype of the Japanese L-type BSE isolates was assessed by intracranial inoculation of cynomolgus macaques with macerate brain from two confirmed cases of L-BSE.

The incubation periods and the duration of the disease were approximately 2/3 shorter than those of C-type BSE, similar to the previous observations made by Comoy and coworkers. This evidence suggests once again that L-type BSE may be more virulent than the C- type BSE in non-human primates (Ono et al., 2011).

With the potential evidence that L-type BSE could be more virulent than C-type BSE, researchers have focused on quantifying the risk that L-type BSE and H-type BSE pose to the humans health. Beringue and colleagues reported that humanized PrP 129 methionine overexpressing mice were susceptible to L-type BSE, showing100% attack rate and shorter incubation periods compared with C-type BSE (Beringue et al., 2008). In contraposition, H-type BSE failed to transmit the disease. The authors suggested a higher theoretical risk of transmission of L-type BSE to humans compared to C-type BSE (Beringue et al., 2008).

Recently, a wide range of atypical animal prion diseases were used to challenge humanized transgenic mice that express physiological levels of the human prion protein (Wilson et al., 2012). In contrast to the findings of Beringue and colleagues, a substantial transmission barrier between the atypical forms of BSE and humans was observed, given that L-type BSE and H-type BSE did not show any signs of disease or pathology (Wilson et al., 2012).

4.1.4 Chronic wasting disease

First recognized as a TSE in the early 1980s by Williams and colleagues(Williams and Young, 1980), chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that affects North American mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus)*, Rocky Mountain elk (*Cervus elaphus nelsoni*), and less frequently moose (*Alces alces shirasi).* After being first identified in Colorado and Wyoming, the disease has been identified in 15 states across USA, two Canadian provinces and 15 states and provinces in South Korea (Reviewed by Saunders S, 2012) (Saunders et al., 2012). Clinical symptoms usually presented in animals affected by CWD are progressive loss of weight, pronounced behavioral changes, excessive salivation, ataxia with head tremors.

4.1.4.1 Surveillance for CWD-related disease in human

The possibility of CWD affecting human health will be a function of exposure to the CWD agent (or agents) and of human susceptibility to these agents. Dose and route could reasonably be expected to be relevant aspects of exposure and as with other human prion diseases the *PRNP* codon 129 polymorphisms may be expected to exert an effect on human susceptibility to CWD. The disseminated nature of PrP^{Sc} and infectivity in animals infected with CWD, coupled with the popularity of deer hunting in CWD endemic areas, suggests the most likely form of human exposure to CWD infectivity is by the oral route. CJD surveillance systems in CWD affected countries have been alert to this possibility and have been vigilant for CJD patients with suspicious symptoms, a young age at onset and potential risk factors such as

game hunting and venison consumption. The literature contains five such cases (Belay et al., 2001, Anderson et al., 2007). The results of intensive investigation provided diagnoses of sporadic CJD, (Belay et al., 2001) familial CJD and early-onset Alzheimer's disease (Anderson et al., 2007) and on balance the authors discounted a link to CWD, but conceded the difficulties involved in coming to this conclusion. It is important to note however that the specific features of an animal prion disease need not be conserved when zoonoses occur. Neither the characteristic vCJD neuropathology nor the pronounced peripheral tissue involvements of vCJD are features of BSE in cattle. The absence of neuropathological similarities between CWD and any individual cases of human prion disease is not sufficient grounds to discount CWD as a human pathogen. Continued surveillance and epidemiology are required. In parallel the susceptibility of humans to the CWD agent has been investigated using *in vivo* and *in vitro* model systems.

4.1.4.2 Transmission and bioassays in cervids

CWD is believed to be horizontally transmitted between deer by direct contact (e.g. by saliva) and indirectly through pasture contamination with urine or faeces (Reviewed by Miller M, 2003) (Miller and Williams, 2003). The experimental exposure of uninfected mule deer to contaminated excreta and decomposed carcasses of infected animals show that CWD prions can persist in the environment for two years, maintaining their infectivity (Miller et al., 2004). Using bioassays in deer Mathiason and colleagues evaluated the presence of CWD prions in body fluids and excretions in the preclinical phase of the infection. They also evaluated the

transmission of the disease using repeated environmental exposure. Positive results were found, and blood and saliva were detected as a potential source of CWD prions. Additionally, the infectious agent was sufficient to transmit the disease from the environment (experimentally shed) to naive deer (Mathiason et al., 2009). These findings reinforce the idea that there is an indirect transmission between deer.

4.1.4.3 Non-human primates model

In order to test the transmissibility of CWD to non-human primates, Marsh and colleagues reported the experimental challenge of two squirrel monkeys (*Saimiri sciureus*) with brain homogenate of CWD infected mule deer. The two animals were euthanized at 31 and 34 months post inoculation. Both animals showed a progressive neurological disease, detection of PrP^{res} by Western blot and histopatological spongiform changes in the brain, all consistent with a TSE (Marsh et al., 2005).

Based on the evidence that non-human primates are susceptible to CWD infection, Race and colleagues evaluated (i) the susceptibility of cynomolgus macaques (*Macaca fasicularis*) and squirrel monkeys to CWD infection, (ii) the different possible routes of inoculation (intracranial and oral), and (iii) various sources of the inoculum (representing wild and captive deer and elk). Intracranial inoculation of squirrel monkeys showed that independent of the origin, 80% of the animals developed signs of a spongiform encephalopathy and accumulated PrP^{res} in the brain. Oral exposure with the infectious agent showed 15% of the squirrel monkeys had the presence of PrP^{Sc} in the brain and in peripheral tissue. In contrast, cynomolgus macaque inoculated orally or intracerebraly failed to show evidence of clinical

disease 70 months post inoculation. Considering the relationship of the two non-human primates species to humans, the authors suguested a pronounced species barrier to CWD in humans (Race et al., 2009).

4.1.4.4 Transgenic mouse models

To model the zoonotic transmission of CWD prions to convert human prion protein, humanized transgenic mice codon 129 methionine lines (overexpressing the transgene PrP one and two fold) were inoculated with CWD elk brain homogenate. After approximately 700 days none of the transgenic mice showed signs of prion disease. The authors concluded that a substantial species barrier to transmission was present (Kong et al., 2005). Sandberg and colleagues confirmed this observation by showing that CWD fails to transmit to transgenic mice irrespective of whether the mice expressed (i) bovine, ovine or human PrP, (ii) the human 129 MM or 129 VV PrP allelic variants, or (iii) whether the CWD isolates were from mule deer, elk or white tailed deer (Sandberg et al., 2010, Tamguney et al., 2006).

In order to evaluate the transmissibility of a range of animal TSEs to transgenic animals that express physiological levels the human prion protein, atypical scrapie, C-type BSE, H-type BSE, L-type BSE and CWD were used to challenge humanized transgenic mice constructed by gene replacement. All TSEs, including the CWD isolate used (derived from white tail deer infected animal), failed to produce disease (or signs of infection) on first experimental passage, whether the mice were homozygous for methionine or valine, or heterozygous at codon 129 of *PRNP*, again

suggesting a substantial species barrier between the atypical TSEs and humans (Wilson et al., 2012).

4.1.4.5 *In vitro* conversion systems: modelling the human PrP susceptibility to chronic wasting disease

One of the earliest attempts to assess molecular barriers of the human prion protein conversion by animal prion diseases was performed by Raymond and colleagues. They compared the ability of CWD, C-type BSE, sheep scrapie, and CJD brain homogenates to convert human prion protein that was metabolically labelled and purified from transfected cells (Raymond et al., 2000). A limited conversion of human PrP by CWD, C-type BSE and scrapie was observed, however, the model system was unable to discriminate between the molecular susceptibility of the two *PRNP* genotypes (*PRNP* 129 M or V polymorphisms) used in the conversion process. These results revealed an overall substantial molecular barrier to conversion of PrP^C by CWD, C-type BSE and sheep scrapie prions (Raymond et al., 2000).

Using PMCA, Kurt and colleagues reported failure to support *in vitro* conversion of the human prion protein (independent of the *PRNP* 129 M or V polymorphisms) when the reaction was seeded with CWD brain homogenate (Kurt et al., 2009). In contrast, Barria and colleagues observed that extensive *in vitro* conditioning of a mule deer-CWD isolate by PMCA in cervid substrate (or passage in cervidised mice) allowed for subsequent efficient in vitro amplification in a humanized transgenic mouse substrate (Barria et al., 2011).

In this chapter, a side-by-side comparison of the zoonotic potential of a wide range of animal prions including BSE, atypical BSE, scrapie, atypical scrapie, and CWD using PMCA was conducted to develop an *in vitro* method of assessing the propensity of human PrP^C to be converted by PrP^{Sc} of a potentially zoonotic strain.

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4.2 Material and methods:

4.2.1 Biological samples

4.2.1.1 Animal prion disease tissues

Ovine, bovine and cervid frozen brain tissue from prion disease affected and unaffected animals were obtained by request from the Animal Health Veterinary Laboratory Agency TSE Archive (AHVLA, Weybridge, UK). The cases and brain regions supplied were selected on the basis of proven disease status and of brain region with an expected high PrP^{Sc} burden, characteristic of the particular prion disease. The prion disease status of the animals involved was determined at AHVLA and/or CFIA Ottawa Laboratory by neuropathology and prion protein immunohistochemistry. The classical scrapie specimen was of brain stem from a field suspect of the *prnp* ARQ/ARQ genotype and a brain stem specimen from an unaffected scrapie suspect of the same genotype was also supplied. The atypical scrapie specimen was of parietal cortex, also from a field suspect, but of the ARQ/AHQ genotype and a corresponding negative control animal specimen was also supplied. The classical or C-type BSE samples were of brain stem from confirmed positive C-type BSE suspects obtained through passive surveillance and the corresponding negative control specimen was similarly obtained. Both the H- and L-type BSE specimens were of frontal cortex from successful experimental bovine transmissions conducted at AHVLA Weybridge. Mid-brain tissue from a confirmed CWD positive and control negative (unaffected) elk (both with *prnp* codon 132MM genotype) was also supplied through the AHVLA. Detailed lists of the analysed samples are shown in the following tables (Tables 4.1, 4.2).

Diagnosis (prion disease)	Species	Thesis Samples ID	Brain region
BSE	Bovine	BSE-1	Brain stem
BSE	Bovine	BSE-2	Brain stem
Scrapie	Ovine	Scrapie-1	Brain stem
CWD	Cervid (elk)	CWD-1	Mid-brain
Atypical scrapie	Ovine	Scrapie-2	Parietal cortex
L-type BSE	Bovine	BSE-3	Frontal cortex
H-type BSE	Bovine	BSE-4	Frontal cortex

Table 4.1 TSE diagnosis, species and brain region for the animal prion disease specimens selected for *in vitro* prion conversion (PrP^{Sc} source).

Species	Thesis Samples ID	Brain region
Bovine	Bovine-1	Brain stem
Bovine	Bovine-2	Brain stem
Bovine	Bovine-3	Frontal cortex
Ovine	Ovine -1	Brain stem
Ovine	Ovine-2	Parietal cortex
Cervid (elk)	Cervid-1	Mid-brain

Table 4.2 Species and brain region of unaffected TSE animal specimens selected for the evaluation of total PrP.

4.2.1.2 Transgenic mouse brain tissue

The entire brain form inbred humanized transgenic mouse lines expressing human PrP^C of the *PRNP* codon 129 methionine (HuMM) and valine (HuVV) were used for substrate preparation and PMCA reactions (Bishop et al., 2006).

4.2.1.3 Human cell lines

The production of PMCA substrates from stably transfected human 293F cells is described in Material and Methods Chapter 3, section 3.2.1.3 (Yokoyama et al., 2011).

4.2.1.4 Human brain tissues

Human brain tissues (frontal cortex) were sampled from a frozen half brain collected at autopsy with the appropriate consent for tissue retention and research use. Selection criteria and ethical approval are described in General Material and Methods, section 2.1.2.1. The vCJD specimen was from a patient (*PRNP* codon 129 MM) with definite variant CJD as defined by established criteria (General Material and Methods, section 2.1.2.1). The non-CJD human brain specimens used for PMCA substrate preparation were frontal cortex from patients with Guillain-Barre syndrome (*PRNP* codon 129 MM) and dementia with Lewy bodies (PRNP codon 129 VV). Ethical approval for the use of these tissues in this chapter is covered by LREC 2000/4/157 (Professor James Ironside). Detailed lists of the non-CJD human brain specimens are shown in the Chapter 3, table 3.4.

4.2.2 Methods

4.2.2.1 Preparation of brain PMCA substrates

Human brain and transgenic mouse brain PMCA substrates were prepared exactly as described in General Material and Methods (Chapter 2)

4.2.2.2 Preparation of prion disease brain PMCA seeds

Homogenates (10% w/v) of C-type BSE, scrapie, CWD, L-type BSE, H-type BSE atypical scrapie and vCJD brain were prepared exactly as described in General Material and Methods (Chapter 2)

4.2.2.3 PMCA procedure

PMCA reactions were prepared exactly as described in General Material and Methods (Chapter 2)

4.2.2.4. Protease digestion and Western blot analysis

4.2.2.4.1 Western blot analysis

Western blotting experiments were prepared exactly as described in General Material and Methods (Chapter 2).

4.2.2.4.2 Detection of PrP total and PrP^{res}

After proteins were electro-transfered to a PVDF membrane, blots were blocked with 2% milk for 1h followed by incubation with 3F4 or 6H4 antibodies exactly as described in General Material and Methods (Chapter 2). The monoclonal antibody 9A2 was obtained from Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands (Jacobs et al., 2007).

The antibody 6H4 recognize an epitope in the protease resistant core of human PrP, which is immunoreactive with amino acids 145-153 and it cross-reacts with bovine, ovine and cervid PrP. The antibody 3F4 also recognize the human PrP (PrP^C and PrP 27-30) presenting immunoreactivity with amino acids 109-112, but 3F4 does not recognize PrP from species other than human and hamster. The combination of protease digestion and detection by 3F4 in these experiments therefore provides a very sensitive method for the detection of newly formed human PrP^{res} (Jones et al., 2009, Jones et al., 2011, Barria et al., 2011). The antibody 9A2 reacts with human PrP amino acids 99-101 and cross-reacts with ovine, bovine and cervid PrP

4.2.2.4.3 Western blot image analysis

Western blot image analyses were performed using, Image Lab software version 2.01, Bio Rad.

4.3 Results

4.3.1 Characterisation of animal prion disease tissues

The animal TSE brain homogenates to be used as seeds in PMCA experiments were first characterised for the presence of total PrP and PrP^{res} (Figure 4.1). The presence of total PrP was observed in all samples in the form of two major bands in the 20-40kDa molecular mass range, probably corresponding to full-length diglycosylated PrP (upper band) and N-terminally truncated diglycosylated or full length monoglycosylated PrP (lower band). The levels of total PrP and the electrophoretic pattern were broadly similar between prion disease samples and the corresponding negative control (unaffected) (Figure 4.1A). However, after proteinase K treatment (50µg/ml), clear differences were noted in the amount of PrP^{res} contained in the TSE specimens (Figure 4.1B). PrP^{res} was most abundant in classical scrapie and CWD samples (Figure 4.1B, lanes 3 and 4), lower levels were detected in C-, H- and L-type BSE samples (Figure 4.1B, lanes 1, 2, 6 and 7) and undetectable level in atypical scrapie (Figure 4.1B, lane 5).
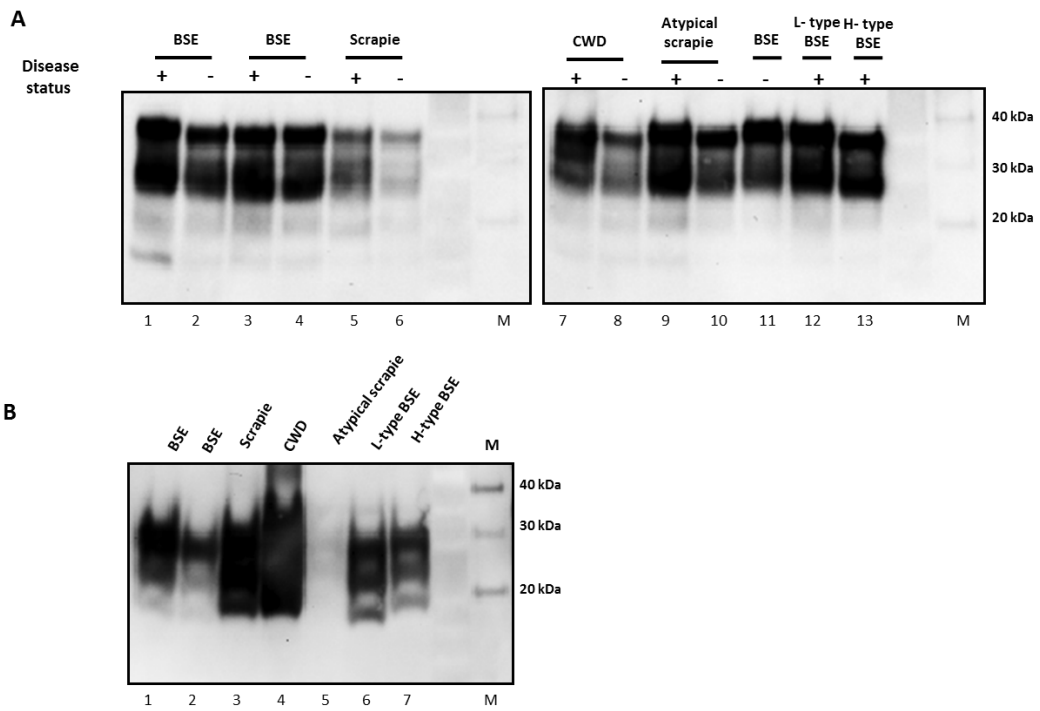
Figure 4.1 Determination of total PrP and PrP^res level present in the animal tissues. (A) To characterize the prion protein expression levels (total PrP), brain homogenates were analysed by Western blot. One µl of 10% brain homogenate for each animal prion disease samples (odd lanes) were loaded in parallel to unaffected animal prion disease control (even lanes) for comparative purposes. **(B)** Nineteen microliters of each 10% weight to volume homogenate was loaded in each lane. To detect the PrP^res present into the samples, proteinase K (PK) digestion (50µg/ml) was performed to remove PrP^C and was then analysed. (+) Samples denotes animal prion disease specimens and (-) the normal animal specimens. M: Molecular marker. The detection antibody was 6H4.

4.3.2 Conversion of human PrP (*PRNP* codon 129 methionine) by animal prion disease samples

The susceptibility of the human PrP (*PRNP* codon 129 MM) to *in vitro* conversion was evaluated by PMCA using BSE, L-type BSE, H-type BSE, scrapie, atypical scrapie, and CWD seeds diluted 1/3 in 10% human brain homogenate substrate. The vCJD seed was diluted 1/100. Western blotting analysis with mAb 3F4 showed formation of human PrP^{res} in the samples seeded with BSE and vCJD. (Figure 4.2A, lanes 2, 4, 14 compared with lanes 1, 3 and 13). Scrapie, L-type BSE, H-type BSE and atypical scrapie did not show detectable human PrP^{res} (Figure 4.2A, lanes 6, 10, 12 and 16). However, 3F4 did detect the presence of human PrP^{res} in the reaction seed with the CWD brain homogenate (Figure 4.2A, lane 8). The corresponding Western blot analysis using the mAb 6H4 (which detects animal PrP^{res} in the animal seeds and newly formed in the human substrate) shows that amplification in the BSE and vCJD seeded samples is marked (Figure 4.2A and B). Repeating a similar experiment with the animal prion disease seeds, but using humanized transgenic mouse brain (*PRNP* codon 129 MM) as the PMCA substrate, BSE and vCJD shows a robust amplification and readily detectable using 3F4 antibody (Figure 4.2B, lanes 2, 4, 14 compared with lanes 1, 3 and 13). Clear PrP^{res} formation was detected in the PMCA reactions seeded with CWD (Figure 4.2B, lane 8). However, reactions seeded with scrapie and H-type and L-Type BSE did not show clear detectable human PrP^{res} formation by the 3F4 antibody (Figure 4.2B).

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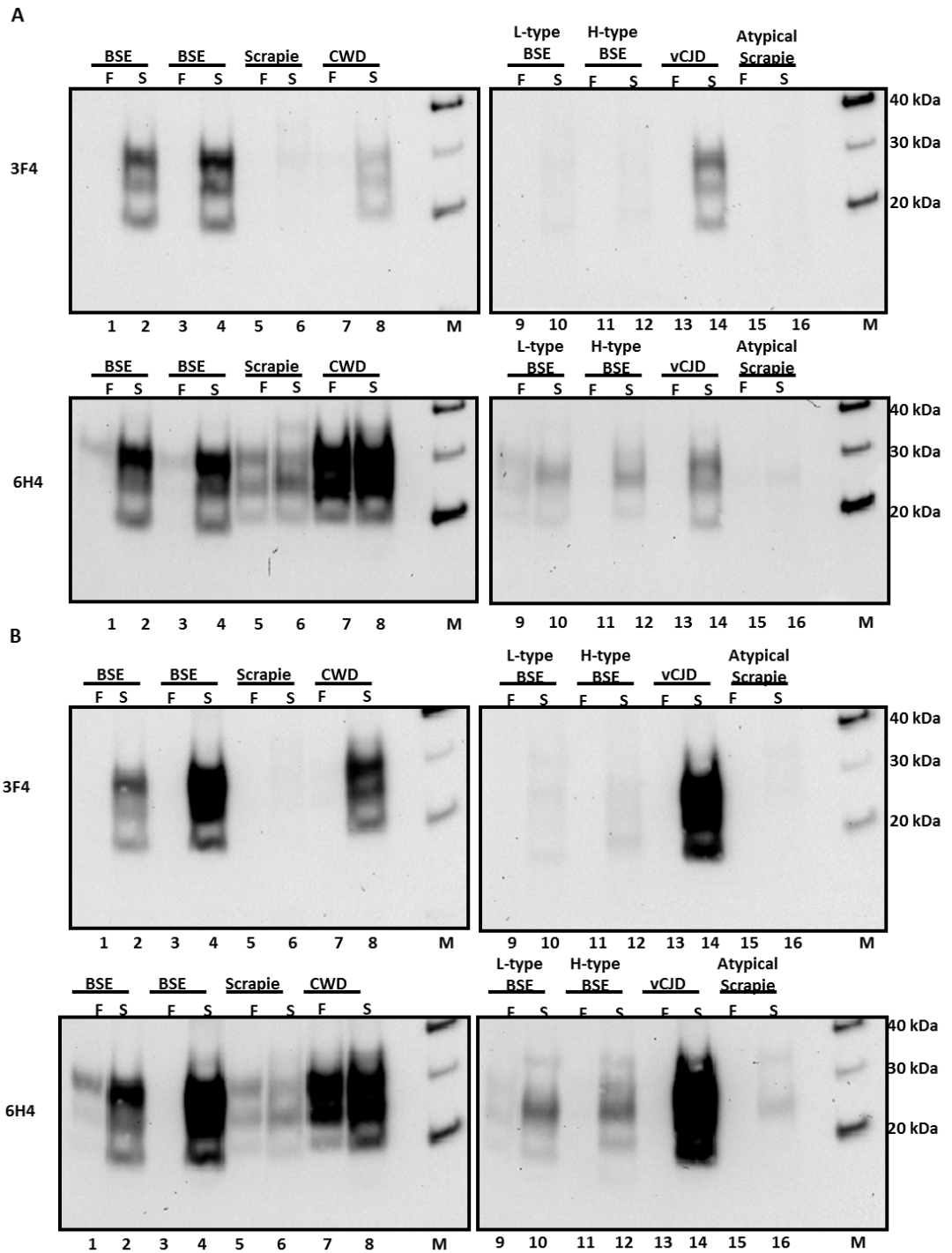
Figure 4.2 PMCA of *PRNP* codon 129 MM human brain homogenate and humanized transgenic mice brain homogenate seeded with BSE, scrapie, CWD, L-type BSE, H-type BSE, vCJD and atypical scrapie. PMCA reactions using *PRNP* 129 MM human brain homogenate **(A)** and *PRNP* 129 MM humanized transgenic mouse brain homogenate **(B)** were seeded TSEs brain homogenate as indicated. Lanes 1, 3, 5, 7, 9, 11, 13 and 15 show the samples without PMCA. Samples in lanes 2, 4, 6, 8, 10, 12, 14 were subjected to PMCA. Western blotting used the mAb 3F4 that allows for the specific detection of human PrP. To compare the PrP^{res} levels present in the seeds (prior to the PMCA) mAb 6H4 was also used. F: Frozen samples; S: Sonicated samples; M: Molecular Marker

4.3.3 Comparison of the conversion of codon 129 MM and codon 129 VV human PrP by CWD brain homogenate

Serial dilutions (1/6, 1/12 and 1/24) of CWD brain homogenate were incubated in substrates prepared from human brain, transgenic mouse brain and 239F cells expressing human PrP with either methionine or valine at codon 129 in order to evaluate their ability to support amplification by PMCA. Irrespective of origin (human post mortem tissue brain, transgenic mouse brain, and 293F human cell line) all three *PRNP* codon 129 methionine substrates supported amplification, albeit with slightly different efficiencies (Figure 4.3A, B and C). Western blotting analysis using mAb 3F4 also indicated *in vitro* prion conversion in all three *PRNP* codon 129 valine PrP substrates (Figure 4.3 D, E and F), even though the level of amplification were lower than the allele codon 129 methionine. As a control, PMCA reactions using vCJD brain homogenate as a source of PrP^{Sc} (seed) were conducted using these same three source of substrates (human post mortem tissue brain, transgenic mouse brain, and 293F human cell line). The vCJD sonicated reactions showed higher levels of PrP^{res} formation compared with non-sonicated samples. Similar to observed in the reaction seeded with CWD PrP^{Sc}, lower level of amplification were observed over the substrate carrying the codon 129 valine (Figure 4.3G).
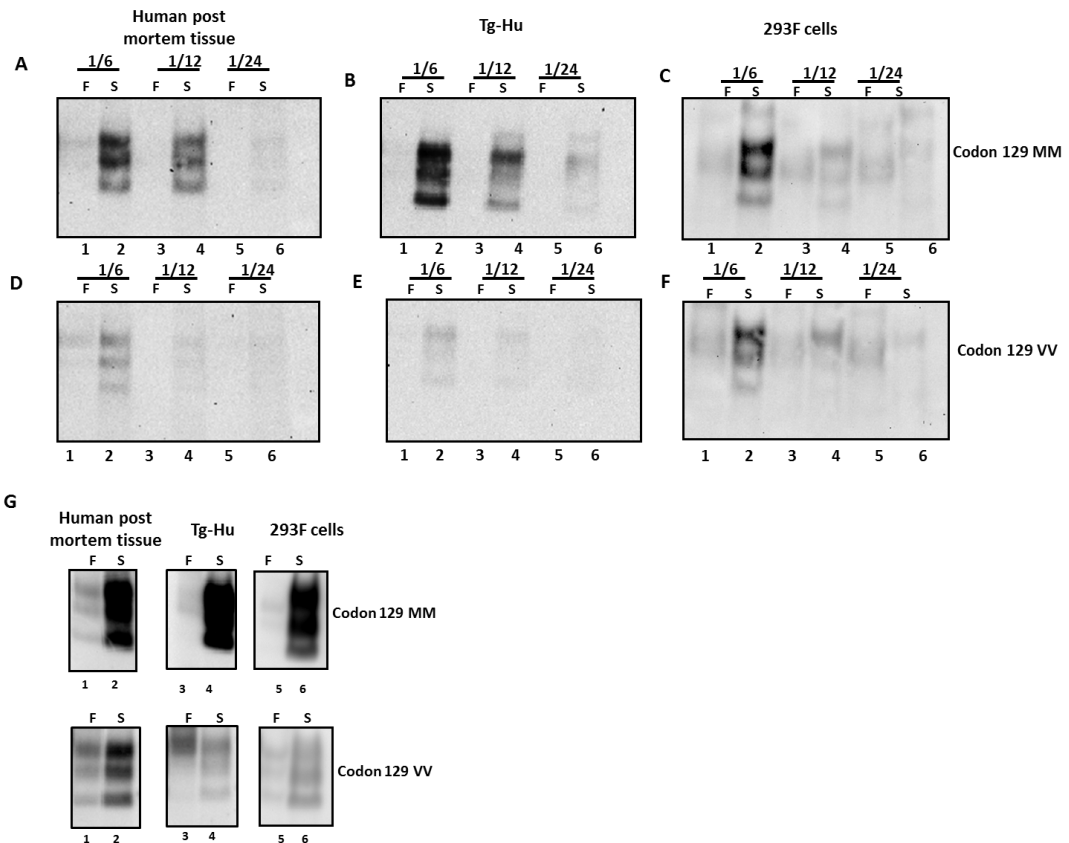
Figure 4.3 Susceptibility of human brain, humanized transgenic mouse brain and 293F cell extract PrP^C to *in vitro* conversion by CWD brain homogenate. PMCA seeded with serial dilutions of CWD brain homogenate (1/6, 1/12 and 1/24) were mixed with three different sources of substrate. **(A** and **D)**: human brain homogenate. **(B** and **E)**: transgenic mouse that express the human prion protein and **(C** and **F)**: 293F human cell extract. The substrates contained human PrP129 MM **(A, B and C)** or PrP 129 VV **(D, E and F).** These same substrates were seeded with vCJD brain homogenate at 1/100 dilution **(G)**. Odd numbers show the samples without PMCA. Even numbered lanes were subjected to PMCA. The PrP detection antibody was 3F4. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

4.3.4 Conversion of human PrP^C by CWD prior to sarkosyl precipitation

In order to discount the possibility that the amplification levels seem in PMCA reactions seeded with CWD brain homogenate depend on the presence of cervid PrP^C in the seed, the CWD PrP^{Sc} in the seed was collected by virtue of its sodium lauroyl sarcosinate (sarkosyl) insolubility properties and compared with an equivalent volume (seed dilution) of CWD brain homogenate. The collected-insoluble CWD material and CWD brain homogenate were used to seed PMCA. Serial dilution of CWD brain homogenate (seed) were performed in PBS (1/12 and 1/24). The diluted CWD samples were mixed with an equal volume of sarkosyl 20% (dissolved in PBS) and incubated for 15 minutes (Morales et al., 2008). Samples were centrifuged at 100000g for 1 hour and resuspended in an equivalent volume of transgenic mouse brain homogenate (substrate) codon 129 MM prior to amplification (100µl). After 96 cycles of PMCA, PrP^{res} formation was detected by 3F4 antibody (Figure 4.4, lanes 2, 4, 6 and 8). The ability of the CWD brain specimen to seed conversion of human PrP^C was found to be retained by this sarkosyl insoluble (PrP^{res} enriched) preparation compared with the respective control (1/12 and 1/24) (Figure 4.4, lanes 6 and 8) and is therefore unlikely to represented cervid PrP^C added to the reaction as part of the seed (Figure 4.3A)

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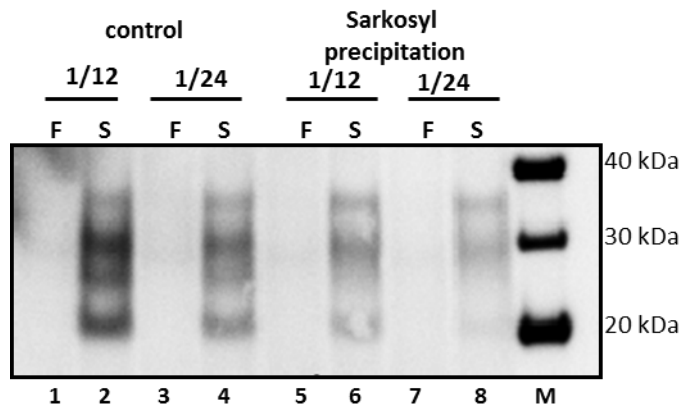
Figure 4.4 Partial purification of CWD PrP^{Sc} prior to the *in vitro* conversion. To confirm human PrP^C *in vitro* conversion by cervid PrP^{Sc}, a sarkosyl insoluble CWD brain homogenate was prepared. The detergent insoluble sample was resuspended in an equivalent volume of 10% humanized transgenic mouse brain homogenate. Serial dilution of CWD seeds (1/12 and 1/24) were performed. Control samples were not fractionated by detergent insolublity. Lanes 1, 3, 5 and 7 show the samples without PMCA. Lanes 2, 4, 6 and 8 show the corresponding samples after PMCA. F: Frozen samples; S: Sonicated samples; M: Molecular Marker. Immunodetection by 3F4 mAb.

