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Characterization of human TSE strains after passage in humanized transgenic mice

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ABBREVIATIONS

aa:	Amino acids
Ala:	Alanine
BSE:	Bovine spongiform encephalopathy
CJD:	Creutzfeldt-Jakob disease
CNS:	Central nervous system
CWD:	Chronic wasting disease
DTT:	Dithiothreitol
DW:	Drowsy
EEG:	Electroencephalogram
FFI:	Fatal Familial Insomnia
FTIR:	Fourier transformed infrared spectroscopy
G:	Gauge
GPI:	Glycosylphosphatidylinositol
GSS:	Gerstmann-Sträussler-Scheinker disease
GT:	Gene targeting
H&E:	Haematoxylin-eosin staining
HuTg:	Transgenic mice expressing human PrP
HuMM:	Transgenic mice expressing human PrP with methionine/methionine genotype at codon 129
HuMV:	Transgenic mice expressing human PrP with methionine/valine genotype at codon 129
HuVV:	Transgenic mice expressing human PrP with valine/valine genotype at codon 129
HY:	Hyper
Iso:	Isoleucine
kDa:	kiloDalton
LDS:	Lithium dodecylsulphate
mAb:	Monoclonal antibody
MM:	Methionine/methionine
MV:	Methionine/valine
NGS:	Normal goat serum
NMR:	Nuclear magnetic resonance
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction

PK: Proteinase K

PRNP: Human gene encoding the prion protein

prnp: Mouse gene encoding the prion protein

PrP: Prion protein

PrP^{C}: Cellular prion protein

PrP^{TSE}: Pathological isoform of prion protein

RGI: Random genomic insertion

sCJD: Sporadic Creutzfeldt-Jakob disease

vCJD: Variant Creutzfeldt-Jakob disease

SDS: Sodium dodecylsulphate

SD: Standard deviation

TBS: Tris buffered saline

TBST: TBS/Tween 20

Thr: Threonine

TME: Transmissible mink encephalopathy

TSE: Transmissible spongiform encephalopathy

Val: Valine

VV: Valine/valine

WB: Western blot

1. INTRODUCTION

1. *1* TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

Transmissible Spongiform Encephalopathies (TSEs) are fatal neurodegenerative diseases affecting human and animals, caused by an infectious agent whose nature is still under debate. The main event characterizing TSEs is the accumulation in the Central Nervous System (CNS) of insoluble fibrils mainly composed of the disease-associated isoform of the host prion protein (PrP^{TSE}), which is produced following a conformational change from the endogenous cellular prion protein (PrP^{C}) (Prusiner, 1998). PrP^{TSE} fibrils can sometimes aggregate into amyloid plaques that are easily detectable by conventional neurohistological examinations, together with other TSE-specific lesions such as spongiosis (i.e. a sponge-like vacuolation of cerebral grey matter), neuron loss, and gliosis (figure 1. *1*). Pathological misfolding of host proteins links TSEs to other human neurodegenerative diseases like Alzheimer's disease (Amyloid β), Parkinson's disease (α-synuclein), or Huntington' s disease (huntingtin). Among these however, only TSEs have been proved to be transmissible either naturally (human to human through medical procedures, or animal to animal via natural secretions), or experimentally (to laboratory animals) via different routes of infection.

According to their origin, human TSEs are classified as sporadic, inherited, or infectious forms (Ironside, 1998) (table 1. *1*). Sporadic forms (including the vast majority of human TSE cases), occur without any apparent episode of infection and are mainly represented by sporadic Creutzfeldt-Jakob disease (CJD), a rapidly progressive dementia commonly associated with myoclonic jerks, pyramidal, extrapyramidal, and cerebellar signs.

Inherited forms, accounting for 10-15% of human cases, are associated to mutations (more than 50 have been described until now) of the prion protein gene (*PRNP*) and, besides a typical Creutzfeldt-Jakob disease picture, they may manifest as peculiar clinical and pathological syndromes (e.g. Fatal Familial Insomnia, FFI, Gerstmann-Sträussler-Scheinker disease, GSS).

Infectious forms reported in the literature originated after accidental inter-human transmission of the infectious agent by medical procedures or have a zoonotic origin through contaminated food.