4.3.5 Relative conversion efficiency of human PrP (129 MM) by different animal prion disease samples

The previous *in vitro* prion conversion experiments were normalised by the amount (weight/volume) brain homogenate used as seed rather than by the amount of PrP^{res} present in the seed sample. It was therefore possible that the abundant PrP^{res} present in the CWD specimen (Figure 4.2B) was responsible for the amplification by human PMCA substrates (Figure 4.2A and B). For this reason the PrP^{res} present in each sample was estimated by densitometry and the amount of 10% brain homogenate from the different animal prion diseases used in the PMCA reaction was adjusted to give roughly equivalent amounts of PrP^{res} seed in each PMCA reaction. The amount of PrP^{res} in the atypical scrapie specimen was so low that a maximum amount of homogenate was used (dilution 1/2). The results of the Western blot analysis of all seed PrP^{res} normalised PMCA reactions using mAb 3F4 (Figure 4.5 A) confirmed that amplification was a function of PrP^{Sc} / PrP^{C} compatibility (seed / substrate) and not simply PrP^{res} abundance. After 96 cycles of PMCA, BSE, vCJD showed higher levels of PrP^{res} formation (Figure 4.5A, lanes 2, 4 and 14). CWD seed also supported the amplification of human prion protein (Figure 4.5A, lanes 8). However, scrapie, and atypical scrapie did not show an increase of PrP^{res} signal by the 3F4 antibody (Figure 4.5A, lanes 6, 10, 12 and 16). L-type BSE, H-type BSE showed a small increase of signal detected on the amplified reactions. In order to assess whether this increase of signal corresponded to human PrP^{C}, a second round of PMCA was performed and lack of amplification for both atypical BSE seeds was observed.

Western blot analysis using mAb 6H4 confirmed that an equivalent amount of PrP^{res} was incorporated to each reaction previous to the PMCA (Figure 4.5B, lanes 1, 3, 5, 7, 9, 11, 13 and 15).
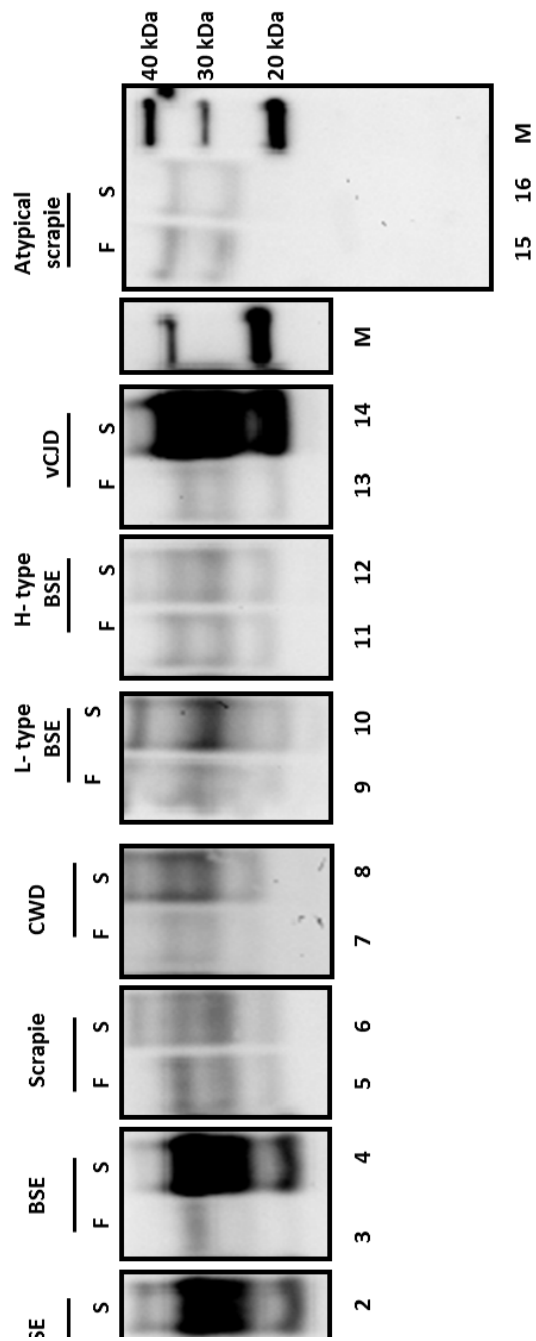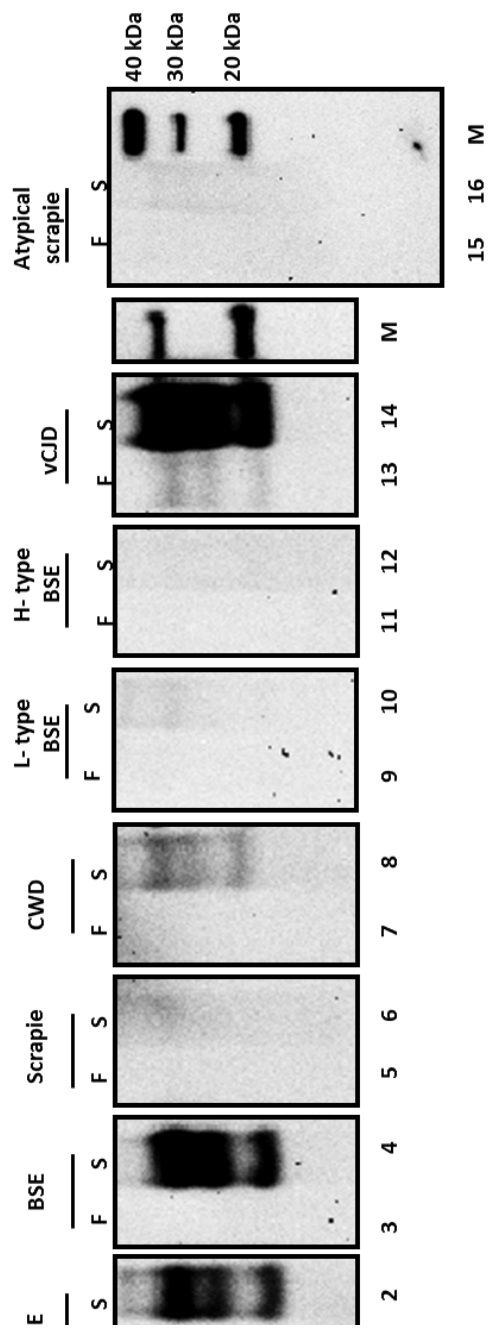
Figure 4.5 Relative conversion efficiency of human PrP (129 MM) by different animal prion disease samples. Brain homogenates from animal prion diseases were seeded at different volumes adjusted to give roughly equivalent amounts of seeding PrP^{res} and amplified using PrP 129 MM containing human brain substrates. Human PrP^{res} formation was detected by the 3F4 antibody **(A)** and seed and newly formed PrP^{res} detected using the 6H4 antibody **(B)**. F: Frozen samples; S: Sonicated samples; M: Molecular Marker

4.3.6 Properties of BSE, CWD and vCJD amplification products in a second round of PMCA

In order to determine whether the PMCA products generated by 96 cycles of PMCA maintained the ability to convert human PrP carrying the amino acid methionine at the position 129, amplification products originally seeded with BSE, vCJD and CWD amplification products were normalized and serially diluted (1/3, 1/6, 1/12, 1/24) in fresh human brain tissue homogenate (PrP 129 MM) and subjected to a second round of PMCA. Densitometric analysis (Figure 4.6) showed that human-PMCA-BSE had an amplification factors of 9.05 fold determined for the first dilutions (Figure 4.6A, lanes 1 and 2). The equivalent amplification factors for CWD were 5.5 fold (Figure 4.6B, lanes 1 and 2). As expected vCJD showed a very much higher propagation efficiency extending to lower seed dilutions (Figure 4.6C, lanes 1,2, 3,4, 5,6, 7,8). The amplification factor was calculated by densitometry measurement, dividing the sonicated (PMCA) value by the value determined for the sample prior to PMCA. The determination of the PMCA amplification factor were following the procedures described in the section: PMCA normalization and quantification in General Material and Methods, Chapter 2
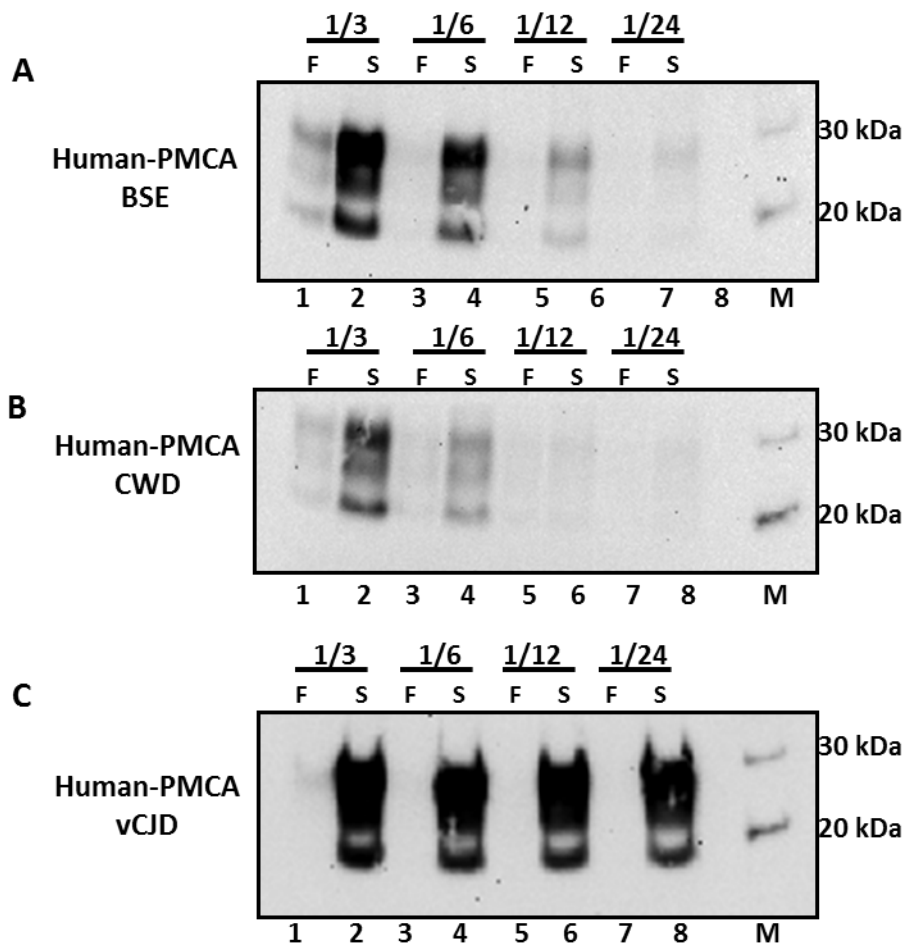
Figure 4.6 Properties of BSE, CWD and vCJD amplification products in a second round of PMCA. (A) human-BSE, **(B)** human-CWD and **(C)** human-vCJD (from a previous round of PMCA) PMCA products were supplemented with fresh human brain homogenate and subjected to a second round of PMCA. The reactions were normalized by PrP^{res} level and the product diluted (1/3, 1/6, 1/12, 1/24) in fresh human brain homogenate (*PRNP* codon 129 MM) prior to PMCA. Odd numbers correspond to samples without PMCA and even numbers to the reactions after PMCA. F: Frozen samples; S: Sonicated samples; M: Molecular Marker

4.3.7 Molecular typing of the CWD-human-PMCA PrP^{res} product

In order to perform a molecular typing analysis of the PrP^{res} material derived from the human CWD amplification product, the electrophoretic mobility and glycosylation profile of CWD-human PMCA product was evaluated against PrP^{res} material derived from cases of sCJD MM1, sCJD VV2 and vCJD. Western blot analysis of PrP^{res} fragments produced by *in vitro* amplification of human post mortem brain homogenate (as a source of PrP^{C}) incubated with CWD brain homogenate (as a source of PrP^{Sc}), showed that after incubation with 3F4 antibody the PrP^{res} fragment share the electrophoretic mobility and glycosylation profile of type 1 PrP^{res}.
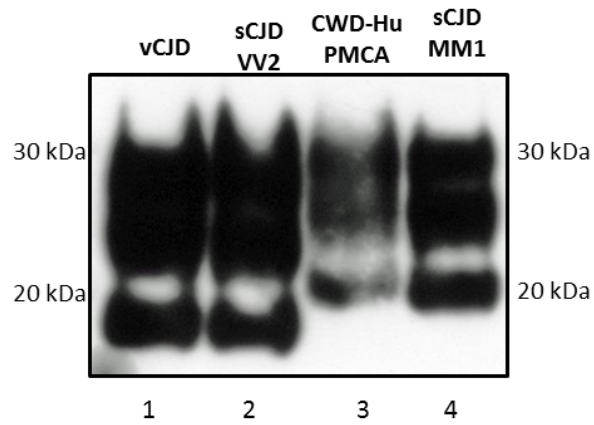
Figure 4.7 Molecular typing of human-CWD-PMCA products: comparison with vCJD, sCJD VV2, sCJD MM1 standards. The CWD-PMCA product was treated with proteinase K (50μg/ml) and analysed by Western blotting. vCJD (lane 1), sCJD VV2 (lane 2) and vCJD (lane 4) were used as a PrP^{res} type standard. The human CWD PMCA product derived from amplification in a human substrate (*PRNP* codon 129 MM) and corresponds to CWD-Hu PMCA (lane 3). Immunodetection by 3F4 mAb.

Donor animal inoculum	In vivo			In vitro			Ref
	Species inoculated	Animal (expression levels)/codon 129	Transmission	PrP source (expression levels)/codon 129	Method	Conversion	
Mule deer White-tail-deer Elk				Human PrP^C expressed in cell culture M V	Cell-free assay	(+)	Raymond G.J *et al*, 2000
Elk	Humanized PrP transgenic mice	Tg-40 (1X)/129MM	(-)				Kong Q *et al*, 2005
		Tg-1 (2X)/129MM	(-)				
Mule deer	Squirrel monkeys	*Saimiri sciureus* (1X)	(+)				Marsh R F *et al*, 2005
Mule deer White-tail-deer Elk	Humanized PrP transgenic mice	Tg(HuPrP)440 (2X)	(-)				Tamgüney G *et al*, 2006
Mule deer White-tail-deer Elk	Squirrel monkeys,	*Saimiri sciureus* (1X)	(+)				Race B *et al*, 2009
	Cynomolgus macaques	*Macaca fascicularis* (1X)	(-)				
Mule deer White-tail-deer				Tg-6816 (X16)/ 129MM	PMCA	(-)	Kurt TD *et al*, 2009
				Tg-7823 (5X) / 129VV		(-)	
Mule deer	Humanized PrP transgenic mice	Tg-45 (4X)/129MM	(-)				Sandberg M K *et al*, 2010
		Tg-35 (2X)/129M	(-)				
		Tg-152 (6X)/129VV	(-)				
White-tail deer	Humanized PrP transgenic mice	HuMM (1X)/129MM	(-)				Wilson R *et al*, 2012
		HuVV (1X)/129VV	(-)				
Mule deer				Tg-440 (2x)/129MM	PMCA	(+)	Barria M A *et al*, 2012
Elk				Human brain (1X)/129MM	PMCA	(+)	Barria M A *et al*, 2014 [*]
				Human brain (1X)/129VV		(+)	
				HuMM(1X)/129MM		(+)	
				HuMM(1X)/129VV		(+)	
				293F cell line(4X)/ 129M		(+)	
				293F cell line(4X)/ 129V		(+)	

Table 4.3 Comparison of the outcomes of experimental transmission and *in vitro* conversion studies of chronic wasting disease in human (PMCA substrate derived from human post-mortem tissue), humanized, and nonhuman primate model systems. Numbers in parentheses denote the stated expression levels of PrP^c in the animal species and cell lines used. PrP, prion protein; (+), positive; (-), negative; PrP^c, host's normal celular PrP. Blank cells indicate that data of this category has not been reported (Barria et al., 2014a). (*) Data presented in this thesis.

4.4 Discussion

TSEs by definition are transmissible. Through experimental investigation it has been shown that prion transmissibility is multifactorial and highly complex. In addition to infectious dose and route, a transmission barrier effect has been described to be involved in the transmission of this infectious agent. Although it is reasonable to suggest – within the context of the prion hypothesis – that the basis for a species barrier would be *prnp* sequence similarity, however, evidence indicates that this factor is a poor predictor of transmissibility. A possible explanation of this is that the interactions between PrP^C and PrP^{Sc} occurs in the form of native PrP^C and misfolded and aggregated PrP^{Sc} conformers, nevertheless, the effect of species-specific sequence difference on PrP^C folding are not well understood. However, there is clear evidence that different prion strains are associated with different PrP^{Sc} conformers and glycotypes which may exist as a quasi-species or molecular cloud (Collinge and Clarke, 2007, Makarava and Baskakov, 2013). Under such a complex scenario molecular compatibility might very well be hard to predict.

With the aim of isolating and studying the molecular effects of compatibility on transmission barriers, Jones and colleagues conducted cell-free prion protein conversion experiments by PMCA using homogenates of bovine and ovine prion disease brain samples to seed brain homogenates containing human PrP, assessing the extent of conversion by detection of human protease-resistant prion protein (PrP^{res}). The results indicated that samples of BSE converted the human PrP^C, with a codon 129 preference similar to that of vCJD. Samples of classical scrapie did not convert human PrP. Remarkably, a sheep BSE isolate resembled BSE and vCJD in its ability to convert human prion protein, thus underscoring influence of strain over

sequence similarity in determining what might be termed a molecular transmission barrier (Jones et al., 2009).

In this chapter, the same approach was applied to study a series of emergent animal prion disease which may pose a risk to human health. Under the PMCA settings used in this thesis, L-type BSE, H-type BSE and atypical scrapie isolates fail to convert the human PrP^C. The CWD seeds utilized did convert human PrP^C, although less efficiently than classical BSE. These observations were confirmed normalising the *in vitro* conversion assay not only by tissue weight but also by PrP^{res} abundance, and whether the PMCA substrate was derived from three sources of substrate (human post mortem tissue, humanized transgenic mouse or human cell line that overexpress the human PrP^C). The conversion of human PrP^C by CWD brain homogenate after PMCA was shown to be dependent on amino acid position at codon 129, with a more efficient amplification for the methionine substrate than the valine one. Furthermore, molecular typing of the CWD amplified products show that the PrP^{res} material resemble in the most common form of human prion disease which is sporadic CJD MM1 type.

Atypical scrapie PrP^{Sc} has shown a reduced PK resistance compared with the classical scrapie (Klingeborn et al., 2006). In the PMCA protocol used in this study, both seed and substrate were treated with 50μg/ml PK prior to Western blot analysis. This pre-treatment may have eliminated protease sensitive PrP^{Sc} associated with atypical scrapie. Therefore, in order to evaluate the conformational properties of the PMCA products derived from the amplification of human PrP^C seeded with atypical scrapie, an alternative method such as the conformation-dependent immunoassay that does not rely on the PK digestion may be preferable (Safar et al., 1998).

Several attempts to determine the transmissibility of these prions disease to humans – thereby enabling to assessment of their zoonotic potential – have employed experimental challenge of non-human primates, humanized PrP transgenic mice, and cell-free assays showing some contradictory results. Marsh and colleagues and Ono and colleagues reported a successful transmission of CWD and L-type BSE to non-human primates, with L-type BSE showing a different pathological profile and a shorter incubation period than C-type BSE (Marsh et al., 2005, Ono et al., 2011). Using a transgenic murine model, Beringue and colleagues reported that humanized overexpressing PrP mice carrying methionine at the position 129, were susceptible to L-type BSE with 100% attack rate and shorter incubation time compared with C-type BSE, suggesting that L-type BSE was more virulent than C-type BSE and may present a higher theoretical risk of transmission to humans (Beringue et al., 2008). Contrary to this finding, H-type BSE failed to transmit the disease in the same animal model.

To assess the potential transmissibility of CWD to humans, Kong and colleagues challenged humanized transgenic mice models. They fail to transit CWD (from elk brain homogenate) to animals expressing human PrP encoding methionine at the position 129 (Kong et al., 2005). Sandberg and colleagues and Tamgüney and colleagues confirmed the previous report of Kong and colleagues that CWD failed to transmit to a transgenic mouse models, irrespective of whether the mice expressed (i) bovine, ovine or human PrP, (ii) the human 129 MM or 129 VV PrP allelic variants, or (iii) whether the CWD isolates were from mule deer, elk or white tailed deer (Sandberg et al., 2010, Tamguney et al., 2006).

In addition to the uses of *in vivo* models to evaluate the potential transmission of animal prions to the humans, *in vitro* conversion approaches have also been reported (Barria et al., 2011, Castilla et al., 2008a, Jones et al., 2009, Kurt et al., 2009). Raymond and colleges compared the ability of CWD, C-type BSE, sheep scrapie, and CJD brain homogenates to convert radioactively labelled PrP^C expressed in transfected cells. They obtained limited conversion of human PrP^C by CWD, C-type BSE and scrapie (Raymond et al., 2000). Using a more efficient *in vitro* conversion system, Kurt and colleagues reported failed to support CWD amplification in human transgenic mouse substrate expressing both methionine and valine codon 129 by PMCA(Kurt et al., 2009). However, extensive *in vitro* conditioning of a CWD isolate by PMCA in cervid substrate (or serial round in substrate from cervidised transgenic mice) was found to be sufficient to cross the species barrier and allow efficient *in vitro* amplification in humanized transgenic mouse substrate for this particular seed (Barria et al., 2011). The comparison of these studies is difficult due to (i) differences between *in vivo* vs. *in vitro* approaches, (ii) different transgenic constructs employed, and (iii) the technical details of the chosen conversion assay (summarised in Table 4.3). Another potential significant difference between these studies is the nature of the CWD isolate employed. Considering that CWD affects different deer species, that interspecies polymorphism in the *prnp* sequence has been detected, and that CWD also occurs in the form of different biological strains of agents (Angers et al., 2010, Perrott et al., 2012), it is reasonable to suggest that different strains of CWD may have a role in determining transmissibility and *in vitro* conversion capacities. Recently Meyerett and colleagues reported the *in vitro* strain

adaptation of a CWD isolate by serial PMCA, similar to that produced by *in vivo* sub-passage (Meyerett et al., 2008).

Among *in vivo* studies, the most directly comparable study to the present research is the one performed by Wilson and colleagues (Wilson et al., 2012), in which a panel of atypical TSEs were used to inoculate transgenic animals expressing a single copy of human PrP^C. C-type BSE, H-type BSE, L-type BSE CWD and atypical scrapie isolates, were unable to produce disease (or signs of infection) on first passage in these mouse model. Potential explanations for this discrepancy are the use of different animal prion disease isolates and the utilization of different cervid species and CWD strain. However, a more fundamental difference between studies may be that the *in vivo* and *in vitro* model systems assess different aspects of the agent and its replication. The *in vitro* cell-free conversion model relies in the molecular compatibility of PrP^{Sc} / PrP^C (seed / substrate), evaluating the potential interaction above the sub-cellular level. Differences between these two models (*in vivo* v/s *in vitro*) are evidenced by the comparison of C-type BSE and vCJD. As presented in this chapter C-type BSE and vCJD seeds amplify efficiently in PMCA using human PrP^C derived from human post mortem tissue and humanized brain homogenate as a substrate, whereas intracranial inoculation of C-type BSE into humanized (129 MM) mice fails to produce disease (Bishop et al., 2006), unless first experimentally transmitted to sheep or goat (Plinston et al., 2011, Padilla et al., 2011, Wilson et al., 2013).

A direct comparison between the two models (*in vivo* and *in vitro*) is difficult and complex. The *in vitro* conversion models offer an attractive opportunity to investigate the mechanisms underlying PrP protein-protein interaction (Barria et al.,

2014a, Castilla et al., 2008a, Jones et al., 2009). Complementary to this, the *in vivo* models have a closer relationship to prion disease in their natural hosts. An adequate interpretation of both models can provide important information for a better understanding of the basis of the transmission barrier to humans.

In summary, this chapter evaluated the susceptibility of human prion protein to different animal prion diseases by PMCA. Human brain and humanized transgenic mouse brain homogenates of the *PRNP* codon 129 MM and VV genotypes, failed to support amplification when the PMCA reactions were seeded with L-BSE, H-type BSE, scrapie and atypical scrapie. In contrast C-type BSE, and vCJD PrP^{Sc} efficiently converted the human PrP^{C} after PMCA, albeit in a codon 129 dependent manner. Surprisingly, a CWD isolate was able to effect the conversion of human PrP^{C}, converting both the 129 MM and 129 VV *PRNP* polymorphic variants, but the conversion of 129 MM was found to be the more efficient. These findings suggest that, at least at the molecular level, atypical scrapie and atypical BSE present a lower level of risk of zoonotic disease than classical BSE. Data also suggest that there is no absolute barrier to the conversion of human PrP^{C} by CWD prions in a protocol using 96 cycles of PMCA. These results would seem to suggest that CWD does indeed have zoonotic potential, at least as judged by the compatibility of CWD prions and their human PrP^{C} target.

This study, using the *in vitro* prion conversion approach has some obvious limitations. First, it does not take account of factors operating above the sub-cellular level. Although the prion hypothesis locates the major determinants of prion disease pathogenesis in prion protein structure it is clear that addition factors affect the zoonotic potential of animal prion diseases. A second limitation is that this study was

conducted with a single specimen of CWD in an elk. CWD affects several cervid species and in some of these species there exist *prnp* polymorphisms and different strains of CWD agent. Before generalizing too broadly from these results, it will be important to test a wide variety of CWD isolates from different cervid species, polymorphic genotypes and geographical locations, raising again the question of CWD as a zoonotic potential (Barria et al., 2014a).

Spontaneous PrP^{Sc} formation using *in vitro* conversion systems as a model for sporadic CJD

5.1 Introduction

5.1.1 Overview

Sporadic CJD is the most common form of human TSEs, affecting approximately 1-2 persons per one million people per year (Ironside and Head, 2008, Ironside et al., 2005). However, important basic questions remain unanswered, such as the molecular mechanisms underlying prion pathology and whether sporadic CJD arises in individuals as a result of a spontaneous conversion of PrP^C to PrP^{Sc}, which then self-propagates. The propagation of the diseases is due to the ability of the misfolded prion protein to convert normal PrP, acting then as a template for further conversions (Prusiner, 1982, Prusiner, 1998). As previously mentioned, structural differences between the two protein isoforms, particularly associated with a higher level of β-sheet content, confer to the pathological isoform a new set of biochemical properties, including detergent insolubility and protease resistance (Baskakov et al., 2004, Legname, 2012, Legname et al., 2004, Prusiner, 1998). Currently, two possible mechanisms have been proposed to generate spontaneous conformational changes of the cellular prion protein: changes in the *PRNP* gene (e.g., mutations and insertions) and spontaneous non-covalent post transcriptional modifications of the expressed PrP (Aguzzi and Calella, 2009). To gain a deeper understanding of this process, both *in vivo* and *in vitro* approaches have been used to assess some of the fundamental aspects of the spontaneous prion conversion and propagation.

5.1.2 Spontaneous prion conversion, *in vivo* approaches

Transgenic mouse models have confirmed that disease-associated mutations of the *PRNP* sequence induce clinical features associated with prion diseases (Hsiao et al., 1990, Jackson et al., 2009, Torres et al., 2013). In this sense, the use of transgenic animals has helped to evaluate the phenotypic-pathological effect of certain *PRNP* mutations. The mutation associated with the GSS syndrome – a substitution of proline for leucine at codon 102 of the *PRNP* gene – was modelled in overexpressing transgenic murine models. (The equivalent codon in the mouse *prnp* gene is 101) After 150 to 200 days P101L transgenic animals developed spontaneously vascular degradation, lethargy and ataxia (Hsiao et al., 1990). Serial transmission experiments using brain homogenate from symptomatic P101L transgenic animals, showed neurological features in a second *in vivo* passage, between 117 and 639 days post inoculation in Syrian hamster and also in the transgenic mouse line which express low levels of the mutated isoform. These results indicated that genetic modification of the prion protein gene can be modelled in experimental animals to evaluate the molecular and histological aspects of pathology, and also its infectivity (Hsiao et al., 1994). A subsequent study using the same animal model indicated that the link between PrP^{Sc} formation, neurodegeneration, and infectivity was not totally clear, and suggested that the potential spontaneous generation might be rather due to acceleration of the disease by promoting the aggregation and accumulation of a pre-existing misfolded PrP isoform (Nazor et al., 2005). Using a similar approach but generating transgenic animals carrying one or two copies of the mutated allele, Manson and colleagues reported lack of spontaneous neurodegeneration in a transgenic rodent model carrying the same mutation. The authors suggested that this

discrepancy could be due to the variation in the expression levels of the mutated gene (Manson et al., 1999).

Jackson and colleagues created a knock-in mouse (Jackson et al., 2009) which express the human PrP carrying the mutation D178N - a change of asparagine (N) instead of the normal aspartic acid (D) - coupled with methionine at the polymorphic codon 129 present in patients who develop FFI. The transgenic animals spontaneously develop clinical signs similar to those described in patients with this particular condition, presenting clinical signs such: ataxia, kyphosis (hunched back posture), pruritus, reduced body weight, and highly unusual paroxysmal hind limb tremors. Intracerebral inoculation of brain homogenate in transgenic animals carrying the wild-type PrP showed transmission of the disease. The authors conclude that a known disease-associated modification of the *PRNP* gene can cause a specific neurodegenerative disease, and be sufficient to create a distinct protein-based infectious element (Jackson et al., 2009).

Also, several attempts to decipher the relationship between penetrance of TSE and the PrP structure have been conducted. Modifications of the prion protein at the octapeptide repeat region, globular domain, and glycosylphosphatidylinositol anchor have shown a variety of effects on the spontaneous generation of prion diseases and their potential transmissibility (Asante et al., 2009, Chiesa et al., 2000, Harris et al., 2000, Sigurdson et al., 2009).

5.1.3 Spontaneous prion conversion by using in *vitro* generated aggregates

If the prion hypothesis is true, then it should be possible to make "synthetic prions" by refolding PrP^C *in vitro*. Legname and colleagues, (Legname et al., 2004) evaluated the infectivity of amyloid fibrils produced from murine truncated recombinant PrP expressed in bacteria (amino acids 89 to 230). Fibrils were intracerebrally inoculated into transgenic animals overexpressing similar truncated murine prion protein 16-times higher than wild-type animals. The mice developed neurologic signs after 380 to 660 days post inoculation, and the brain extract showed spongiform change and deposition of the protease resistant PrP^{Sc}. Secondary transmission experiment in transgenic and wild-type animals revealed a reduction in the incubation time, with a mean incubation time of 90 and 154 days respectively (Legname et al., 2004). These results suggest that synthetic PrP fibrils-alone were sufficient to generate infectivity in transgenic animals, and even more importantly, structural modifications of the recombinant PrP protein were sufficient to produce a new synthetic strain that was able to propagate. Later, in order to evaluate conformational stability of PrP amyloids and its potential effect over the replication and propagation of prions, Colby and colleagues created several synthetic prion strains using truncated recombinant mouse PrP. They produced a spectrum of synthetic prions with different conformational stabilities, which subsequently were inoculated into transgenic animals. The most stable recPrP amyloid conformations exhibit the longest incubation periods, whereas the less stable PrP amyloids produce strain which generated a shorter survival time. Thereby direct correlation between stability and incubation time was proposed (Colby et al., 2009). More recently, the same authors evaluated the infectivity associated to protease sensitive synthetic

prions resulting from the polymerisation of recPrP (Colby et al., 2010). PrP^{Sc} is usually associated with resistance to protease; however PrP^{Sc} sensitive to the proteolytic treatment has been isolated and characterized in certain animal or human prion pathologies (Colby et al., 2010, Gambetti et al., 2008, Nazor et al., 2005). The recPrP protease sensitive preparations were inoculated into transgenic mice that overexpress N-terminally truncated PrP. Interestingly, only recPrP amyloid preparations, but not recPrP monomers or oligomers were able to generate a prion-like disease phenotype to the transgenic animals. When prion isolates (from infected mice challenged with recPrP protease sensitive) were serially transmitted in the same transgenic mouse line, or when the synthetic PrP amyloid was inoculated in a transgenic model that overexpresses full-length PrP, both murine models exhibited accumulation of protease sensitive PrP^{Sc} and extensive neurodegeneration. The inoculation of new *in vitro* generated synthetic prions demonstrate that a conformational change of murine wild-type PrP was capable of producing abnormal prions, composed exclusively of sensitive PrP^{Sc} (Colby et al., 2010).

Attempts to use the full-length recombinant hamster PrP to evaluate the spontaneous prion conversion and infectivity have also been conducted. Full-length hamster recombinant PrP, mixed with normal brain homogenate and bovine serum albumin (BSA), were used to yield β-sheet-rich aggregates. These synthetic hamster amyloid prion preparations were inoculated intracerebrally in golden Syrian hamsters, demonstrating a lack of infectivity in the first *in vivo* passage. Nevertheless, animals developed neurological signs after ~550 days, presenting higher levels of protease resistant PrP in the second transmission. Several conclusions can be drawn from this work. First, full-length recombinant PrP is able to generate a TSE-like disease in

wild-type animals in consecutive transmission experiments. Second, the disease generated by the inoculation of recPrP amyloids seems to show a distinct clinical presentation, pathology and biochemistry compared to other rodent prions strains (Makarava et al., 2010).

5.1.4 Cell free conversion systems and spontaneous prion generation

The *in vitro* conversion assays considered thus far were developed to reproduce the seeded conversion process of the normal prion protein into its misfolded isoform. In the earliest attempts, large amounts of radioactively labelled PrP^C incubated with brain-derived PrP^{Sc} were required to achieve only limited prion conversion (Kocisko et al., 1994). More recently, with the development of two different *in vitro* conversion assays, named protein misfolding cyclic amplification (PMCA) and real-time quaking induced conversion (RT-QuIC), the detection of PrP^{Sc} has experienced dramatic increase in sensitivity, with these techniques being able to amplify and therefore detect minute amounts (around 1≥ femtogram) of PrP^{Sc}. Both technologies have proved to be suitable candidates for diagnostic test development (Atarashi et al., 2011a, Atarashi et al., 2011b, Castilla et al., 2006, Chen et al., 2010, McGuire et al., 2012, Morales et al., 2012, Peden et al., 2012). An important issue in the development of potential *in vitro* conversion tests for TSEs has been the detection of "unwanted" false positives in unseeded control reactions. This important issue has been utilized to explore basic questions concerning protein conversion and replication. Indeed, both systems have been used as experimental strategies to elucidate the spontaneous PrP misfolding (Barria et al., 2009, Deleault et al., 2007, Supattapone, 2013, Vieira et al., 2014, Wang et al., 2010). A central issue in the generation of infectious prions from recombinant PrP have been the associated low

titres of infectivity, long incubation times, and lack of infectivity in wild-type animals (Colby and Prusiner, 2011). There are possible explanations for this: (i) during the *in vitro* synthetic prion amyloid preparation a heterogenous mixture of conformers is generated, in which only some have the appropriate folding to propagate successfully; (ii) maturation process: perhaps, some of the aggregates require a molecular adaptation to acquire the appropriate conformation to become fully infectious (Colby and Prusiner, 2011, Vanni et al., 2014).

5.1.4.1 *In vitro* generation of prions by PMCA

Apparently, PMCA through the continuous amplification cycles appears to select certain PrP^{Sc} conformations (Makarava et al., 2013, Vanni et al., 2014). The "new formed" PMCA products through the consecutive cycles of sonication / incubation and subsequently with serial amplification rounds, appear to select PrP conformers capable of propagating efficiently *in vitro*. Because of this, PMCA has been used by several laboratories to explore the generation of prions *in vitro* (Barria et al., 2009, Deleault et al., 2007, Kim et al., 2010, Wang et al., 2010, Zhang et al., 2013). Using purified native PrP^C, co-purified lipids, and synthetic polyanions, Deleault and colleagues showed that these were the minimal components required to generate *de novo* infectious prions by PMCA. After serial rounds of amplification they were able to produce, apparently in a stochastic manner, PrP^{res} fragments detectable by PK treatment and Western blot. This spontaneous PrP^{res} PMCA material was able to infect wild-type hamsters, with incubations periods around 130 to 170 days, presenting typical signs of scrapie indistinguishable from two well characterized hamster prions strains used as a control (Sc237 and 139H). The similarity to existing

strains meant that the investigators were unable to entirely discard the effect of a potential contamination (Deleault et al., 2007).

In order to evaluate whether murine amyloid, generated spontaneously in the absence of any additional cofactors, was able to produce TSE pathology in wild-type animals, Kim and colleagues (Kim et al., 2010) used a modified version of PMCA to propagate synthetic hamster prions, which were then inoculated into wild-type animals. The modified version of the PMCA used bacterially expressed recombinant PrP, instead of brain homogenate PrP^C, as substrate. The inoculated PMCA products resulted in the development of clinical signs of scrapie in the inoculated Syrian hamsters, showing a long and varied incubation period in the first passage and a substantial decrease on serial passage (Kim et al., 2010).

The susceptibility to generate *de novo* prion in murine brain derived tissue has also been evaluated (Barria et al., 2009). Brain extract from healthy hamster, wild-type mice, and transgenic mice that overexpress human PrP^C were used in serial rounds of PMCA, under standard and prolonged sonication / incubation cycles. None of the specimens were able to produce PrP^{res} under standard PMCA conditions. However, after extensive PMCA cycles, PrP^{res} fragments were infrequently observed, but only in the samples incubated with rodent PrP^C, with no evidence of spontaneous PrP^{res} formation of human PrP under the same conditions. After inoculation of the PMCA spontaneous generated material in wild-type hamster, a new TSE-disease phenotype was observed, suggesting the emergence of a new strain derived from the spontaneous conversion of the normal prion protein (Barria et al., 2009). These results suggest that different animal species have a differential propensity to initiate prion misfolding *in vitro,* and also that PMCA technology can be used as a powerful

tool to investigate the spontaneous formation and propagation of *de novo* prions (Colby and Prusiner, 2011, Jones et al., 2011, Morales et al., 2012).

Recently, Wang and colleagues (Wang et al., 2010, Zhang et al., 2013) have employed PMCA to generate spontaneously infectious prions using only a mixture of recombinant murine PrP, synthetic anionic phospholipids and RNA isolated from liver. After 17 serial PMCA rounds, proteinase K resistant recombinant PrP fragments were detected by Western blot and subsequently inoculated into CD-1 wild-type mice. The *in vitro* generated material induced neurological signs in the animals after about 130 days, reaching the terminal phase of disease around 150 days post inoculation, with minimal variation in the incubation time between animals (Wang et al., 2010). Thus, for the first time it was possible to produce *in vitro* infectious prions, which in terms of infectivity behave similarly to a natural occurring strain.

To further clarify the equation that represents the spontaneous *in vitro* prion propagation proposed by Wang (recPrP + lipids + RNA $\rightarrow$ prions), Deleault and colleagues, investigated the effect of the phospholipid phosphatidylethanolamine (PE) as a sole cofactor for prion propagation (Deleault et al., 2012, Supattapone, 2013). This membrane phospholipid seemed to facilitate the propagation of recPrP^{res} (protease resistant prion protein derived from recPrP) in PMCA of several prion strains in seeded reactions, acting as an endogenous factor in the propagation of infectious prions (Deleault et al., 2012).

The large majority of studies that have investigated the spontaneous conversion of the prion protein have been using murine PrP^C. Only a very few attempts have been

made using human prion protein, and with no success. As a result, currently, there is no information concerning the *in vitro* susceptibility and spontaneous formation of human prions.

This chapter directly addresses the potential of human prion protein to generate spontaneously misfolded PrP using *in vitro* conversion models. RT-QuIC and PMCA were both used to model the spontaneous generation of human PrP.

The aims of this chapter were (i) to establish a protocol for the spontaneous generation of the abnormal form of the human PrP *in vitro*, (ii) to determine whether the spontaneous *in vitro* generated human PrP^{res} can propagate *in vitro* and (iii) to determine whether PrP^{C} methionine or valine at the position 129 conferred greater susceptibility to the spontaneous conversion.

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5.2 Material and methods

5.2.1 Methods

5.2.1.1 Expression and purification of recombinant PrP

The full-length human recombinant prion proteins used in this chapter (residues 23-231), named recPrP 129 M or recPrP 129 V (with either methionine or valine at codon 129) were produced using the procedures described in Wilham and colleagues (Wilham et al., 2010) with minor modifications and were generously provided by Dr G Malinson (Bristol Institute of Transfusion Sciences, Bristol, UK). Briefly, the PCR amplification products of the human prion gene (GenBank accession number No. M13899) were ligated into the pRSET A vector (Cat. No. V351-20, Invitrogen). The ligation products were transfected into *Escherichia coli* Rosetta cells (Invitrogen). Protein expression was achieved by using Overnight Express Autoinduction system (Novagen- Merck Millipore). To isolate inclusion bodies, 0.5 litres of cells culture were centrifuged and subsequently lysed with BugBuster master mix (Cat. No. 71456, Merck Millipore). The inclusion bodies were washed two times with 0.1X BugBuster and then recovered by centrifugation and frozen. Pellets were resuspended and denatured in 8M guanidine/HCl (pH 8.0). Protein extracts were recovered by centrifugation at 16,000 g for 5 minutes. Supernatant were bound to 20 ml Ni-NTA Superflow resin (Qiagen) which was previously equilibrated in denaturing buffer (100mM sodium phosphate (pH 8.0), 10mM Tris/HCl, 6M guanidine/HCl). The Superflow resin coupled to the protein extract was loaded onto a column. The denatured protein was refolded using a linear gradient of refolding buffer (100mM sodium phosphate (pH 8.0), 10mM Tris/HCl) over 5 hours at a flow

rate of 1 ml min^{-1}. Protein was eluted with a linear gradient of sodium phosphate/imidazole buffer (100mM sodium phosphate (pH 5.8), 10 mM Tris/HCl, 500mM Imidazole) at 1 ml min^{-1} over 60 min. Recovered protein was diluted 1/2 into dialysis buffer (10mM sodium phosphate (pH 5.8)) filtered with a 0.2 mm syringe filter and dialysed. Comassie blue staining of SDS gels revealed that both recombinant PrP proteins purity (methionine and valine at the codon 129) were greater than 90%. Finally, recPrP protein concentration was determined by bicinchoninic acid assay (Thermo Scientific) and aliquoted in a final concentration of 0.2–0.5 mg/ml^{-1}. Aliquots were stored at -80°C.

5.2.1.2 RT-QuIC procedure

Tubes containing frozen aliquots of bacterially expressed full-length human recombinant PrP, with either methionine or valine at codon 129, were allowed to thaw in water at room temperature for 10 minutes and then kept on ice. Prior to determination of the protein concentration, recPrP aliquots were filtered through 100 kDa Nanosep centrifugal concentrators (VWR). Protein concentration was determined by absorbance spectroscopy. To avoid saturated measurements, three independent aliquot of the filtrated protein solution were diluted 1/10 in PBS, containing 0.1% SDS. Protein concentration was determined by absorbance at 280 nm in an Ultrospec 3000 spectrophotometer (GE Life Sciences). The final protein concentration was calculated by multiplying the dilution factor with the extinction coefficient of full-length recombinant PrP (2.6 l g^{-1} cm^{-1}). A master mix reaction, named "RT-QuIC master mix" was then prepared using the following

concentrations: 10mM phosphate buffer (pH 7.4), 324mM NaCl, 1mM EDTA, 10μM ThT and 0.1mg/ml recPrP. Aliquots of RT-QuIC master mix reactions were added to a 96 wells clear-bottomed microplate in a final volume of 100 μl per reaction. The microplates were sealed with Adhesive PCR Film and incubated in the FLUOstar Omega at 42°C for 100 hours with 87 seconds of consecutive double orbital shaking at 900rpm followed by 33 seconds of rest. ThT fluorescence was measured every 15 minutes at 480nm, after excitation at a wavelength of 450nm. The ThT activity was evaluated in real time by relative fluorescence units (RFU) with a maximum plateau at 260000 RFU. The protocol was that of Peden and colleagues, and McGuire and colleagues, with minor modifications (McGuire et al., 2012, Peden et al., 2012). All RT-QuIC reactions performed for this thesis chapter were unseeded.

5.2.1.3 Preparation of brain PMCA substrates

Transgenic mouse brain PMCA substrates were prepared exactly as described in General Material and Methods (Chapter 2)

5.2.1.4 PMCA procedure

PMCA reactions were prepared exactly as described in General Material and Methods (Chapter 2)

5.2.1.5 Protease digestion and Western blot analysis

Western blotting experiments were prepared exactly as described in General Material and Methods (Chapter 2)

5.2.1.6 Image Analysis

Image analyses were performed using, Image Lab software version 2.01, Bio Rad.

5.2.1.7 RT-QuIC Fluorescence readings

Fluorescence measurements were collected by BMG Omega Data Analysis software and further exported into Microsoft Excel 2010.

5.2.1.8 Data analysis

All statistical analysis, graphs and further analysis were completed using GraphPad Prisms 6.

5.3 Results

5.3.1 Determination of cut-off criteria for the unseeded amyloid formation of recombinant PrP by RT-QuIC

RT-QuIC involves the use of soluble recombinant prion protein (recPrP) as a substrate for the formation of amyloid. Thioflavin T is a dye that binds amyloid resulting in enhanced fluorescence. Consequently Thioflavin T it has been used in RT-QuIC as a reporter molecule to evaluate amyloid formations. In order to evaluate the unseeded amyloid formation using recombinant PrP, an *in vitro* conversion system (RT-QuIC) was used to mimic the spontaneous misfolding of bacterially expressed recombinant human PrP (recPrP). In order to establish a definition of positive and negative reactions, a cut-off point or exclusion value was first determined. Thioflavin T activity of each sample was measured in relative fluorescence units (RFU) every 15 minutes for a total time of 100 hours. Twelve replicates were tested for each experimental condition and the mean and standard deviation (SD) of the first readings were used to establish the baseline. The cut-off was calculated by the mean of the tested samples, plus 3 times the standard deviation (mean + 3 x SD) (Figure 5.1). All samples below the cut-off level were considered negative (Figure 5.2A). Samples that rose above the cut-off level were considered positive (Figure 5.2B). Positive samples had a fluorescence level between 15,000 to 260,000 RFU, typically with a sigmoidal trace (Figure 5.2 C).
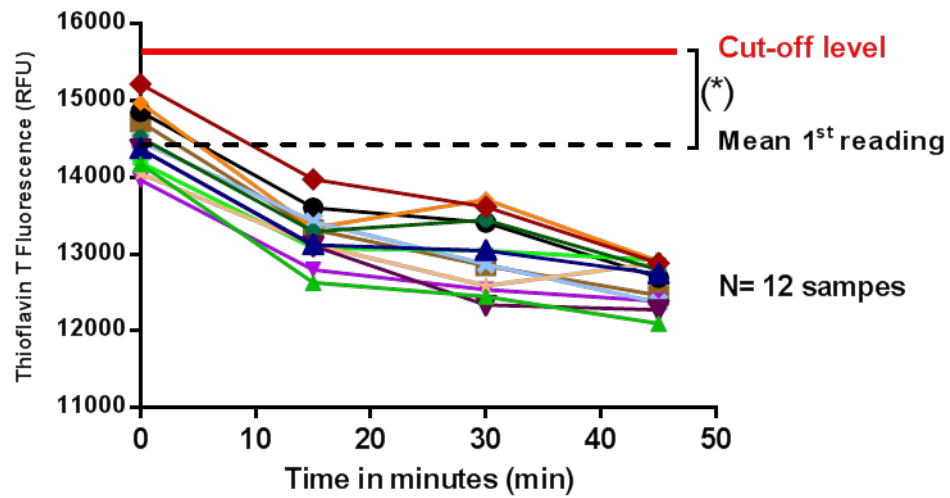
Figure 5.1 Determination of cut-off value for the unseeded amyloid formation of recombinant PrP by RT-QuIC. Formation of PrP amyloid was monitored by detection of ThT fluorescence. The ("Y") axis represents the ThT fluorescence (relative fluorescence units, RFU) and the ("X") axis represents time. The mean and standard deviation (SD) of 12 replicates (colour lines) were calculated. A cut-off level (solid red line) was chosen based on the mean of the total number of samples (segmented black line), plus 3 times the standard deviation (represented by the *).
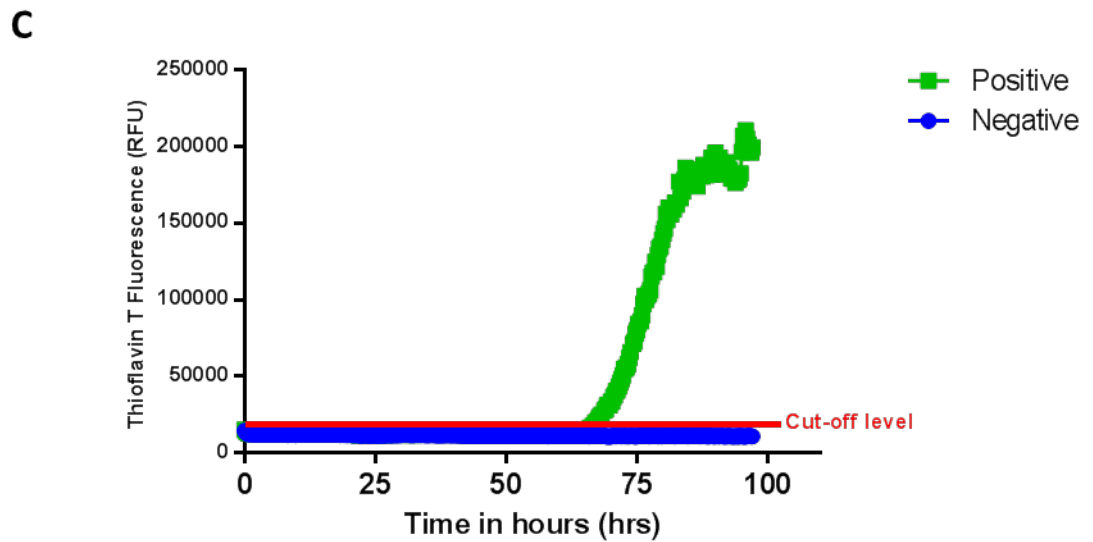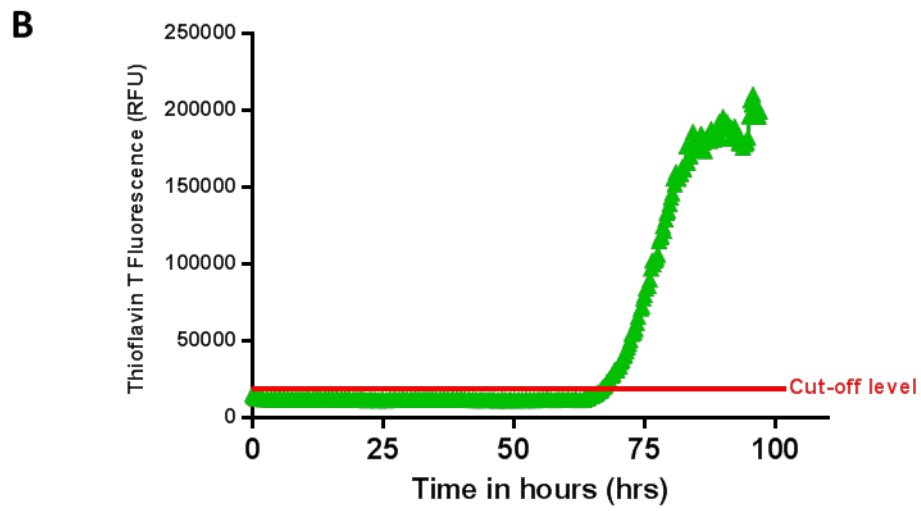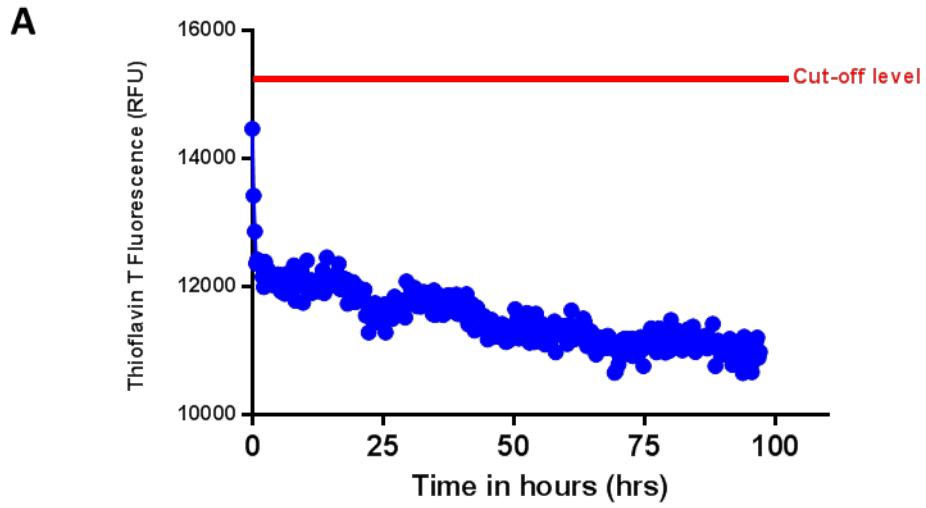
Figure 5.2 RT-QuIC response generated by recombiant PrP in unseeded reactions. The detection of recombinant PrP amyloid formation was monitored by ThT activity every 15 minutes for 100 hours. The ("Y") axes represents the ThT activity in relatives flouresence units (RFU). The ("X") axes represent time. **(A)** An example of a typical negative trace (blue line). **(B)** A typical sigmoidal positive trace (green line). **(C)** Overlaying of a positive (green line) and negative curves (blue line). Red line represent the cut-off level.

5.3.2 Unseeded amyloid formation in recombinant human prion protein with either methionine or valine at the codon 129

RT-QuIC is an amyloid seeding assay, currently under development for use as a diagnostic assay for sCJD, which aims to detect prions in human or animal tissues. Based on features of agitation and ThT reporter activity, RT-QuIC technology was used to evaluate the unseeded amyloid formation of full-length recombinant human prion methionine or valine at position 129 of the *PRNP* gene (recPrP 129 M or recPrP 129 V). Eight independent experiments were performed in which twelve replicates were performed for each assay. The number of positive samples was expressed as a percentage for each experimental group (n=12). By 100 hours, unseeded amyloid formation was observed in a subset of samples of both groups (M or V at the codon 129). A higher percentage of positive samples were found in samples that had valine at the codon 129, within a range of 58 to 100% of samples becoming positive. In contrast, the samples that had methionine at codon 129 showed lower levels of amyloid formation, with the percentage of positives less than or equal to 50% (Figure 5.3). The unseeded conversion of recPrP (129 M or V) by RT-QuIC showed a degree of consistency: in eight independent experiments, the unseeded human recPrP 129 M amyloid formation samples never exceeded the 50% rate. In contrast, human recPrP 129 V samples were always over 58%. In general, the time required for samples to reach the cut-off level (to be considered positive) was reached after 24 hours, but with a broad range between 30 to 100 hours.
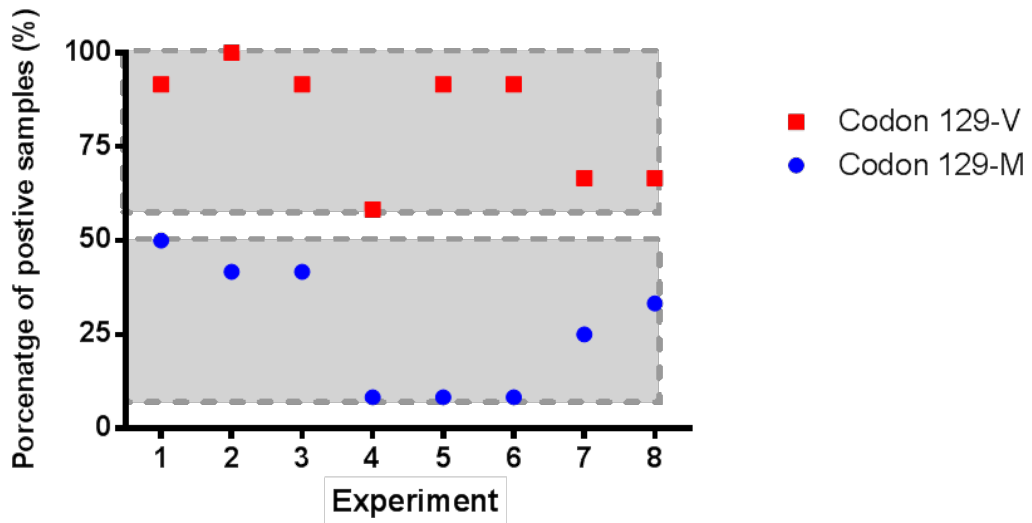
Figure 5.3 Determination of unseeded amyloid formation in full length human recombinant prion protein (methionine or valine at the codon 129) by RT-QuIC. Eight independent experiments were performed in which twelve replicates were conducted for each experiment (coloured symbols correspond to the mean of 12 replicates). The number of samples with unseeded amyloid formation was calculated in each experiment, and the final percentages of positive samples were plotted ("Y" axis). Full-length recombinant human prion [methionine (blue dots) or valine (red squares) at position 129 of the *PRNP* gene] was used in the RT-QuIC reactions. The ("X") axis indicates the 8 independent experiments.

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5.3.3 Effect of protein concentration and batch-to-batch variation on the unseeded recombinant human PrP conversion

It is possible that recombinant human PrP concentration or batch-to-batch variability may affect unseeded amyloid formation in RT-QuIC assays. In order to evaluate (i) the effect of protein concentration and (ii) batch-to-batch variability on unseeded PrP conversion, two protein concentrations and two different full length recombinant human prion protein batches were tested. The standard RT-QuIC procedures recommend an optimal recombinant PrP final concentration of 100µg/ml. To assess whether the protein concentration has an effect on the unseeded conversion of the recombinant PrP, 100 and 200µg/ml of recPrP substrate were added to twelve technical replicates that encode methionine or valine at codon 129. After 100 hours of incubation and shaking, RT-QuIC reactions showed variations in the percentage of positive samples associated to the two polymorphic variants. Similar to the previous findings, in which under standard conditions the recombinant PrP methionine substrate showed a rate of spontaneous formation under 50% (Figure 5.3 and 5.4), a new set of samples carrying the same amino acid at the position 129 had a positive signal for around 10% of the samples. The recombinant PrP valine substrate presented consistency with the previous data (Figure 5.3), showing a higher number (90%) of positive reactions (Figure 5.4).

Using a double concentration of recombinant PrP substrate (200µg/ml of recPrP), from twelve samples that had methionine at codon 129, five had positive reactions (41% of the samples). The other polymorphic substrate (recPrP 129 V) had a positive signal in nine out of twelve reactions, corresponding to 75%.

In order to evaluate consistency of the results, two recombinant PrP batches, designated batch "A" and "B" were utilized. Two independent set of experiments were carried out. Similar to the control reactions the samples prepared with the batch B showed 0 and 25% of positive samples in the reactions incubated with recPrP 129 M in two independent experiments. The recPrP 129 V reactions were 91 and 100% positive. Reactions using recombinant PrP carrying valine at the position 129 had a higher predisposition to form PrP amyloids compared with reactions using recombinant PrP methionine at the same position (Figure 5.4). Given the limited number of batches available, only two sets of recombinant PrP substrates carrying codon 129 M and V were tested. Further experiments must be performed using a larger number of batches in order to evaluate the variability between batches. Nevertheless, the results presented in this chapter suggest that the protein concentration and batch-to-batch variability had no effect on the trend shown by recPrP 129 M or recPrP 129 V to form amyloids.
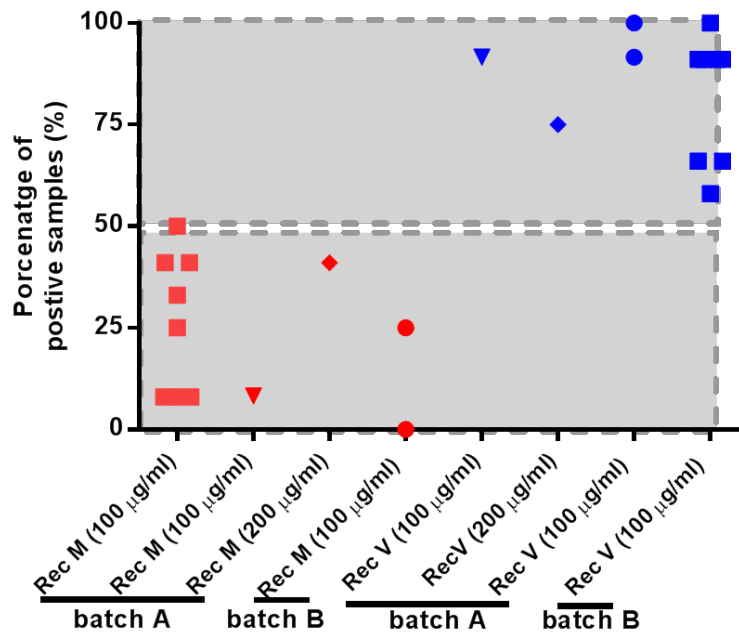
Figure 5.4 The effect of protein concentration and batch-to-batch variability on the unseeded conversion of rec PrP methionine or valine at the codon 129. Two recombinant PrP batches, designated batch "A" and "B, were used to test for the unseeded recPrP amyloid formation. The concentration of rec human PrP were 100μg/ml and 200μg/ml of recPrP. recPrP M (red square, triangle, diamond and circle) or recPrP V (blue square, triangle, diamond and circle) at codon 129 were utilized. The proportion of samples with unseeded amyloid formation was calculated in each experiment and the final percentages of positive samples were plotted. The Y axis indicates percentage of positive samples. X axis indicates recPrP protein concentration and the two recombinant PrP batches utilized, named batch A and B. Results obtained in previous trials were imported into the chart for comparative purposes (blue and red squares represent methionine or valine PrP recombinant substrate respectively).

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5.3.4 Determination of the effect of phosphatidylethanolamine on the unseeded PrP^C conversion of recombinant PrP methionine or valine at the codon 129 by RT-QuIC

To investigate conditions which may promote or inhibit the unseeded amyloid formation of recombinant PrP (methionine or valine at *PRNP* codon 129), different concentrations of phosphatidylethanolamine (PE) were incorporated to the RT-QuIC reactions. PE is one of the most abundant phospholipids present in the cell membrane, frequently found in nervous and other tissues. In the brain, PE accounts for around 45% of the total phospholipid content. Recently, PE has been described as an endogenous cofactor for the *in vitro* prion propagation (Deleault et al., 2012). In order to evaluate the effect of PE on unseeded recPrP conversion, concentrations of 0, 1, 10 and 100μM PE were introduced into the RT-QuIC reactions.

RT-QuIC reactions were performed with recombinant PrP substrate with either methionine or valine at the codon 129. Recombinant PrP amyloid formation was not observed when the two polymorphic substrates were incubated with PE at 100 and 10μM. In contrast, comparable levels of unseeded amyloid formation were observed over the samples incubated with 0 and 1μM of PE. Samples incubated with recPrP 129 M substrate showed 4 and 3 reactions with amyloid formation in the samples containing 1μM or no PE respectively. The reactions incubated with recPrP 129 V containing 1μM or no PE had 6 and 8 positive reactions.

These results suggest that higher levels of PE (100 and 10μM) inhibit the PrP *in vitro* amyloid formation. Nevertheless, a concentration of 1μM does not have an obvious effect on unseeded human recombinant PrP amyloid formation.
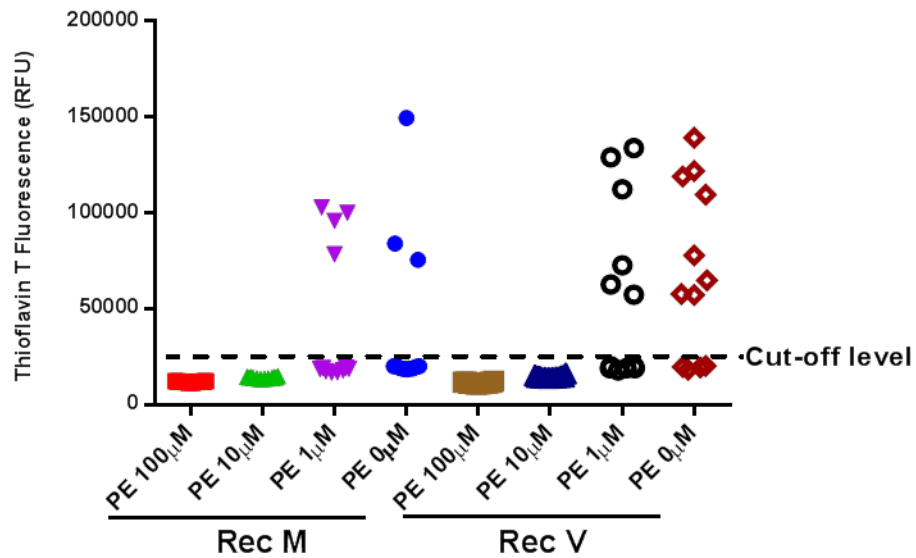
Figure 5.5 Effect of phosphatidylethanolamine on the unseeded human recPrP amyloid formations by RT-QuIC. Concentrations of 100, 10, 1µM or non PE were used in RT-QuIC unseeded reactions. The amyloidogenic activity was monitored by detection of ThT fluorescence in relative fluorescence units ("Y") axis. The measurements were taken every 15 minutes for a total of 100 hours. Recombinant PrP 129 M or V were used as a source of PrP^{C}. ("X") axis indicates PE concentration on the two human recPrP substrates.

5.3.5 Effect of heparin on the unseeded amyloid formation of recombinant PrP methionine or valine at the codon 129 by RT-QuIC

Heparin is a glycosaminoglycan that has been reported to be associated with varied types of amyloids. Several lines of evidence have shown that heparin can act as a ligand for the prion protein inhibiting or enhancing the prion conversion / replication (Vieira et al., 2014, Yokoyama et al., 2011). Heparin, particularly low molecular weight heparin (LMWH), has been reported to act as an enhancer of *in vitro* vCJD PrP^{Sc} conversion in PMCA, with an effective concentration of 100μg/ml (Yokoyama et al., 2011). To evaluate the effect of LMWH on the *in vitro* unseeded amyloid formation, 100μg/ml of LMWH was added to recombinant PrP, either methionine or valine at codon 129, for 100 hours. Amyloid formation was measured by ThT fluorescence every 15 minutes. Twelve technical replicates were performed for each experimental condition. The levels of ThT fluorescence were evaluated for the two substrates (recPrP 129 M or V), both in presence or absence of LMWH. Samples incubated with heparin and recPrP 129 M showed 11/12 positive reactions, compared with 5/12 samples in the absence of heparin. This 50% increase in positive samples was found to be significant using a one way ANOVA-Tukey test ($P = 0.0200$). The recPrP 129 V substrate showed 11/12 positive replicates whether or not heparin was in the reaction. Statistical analyses showed significant differences in the level of fluorescence among the two experimental conditions ($P = 0.0001$).

Considering the number of positive samples observed in the reactions incubated with recPrP V, it seems that the statistical differences was not a reflection of the number of samples above the cut-off, but rather, it was a reflection of the ThT fluorescence level reached by each sample.

Heparin seems to enhance the spontaneous amyloid formation in samples carrying methionine at the position 129 of the recombinant prion protein. However, the recombinant PrP valine substrate did not show a clear effect on the number of positive samples, although a significant difference in the level of fluorescence was observed. The potential impact of heparin on the *in vitro* spontaneous prion conversion is addressed at the discussion section, Chapter 5, pages 200-202.
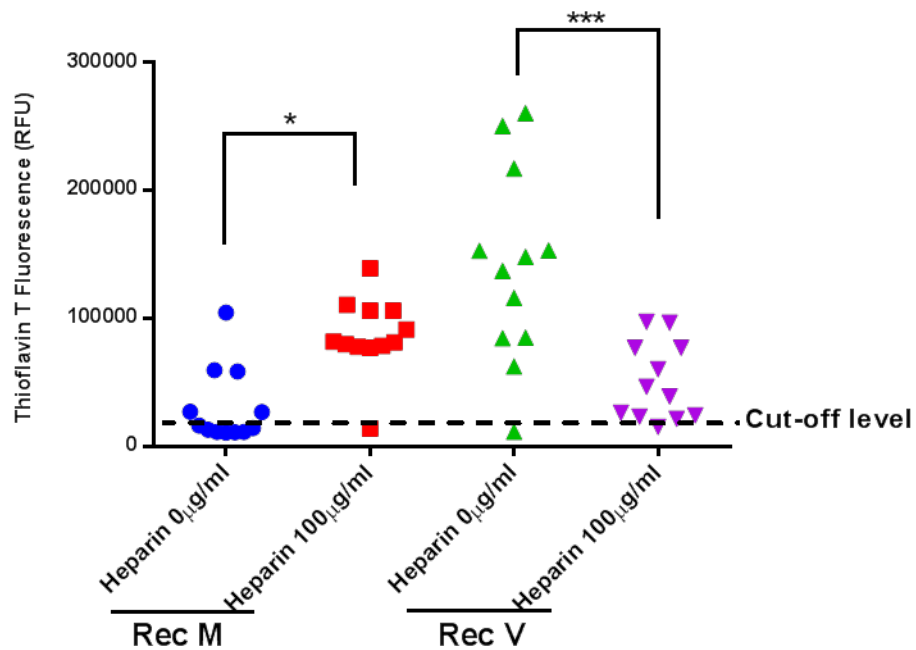
Figure 5.6 Effect of heparin on the unseeded human recPrP amyloid formations in RT-QuIC. Unseeded RT-QuIC reactions without heparin and with heparin at 100μg/ml reactions were used to evaluate the unseeded amyloid formation of recPrP. The amyloidogenic PrP activity was monitored by detection of ThT fluorescence in relative fluorescence units ("Y" axis). The measurements were taken every 15minutes over 100 hours. Statistical analysis was performed by a one-way ANOVA Tukey test. recPrP M or V were used as a source of PrP[C]. Asterisk (*) indicate significantly different between the experimental conditions. Highly significant (***).

5.3.6 Can unseeded amyloid RT-QuIC reaction products be amplified in PMCA?

The results presented so far have shown that RT-QuIC was able to produce recPrP amyloid spontaneously after 100 hours of continuous incubation and shaking. Additional components such as PE or heparin were able to inhibit and promote the unseeded formation of prion protein amyloid. recPrP amyloid derived from several RT-QuIC reactions were selected to seed serial PMCA reactions. To analyse the potential capacities of RT-QuIC unseeded amyloid products to further propagate conversion *in vitro*, several unseeded recPrP amyloid RT-QuIC products were used to seed PMCA reactions. recPrP amyloids with either methionine or valine at codon 129 were used in serial PMCA to maximise the chances of detecting amplification. Seed and substrate matching of the polymorphism at codon 129 was made between the PMCA and RT-QuIC. The PMCA substrates used were humanized transgenic mouse brain homozygous for PrP with methionine or valine at codon 129 (Tg-MM or Tg-VV). Several conditions that promote the recPrP amyloid formation were selected for PMCA analysis as follows:

1) Condition "A": recPrP amyloid product derived from standards RT-QuIC protocol (see Material and Methods Chapter 5). For this condition, two samples were selected: a negative sample (under the RT-QuIC cut-off level), and a positive sample (above the cut-off level) (Figure 5.7, lanes 1 and 2 respectively).

2) Condition "B": recPrP amyloid product derived from RT-QuIC in presence of heparin (100μg/ml). Both selected samples presented amyloid formation above the RT-QuIC cut-off level (Figure 5.7, lanes 3 and 4).

3) Condition "C": recPrP amyloid product derived from RT-QuIC reactions in which humanized transgenic mouse brain was present. In order to promote the amyloid formation from wild-type human PrP^C (rather than recombinant human PrP), a dilution 1/1000 (regarding the 10% homogenate) of humanized transgenic mouse brain homogenate (Tg-MM or Tg-VV) was incorporated to the RT-QuIC reactions. Both selected samples showed amyloid formation (Figure 5.7, lanes 5 and 6).

4) Condition "D": Rec-PrP amyloid product derived from RT-QuIC in the presence of PE (1μM). Both selected samples showed amyloid formation (Figure 5.7, lanes 7 and 8).

5) Condition "E": PMCA reactions performed in absence of any recPrP seed (Figure 5.7, lanes 9 and 10)

6) Condition "F": PMCA reactions performed in absence of any recPrP seed and without heparin added. The standard PMCA protocol (used in this thesis) employed heparin at 100μg/ml. In order to evaluate the effect of heparin over unseeded PMCA reactions, two samples without heparin were serial amplified (Figure 5.7, lanes 11 and 12)

Amplification reactions incorporated five consecutive rounds of PMCA. After each PMCA round, a 1/10 dilution of the previous PMCA product was diluted in fresh substrate in order to perform the subsequent round of PMCA. All PMCA reactions were performed in presence of heparin 100μg/ml, except the samples that correspond to the condition "F". All PMCA samples were treated with PK and analysed by western blotting.

After five rounds of PMCA higher levels of PrP^{res} formation were detected for the samples seeded with recPrP 129 M amyloids (Figure 5.7, Tg-MM; conditions A, B, C, and D). One of the unseeded samples showed PrP^{res} at the fifth rounds of PMCA (Figure 5.7, Tg-MM; condition E). The detection of PrP^{res} in one unseeded control may be due to several factors, including a specific effect of heparin on the PrP amplification in substrate carrying methionine at the codon 129 or cross-contamination between samples. Further amplification experiments utilizing a larger number of unseeded controls (carrying the two possible polymorphisms) and incorporating molecular cofactors such as heparin may help to discriminate at least one of these possible scenarios.

The other two unseeded samples did not show PrP^{res} formation (Figure 5.7, Tg-MM; condition F). No amplification was detected in samples seeded with recPrP 129 V under any conditions (Figure 5.7, Tg-VV; conditions A, B, C, D, E and F).
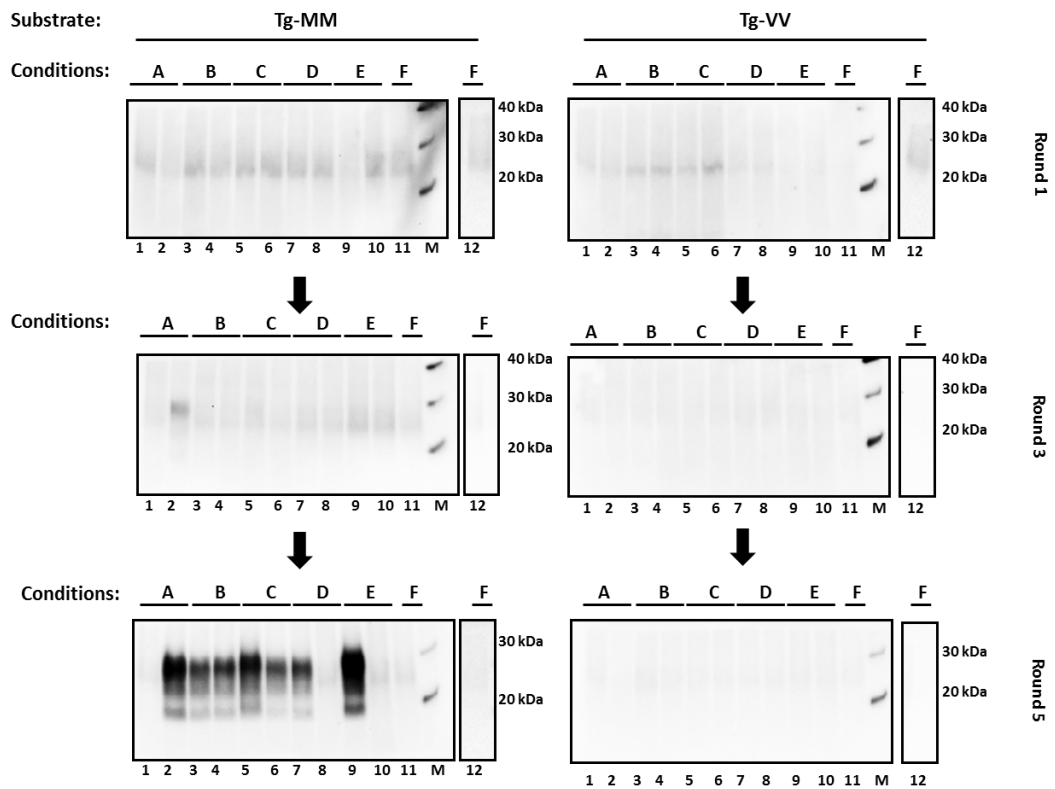
Figure 5.7 PMCA reactions seeded with RT-QuIC unseeded amyloid products. Four different RT-QuIC conditions that promoted the recPrP amyloid formation, were diluted (1/10) to seed serial rounds of PMCA using transgenic mouse brain homogenate substrate methionine (tg-MM) or valine (tg-VV) at the codon 129 of the *PRNP* (Conditions A, B, C and D). Two groups, of two samples each, were used as unseeded control (Conditions E and F). The condition "E" corresponds to PMCA following the standard amplification procedure. The condition "F" uses the same PMCA procedure, but in absence of low molecular weight heparin. The amplification procedure was performed using 5 rounds of PMCA, each corresponding to 96 PMCA cycles. All samples were digested with PK (under standard conditions at a concentration of 50µg/ml) before Western blotting. M: Molecular Marker. Immunodetection by 3F4 mAb.

5.4 Discussion

The autocatalytic PrP^{Sc} conversion, which is generated at the expense of the normal prion protein, is considered a key event in the prion replication process. However, the mechanism behind the prion spontaneous conversion remains unknown. Different technical approaches have been used to address these questions: cell-free conversion systems, cell culture models or genetic modifications of the prion protein gene (Benetti et al., 2010, Benetti and Legname, 2009, Colby and Prusiner, 2011, Morales et al., 2012). The use of recombinant prion protein and purified wild-type brain derived PrP^C has been important in the study of this phenomenon. Using cell-free conversion systems Supattapone and colleagues explored the minimal components for spontaneous PrP^{Sc} generation. Lipids and nucleic acids were found to be essential in the formation and propagation of new prions (Supattapone, 2013). Using cell culture models Edgeworth and colleagues showed that the incubation of metal wires with susceptible cells was sufficient to generate PrP^{Sc} spontaneously and to produce a new disease phenotype in recipient wild-type mice (Edgeworth et al., 2010). Recently, Wang and colleagues have reported spontaneous generation of infectious prion by PMCA using a mixture of recombinant PrP, purified RNA from mice liver and synthetic lipids (Wang et al., 2010, Zhang et al., 2013). Thus far, the vast majority of groups that investigate the *in vitro* generation of prions in the absence of a PrP^{Sc} seed have used either mouse or hamster prion protein. In the cases in which human prion protein has been used, the spontaneous *in vitro* conversion of PrP^{Sc} has been unsuccessful (Barria et al., 2009, Colby and Prusiner, 2011). In this thesis, RT-QuIC technology combined with PMCA reactions were used to establish conditions that promote the spontaneous generation of human misfolded prion protein. The aims

were (i) to establish a protocol for the spontaneous generation of the abnormal form of the human PrP protein *in vitro*, (ii) to determine whether *the spontaneous* and *in vitro* generated human PrP^{res} can propagate *in vitro* and (iii) to determine whether PrP^C with methionine or valine at the position 129 conferred greater susceptibility to the spontaneous conversion.

The process of amyloid formation is associated with several human neurodegenerative diseases including Alzheimer disease, Parkinson diseases and Creutzfeldt-Jakob disease, among others. Amyloid formation has been described to be dependent on several factors including protein concentration, pH, temperature and sometimes the effect of cofactors (Eisenberg and Jucker, 2012). The kinetic of amyloid formations may be influenced by physical conditions, such as shaking or sonication. These mechanical processes can lead the acceleration and formation of different fibril morphologies (Buttstedt et al., 2013). Considering the amyloidogenic property of the misfolded isoform of the human prion protein, and the enhancing effect of agitation on fibril formation, RT-QuIC amplification technology was used to evaluate the spontaneous recombinant PrP conversion, using full-length recombinant human prion protein with either methionine or valine at position 129.

RT-QuIC is a relatively new recPrP conversion assay, in which nucleation and polymerisation occur to convert synthetic recombinant prion protein. It also incorporates real time monitoring of the formation of recPrP amyloid using ThT. RT-QuIC has been developed as a potential diagnostic test for the detection of PrP^{Sc} in different human and non-human tissues and biological fluids. In order to discriminate between positive and negative samples in RT-QuIC, clear criteria are needed. Atarashi and McGuire and colleagues have tested for RT-QuIC seeding activity in

clinical human CSF samples from sporadic CJD patients and controls in which all three 129 codon genotypes were represented. Eighty to 100% sensitivity was obtained for the different genotypic groups (Atarashi et al., 2011a, Atarashi et al., 2011b, Capellari et al., 2011, McGuire et al., 2012). The samples were considered positive if the average of the two highest readings were greater than 10000rfu (relative fluorescence unit). This cut-off value (10000rfu) was chosen based on the mean reading of the first 20 CSF non-CJD RT-QuIC reactions performed, added to three times the value of the standard deviation of the mean (McGuire et al., 2012).

Considering that in this study only unseeded RT-QuIC reactions were utilized to investigate the spontaneous conversion of the recombinant human PrP, a similar cut-off criteria was chosen as follows: the mean value of the initial RFU readings in all samples (for each condition) plus three times the standard deviation. Using this criteria, spontaneous amyloid formation was observed for human recombinant prion protein, either M or V at codon 129, by RT-QuIC.

By comparing a total of eight separate experiments, spontaneous amyloid formation of recPrP 129 M was found to have a lower frequency of spontaneous conversion compared to its recPrP 129 V counterpart. This was a surprising outcome. Previously, it has been reported that the polymorphism at the codon 129 may affect β-sheet-rich oligomer formation, with a higher propensity of the 129 M protein to form oligomers (Tahiri-Alaoui et al., 2004). All these analyses were performed using low pH and high protein concentration. In contrast, the kinetics of amyloid formation (not oligomer formation) seems to show a different pathway compared to the initial folding states of the PrP. The polymerisation of recPrP into amyloid forms (fibrils) seems to exhibit a different trend. Analysis performed to determine the kinetics of

amyloid formation in partially folded human PrP carrying methionine or valine at the position 129, and under continuous shaking, showed that recombinant PrP (valine at codon 129) exhibits a considerable shorter lag phase, with a rapid acquisition of β-sheet conformation compared with recombinant PrP methionine under both spontaneous and seeded conditions (Baskakov et al., 2005).

Considering the methodology employed throughout this thesis, being able to distinguish the different stages and pathways associated with PrP aggregation is fairly complex. However, the *in vitro* conversion systems used here (RT-QuIC) seems to show that both polymorphic forms of the prion protein are able to generate spontaneous PrP amyloids. Moreover, PrP carrying valine at position 129 appears to have a higher predisposition to form PrP aggregates compared with PrP carrying methionine at this position. This observation is consistent with the work reported by Baskakov and colleagues (Baskakov et al., 2005) in which the presence of valine at the position 129 accelerates the formation of amyloids under spontaneous and seeded conditions.

It has been reported that the aggregation of several amyloidogenic proteins is affected by the protein concentration and shaking (Atarashi et al., 2011a). Commonly, agitation is considered to cause aggregation of proteins by increasing the air-water interaction. However, under specific circumstances in which the protein concentration is elevated, the aggregation rate seems to have an inverse correlation with the protein concentration (Atarashi et al., 2011a). Atarashi and colleagues tested the effects of recombinant PrP protein concentration on the amyloid formation using RT-QuIC. They found an inverse correlation between the rate of PrP amyloid formation and the concentration of human recombinant PrP (Atarashi et al., 2011b).

Moreover, others studies using cell-free conversion have shown that the rate of PrP^{Sc} conversion and the amyloid formation is directly proportional to the concentration of PrP^{C} (Baskakov et al., 2000, Caughey et al., 1995, Stohr et al., 2008). In order to evaluate if the amount of recombinant human prion protein has an effect on the spontaneous *in vitro* amyloid formation, two concentrations of recombinant PrP methionine or valine at the codon 129 were utilized in unseeded RT-QuIC reactions. The proportion of samples that showed spontaneous PrP amyloid formation did not change, either in the controls or in the samples with double protein concentration. A greater predisposition to amyloid formation (over 70% of the samples) was detected for the recPrP 129 V, whereas the samples with the methionine at the codon 129 remained with a spontaneous amyloid formation below that at 50%. In order to test consistency and reproducibility of the previous results (batch-to-batch variability), a second batch of full-length recombinant PrP methionine or valine at the 129 codon was used. Two independent experiments, performed at different times, showed that the percentage of spontaneous amyloid formation maintained the same trend, showing a highest percentage of positive samples for recPrP 129 V compared to recPrP 129 M. These results confirm the potential for human recPrP to form spontaneous amyloid under the experimental *in vitro* procedure used here. Furthermore, valine at the position 129 of the *PRNP* appears to confer a greater susceptibility to spontaneous amyloid formation.

The mechanism by which the normal prion acquires a β-sheet-rich conformation remains unclear. Similarly, whether the conversion or the propagation of PrP^{C} / PrP^{Sc} requires a cofactor remains as an open question. The use of simplified *in vitro* conversion models has helped to clarify important role of cofactors in facilitating this

process. Nucleic acids, other polyanions and lipids have been proposed as fundamental cofactors required for *in vitro* propagation and for the production of infectivity. Recently, Supattapone and colleagues isolated and identified a phospholipid cofactor involved in the *in vitro* prion conversion (Deleault et al., 2012). In a seeded reaction phosphatidylethanolamine (PE) facilitates the conversion of recombinant PrP into infectious PrP^{Sc} (Deleault et al., 2012).To investigate the effect of this membrane phospholipid on *in vitro* human PrP amyliod formation by RT-QuIC, a range of concentrations of synthetic PE were incubated with recPrP (methionine and valine) for 100 hours. Inhibition of amyloid formation was observed for concentration higher that 1μM of PE. However, the reactions incubated with 1μM of PE showed similar number of positive samples that the samples without PE. A predominant spontaneous amyloid formation was observed in the recPrP 129 V samples, thereby retaining the original trend of the recPrP 129 V. In serial rounds of PMCA, PE successfully propagated recPrP (into the misfolded form) in seeded reactions with optimal concentrations ≥ 100μM. Here, using the same concentration or 10 times less, PE exhibits an apparent inhibition on the spontaneous human recPrP amyloid formation. Many factors can be attributed to this discrepancy, (i) the use of different *in vitro* conversion systems, which are based on two different physical properties (sonication for the PMCA or shaking for RT-QuIC), (ii) the use of seeded or unseeded reactions, (iii) the use of different substrates, etc. It seems that cofactors appear to have a degree of specificity in their action, with a predisposition to propagate certain type of seed / substrates conventions depending on whether the cofactor are lipids or polymeric macromolecules such as RNA or glycosaminoglycans.

Glycosaminoglycans (GAGs) are a group of polysaccharide commonly associated with β-sheet-rich aggregates, including prions (Vieira et al., 2014). GAGs have been show to act as potential physiological ligands of PrP[C], sometimes promoting or inhibiting the PrP conversion / propagation (Gonzalez-Iglesias et al., 2002, Vieira et al., 2014). Heparin is a highly sulphated glycosaminoglycan, widely investigated in several amyloidoses with some contradictory effects. For example, in order to develop a highly sensitive *in vitro* screening tests for vCJD, low molecular weight heparin (LMWH) can be added to PMCA; PMCA in conjunction with heparin achieved an increase of 100-fold of vCJD PrP[Sc] detection, using human *PRNP* transfected cell lysate substrate. On the other hand, incubation of heparin with recombinant murine prion protein decreased the polymerisation of recPrP. Additionally, RT-QuIC reactions seeded with RML and 263K prion strains incubated with LMWH, have shown a delay on the fibrillation of recPrP in reactions seeded with RML, whereas 263K showed delay and also a decrease in fibril formation, suggesting a potential species-specific effect in its action (Vieira et al., 2014). As shown here unseeded RT-QuIC reactions using recombinant human PrP, appear to have an inherent tendency to amyloid formation. Heparin seems to increase the formation of amyloid in samples with methionine at codon 129 of *PRNP*. This difference was not evident in samples with valine at codon 129, perhaps because the rates of spontaneous amyloid formation were already elevated. However, differences in the level the ThT fluorescence were observed. The cause of this difference may be due to a specific target effect of heparin on human prion protein, particularly associated to the presence of methionine or valine at the codon 129. It is well known that polymorphic variations in this codon has a strong influence on susceptibility to

prion diseases, therefore differences in the effect of heparin on these two human polymorphic substrates, is not completely surprising and might be biologically relevant.

To assess whether the recPrP amyloids generated by RT-QuIC have the property to convert full-length wild-type PrP^C, serial PMCA round were performed. Previously, Soto and colleagues reported that as a result of many round of PMCA, minimal amounts of PrP^{Sc} can convert "fresh" wild-type PrP^C found in brain homogenate (Castilla et al., 2005, Castilla et al., 2006). recPrP^{res} amyloids generated by RT-QuIC, were diluted 1/10 in substrate derived from transgenic animals that express physiological levels of the human PrP^C. It was decided to match seed and substrate the codon 129 genotype to maximise the chances of amplification. Hence, amyloid generated by RT-QuIC from recPrP carrying methionine at the codon 129 was propagated using transgenic brain homogenate homozygous for methionine at codon 129. Similar considerations were made for samples possessing valine at the same codon. After five consecutive rounds of PMCA, samples seeded with RT-QuIC amyloid products, with methionine at the position 129, produced PrP^{res} in all seeded amplified reactions, in addition to one of the unseeded reactions prepared in presence of heparin. Furthermore, the samples seeded with a RT-QuIC product (which showed ThT fluorescence levels below the cut-off) did not exhibit PrP^{res} formation), in addition to the unseeded reactions prepared in absence of heparin, which also failed to propagate PrP^{res}. No amplification was detected for the samples incubated with RT-QuIC amyloid products carrying valine at the codon 129.

The results appear to show that the PMCA reactions seeded with recPrP 129 M amyloid fibrils have an increased susceptibility to propagate relative to the reactions

seeded with recPrP 129V. This contrasts with the higher levels of spontaneous amyloid formation shown by recPrP 129 V compared with recPrP 129 M, by RT-QuIC. The absence of amplification of recPrP 129 V seeded PMCA reactions may be due to several factors, including inhibition or incompatibility between seed and substrate. However, this is unlikely, because the volume of "seed" were apparently the same over the reactions incubated with recPrP M or V. One possible interpretation is that heparin can act as a specific cofactor for spontaneous prion formation in PMCA, being specific for recPrP codon 129 M. This could be the reason why one unseeded samples (prepared in presence of heparin) formed PrP^{res} after five rounds of PMCA. Several lines of evidences have shown that cofactors can act specifically on the amplification of one particular strain. RNA for example has been described to be critical cofactor for hamster prion formation *in vitro*, whereas heparin has a profound effect on the amplification of vCJD, but limited on the amplification of sCJD (Yokoyama et al., 2011). Given that the amplification and the spontaneous formations of prions have the potential selection of cofactors according to the structure of the prion protein, is not so unreasonable to think that the human prion protein, and its different polymorphic variations, has the ability to behave differently against distinct factors.

A possible role for molecular chaperones in prion protein conversion

6.1 Introduction

Many neurodegenerative diseases share common features and molecular patterns. Diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and prion diseases share features including specific protein aggregation and inclusion body formation in the brain. Although the proteins characteristic of the above mentioned diseases lack significant primary sequence homology, they share similarities in their ability to undergo conformational changes and form fibrillar aggregates, alongside with comparable pathways of aggregation (Oliveira et al., 2013, Trzesniewska et al., 2004, Zanuy et al., 2004). One particular example of a neurodegenerative disease associated with protein aggregation is CJD, a lethal and infectious disease associated with the misfolding and accumulation of abnormal prion protein in brain regions (Head and Ironside, 2012b). The underlying pathogenesis is thought to be by the conformational change of a normal brain protein that changes its native conformation to a misfolded isoform. This misfolding process can result in the malfunction of the normal protein, toxicity, cell death and inflammation, all of which are also common aspects of AD, PD and motor neurone disease (Prusiner, 2013).

The heat shock response is a set of conserved reactions of cells and organisms to stress. It was one of the first examples of inducible gene expression amenable to study and has allowed the identification of a series of protein families with constitutive and stress-regulated functions. The response to stress is represented at the molecular level by the induced expression of heat shock proteins (HSPs), of which many are chaperones. The function of molecular chaperones is to mediate the proper folding of proteins and to ensure proteins maintain their native conformation

during stress stimulus (Lindquist and Kelly, 2011, Morimoto, 2011). The members of the small heat shock protein/ α-crystallin gene family (e.g., HSP27, αA-crystallin and αB-crystallin) are of particular interest because of their evolution and versatile functions (Wistow, 2012) . α-crystallin is a protein composed of two subunits "A" and "B", and is a major soluble protein (up to 40%) of the vertebrate eye lens (Horwitz, 2003), although it can also be found at intermediate levels in various tissues and cell types (e.g., in oligodendrocytes in the brain). α-crystallin protein expression is upregulated in neurological disease states and physiological stress models (Head et al., 1994, Iwaki et al., 1993, Kato et al., 1993). At present it is unclear whether α-crystallin accumulation is part of a particular (perhaps adaptive) response to misfolded proteins, or whether this feature is part of a more generalised response to the neurodegenerative process (i.e., neuronal dysfunction and gliosis) or stress.

In order to test whether the chaperone activity of α-crystallin affect the conversion and propagation of a neurological disease-specific protein, two well characterised cell-free conversion models were used to evaluate the *in vitro* propagation of vCJD and the spontaneous formation of recombinant human β-rich-sheet aggregates. Although both conversion systems appear similar, it has been suggested that protein misfolding cyclic amplification (PMCA) and real-time quaking induced conversion (RT-QuIC) may differ in their relationship to prion protein conversion *in situ* (Jones et al., 2011, Peden et al., 2012). PMCA generally uses brain homogenate as a source of PrP^C and ultrasound to fragment the PrP^{Sc} aggregates. In contrast, RT-QuIC utilizes recombinant PrP derived from bacterially expressed and shear forces. The aims of this chapter were: (i) determine the effect of α-crystallin in a seeded vCJD

PMCA reaction, and (ii) evaluate if there is any effect on the formation of spontaneous amyloids by RT-QuIC.

6.2 Material and methods

6.2.1 vCJD brain tissue

Human brain tissues (frontal cortex) were sampled from a frozen half brain collected at autopsy with the appropriate consent for tissue retention and research use. Selection criteria and ethical approval are described in General Material and Methods, section 2.1.2.1. The vCJD specimen was from a patient (*PRNP* codon 129 MM) with definite variant CJD as defined by established criteria (General Material and Methods, section 2.1.2.1)

6.2.2 Transgenic mouse brain tissue

The entire brain from inbred humanized transgenic mouse lines expressing human PrP^C of the *PRNP* codon 129 methionine (HuMM) was used for substrate preparation and PMCA reactions (Bishop et al., 2006).

6.2.3 PMCA procedure

6.2.3.1 Preparation of brain PMCA substrates

Human brain and transgenic mouse brain PMCA substrates were prepared exactly as described in General Material and Methods (Chapter 2)

6.2.3.2 Preparation of prion disease brain PMCA seeds

Homogenates (10% w/v) vCJD brain was prepared exactly as described in General Material and Methods (Chapter 2)

6.2.3.3 PMCA procedure

PMCA reactions were prepared exactly as described in General Material and Methods (Chapter 2)

6.2.4 Protease digestion and Western blot analysis

6.2.4.1 Western blot analysis

Western blotting experiments were prepared exactly as described in General Material and Methods (Chapter 2).

6.2.4.2 Western blot image analysis

Western blot image analyses were performed using, Image Lab software version 2.01, Bio Rad.

6.2.5 RT-QuIC procedure

6.2.5.1 Expression and purification of recombinant PrP

Expression and purification of recombinant PrP was exactly as described in Material and Methods Chapter 5, section 5.2.1.1.

6.2.5.2 RT-QuIC procedure

RT-QuIC procedure was performed as exactly as described in Material and Methods Chapter 5, section 5.2.1.2.

6.2.5.3 RT-QuIC Fluorescence readings

Fluorescence readings measurements were collected by BMG Omega Data Analysis software and further exported into Microsoft Excel 2010.

6.2.5.4 Data analysis

All statistical analysis, graphs and further analysis were completed using GraphPad Prisms 6.

6.3 Results

6.3.1 Effect of α-crystallin on PMCA seeded with variant CJD brain

PMCA was conducted using humanized transgenic mouse brain homogenate (codon 129 MM) as substrate, and variant CJD brain homogenate as the PrP^{Sc} (seeds). The PMCA reactions resulted in a robust amplification detected by Western blots in which PrP^{res} appears in sonicated lanes (marked S) compared to frozen lanes (marked F) (Figure 6.1A, B, C and D). In order to evaluate the effect of α-crystallin on PMCA, amplification reactions were seeded with a relatively low dilution of PrP^{Sc} (1/1000) and different concentration of α-crystallin were added: 10μg/ml, 1μg/ml, 0.1μg/ml and 0.01μg/ml (Figure 6.1A). Amplification products derived from the amplification of vCJD (detected after proteinase K digestion and Western blots) suggested a partial inhibitory effect of α-crystallin at 10μg/ml (Figure 6.1A, lane 2), but an enhancing effect at the lower concentrations, 0.1μg/ml and 0.01μg/ml (Figure 6.1A, lanes 4, 6 and 8), compared to the control reaction (without α-crystallin) (Figure 6.1A, lane 10). To further test whether this enhancing effect was reproducible, a second experiment was performed using lower concentration of variant CJD brain homogenate (1/100000), but maintaining the α-crystallin concentration range (Figure 6.1B). At this high dilution factor, PrP^{res} was undetectable after PMCA (Figure 6.1B, lane 10). The addition of α-crystallin resulted in detectable PrP^{res} after PMCA at the concentrations 1, 0.1, and 0.001 (Figure 6.1B, lanes 4, 6 and 8), suggesting that the addition of α-crystallin increases the limit of detection (sensitivity) of vCJD brain PrP^{res} greater than 10 fold, with an optimal enhancing effects at a concentration of around 0.1μg/ml (Figure 6.1B, lane 6). In order to test whether the enhancing effect was specific to transgenic mouse brain

homogenate, an alternative substrate, non-CJD human brain homogenate, was used to perform the PMCA assay. The human post mortem brain homogenate was able to support vCJD brain homogenate PrP^{Sc} amplification, but is consistently less potent than the transgenic mouse brain substrate. Consequently, lower vCJD brain homogenate seed dilutions (1/1000 and 1/5000) were used. Similar enhancing effects were observed, but higher concentrations of compound were required (10µg/ml, 100µg/ml and 1000µg/ml) (Figure 6.1C and D). The inclusion of α-crystallin in the PMCA reaction did not appear to modify the typical mobility and glycoform ratio of vCJD brain PrP^{res} (known as type 2B) after PMCA.

A

α-crystallin
(μgr/ml)

10	1	0.1	0.01	0
F S	F S	F S	F S	F S

1   2   3   4   5   6   7   8   9   10   M

Seed: vCJD 1/1000

B

α-crystallin
(μgr/ml)

10	1	0.1	00.1	0
F S	F S	F S	F S	F S

1   2   3   4   5   6   7   8   9   10   M

Seed: vCJD 1/100000

C

α-crystallin
(μgr/ml)

10	100	1000	0
F S	F S	F S	F S

40 kDa
30 kDa
20 kDa

1   2   3   4   5   6   M   7   8

Seed: vCJD 1/1000

D

α-crystallin
(μgr/ml)

10	100	1000	0
F S	F S	F S	F S

40 kDa
30 kDa
20 kDa

1   2   3   4   5   6   M   7   8

Seed: vCJD 1/5000

215
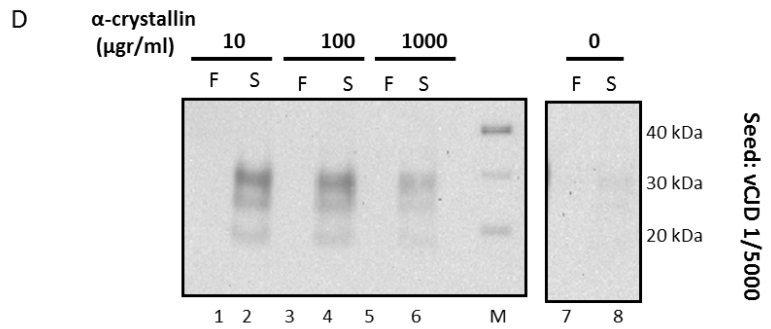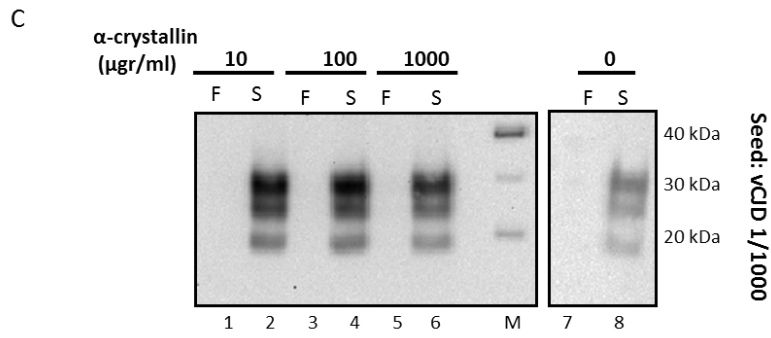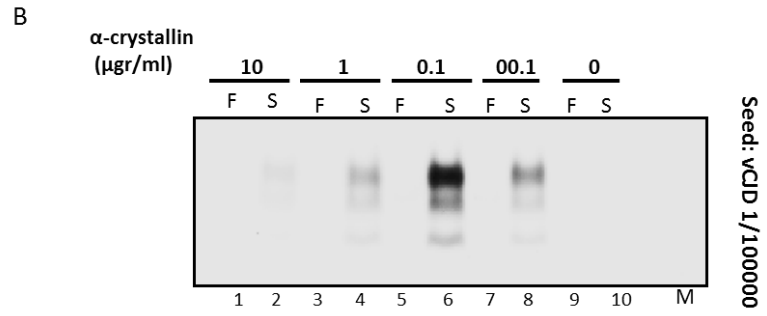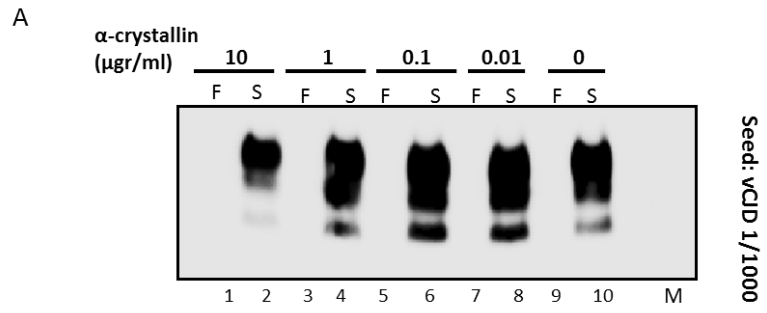
Figure 6.1 Effect of α-crystallin on PMCA seeded with variant CJD brain.
Bovine lens α-crystallin was added to PMCA reactions at the concentrations shown
using *PRNP* humanised transgenic mouse brain (*PRNP* 129 MM) as substrate (A and
B) or using non-CJD human brain homogenate (*PRNP* 129 MM) as substrate (C and
D). The seed was variant CJD (vCJD) brain homogenate added at 1/1000 (A) and
1/100000 (B) in the reactions using humanised transgenic mouse brain, and added at
1/1000 (C) and 1/5000 (D) in the non-CJD human brain homogenate. The samples
were subjected to a single round of PMCA. Control PMCA reactions (C and D) were
blotted from the same gel and given equivalent exposures; however they were not
consecutive lanes. All samples were digested with proteinase K digestion and PrP^res
detected by Western blotting. F: Frozen samples; S: Sonicated samples; M:
Molecular Marker. Immunodetection by 3F4 mAb.

6.3.2 Effect of α-crystallin on unseeded RT-QuiC reactions

In Chapter 5, RT-QuIC reactions using recombinant human prion protein were shown to display spontaneous amyloid formation as monitored by thioflavin T fluorescence. Therefore unseeded RT-QuIC reactions were utilized to evaluate the effect of α-crystallin on the spontaneous recPrP amyloid formation. The unseeded RT-QuIC reactions were performed using the full length human recombinant PrP, with either methionine (M) or valine (V) at the position of codon 129. A total of twelve replicates were used for each genotype, and the assay was run for 100 hours. Under these experimental conditions spontaneous amyloid formation was observed in both 129 M and 129 V, with a greater number of events occurring in the unseeded 129 V genotype (11/12) versus 2/12 in 129 M (Figure 6.2). Considering the results presented in the previous chapter, and the results presented here, the general observation is that spontaneous amyloid formation occurs more frequently in unseeded 129 V than 129 M recPrP RT-QuIC reactions. To determine whether unseeded PrP amyloid formation was affected by the presence of α-crystallin in the reaction, unseeded RT-QuIC reactions were performed in presence or absence of α-crystallin. The addition of α-crystallin at 10μg/ml completely suppressed spontaneous PrP amyloid formation in both unseeded 129-M and 129-V recombinant human PrP RT-QuIC reactions (Figure 6.2).
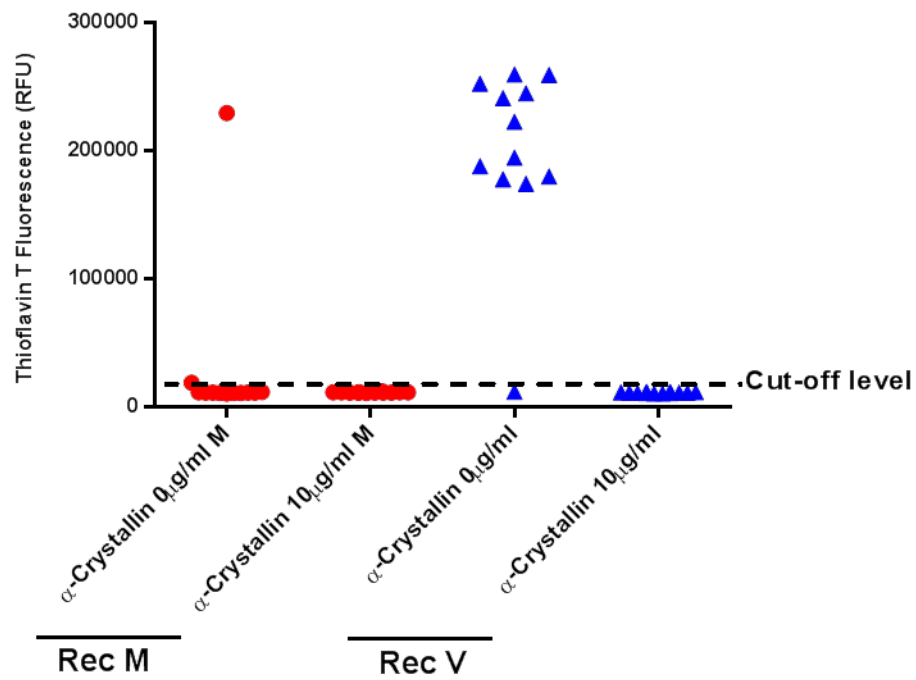
Figure 6.2 Effect of α-crystallin on unseeded RT-QuIC reactions. Twelve unseeded replicate reactions using full length recombinant human prion protein with either methionine (PrP 129M) or valine (PrP 129V) at codon 129 were run for 100 hours by RT-QuIC. Amyloid formation was monitored by thioflavin T fluorescence. Fluorescence traces for individual PrP 129M and PrP 129V reactions are shown in red and blue respectively. Bovine lens α-crystallin was added at 10μg/ml to 12 replicates for each genotype. The ("Y") axis represents the ThT fluorescence (relative fluorescence units, RFU). The ("X") axis indicates α-crystallin concentration on the two human recPrP substrates.

6.4 Discussion

Although the two methods tested here are superficially similar, PMCA and RT-QuIC differ in a number if relevant aspects, including their response to seeding with brain homogenates from different forms of CJD (Atarashi et al., 2011b, Jones et al., 2007, Jones et al., 2008, McGuire et al., 2012, Peden et al., 2012). On one hand, RT-QuIC provides a sensitive and rapid method for the detection of seeding activity in sporadic CJD brain homogenates independent of the disease subtype and substrate genotype, but performs very poorly with variant CJD (Peden et al., 2012). On the other hand, PMCA displays a strong amplification of PrP^{Sc} from vCJD brain homogenates (Chapter 3 and 6). These distinctive features placed constraints on the design of the experiments presented here, in which the effects of α-crystallin on PMCA reactions seeded with variant CJD brain homogenate, and the spontaneous amyloid formation in unseeded reactions by RT-QuIC was evaluated.

As examples of molecular chaperones, members of the α-crystallin / small heat shock protein family are known assist in protein folding and maintenance of the proteome, it is therefore not surprising that they would have an effect in cell-free conversion systems such as PMCA and RT-QuIC. The propensity to produce dysfunctional amyloidogenic conformations can be considered as an intrinsic weakness of protein structure. However, the exact molecular mechanism involved in this process is unclear.

The data presented in this chapter seems to suggest that a molecular chaperone, α-crystallin, may be a useful additive to increase sensitivity of detection of vCJD PrP^{Sc} by PMCA, and that it might also serve to inhibit the human recombinant PrP

spontaneous amyloid formation by RT-QuIC. Given that a single concentration of α-crystallin was considered to perform the RT-QuIC experiments, higher and lower concentrations than 10μg/ml of the molecular chaperone should be used to assess the inhibitory *in vitro* effect on unseeded RT-QuIC reactions. The inhibitory effect on spontaneous PrP amyloid formation in RT-QuIC is consistent with the reports that αB-crystallin inhibits both α-synuclein and Aβ fibril growth in solution (Shammas et al., 2011, Waudby et al., 2010).

In principle there are three different mechanisms that could explain the promotion of vCJD amplification by in PMCA by a molecular chaperone, (i) α-crystallin might preserve PrP^C availability and its ability to be converted in the misfolded isoform, (ii) α-crystallin might interact with PrP^{Sc} to enhance the *in vitro* conversion, or (iii) α-crystallin may interact with a third factor to influence, directly or indirectly, the PrP *in vitro* conversion.

In the context of prion disorders, a basic requirement to link α-crystallin chaperone function to prion pathology *in situ* is that both molecules co-occur in the same cellular compartment. It has been previously reported that αB-crystallin and HPS27 can accumulate in astrocytes and neurons in prion diseases (including CJD), however, within the cell αB-crystallin and PrP show a disjunct distribution. αB-crystallin and HPS27 are cytoplasmatic proteins, which can translocate to the nucleus. Whereas PrP is a GPI anchored protein, secreted through the ER and Golgi apparatus, located in lipid raft membrane microdomains and usually excluded from the cytoplasm (Aguzzi and Calella, 2009, Horwitz, 2003). Nevertheless, cytoplasmatic localization of PrP has been observed in different species and cell types, and has been proposed as a disease-associated toxic species (Chakrabarti and

Hegde, 2009). Under this scenario interaction between αB-crystallin and PrP^{Sc} is possible, however, to analyse whether this interaction is hypothetical or not, further experimental investigations are needed. Evaluating for instance a larger number of concentrations of α-crystallin in RT-QuIC and, the effect of seed and chaperone specificity by using sCJD seeds in both *in vitro* conversion systems will offer insights into the molecular mechanism involved in prion conversion, which in turn could be used as a practical outcome to improve the sensitivity and specificity of these two test methods.

Chapter Seven

General discussion and conclusion

7.1 Overview

A key molecular event in the transmissible spongiform encephalopathies (TSEs) or prion diseases is the conformational change of the cellular prion protein PrP^C into the misfolded and pathogenic isoform, PrP^{Sc}. This simple but still cryptic phenomenon triggers a series of concatenated events associated with protein aggregation, neuronal loss, toxicity, generation of infectivity and death.

Over the course of this thesis, I have addressed several aspects linked with human prion replication, in particular those associated with the genetic compatibility, molecular susceptibility, spontaneous formation, and the enhancing or inhibitory effect of other molecules on prion protein (PrP) conversion. The topics were investigated in order to obtain a better understanding of the molecular mechanisms that form the basis of prion replication. To approach these questions, PrP conversion assays (protein misfolding cyclic amplification and real time quaking-induced conversion) were used to model *in vitro* the replication and the spontaneous conversion of the normal prion protein into the disease associated isoform.

Human prion diseases are classified according to their aetiology into sporadic, inherited and acquired forms, which show clinical, genetic and neuropathological variation within each group. To model the human PrP protein-protein interaction associated with the genetic compatibility of the *PRNP* codon 129 genotypes by PMCA, normal human prion protein incubated with disease associated seeds (sCJD, VPSPr, FFI, GSS, GSS with 8kDa PrP^{res} fragment and vCJD PrP^{Sc}) were used to seed *in vitro* conversions in order to analyse the molecular compatibility of the PrP^C / PrP^{Sc} interactions.

7.2 Homologous (human:human) amplification of PrP^{Sc} from different human prion diseases (Chapter 3)

The results obtained in this chapter confirmed the competence of human prion protein to act as a substrate for the amplification of vCJD and sCJD PrP^{Sc} seeds in a cell-free conversion system. They showed also that among the six sCJD subtypes analysed (MM/MV1, MM2 thalamic, MM2 cortical, MV2, VV1 and VV2), only three were able to convert the human prion protein after a single round of PMCA (MM1/MV1, MV2 and VV2). These observations suggest that the PrP^{Sc} types, found in the most prevalent sCJD subtypes, have a greater ability to convert PrP^{C} *in vitro*, whereas the subtypes with a lower prevalence have poorer amplification potential. Differences in the ability to convert the normal prion protein *in vitro* have been reported previously in synthetic and natural prion strains by PMCA (Makarava et al., 2013, Yokoyama et al., 2011). The explanation for this is not clear, however the presence of several PrP^{Sc} structural conformations - or intermediate PrP^{Sc} conformers - in sCJD subtypes (MM2 thalamic, MM2 cortical, and VV1) might be considered as one of the reasons (Safar, 2012). Perhaps PrP^{Sc} samples derived from MM2 thalamic, MM2 cortical and VV1 cases require a step of molecular maturation or adaption before converting in an efficient manner, however, this hypothesis remains to be tested in future studies. Another possibility relates to the molecular environment in which the PrP conversion occurs. Prion conversion in mammals has been proposed to take place at the cell surface or at intracellular loci (endocytic vesicles), suggesting that the PrP^{C} / PrP^{Sc} interaction may occur in different micro-environments. Considering that the identities of the cofactors involved in the conversion process are largely unknown, we cannot exclude the possibility that there

is an appropriate molecular environment that is necessary for *in vitro* PrP^C misfolding. If so, the investigation of different cofactors such as lipids, RNA, polymers, or molecules with biological activity are necessary to understand the relatively poor *in vitro* conversion of the human prion subtypes.

PrP^{Sc} from samples of brain from sCJD MV1 and MV2 cases was able to amplify *in vitro*. Furthermore, the efficiency of amplification of both subtypes was dependant on the codon 129 genotype of the substrate. In PMCA, MV1 PrP^{Sc} seeds amplified most efficiently in MM substrates, whereas MV2 seeds displayed a preference for VV substrate. These observations are similar to those reported by Jones and colleagues. However, the results obtained here show that the ability of the PrP^{Sc} seed to convert a particular allelic substrate exhibits variation between case samples. Several questions arise from these observations. For example: (i) Considering that there may be differential expression of the two PrP alleles in heterozygous individuals, are the allelic contributions of both *PRNP* codon 129 genotypes the same in the formation of PrP^{Sc}? (ii) Also, considering that an optimization process of the *in vitro* prion conversion is needed to amplify all subtypes that show a lack of amplification - are these modifications informative about the conversion of PrP^{Sc} *in vivo*? These are questions that should be addressed in future research.

To evaluate *in vitro* conversion properties of non-standard PrP^{Sc} types, such as those found in GSS, FFI and VPSPr cases, PMCA experiments were performed. Low amplification was observed in the samples seeded with GSS brain homogenate. Nevertheless, amplification was evident in reactions seeded with FFI brain

homogenates. Both GSS and FFI are associated with mutations in the prion protein gene. GSS is linked to a substitution of proline for leucine at codon 102, whereas FFI is linked to a substitution of aspartic acid for asparagine at codon 178, and in these genetic forms both mutations are in cis with methionine at polymorphic 129. Polymorphisms and specific mutations in *PRNP* are major determinants of phenotype variability in human prion diseases. The same *PRNP* mutation (e.g. D178N) coupled with methionine or valine at the position 129 can drive the prion pathology to two different conditions (FFI, if physically coupled to 129 MM and gCJD if physically coupled to 129 VV). Chen and colleagues evaluated the allelic contribution to PrP^{Sc} in the brain of FFI cases. They observed that the majority of PrP^{res} produced in FFI cases derived from the mutated allele, with only a small contribution from the wild-type allelic product (Chen et al., 1997). In GSS cases, the contribution of wild-type PrP^{Sc} has been detected using a monoclonal antibody that recognizes wild-type PrP, but not PrP with the mutation (Wadsworth et al., 2006). Nearly all cases of genetic prion disease, such GSS and FFI, are heterozygous for the gene associated with the pathogenic mutation, and are therefore likely to express both the mutated and the wild-type form of the prion protein. The contributions of the different allelic forms of PrP into the prion conversion are not clear. However, as presented in this thesis, the participation of the wild-type PrP^{C} appears to be important factor in forming PrP^{res}. Whether the formation of FFI PrP^{res} derived from the amplification of PrP^{Sc} with or without the D178N mutation is unclear, and remains to be determined.

It is noteworthy that - for the first time - VPSPr samples derived from two different brain regions can seed the conversion of PrP^C to PrP^{Sc} in PMCA. Both frontal cortex and cerebellum amplified PrP^{Sc} *in vitro*, albeit with differences amplification efficiencies, and there was a notable increase in the formation of diglycosylated PrP^{res}. Interestingly, no amplification was observed for the 8 kDa fragment characteristic of VPSPr cases, similar to the reaction seeded with GSS with the 8 kDa fragment. Whether the conversion of the different PrP^{Sc} conformations has distinctive conversion properties, kinetics, or competence to propagate *in vivo* or *in vitro* is unclear and remains to be investigated. What is clear is that the heterogeneity of prion conformations derived from the mutations or the polymorphisms in the prion protein gene or the spontaneous conversion of the PrP^C is unpredictable and difficult to estimate. Molecular adaptation, selection and evolution of prions, could at least partially explain the *in vitro* different amplification efficiencies, and seed / substrate compatibilities of different PrP^{Sc} types *in vitro* summarized in Table 7.1.

Seed source (PrP^{Sc})	PMCA substrate (PrP^{C} codon 129)		
	MM	MV	VV
Idiopathic			
sCJD			
MM1	✓✓	✓	✓
MV1	✓	✓	✓
VV1	*nt*	*nt*	✗
VV2	✓✓	✓	✓✓✓
MV2	✓	✓	✓✓
MM2 thalamic	✗	*nt*	*nt*
MM2 cortical	✗	*nt*	*nt*
VPSPr	*nt*	*nt*	✓
Genetic			
GSS	✗	*nt*	*nt*
GSS with 8kDa fragment	✗	*nt*	*nt*
FFI	✓	*nt*	*nt*
Acquired			
vCJD	✓✓✓	✓✓	✓

Table 7.1 Summary of PMCA reactions conducted using brain homogenates from different human prion diseases as a source of PrP^{Sc} and humanised transgenic mouse brain homogenate containing PrP^{C} as a substrate.
(✓✓✓) Correspond to a robust amplification (over 10 fold of conversion)
(✓✓)　 Correspond to a moderate amplification (between 4 – 10 fold of conversion)
(✓)　　 Correspond to a low amplification (between 1 – 4 fold of conversion)
(✗)　　 Corresponds to an absence of conversion
(*nt)*　 Not tested

7.3 Heterologous (animal:human) amplification of PrP^{Sc} from different animal prion diseases (Chapter 4)

In order to study the molecular basis of transmission barriers, a panel of animal prion diseases were used to assess the susceptibility of conversion of the human prion protein by the *in vitro* conversion assay, PMCA. Amplification of the human PrP^C, derived from different biological sources (normal human brain, humanized transgenic mouse brain or cell cultures), incubated with BSEs (C-type BSE, L-type BSE, H-type BSE), classical and atypical scrapie, and chronic wasting disease (CWD), showed that the known susceptibility of humans to BSE infection and the presumed resistance to scrapie infection are reflected in the susceptibility of human PrP^C to be converted in PMCA by BSE and scrapie brain homogenates. Furthermore, the atypical forms of BSE and scrapie failed to produce human PrP conversion; nonetheless, the CWD isolate did convert human PrP^C independent of the origin of the substrate (human brain, transgenic mouse brain or human cells) and in a codon 129 dependent manner, showing a more efficient amplification for the methionine substrate than the valine. Comparing the CWD amplification efficiency alongside C-type BSE and vCJD, a robust amplification was seen with vCJD, followed by C-type BSE and lastly CWD. These findings suggest that, at least at the molecular level, atypical scrapie and atypical BSE present a lower level of risk of zoonotic disease than classical BSE. They also suggest that there is no absolute barrier to the conversion of human PrP^C by CWD prions in a single round of PMCA.

It has been previously reported that mule deer CWD require an *in vitro* conditioning before to convert humanized transgenic mouse substrate (Barria et al., 2011). However, as described in this chapter a single round of PMCA using an elk CWD

specimen was sufficient to convert the human prion protein. The reason behind this difference is unclear; nonetheless based on the available knowledge of prion transmission, adaptation, and stabilization – *in vivo* and *in vitro* - it is likely that molecular prion protein diversity it can lead to the selection of a prion strain, able to propagate efficiently in the same, or in a different species (Castilla et al., 2008a, Meyerett et al., 2008). Before outlining a more definitive conclusion, it will be important to test a wide variety of CWD isolates from different cervid species, polymorphic genotypes and geographical locations. The results presented here, raise again the question about CWD and its zoonotic potential.

7.4 Spontaneous PrP^{Sc} formation using *in vitro* conversion systems as a model for sporadic CJD (Chapter 5)

This chapter focussed on the use of the cell-free conversion systems protein misfolding cyclic amplification and real time quaking-induced conversion to model the spontaneous misfolding of the human prion protein *in vitro*. A key event associated with all amyloidogenic diseases is the molecular conversion of wild-type cellular proteins into disease associated isoform. In this respect, the misfolded PrP, or PrP^{Sc}, is perhaps the prime example. Currently, two possible mechanisms have been proposed to generate spontaneous conformational changes of the cellular prion protein: somatic mutation of the *PRNP* gene sequence and spontaneous post-transcriptional conversion of the cellular PrP conformation. To gain a deeper understanding of the second mechanism, a combination of two *in vitro* conversion assays were used to model the spontaneous misfolding of the human prion protein. The ultimate goal was evaluating the propensity of the human prion protein to spontaneous acquire a β-sheet-rich conformation *in vitro*. Using RT-QuIC spontaneous amyloid formation was detected in unseeded samples incubated with bacterially expressed recombinant human protein, with either methionine or valine at the codon 129. Human recombinant PrP carrying the amino acid valine at the position 129 showed a greater predisposition to spontaneously form amyloid in comparison to its allelic counterpart with methionine at the same position. The presence of methionine or valine at the codon 129 of the *PRNP* is the most important polymorphism associated with CJD. Considering that 70% of the sCJD cases have methionine homozygosity at the codon 129, the finding presented in this thesis that recombinant PrP with valine at codon 129 is more predisposed to amyloid formation

seems surprising. However, if we take into consideration one of the four different hypothesised PrP folding pathways; this observation is not so unreasonable. Four major folding pathways of recombinant human PrP have been proposed (Baskakov et al., 2005). In one of them, the transformation of partially unfolded PrP to multimeric β-sheet-rich amyloid forms is hypothesised (Figure 7.1A). Aggregation studies using recombinant prion protein have shown that allelic variation at the codon 129 of *PRNP* impacts on the formation of misfolded PrP amyloids. The presence of valine at the residue 129 accelerates the PrP amyloid formation in spontaneous and seeded reactions showing a considerable reduction on the lag phase to produce amyloid as compared with the methionine 129 allelomorph under the same condition (Baskakov et al., 2005). This observation provides an explanation of the difference in the susceptibility to spontaneous generation of PrP aggregates by the two recombinant PrP polymorphic substrates in this study.

It is widely accepted that one of the possible pathways to the conversion of PrP^C to the β-sheet-rich isoform requires a certain degree of unfolding of PrP^C (Baskakov et al., 2005). It is also well accepted that the air / water interphase - through a denaturation boundary that exists between the hydrophilic water environment and the hydrophobic air environment - could induce unfolding of recombinant PrP^C (Reviewed by Atarashi R, 2011) (Atarashi et al., 2011a). Such 'boundary-induced-unfolded' condition can be increased due the mechanical agitation characteristic of RT-QuIC (Atarashi et al., 2011b). The combination of these two phenomena: the partial denaturation of the recombinant PrP by the exposition to the air / water interfaces, and the effect of the codon 129 polymorphism in the predisposition to

produce spontaneous PrP amyloids could explain, at least partially the results presented in the chapter 5 (Figure 7.1C).

A Model of multiple folding pathways of recombinant human PrP

(intermediate state?)

(intermediate state?)

(3)

(4)

(1)

(2)

α-helix-rich-PrP

unfolded-PrP

β-sheet-rich-PrP oligomers/amyloids

B Denaturant-free solution

α-helix-rich-PrP

unfolded-PrP

β-sheet-rich-PrP oligomers/amyloids

C Denaturant-free solution + air/shear forces

α-helix-rich-PrP

unfolded-PrP

β-sheet-rich-PrP oligomers/amyloids

D Denaturant-free solution + air/shear forces + chaperon (α-Crystallin)

α-helix-rich-PrP

unfolded-PrP

β-sheet-rich-PrP oligomers/amyloids

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Figure 7.1 **Hypothetical model of the folding and the spontaneous conversion of PrP** *in vitro*. **(A)** Four routes of PrP folding from denatured recombinant human PrP have been proposed by Baskakov and Tahiri-Alaoui and colleagues (Baskakov et al., 2005, Tahiri-Alaoui and James, 2005). The first pathways produce α-helix-rich, soluble and monomeric PrP from PrP in a denatured state (1). The second pathway results in the formation of soluble, β-sheet-rich and PK resistant PrP oligomeric forms (2). The third pathways produce β-sheet-rich-PrP amyloids from PrP α-helix-rich isoform (3) and the last pathway produce PrP β-sheet-rich amyloids, from partially unfolded PrP (4). **(B)** Homogeneous folded recombinant PrP^C in a denatured free condition. **(C)** Exposure of folded recombinant PrP^C to the air-water interfaces and shearing forces can induce unfolding of the recombinant PrP^C. **(D)** Increased levels of unfolded (or partially unfolded) recombinant PrP , induced by the condition (C), can be redirected to the pathway (1) by molecular actions of the chaperones *in vitro* (α-crystallin). Low levels of unfolded or partial unfolded PrP α-helix-rich, can lead to a decrease of β-sheet-rich PrP oligomeric and amyloids formations. Arrows represent the potential direction of prion folding routes.

Serial rounds of PMCA seeded with spontaneous recombinant PrP amyloid aggregates - created by RT-QuIC, either methionine or valine at the codon 129 - showed an efficient *in vitro* amplification when PrP homozygous for methionine at codon 129 was used as a substrate. In contrast, there was a lack of conversion in similarly seeded PMCA reactions using PrP homozygous for valine at codon 129. Multiple factors might explain this finding, including inhibition or incompatibility PrP seed / substrate, enhancing or blocking effect of cofactors (such lipids, RNA or glycosaminoglycans), among others. It has been proposed that the amplification and spontaneous formation of prions *in vitro* may involve the potential selection of specific cofactors according to the architecture of the amplified products (Supattapone, 2013, Yokoyama et al., 2011), and that cell-free conversion systems can model the spontaneous prion formation. The combined progress in the development of new strategies to understand the molecular structure of the prion protein along the systematic investigation of the *in vitro* prion replication are essential to establish a mechanistic model to explore the spontaneous formation and replication of prions.

7.5 A possible role for molecular chaperones in prion protein conversion (Chapter 6)

Protein folding defines the three-dimensional structures of polypeptides and how they acquire their functional shape. In prion diseases, the conformational change of the normal folded PrP^C is thought to trigger a series of events associated with accumulation of the abnormal prion protein in the brain, toxicity, and cell death. Heat shock proteins (HSP) are synthetized in response to heat shock or other kinds of physiological stress. Many HSPs are molecular chaperones that mediate the proper folding and translocation of proteins under normal physiological conditions and preserve native conformation during physiological stress and disease states. In order to test whether the chaperone activity of α-crystallin affects prion protein conversion, PMCA and RT-QuIC were used to evaluate the *in vitro* amplification of vCJD, and the spontaneous formation of recombinant human β-rich-sheet aggregates respectively.

The inclusion of α-crystallin in the PMCA reaction enhanced the vCJD amplification greater than 10 fold compared to the control reaction, showing an optimal effect at concentration around 0.1μg/ml. Conversely, the incorporation α-crystallin on unseeded RT-QuIC reactions suppressed the spontaneous PrP amyloid formation in reactions carrying the amino acid methionine or valine at the position 129 of the prion protein gene. Taken together these data suggest that α-crystallin may have both direct and indirect effects on the conversion of the prion protein. Assuming that shear forces and air-water interfaces induce unfolding or partially unfolding of PrP in RT-QuIC (Figure 7.1C), one possible explanation is that α-crystallin interacts with denatured PrP reverting the conformation to its native rich-α-helix state (Figure

7.1D). On the other hand, the PrP^C able to be converted into the misfolded isoform in PMCA is usually derived from tissue homogenate; therefore the complexity arising from the heterogeneity of protein interactions is higher than in RT-QuIC. In this situation, α-crystallin can be interacting with PrP^C promoting the substrate availability (or competence), or interacting with third factors that influence the prion conversion. Considering that α-crystallin increases the amplification efficiency in a seeded reaction, it is also conceivable that the interaction of the α-crystallin protein might be through the interaction with the PrP^{Sc}, promoting the seed competence. Whether these properties are shared among others members of the α-crystallin / small heat shock proteins family, or others molecular chaperons, is an open question. The role of the molecular chaperones on the prion conversion in mammalian cells is unknown, but using cell-free conversion systems it might possible to gain a deeper understanding of the role of these molecules over the folding and misfolding of the prion protein (Meehan et al., 2007, Narayan et al., 2012, Shammas et al., 2011). Finally, the information obtained in the chapter 6, could be used in the optimization of *in vitro* prion protein conversion assay to improve the actual developing of a diagnostic test.

7.6 General conclusion

In vitro conversion assays can recapitulate and model fundamental aspects of the human prion phenomena. Through the research performed in this thesis it has been possible to (i) evaluate the combined effects of different PrP type and genotypes, (ii) replicate aspects of cross-species transmission potential and provide new information about molecular barrier to zoonotic transmission, (iii) model the spontaneous PrP^{Sc} formation, and (iv) assess the potential impact of chaperones on conversion of the human prion protein. In summary, this work provides molecular evidence about the origin, replication and transmission of human prions.

Appendix

Data generated form this thesis was published in two scientific manuscripts listed below. Full permission has been granted form the publishing journals. Both are included in this Appendix.

Barria, M. A., Balachandran, A., Morita, M., Kitamoto, T., Barron, R., Manson, J., Knight, R., Ironside, J. W. & Head, M. W. (2014a). Molecular barriers to zoonotic transmission of prions. *Emerg Infect Dis,* 20**,** 88-97.

Barria, M. A., Ironside, J. W. & Head, M. W. (2014b). Exploring the zoonotic potential of animal prion diseases: In vivo and in vitro approaches. *Prion,* 8**,** 85-91.

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Molecular Barriers to Zoonotic Transmission of Prions

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Jean Manson, Richard Knight, James W. Ironside, and Mark W. Head

The risks posed to human health by individual animal prion diseases cannot be determined a priori and are difficult to address empirically. The fundamental event in prion disease pathogenesis is thought to be the seeded conversion of normal prion protein to its pathologic isoform. We used a rapid molecular conversion assay (protein misfolding cyclic amplification) to test whether brain homogenates from specimens of classical bovine spongiform encephalopathy (BSE), atypical BSE (H-type BSE and L-type BSE), classical scrapie, atypical scrapie, and chronic wasting disease can convert normal human prion protein to the abnormal disease-associated form. None of the tested prion isolates from diseased animals were as efficient as classical BSE in converting human prion protein. However, in the case of chronic wasting disease, there was no absolute barrier to conversion of the human prion protein.

Prion diseases are rare fatal neurodegenerative conditions that affect humans and animals. The human diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia. Most cases of human prion disease are apparently spontaneously occurring (sporadic CJD [sCJD]) or are associated with mutations in the human prion protein gene, designated *PRNP* (genetic CJD, Gerstmann-Sträussler-Scheinker disease, or fatal familial insomnia). A small minority of cases are acquired by inadvertent human-to-human transmission during medical or surgical treatments (iatrogenic CJD).

In contrast, animal prion diseases are generally acquired. This applies to scrapie in sheep, transmissible mink encephalopathy, and chronic wasting disease (CWD) in deer and elk. No credible evidence exists of a link between scrapie and any human prion disease, despite the endemicity of scrapie in many parts of the world and the consequent likely human exposure to the scrapie agent, which has been attributed partly to a species barrier between sheep and humans. However, strong epidemiologic, pathologic, and molecular evidence does indicate that the epidemic of bovine spongiform encephalopathy (BSE), primarily in the United Kingdom during the 1980s, resulted in a zoonotic form of CJD termed variant CJD (vCJD). BSE/vCJD is the only known zoonotic prion disease strain.

After identification of BSE and vCJD, active surveillance for animal prion diseases in Europe and elsewhere has identified rare atypical prion diseases in sheep and cattle. These include Nor98 or atypical scrapie in sheep (*1*) and 2 prion diseases of cattle, bovine amyloidotic spongiform encephalopathy or L-type BSE (*2*) and H-type BSE (*3*), both of which have a pathology and epidemiology distinct from classical or C-type BSE (*4*). In addition to these new (or newly described) diseases of farmed sheep and cattle, CWD in cervids is an acquired, probably contagious disease that affects captive and free-ranging deer and elk populations primarily in North America (*5*).

Their distinctive epidemiology, clinical features, neuropathology, PrP biochemistry, and transmission characteristics suggest that scrapie, atypical scrapie, C-type BSE, H-type BSE, L-type BSE, and CWD represent distinct prion strains in their respective species (*6,7*). Within scrapie and CWD, natural strain variation also occurs. The prion hypothesis posits that the posttranslational conformational conversion of a host's normal cellular prion protein (PrP^C) by the abnormal form of the prion protein (PrP^{Sc}) is the fundamental event in prion disease pathogenesis and that PrP^{Sc} itself constitutes the infectious agent. It follows that an aspect of prion host range may be a species barrier operating at the molecular level that depends on compatibility between the PrP^{Sc} from 1 species and the PrP^C from another. Similarities in the species-specific primary *PRNP* sequences may account for part of this effect, but prion strain and host *PRNP* polymorphic genotype, both of which

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probably find expression in the conformation of PrP, affect susceptibility in ways not yet fully understood.

A relatively simple empirical approach to assessing this molecular barrier is to use cell-free PrP conversion assay techniques (8,9) to determine the relative efficiency of PrP conversion using natural "seeds" from an infectious prion source from the brain of 1 species and a normal brain "substrate" from another species. We have previously reported the use of protein misfolding cyclic amplification (PMCA) as a model of cross-species prion transmission of C-type BSE in cattle and sheep to humans (10). Here we report a comparative study of the ability of sheep, cattle, and deer prions to convert normal human PrP in this same cell-free system.

Methods

Ovine, bovine, and cervine frozen brain tissue from prion disease–affected and –unaffected animals were obtained by request from the Animal Health Veterinary Laboratory Agency TSE Archive (AHVLA, Weybridge, UK). The cases and brain regions supplied were selected on the basis of proven disease status and of brain region with an expected high PrP^{sc} load, characteristic of the particular prion disease. The prion disease status of the animals involved was determined at AHVLA and/or the Canadian Food Inspection Agency's Ottawa laboratory (Ottawa, ON, Canada) by neuropathology and PrP immunohistochemistry. The classical scrapie specimen was of brain stem from a field suspect of the animal prion protein gene *Prnp* ARQ/ARQ genotype, and a brain stem specimen from an unaffected scrapie suspect of the same genotype was also supplied. The atypical scrapie specimen was of parietal cortex, also from a field suspect, but of the ARQ/ AHQ genotype, and a corresponding negative control animal specimen was also supplied. The C-type BSE samples were of brain stem from confirmed positive C-type BSE suspects obtained through passive surveillance, and the corresponding negative control specimen was similarly obtained. Both the H- and L-type BSE specimens were of frontal cortex from successful experimental bovine transmissions conducted at AHVLA. Mid-brain tissue from a confirmed CWD-positive and control-negative (unaffected) elk (both with *Prnp* codon 132MM genotype) was also supplied through the AHVLA.

Frozen half brains from inbred transgenic mouse lines expressing human PrP^{C} of the *PRNP* codon 129 methionine (129MM) and valine (129VV) genotypes (11–13) were used for PMCA substrate preparation. The production of PMCA substrates from stably transfected human 293F cells overexpressing human PrP^{C} (exogenous *PRNP* codon 129M and endogenous *PRNP* codon 129MM) has been described (14). A *PRNP* codon 129 valine expressing counterpart was engineered by suppressing expression of

endogenous *PRNP* codon 129MM expression with RNAi and transient transfection with a *PRNP* codon 129 valine expression vector (designated 129V).

Human brain tissues (frontal cortex) were sampled from frozen half brains collected at autopsy with the appropriate consent for tissue retention and research use. The vCJD specimen was from a patient (*PRNP* codon 129MM) with definite vCJD as defined by established criteria. The non-CJD human brain specimens used for PMCA substrate preparation were frontal cortex from patients with Guillain-Barré syndrome (129MM) and dementia with Lewy bodies (129VV). sCJD specimens from patients with the MM1 and VV2 subtypes of the disease were used as reference standards in certain Western blotting experiments. Ethical approval for the use of these tissues in this study is covered by Local Research Ethics Committee 2000/4/157.

Brain homogenates were prepared by using a manual homogenizer and chilled conversion buffer (150 mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail in 1× phosphate-buffered saline) to obtain a final 10% wt/vol solution. The homogenized tissue was cleared by centrifugation at 2,000 rpm for 40 s in a refrigerated centrifuge (4°C), and the supernatant was aliquoted and stored at -80°C (15).

We prepared homogenates (10% wt/vol) of C-type BSE, scrapie, CWD, L-type BSE, H-type BSE atypical scrapie, and vCJD brain. We followed the same method used for the substrate brain homogenate.

PMCA experiments were carried out in PCR tubes. Aliquots of 10% substrate brain homogenate (or 20% cell extracts) were mixed with 10% prion disease brain seeds in a final volume of 120 μL. Low molecular weight heparin was included at 100 μg/mL in all PMCA reactions (15). Before sonication, 19 μL of the PMCA reaction mixture (termed "frozen" sample) was taken for comparison with the amplified sample (termed "sonicated" sample). The reactions were incubated into the microplate horn of a programmable sonicator (Misonix 4000, Misonix, Farmingdale, NY, USA) at 37°C. A total of 96 PMCA cycles were performed comprising 20 s of sonication (at an amplitude of 90%) followed by 29 min 40 s of incubation for every cycle (16).

Tissue homogenates and PMCA reaction products were digested with proteinase K (50 μg/mL for 1 h at 37°C) and detected by Western blotting (10). Detection was with 3F4 or 6H4 antibodies diluted in 1× phosphate-buffered saline, 0.05% Tween 20 (10). The monoclonal antibody 9A2 was obtained from Central Veterinary Institute Wageningen UR (Lelystad, the Netherlands) (7). Membranes were developed by using peroxidase conjugated secondary antibody and a luminescent peroxidase substrate ECL-Plus (10). Finally, blots were exposed to a photographic film and the image acquired using the XRS digital CCD camera system (Bio-Rad Laboratories, Hercules, CA, USA). The antibody 6H4 recognizes an epitope in the protease-resistant

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core of human PrP spanning amino acids 145–153, and it cross-reacts with bovine, ovine, and cervine PrP. 3F4 also reacts with a sequence in the protease resistant core of the human PrP spanning positions 106–112, but 3F4 does not recognize PrP from species other than humans and hamsters. The combination of protease-digestion and detection by 3F4 in these experiments therefore provides a very sensitive method for detecting newly formed human protease-resistant prion protein (PrP^{res}) (10,17). The antibody 9A2 reacts with human PrP amino acids 99–101 and cross-reacts with ovine, bovine, and cervine PrP.

Results

PrP was confirmed in all animal brain samples in the form of 2 major bands in the 20–40-kDa molecular mass range, probably corresponding to full-length diglycosylated PrP (upper band) and N terminally truncated diglycosylated or full-length monoglycosylated PrP (lower band). The levels of PrP and the electrophoretic pattern were broadly similar between prion disease and corresponding negative control (unaffected) animal brain samples (Figure 1, panel A). However, proteinase K digestion showed differences in the amount of PrP^{res} contained in these samples. PrP^{res} was most abundant in classical scrapie and CWD samples; lower levels were seen in C-, H- and L-type BSE samples and were barely detectable at this level of sensitivity in atypical scrapie (Figure 1, panel B). Normalizing the Western blot PrP^{res} signal by adjusting sample loading volumes demonstrated the expected PrP^{res} relative mobilities and glycosylation types (data not shown). When larger volumes of brain homogenate were analyzed, a characteristic <10-kDa band was present in the atypical scrapie specimen (Figure 1, panel C).
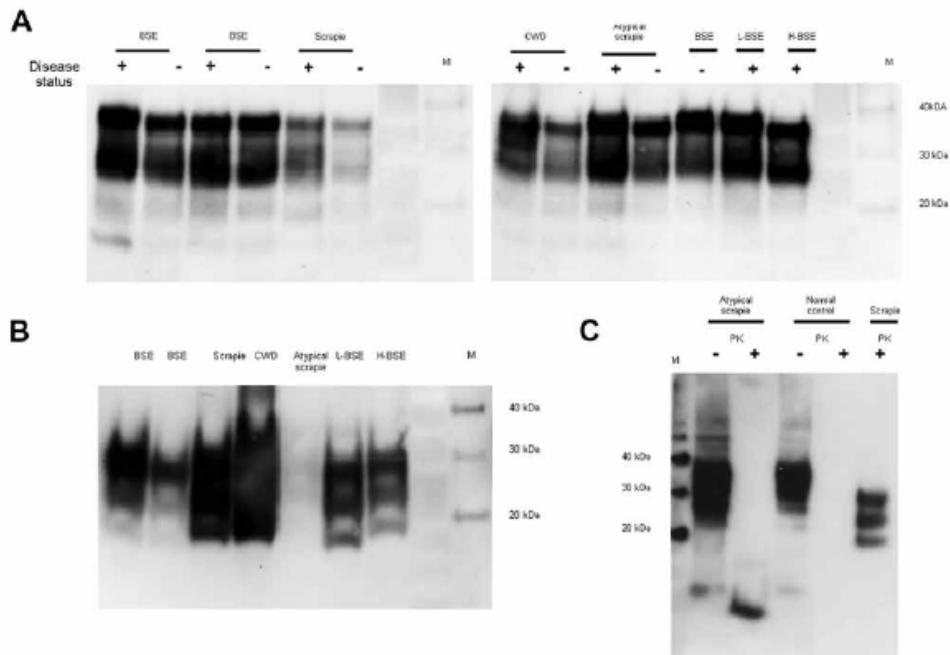
Figure 1. Determination of total PrP and PrP^{res} level in animal tissues. To characterize the PrP expression levels (total PrP), brain homogenates were analyzed by Western blot without digestion with PK. Nineteen microliters of each 10% wt/vol homogenate was loaded in each lane (A). To detect the PrP^{res} in the samples, PK digestion (50 µg/mL) was performed to remove PrP^C, and the samples were then reanalyzed (B). The atypical scrapie and matched normal control animal samples were further analyzed by Western blot both with (+) and without (–) prior PK digestion (C) by comparing 3 µL of undigested homogenates with 100 µL of the PK-digested sample concentrated by centrifugation. Five microliters of a PK-digested classical scrapie brain homogenate was analyzed in parallel for comparison. The detection antibody was 6H4 in (A) and (B) and 9A2 in (C). PrP, prion protein; PrP^{res}, protease-resistant PrP; PK, proteinase K; PrP^C, normal cellular PrP; M, molecular marker; BSE, bovine spongiform encephalopathy; +, animal prion disease sample; –, matched normal animal control sample; CWD, chronic wasting disease; L-BSE, L-type BSE; H-BSE, H-type BSE.

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We evaluated the susceptibility of the human PrP (129MM) to in vitro conversion first using the human brain homogenate substrate. Western blotting with antibodies 3F4 and 6H4 both showed readily detectable amplification in the samples seeded with C-type BSE and vCJD. (Figure 2, panel A, lanes 2, 4, and 14, compared with lanes 1, 3, and 13). Scrapie, L-type BSE, H-type BSE, and atypical scrapie reactions did not show detectable human PrP^res formation with the 3F4 antibody (Figure 2, panel A, lanes 6, 10, 12, and 16). However, 3F4 detected human PrP^res in the reaction seeded with the CWD brain homogenate (Figure 2, panel A, lane 8). Humanized transgenic mouse (129MM) brain substrate similarly showed efficient amplification of vCJD and C-type BSE and readily detectable amplification

of CWD PrP^res using 3F4 (Figure 2, panel B). Faint bands were seen in L-type BSE and H-type BSE PMCA reactions when the 6H4 antibody was used. These bands most likely represent conversion of endogenous bovine PrP^C from the inoculum converted to PrP^res, rather than conversion of human PrP^C from the substrate (compare lanes 11 and 12 for 6H4 and 3F4 in Figure 2, panels A, B).

Dilutions of CWD brain homogenate in substrates prepared from human brain, transgenic mouse brain, and 239F cells expressing human PrP (129M or 129V) were compared for their ability to support amplification. Irrespective of origin, all 3 *PRNP* 129M-containing substrates supported amplification, albeit with slightly different efficiencies (Figure 3, panels A, B, C). All three *PRNP*
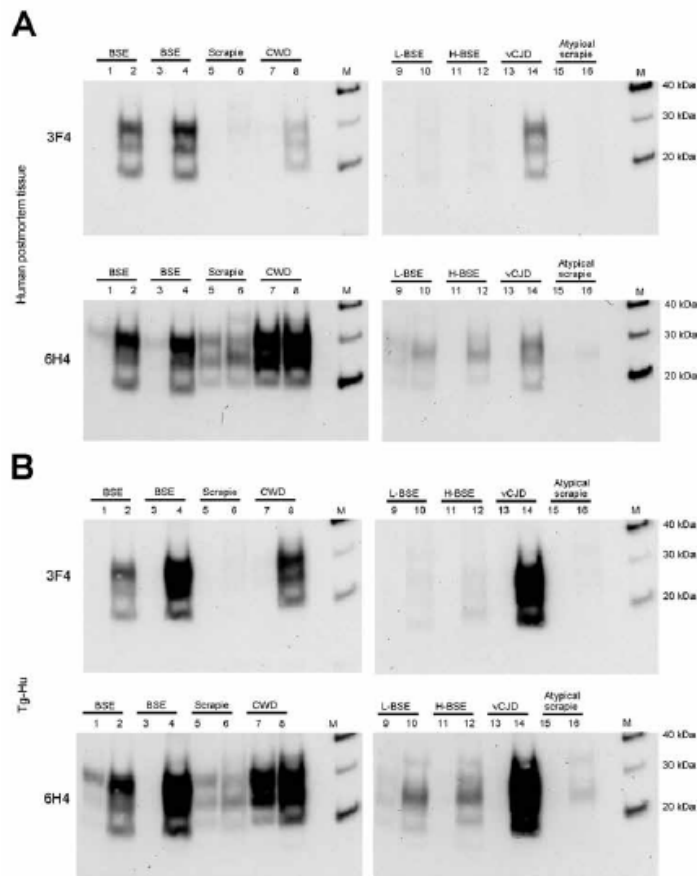
Figure 2. PMCA of *PRNP* codon 129MM human brain homogenate and humanized transgenic mice brain homogenate seeded with C-BSE, scrapie, CWD, L-BSE, H-BSE, vCJD, and atypical scrapie. PMCA reactions using *PRNP* 129MM human brain homogenate (human postmortem tissue) (A) and *PRNP* 129MM humanized transgenic mouse brain homogenate (Tg-Hu) (B) were seeded (1:3) with animal prion disease brain as indicated. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 show the samples without PMCA. Samples in lanes 2, 4, 6, 8, 10, 12, and 14 were subjected to PMCA. Western blotting used the antibody 3F4 that enables the specific detection of human PrP. To compare the PrP^res levels into the seeds (before the PMCA), antibody 6H4 was also used. PMCA, protein misfolding cyclic amplification; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; vCJD, variant Creutzfeldt-Jakob disease; PrP, prion protein; PrP^res, protease-resistant PrP; M, molecular marker.

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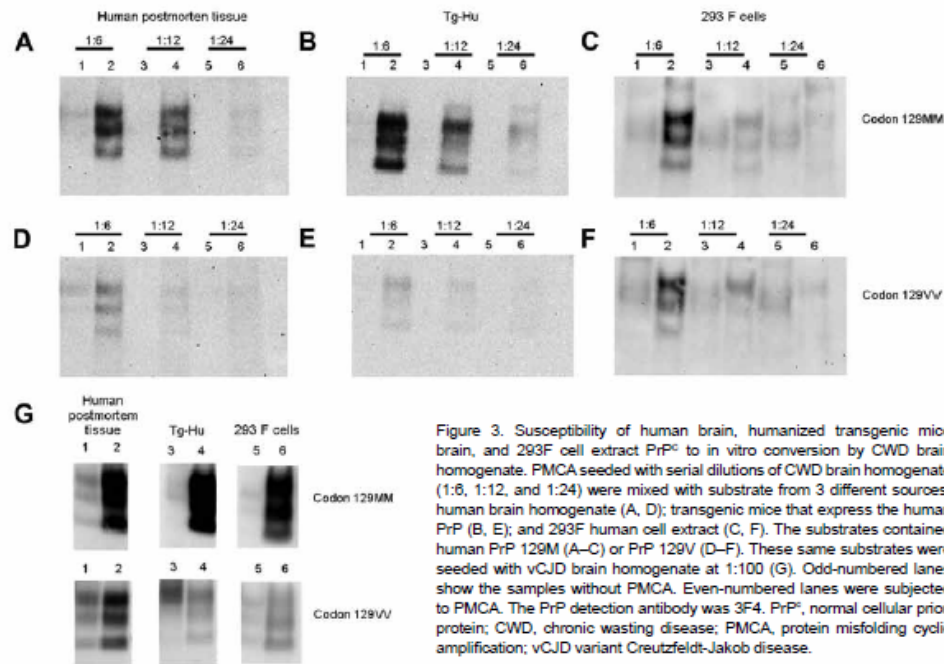
Figure 3. Susceptibility of human brain, humanized transgenic mice brain, and 293F cell extract PrP^C to in vitro conversion by CWD brain homogenate. PMCA seeded with serial dilutions of CWD brain homogenate (1:6, 1:12, and 1:24) were mixed with substrate from 3 different sources: human brain homogenate (A, D); transgenic mice that express the human PrP (B, E); and 293F human cell extract (C, F). The substrates contained human PrP 129M (A–C) or PrP 129V (D–F). These same substrates were seeded with vCJD brain homogenate at 1:100 (G). Odd-numbered lanes show the samples without PMCA. Even-numbered lanes were subjected to PMCA. The PrP detection antibody was 3F4. PrP^c, normal cellular prion protein; CWD, chronic wasting disease; PMCA, protein misfolding cyclic amplification; vCJD variant Creutzfeldt-Jakob disease.

129V-containing substrates also supported amplification (Figure 3, panels D, E, F), although the level of amplification was lower than for the 129M equivalent. PMCA reactions using vCJD brain homogenate as a seed were conducted by using these same substrates and are shown for comparison (Figure 3, panel G).

Next, we estimated the relative PrP^{res} amount in each sample by densitometry and adjusted the volume of 10% brain homogenate from the different animal prion diseases used in the PMCA reaction to give roughly equivalent amounts of PrP^{res} seed in each reaction (Figure 4). The amount of PrP^{res} in the atypical scrapie specimen was so low that a maximum volume of homogenate was used. The results of the Western blot analysis of these seed PrP^{res} normalized PMCA reactions, using antibody 3F4, confirmed that amplification efficiency was a function of seed/substrate compatibility and not simply PrP^{res} abundance.

C-type BSE, vCJD, and CWD amplification products were normalized and diluted (1:3, 1:6, 1:12, 1:24) in fresh human brain tissue homogenate (129MM) and subjected to a second round of PMCA. The CWD and C-type BSE PMCA reaction products retained their ability to convert

further human 129M PrP, albeit at a lower efficiency than vCJD (Figure 5).

Western blot analysis of PrP^{res} produced by a PMCA reaction using human brain homogenate (129MM) seeded with CWD brain homogenate (Figure 6, lane 2) showed that this PrP^{res} shared the mobility and general glycosylation profile of type 1 PrP^{res} from sCJD brain (MM1 subtype) (Figure 6, lane 1). It was distinct from that of type 2 PrP^{res} characteristic of sCJD (VV2 subtype) and vCJD (Figure 6, lanes 3 and 4, respectively).

Discussion

Multiple factors govern the transmission of prions in experimental settings. In addition to infectious dose and route, a species or transmission barrier phenomenon is well recognized. Within the theoretical confines of the prion hypothesis, the most obvious basis of a species barrier effect would be dissimilarity in *PRNP* sequence between the infectious source and the exposed individual. However, *PRNP* sequence similarity alone does not seem to accurately predict whether prions are transmissible between species, perhaps because interactions between PrP^C and PrP^{Sc} occur as native PrP^C and misfolded and aggregated PrP^{Sc}
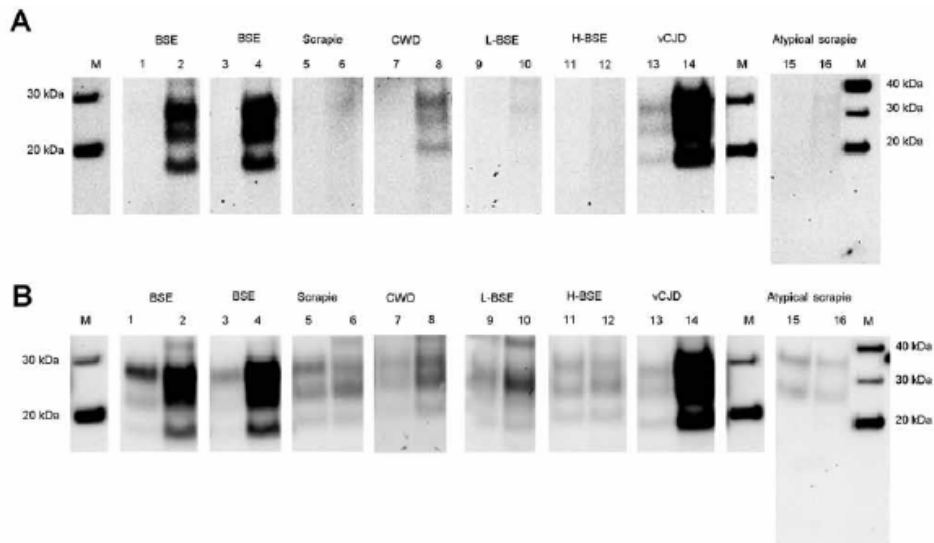
Figure 4. Relative conversion efficiency of human PrP (129M) by different animal prion disease samples. Brain homogenates from animal prion diseases were seeded at different volumes adjusted to give roughly equivalent amounts of seeding PrP^{res} and amplified by using PrP 129M-containing human brain substrate. Human PrP^{res} formation was detected by the 3F4 antibody (A) and seed and newly formed PrP^{res} detected using the 6H4 antibody (B). PrP, protein prion; PrP^{res}, protease-resistant PrP; M, molecular marker; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; L-BSE, L-type BSE; H-BSE, H-type BSE; vCJD variant Creutzfeldt-Jakob disease; M, molecular marker.

conformers. The possible effects of species-specific sequence difference on PrP^{C} folding are not well understood. Neither is the secondary and higher order structure of PrP^{Sc} except for clear evidence that different prion strains are associated with different PrP^{Sc} conformers and glycotypes (reviewed in *4,18*) and that these might exist as a quasispecies or molecular cloud (*19*). Under such a scenario molecular compatibility might be difficult to predict.

To isolate and study molecular effects, we have previously conducted cell-free PrP conversion experiments by PMCA using homogenates of bovine and ovine prion disease brain samples to seed brain homogenates containing human PrP, assessing the extent of conversion by detection of human PrP^{res}. These studies showed that samples of C-type BSE (which is a known human pathogen and the cause of vCJD) efficiently converted human PrP, with a codon 129 preference similar to that of vCJD (MM>MV>VV), whereas samples of classical scrapie (which is not thought to be a human pathogen) failed to convert human PrP to a measurable extent. Equally importantly, a sheep BSE isolate resembled C-type BSE and vCJD in its ability to convert human PrP, thus underscoring influence of strain over sequence similarity in determining what might be termed a molecular transmission barrier (*10*).

Here we applied the same approach to a series of animal prion diseases whose risk to human health is poorly characterized. Our results show that under the PMCA conditions used, L-type BSE, H-type BSE, and atypical scrapie isolates fail to produce detectable human PrP^{res}. The CWD isolate used converted human PrP^{C}, albeit less efficiently than C-type BSE. This observation remained true whether the input animal prion disease brain homogenate was normalized by tissue weight or by PrP^{res} abundance and whether the PMCA substrate was from human brain, *PRNP* humanized murine brain, or a human-derived and human PrP^{C} overexpressing cell line. The conversion of human PrP^{C} by CWD brain homogenate in PMCA reactions was less efficient when the amino acid at position 129 was valine rather than methionine. Furthermore, the form of human PrP^{res} produced in this in vitro assay when seeded with CWD, resembles that found in the most common human prion disease, namely sCJD of the MM1 subtype.

Previous attempts to determine the transmissibility of these prion diseases to humans and thus assess their zoonotic potential have used experimental challenge of nonhuman primates, humanized PrP transgenic mice, and cell-free assays with sometimes conflicting results. Successful

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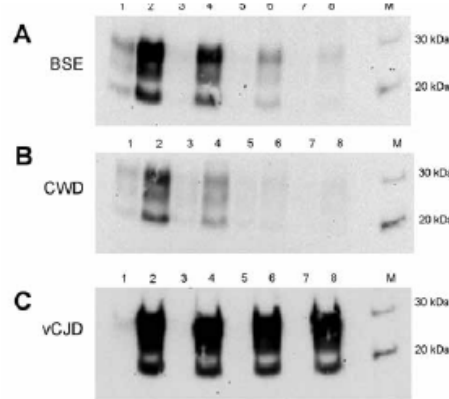
Figure 5. Properties of C-BSE, CWD, and vCJD amplification products in a second round of PMCA. Hu-C-BSE, hu-vCJD, and hu-CWD (from a previous round of PMCA) were supplemented with fresh human brain homogenate and subjected to a second round of PMCA. The reactions were normalized by PrP^res level and the product diluted (1:3, 1:6, 1:12, 1:24) in fresh human brain homogenate (PRNP codon 129MM) before PMCA. Odd numbers correspond to samples without PMCA; even numbers correspond to the reactions after PMCA (A, B, and C). The PrP detection antibody was 3F4. C-BSE, C-type bovine spongiform encephalopathy; CWD, chronic wasting disease; vCJD variant Creutzfeldt-Jakob disease; PMCA, protein misfolding cyclic amplification; hu, human; PrP^res, protease-resistant prion protein; M, molecular marker.

transmission of CWD and L-BSE to certain nonhuman primates has been reported: L-type BSE showing a different pathologic profile and a shorter incubation period than C-type BSE (20–23). However, Kong et al. (24) reported that CWD failed to transmit to humanized PrP 129M overexpressing mice inoculated with an elk brain homogenate. In contrast, Beringue et al. (25) reported that humanized PrP 129M overexpressing mice were susceptible to L-type BSE and suggested that L-type BSE was more virulent than C-type BSE and presented a zoonotic risk. H-type BSE reportedly failed to transmit to these same mice. Sandberg et al. (26) and Tamgüney et al. (27) confirmed the previous report of Kong et al. that CWD fails to transmit to transgenic mice, irrespective of whether 1) the mice expressed bovine, ovine, or human PrP; 2) the mice expressed the human 129M or 129V PrP allelic variants; or 3) the CWD isolates were from mule deer, elk, or white-tailed deer.

Cell-free approaches to modeling human susceptibility to animal prion prion diseases also have been published (8,10,28–31). Raymond et al. (28) compared the ability of CWD, C-type BSE, sheep scrapie, and CJD brain homogenates to convert human PrP^C metabolically labeled and purified from transfected cells. These experiments obtained

limited conversion of human PrP^C by CWD, C-type BSE, and scrapie. In contrast to our study, this early cell-free system failed to distinguish between scrapie and C-type BSE in their ability to convert human PrP^C; however, it indicated a substantial molecular barrier to conversion of human PrP^C by CWD PrP^Sc (28,29), which agrees with this report. Kurt et al. (31) reported that PMCA using human PrP^C overexpressing transgenic mice brain (both 129M and 129V lines) as substrate failed to support amplification when seeded with CWD cervine brain homogenate. Cervidized Prnp transgenic mouse brain homogenate can support CWD prion replication (32), and extensive in vitro conditioning of a CWD isolate by PMCA in a cervidized substrate (or passage in cervidized mice) was sufficient to overcome the barrier and enable efficient in vitro amplification in a humanized transgenic mouse substrate (33). Direct comparison of these studies is made difficult by the differences in approach (in vivo vs. in vitro), the different transgenic constructs used, and the technical details of the cell-free conversion assays undertaken (Table). An additional possibly significant difference between these studies is the nature of the CWD isolate used. CWD affects different deer species (some of which show allelic variation in their Prnp sequence), but CWD also occurs as different biologic strains of agent (34–36). Different strains of CWD may have a role in determining transmissibility and conversion efficiency. Recently, Meyerett et al. (37) reported the in vitro strain adaptation of a CWD isolate by serial PMCA, similar to that produced by in vivo subpassage.
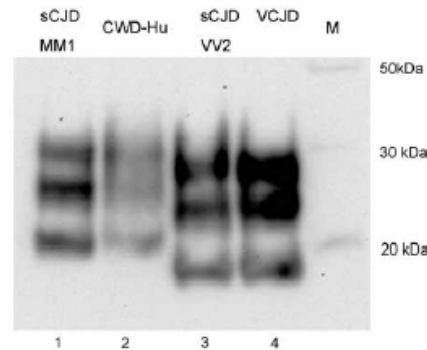
Figure 6. PrP^res typing of the CWD amplification product. The CWD PMCA product derived from amplification in a human brain homogenate substrate (PRNP codon 129MM) was compared by Western blotting with PrP^res from human brain samples from cases of sCJD of the MM1 subtype, sCJD of the VV2 subtype, and variant CJD. The PrP detection antibody was 3F4. PrP^res, protease-resistant prion protein; CWD, chronic wasting disease; PMCA, protein misfolding cyclic amplification; sCJD sporadic Creutzfeldt-Jakob disease; vCJD, variant CJD; hu, human; M, molecular marker.

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Table. Comparison of the outcomes of experimental transmission and in vitro conversion studies of chronic wasting disease in human, humanized, and nonhuman primate model systems*

Donor animal inoculum	In vivo			In vitro			
	Species inoculated	Animal (expression levels)/codon 129	Transmission	PrP source (expression levels)/codon 129	Method	Conversion	Ref
Mule deer, white-tailed deer, and elk				Cells 129M and 129V	C-FA	Pos	(28,29)
Elk	Humanized PrP transgenic mice	Tg-40 (1×)/129MM	Neg				(24)
		Tg-1 (2×)/129MM	Neg				
Mule deer	Squirrel monkeys	Saimiri sciureus (1×)	Pos				(20)
Mule deer, white-tailed deer, and elk	Humanized PrP transgenic mice	Tg(HuPrP)440 (2×)	Neg				(27)
Mule deer, white-tailed deer, and elk	Squirrel monkeys	Saimiri sciureus (1×)	Pos				(22)
	Cynomolgus macaques	Macaca fascicularis (1×)	Neg				
Mule deer and white-tailed deer				Tg-6816 (16×)/129M	PMCA	Neg	(31)
				Tg-7823 (5×)/129V	PMCA	Neg	
Mule deer	Humanized PrP transgenic mice	Tg-45 (4×)/129MM	Neg				(26)
		Tg-35 (2×)/129M	Neg				
		Tg-152 (6×)/129VV	Neg				
White-tailed deer	Humanized PrP transgenic mice	HuMM (1×)/129MM	Neg				(11)
		HuVV (1×)/129VV	Neg				
Mule deer				Tg-440 (2×)/129MM	PMCA	Pos (after in vitro conditioning)	(33)
Elk				Human brain (1×)/129MM	PMCA	Pos	This article
				Human brain (1×)/129VV	PMCA	Pos	
				HuMM (1×)/129MM	PMCA	Pos	
				HuVV(1×)/129VV	PMCA	Pos	
				293F cell line(4×)/129M	PMCA	Pos	
				293F cell line(2×)/129V	PMCA	Pos	

*Numbers in parentheses denote the stated expression levels of PrP^C into the animal species and cell lines used. PrP, prion protein; C-FA, cell-free assay; pos, positive; neg, negative; PrP^C, host's normal cellular PrP. Blank cells indicate no reported data.

The most directly comparable in vivo study to that reported here is Wilson et al. (11), in which a similar series of atypical animal prion diseases were used to challenge transgenic mice expressing physiologic levels of human PrP^C. Atypical scrapie; C-, H-, and L-type BSE; and CWD all failed to produce disease (or signs of infection) on first passage in these mice (11). The use of different animal prion disease isolates (and possibly differing species and strains of CWD) might explain this discrepancy; however, a more fundamental difference might be that the in vivo and in vitro model systems assess different aspects of the agent and its replication. The in vivo model is undoubtedly more complex and arguably more physiologically relevant, and the readout is disease; however, it remains disease in a mouse, in which the PRNP sequence alone is human. The in vitro cell-free model does not assess disease as such, only the compatibility of particular combinations of seed and substrate homogenates (some of which, in these examples, were entirely of human origin) to produce PrP^{res}.

Differences between the in vivo and in vitro models are exemplified by the comparison of C-type BSE, and vCJD. Both amplify well in PMCA using humanized (129MM) brain homogenate as a substrate (10), whereas intracranial inoculation of C-type BSE into humanized (129MM) mice fails to produce disease (12), unless first experimentally transmitted to sheep or goats (13,38,39).

The interpretation of different amplification efficiencies as a semiquantitative measure of relative risk is tempting but is probably premature and almost certainly an oversimplification. The testing of more isolates, especially of CWD in deer and elk, is advisable before any firm conclusions can be drawn. Additionally, possible strain-specific effects on amplification efficiency by the precise PMCA experimental conditions are difficult to discount and might complicate interpretation. The relative amplification efficiencies of C-, H-, and L-type BSE might differ intrinsically because certain strains of sheep scrapie appear to, even when amplified in homologous sheep substrates (40). However, we can say

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with confidence that under the conditions used here, none of the animal isolates tested were as efficient as C-type BSE in converting human PrP^C, which is reassuring. Less reassuring is the finding that there is no absolute barrier to the conversion of human PrP^C by CWD prions in a protocol using a single round of PMCA and an entirely human substrate prepared from the target organ of prion diseases, the brain.

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Exploring the zoonotic potential of animal prion diseases
In vivo and in vitro approaches

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Following the discovery of a causal link between bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt–Jakob disease (vCJD) in humans, several experimental approaches have been used to try to assess the potential risk of transmission of other animal transmissible spongiform encephalopathies (TSEs) to humans. Experimental challenge of non-human primates, humanised transgenic mice and cell-free conversion systems have all been used as models to explore the susceptibility of humans to animal TSEs. In this review we compare and contrast in vivo and in vitro evidence of the zoonotic risk to humans from sheep, cattle and deer prions, focusing primarily on chronic wasting disease and our own recent studies using protein misfolding cyclic amplification.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal transmissible neurodegenerative conditions affecting humans and other mammals. TSEs are thought to result from a change in the conformation of the normal prion protein (termed PrP^C) into an abnormal form of the protein termed PrP^{Sc}.[1] They have been described in a range of farmed, captive and free-ranging animal species, including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and chronic wasting disease (CWD) in deer and elk. In humans, TSEs occur as sporadic, genetic and acquired diseases, the most common of which is sporadic Creutzfeldt–Jakob disease (sCJD). TSE pathology is characterized by neurological degeneration and death, gliosis and the deposition of PrP^{Sc} in the brain. TSEs are (as the name indicates) transmissible, and the causal infectious agents are termed prions.[2]

Over the past 30 years, substantial efforts have been made to establish the biology of prions. In 1967 the first theoretical model of a self-replication scrapie agent was proposed by Griffith and coworkers.[3] In the same year, Pattison and coworkers considered the nature of sheep prions, suggesting a proteinaceous nature.[4] At the beginning of the 1980s Stanley Prusiner coined the term "prion", distinguishing the infectious agent causing TSE from viruses and viroids, and proposing a protein only mechanism of agent replication, which is now known as the "prion hypothesis."[2]

The increasing evidence in favor of the prion hypothesis has involved epidemiological, clinical and pathological research on TSEs in human and animals. Increasingly sophisticated experimental models, including non-human primates, wild-type and humanized transgenic mice and cell-free molecular conversion systems, have each contributed to a better understanding of the nature of prions.

Transgenic Mouse Models

Five years after the initial development of transgenic animals, the cellular gene which encodes the prion protein in mammals (*PRNP* in humans, *Prnp* in other animals) was identified.[5] Five years later, a transgenic murine model with a single amino acid substitution (P101L) in the murine prion protein gene was created. This transgenic mouse model showed a neurodegenerative process, similar to that in humans affected by a corresponding P102L mutation in the *PRNP* gene.[6] This suggested that a human prion disease can be genetically modeled in animals and introduced the use of transgenic animals into the TSE research, although subsequent studies using this model have shown that the relationship between PrP^{Sc} formation, neurodegeneration and prion infectivity is not a straightforward one.[7] In order to evaluate the association between *Prnp* gene expression and prion transmission, Bueler and coworkers intracerebrally challenged *Prnp* knockout mice with a high dose of scrapie prions. The animals failed to develop any clinical signs of a TSE, and showed no TSE pathology in the brain. The authors concluded that development of clinical signs and pathology is strictly dependent on the presence of PrP^C, and that incubation time and disease progression are inversely related to the levels of PrP^C.[8]

Transgenic animals have not only been relevant to understanding the biology of prions, they have also contributed to our understanding of their transmission between species. In this regard, the concepts of strain and species barrier acquire critical

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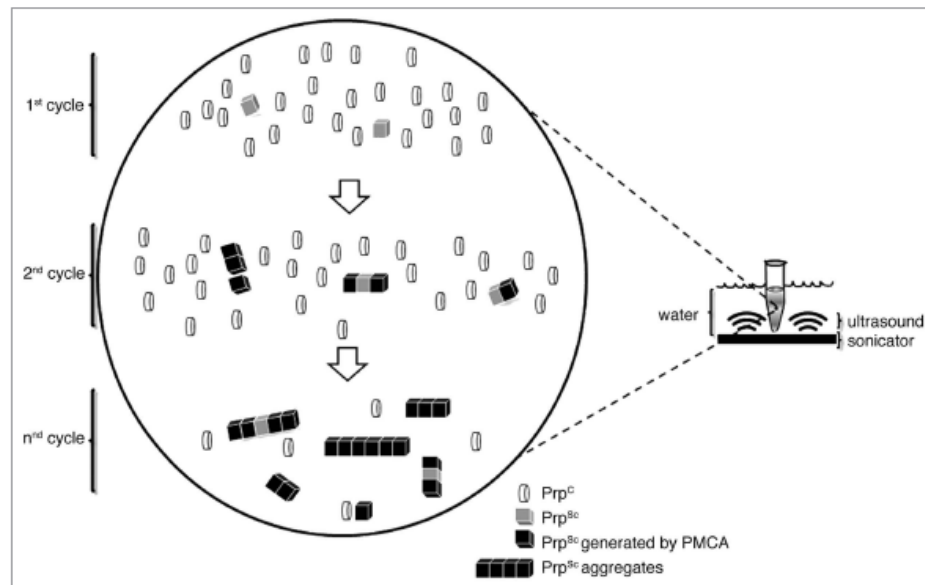
Figure 1. Schematic representation of protein misfolding cyclic amplification (PMCA) showing the presumed mechanism of molecular conversion. A large excess of PrP^C is incubated with minimal quantities of PrP^{Sc} followed by cycles of incubation and sonication. PrP^{Sc} aggregates are shown to grow, converting and incorporating PrP^C molecules. With each cycle of sonication and incubation (represented by arrows) PrP^{Sc} aggregates are broken, creating further seeds. After several cycles of sonication and incubation most of the PrP^C will have been converted into the PrP^{Sc}.

importance. Briefly, a prion strain is defined as an infectious isolate that causes a distinct prion disease phenotype (incubation time, lesion profile, etc.), which is maintained upon serial transmission when it is inoculated to another host.[9] A species barrier can be defined as the difficulty in transmitting a prion disease from one species to another in a primary transmission. In practice this can be recognized by an adaptation of the agent to the second species, producing a shorter incubation periods and uniform transmission properties in a secondary transmission in the second species. The ability of a prion strain to be transmitted to a new host is affected by the differences in primary sequence of the prion protein between donor and host species.[10] Additionally, it is affected by the conformational properties associated with the PrP^{Sc}.[11]

In Vitro Conversion Systems

Caughey and coworkers developed an in vitro system in which recombinant hamster PrP^C, incubated with the misfolded form of the prion protein and a chaotropic agent, generated "new" protease-resistance material derived from the original PrP^C.[12] Although this represented a major development in prion biology, a large excess of input material (PrP^{Sc} > 50 fold) was required

to perform the conversion of the normal prion protein, placing constraints on its utility.

Subsequently, and based on the nucleation and polymerization model, Soto and colleagues described an in vitro procedure that mimics the in vivo PrP^{Sc} conversion process. This approach allows amplification of minute quantities of PrP^{Sc} in a sample, and is more efficient than the previous cell-free conversion assay reported by Caughey and coworkers. The technique, called protein misfolding cyclic amplification (PMCA), consists of cycles of sonication and incubation periods of a mixture of a normal brain homogenate (which is rich in PrP^C) and a source of PrP^{Sc}, in the presence of detergents (Fig. 1).[13]

PMCA has proved to be a rapid, versatile and inexpensive method to investigate fundamental molecular aspects of prion protein conversion. For example, a species barrier is known to exist for sheep scrapie strains adapted to mice and to hamsters. Castilla and coworkers reported the use of murine scrapie strains and hamster PrP^C to mimic the in vivo adaptation process in vitro, generating new prions by serial rounds of PMCA. Similar results were obtained using hamster seeds and murine substrate.[14] This provided the proof of concept that PMCA can recapitulate a biological process in days what would normally take months or even years in vivo.

vCJD and BSE: The First Example of a Zoonotic Prion Disease

In 1985 the emergence of a new TSE affecting cattle was reported in the United Kingdom. The disease, bovine spongiform encephalopathy or BSE, eventually affected more than 180 000 animals in the United Kingdom with more than 70 000 confirmed clinical cases between 1992 and 1993.[15] The affected animals suffered a slow progressive neurological disorder, changes in sensation, posture and movement, weight loss and reduced milk yield. Affected cattle were pathologically characterized by brain stem gray matter vacuolation.[16]

At this time it was unclear whether BSE would prove a threat to human health. BSE is now known to be the pathogen that causes variant CJD (vCJD) in humans, whereas scrapie in sheep is thought to pose little or no risk to human health. In retrospect, the connection between BSE and vCJD seems obvious, but at the time it required careful surveillance and research for the connection to be established.

In 1996 Will and colleagues reported the identification of 10 unusual cases of CJD in young individuals in the UK. Active surveillance identified no obvious increase in the incidence of CJD in young patients in other European countries. Taking into account the young age at onset of the reported cases, a distinctive neropathological profile, the presence of "florid" PrP plaques and the apparent absence of cases with a similar profile in Europe, the authors suggested that the appearance of vCJD in the UK was linked to the earlier BSE epidemic.[17]

All definite cases of vCJD that have undergone *PRNP* analysis are methionine homozygotes at codon 129.[18] *PRNP* polymorphisms appear to influence clinical and pathological phenotypes in the human prion diseases. The most important polymorphism resides at codon 129, which results in three polymorphic combinations: the homozygous methionine/methionine and valine/valine, and the heterozygous methionine/valine.

In order to test the link between BSE and vCJD, non-human primates were intracerebral inoculated with BSE brain homogenate. All three BSE-inoculated *Cynomolgus macaques* developed abnormal behavior similar to that observed in the vCJD cases and had a neuropathology including PrP^{Sc} deposition reminiscent of that seen in vCJD patients. This supported the hypothesis that BSE is the agent responsible for vCJD.[19] Transmission studies in inbred mouse strains reinforced this view, and strongly suggested that vCJD is caused by the same strain of agent that causes BSE, feline spongiform encephalopathy (FSE) and TSEs in exotic ruminants.[20,21] More compiling evidence of a link between BSE and vCJD was presented by Scott and coworkers who inoculated bovinized transgenic mice with BSE, scrapie, and vCJD.[22] All three inocula propagated efficiently. BSE and vCJD were indistinguishable in terms of incubation time and neuropathology, but were distinct from scrapie, reinforcing the conclusion that vCJD is caused by the same strain of agent as that causing BSE.

In order to model the transmissibility of vCJD, sCJD and BSE to humans, transgenic animals that expressed human *PRNP* valine (V)[23] or methionine (M)[24] at codon 129 were inoculated. The transgenic mice presented evidence of transmission for each inoculum. However, the molecular signature of the vCJD-PrP^{Sc} generated by the 129–V infected animals did not share the same electrophoretic mobility of the original inoculum.[23] In contrast, the animals carrying the polymorphism 129-M (inoculated with vCJD) conserved the molecular pattern of the original inoculum.[24] To further evaluate the transmissibility of BSE and vCJD and to assess the effect of codon 129 polymorphism on human susceptibility, Manson and coworkers produced "knock in" humanized transgenic mice carrying the three *PRNP* codon 129 genotypes: methionine and valine homozygous and methionine/valine heterozygous. After inoculation with BSE and vCJD prions, different pathological characteristics and transmission efficiency was detected among the three genotypes. In terms of vCJD propagation, the MM genotype at codon 129 was more susceptible than MV, which in turn was more susceptible than VV.[25] The authors cited a previous publication in which a cell-free assay showed that human purified PrP^C with M at position 129 was converted to PrP^{Sc} more readily by vCJD and BSE than the 129-V equivalent.[26]

In order to test whether inter-species transmission barriers could be modeled using PMCA, Jones and coworkers tested the ability of BSE and scrapie to convert human prion protein of the three major human prion protein polymorphic variants (*PRNP* codon 129 MM, MV and VV) expressed in humanised transgenic mouse brain. The results showed that cattle BSE effected efficient conversion of human PrP with a human *PRNP* genotypic preference similar to that of human vCJD (MM > MV > VV) and that scrapie failed to convert the human substrate.[27] These results suggest that PMCA can faithfully replicate aspects of cross-species transmission potential and might provide useful additional information concerning the molecular barrier to zoonotic transmission.

The establishment of a causative link between BSE and vCJD clearly involved epidemiology, clinical and neuropathological investigation, but as shown above, it also involved the use of carefully selected in vivo and in vitro model systems. The history of BSE/vCJD can be used as a paradigmatic example of how to consider possible links between other animal prion diseases and how to investigate zoonotic potential.

Atypical TSEs

Following the BSE epidemic in the UK, active surveillance programs for animal prion diseases in Europe and elsewhere led to the identification of rare atypical forms of prion diseases in sheep and cattle. These included Nor98 or atypical scrapie in sheep[28] and two prion diseases of cattle: bovine amyloidotic spongiform encephalopathy or L-type BSE[29] and H-type BSE,[30] both of which have a pathology and epidemiology distinct from classical (C-type) BSE.

Atypical scrapie: Nor98

First identified in Norway in 1998, Nor98 affected animals presented with progressive ataxia and showed vacuolar lesions and PrP^{Sc} accumulation in the cerebral and cerebellar distinct from classical scrapie. Nor98 affected sheep are of *Prnp* genotypes rarely affected by the classical scrapie. Molecular analysis

of protease-resistant prion protein (PrP^res) in the brain revealed the presence of a low molecular weight PrP^res band hitherto not described in animal TSE. These features suggested the identification of a novel scrapie phenotype or strain.[28,31]

Transmission of scrapie and atypical scrapie to a transgenic mice model that overexpresses the ovine prion protein of the ARQ/ARQ *Prnp* genotype showed differences in the transmission rate between these two sheep TSEs, suggesting that they represent different strains and that the risk of transmission of atypical scrapie (between sheep) is lower than that of classical scrapie.[32] Recently, Wilson and coworkers reported failure of transmission of Nor98 in humanized transgenic model mice, arguing for a substantial transmission barrier between atypical scrapie and humans.[33]

L-BSE/ H-BSE: in vivo and in vitro models

Intracranial inoculation of cynomolgus monkeys (*Macacca fasicularies*) with C-BSE, L-type BSE and vCJD revealed a distinctive pathological profile for L-type BSE. The animals challenged with L-type BSE had a shorter survival time compared with that for the C-BSE inoculated macaques, even although the dose was 4 times less (in terms of the amount of tissue used) compared with the C-BSE inoculum. Furthermore, the C-BSE inoculum was 10-fold times more concentrated in terms of PrP^res than the L-BSE material. These results demonstrate the susceptibility of non-human primates to L-BSE and suggest greater pathogenicity of L-BSE than C-BSE to non-human primates.[34]

Active animal TSE surveillance programs worldwide have identified H-BSE and L-BSE in countries outside the European Union (e.g., Japan). To evaluate the infectivity and the prion disease phenotype of the Japanese L-BSE isolates, cynomolgus macaques were intracranially inoculated with a brain macerate from two confirmed cases of L-BSE. Similar to the report of Comoy and coworkers,[34] the incubation periods and the duration of the disease were approximately 2/3 shorter than those of C-BSE, suggesting once again that L-BSE may be more virulent than the C-BSE in non-human primates.[35]

Following the suggestion that L-type BSE could be more virulent than C-BSE, researchers have made pointed efforts to quantify the risk associated with L-BSE and H-BSE to the human health. Beringue and coworkers reported that humanized PrP 129-M overexpressing mice were susceptible to L-type BSE with 100% attack rate and shorter incubation periods compared with C-BSE. Contrary to this finding, H-BSE failed to transmit in the same mice model. The authors suggested a higher theoretical risk of transmission of L-BSE to humans compared with C-BSE.[36]

Recently, a full range of atypical animal prion diseases were used to challenge humanized transgenic mice that express physiological levels of the human prion protein.[33] In contrast to the findings of Beringue and coworkers, L-BSE and H-BSE failed to show any signs of clinical disease or prion pathology, suggesting a substantial transmission barrier between the atypical forms of BSE and humans.[33]

To our knowledge, the only evidence comparing L-BSE and H-BSE in a cell free system comes from work by our group. A panel composed of several animal TSEs were tested for their ability to convert the human prion protein by PMCA. Human brain and humanized transgenic mouse brain homogenates of the *PRNP* codon 129 MM and VV genotypes all failed to support amplification when the PMCA reactions were seeded with L-BSE, H-BSE, scrapie and atypical scrapie. In contrast C-BSE and vCJD PrP^Sc efficiently converted the human PrP^C, albeit in a codon 129 (M allele) dependent manner. These findings suggest that, at least at the molecular level, atypical scrapie and atypical BSE present a lower level of risk of zoonotic disease than classical BSE.[37]

Chronic Wasting Disease:
A Risk As Yet Unquantified?

First recognized as a TSE in the early 1980s by Williams and coworkers, chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that affects North American mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), Rocky Mountain elk (*Cervus elaphus nelsoni*), and less frequently moose (*Alces alces shirasi*).[38] After being first identified in Colorado and Wyoming, the disease has been identified in 15 states across the USA, two Canadian provinces and 15 states and provinces in South Korea .[39] Clinical signs in animals affected by CWD include progressive loss of weight, pronounced behavioral changes, excessive salivation, and ataxia with head tremors.

Surveillance for CWD-related disease in human

The possibility of CWD affecting human health will be a function of exposure to the CWD agent (or agents) and of human susceptibility to these agents. Dose and route could reasonably be expected to be relevant aspects of exposure and as with other human prion diseases the *PRNP* codon 129 polymorphisms may be expected to exert an effect on human susceptibility to CWD. The disseminated nature of PrP^Sc and infectivity in animals infected with CWD, coupled with the popularity of deer hunting in CWD endemic areas, suggests the most likely form of human exposure to CWD infectivity is by the oral route. CJD surveillance systems in CWD affected countries have been alert to this possibility and have been vigilant for CJD patients with suspicious clinical signs and symptoms, a young age at onset and potential risk factors such as game hunting and venison consumption. The literature contains five such cases.[40,41] The results of intensive investigation provided diagnoses of sCJD,[40] familial CJD and early-onset Alzheimer disease[41] and on balance the authors discounted a link to CWD, but conceded the difficulties involved in coming to this conclusion. It is important to note however that the specific features of an animal prion disease need not be conserved when zoonoses occur. Neither the characteristic vCJD neuropathology nor the pronounced peripheral tissue involvements of vCJD are features of BSE in cattle. The absence of neuropathological similarities between CWD and any individual cases of human prion disease is not sufficient grounds to discount CWD as a human pathogen. Continued surveillance and epidemiology are required. In parallel the susceptibility of humans to the CWD

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agent has been investigated using in vivo and in vitro model systems.

Transmission and bioassays in cervids

CWD is thought to be horizontally transmitted between deer by direct contact (e.g., by saliva) and indirectly through pasture contamination with, urine or faeces (reviewed by Miller et al.).[42] Experimental exposure of uninfected mule deer to contaminated excreta and decomposed carcasses (of infected animals) shows that CWD prions can persist in the environment for two years.[43] Using bioassays in deer, Mathiason and coworkers evaluated the presence of CWD prions in body fluids and excretions in the preclinical phase of the infection. They also evaluated the transmission of the disease using repeated environmental exposure. Positive results were found. Blood and saliva were detected as a potential source of CWD prions. Additionally, the infectious agent (experimentally shed in the environment) was found to be sufficient to transmit the disease to naive deer.[44] These observations underline the risks associated with both direct and indirect transmission between deer.

Non-human primate models

In order to test the transmissibility of CWD to non-human primates, Marsh and coworkers reported the experimental challenge of two squirrel monkeys (*Saimiri sciureus*) with brain homogenate of CWD infected mule deer. The two animals were euthanized at 31 and 34 months post inoculation. Both animals showed a progressive neurological disease, detection of PrP^{res} by western blot and histopatological spongiform changes in the brain, all consistent with a TSE.[45]

Based on the evidence that non-human primates are susceptible to CWD infection, Race and coworkers evaluated (1) the susceptibility of cynomolgus macaques (*Macaca fasicularis*) and squirrel monkeys to CWD infection, (2) the different possible routes of inoculation (intracranial and oral) and (3) various sources of the inoculum (representing wild and captive deer and elk). Intracranial inoculation of squirrel monkeys showed that independent of the origin, 80% of the animals developed signs of a spongiform encephalopathy and accumulated PrP^{res} in the brain. Oral exposure with the infectious agent showed 15% of the squirrel monkeys had the presence of PrP^{Sc} in the brain and in peripheral tissue. In contrast, cynomolgus macaque inoculated orally or intracerebraly failed to show evidence of clinical disease 70 months post inoculation.[46] Considering relationship of these two non-human primates species to humans, the authors suggested a pronounced species barrier to CWD in humans.

Transgenic mouse models

Modeling the zoonotic transmission of CWD prions to humans, Kong and coworkers reported the inoculation of humanized transgenic mice 129-M lines (expressing the transgene PrP one and 2-fold compared with physiological levels) with CWD elk brain homogenate. After approximately 700 days none of the transgenic mice showed signs of prion disease, and the authors concluded that a substantial species barrier to transmission must be present.[47] Sandberg and coworkers confirmed the previous report of Kong et al., that CWD fails to transmit to transgenic mice, irrespective of whether the mice expressed (1) bovine, ovine, or human PrP, (2) the human 129-M or 129-V

PrP allelic variants, or (3) whether the CWD isolates were from mule deer, elk, or white tailed deer.[48,49]

In order to evaluate the transmissibility of a range of animal TSEs to transgenic animals that express physiological levels the human prion protein, atypical scrapie, C-BSE, H-BSE, L-BSE, and CWD were used to challenge the humanised transgenic mice constructed by gene replacement.[33] All TSEs, including the CWD isolate used (derived from white tail deer infected animal), failed to produce disease (or signs of infection) on first experimental passage, whether the mice were homozygous for methionine or valine, or heterozygous at codon 129 of *PRNP*, again suggesting a substantial species barrier between the atypical TSEs and humans.[33]

In vitro conversion systems: modeling human PrP susceptibility to conversion by chronic wasting disease

In the earliest attempt to assess molecular barriers of the human prion protein conversion by animal prion diseases, Raymond and coworkers compared the ability of CWD, C-BSE, sheep scrapie, and CJD brain homogenates to convert human prion protein metabolically labeled and purified from transfected cells.[26] Limited conversion of human PrP by CWD, C-BSE and scrapie was observed, but the model system was unable to discriminate between the molecular susceptibility of the two *PRNP* genotypes (129-M and 129-V) used in the conversion process. These results revealed a substantial molecular barrier to conversion of PrP^C by CWD, C-BSE and sheep scrapie prions.

Using PMCA, Kurt and coworkers reported failure to support in vitro conversion of the human prion protein (independent of the *PRNP* 129 M or V polymorphisms) when the reaction was seeded with CWD brain homogenate.[50] In contrast, Barria and coworkers observed that in vitro conditioning of a mule deer-CWD isolate by PMCA in cervid substrate (or passage in cervidised mice) allowed for subsequent efficient in vitro amplification in a humanised transgenic mouse substrate.[51]

We have recently conducted a side-by-side comparison of the zoonotic potential of a wide range of animal prions including BSE, atypical BSE, scrapie, atypical scrapie, and CWD using PMCA.[37] We found that, unlike atypical scrapie and H- and L-BSE, the CWD specimen tested was able to effect the conversion of human PrP^C. The efficiency of conversion was less than that of C-BSE, but it was detectable whether human brain, humanised transgenic mouse brain or human cell homogenates were used as the PMCA substrate. Both the 129-M and 129-V *PRNP* polymorphic variants were converted by the CWD brain homogenate, but the conversion of 129-M was found to be the more efficient. The CWD-PMCA reaction product retained the ability to propagate in the human PrP^C-containing substrate in a second round of PMCA. The human PMCA reaction product shared features with type 1 PrP^{res} (Fig. 2).[37]

These results would seem to suggest that CWD does indeed have zoonotic potential, at least as judged by the compatibility of CWD prions and their human PrP^C target. Furthermore, extrapolation from this simple in vitro assay suggests that if zoonotic CWD occurred, it would most likely effect those of the *PRNP* codon 129-MM genotype and that the PrP^{res} type would
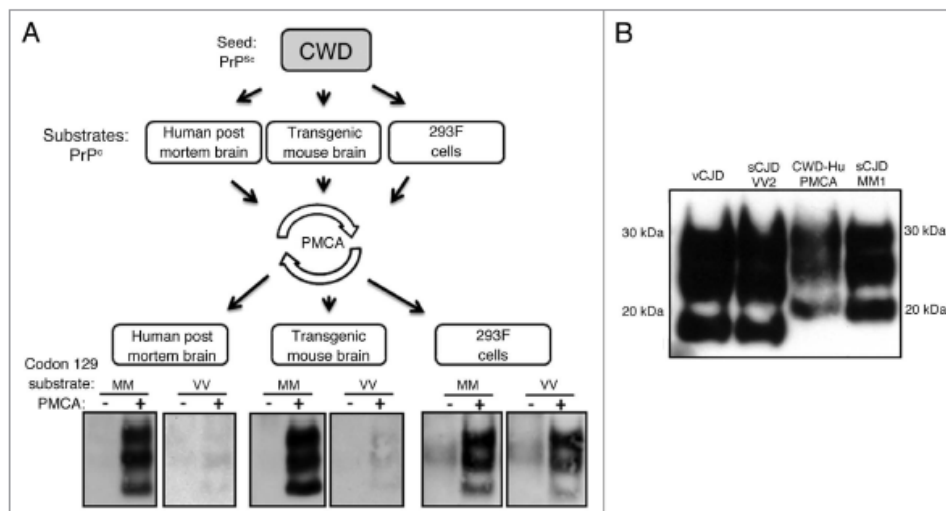
Figure 2. Human PMCA reactions with CWD prions. (A) CWD PrPˢᶜ amplification was conducted with substrates from three different sources (human brain, humanized transgenic mouse brain, human cell line,), each with both the *PRNP* codon 129 MM and the VV genotypes. The susceptibility of human PrPᶜ to conversion was evaluated after a single round of PMCA. Irrespective to the origin, the three substrates supported amplification after one round (96 cycles) of PMCA. CWD amplification showed a preference for the MM genotype, with a robust amplification, compared with the VV genotype. Amplified sample (+), non-amplified sample (-). (B) PrPʳᵉˢ typing of the CWD PrPˢᶜ amplified in a human brain substrate. vCJD, sCJD VV2 subtype and sCJD MM1 subtype were used as PrPʳᵉˢ type reference standards. The CWD PrPʳᵉˢ material produced by PMCA (CWD-Hu PMCA) resemble type 1 human PrPʳᵉˢ. PrP detection antibody was 3F4. M, Molecular marker.

be similar to that found in the most common subtype of sCJD (MM1).

Our study has obvious limitations. First of all, it does not take account of factors operating above the sub-cellular level. Although the prion hypothesis locates the major determinants of prion disease pathogenesis in prion protein structure it is clear that additional factors affect the zoonotic potential of animal prion diseases, such as route of exposure, dose, host genetics, age and co-existing morbidities. A second limitation is that our study was conducted with a single specimen of CWD in an elk. CWD affects several cervid species and in some of these species there exist *Prnp* polymorphisms and different strains of CWD agent. Before generalizing too broadly from these results, it will

be critical to test a wide variety of CWD isolates from different cervid species, polymorphic genotypes and geographical locations to determine whether heterogeneity of zoonotic potential exists within CWD.

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No potential conflicts of interest were disclosed.

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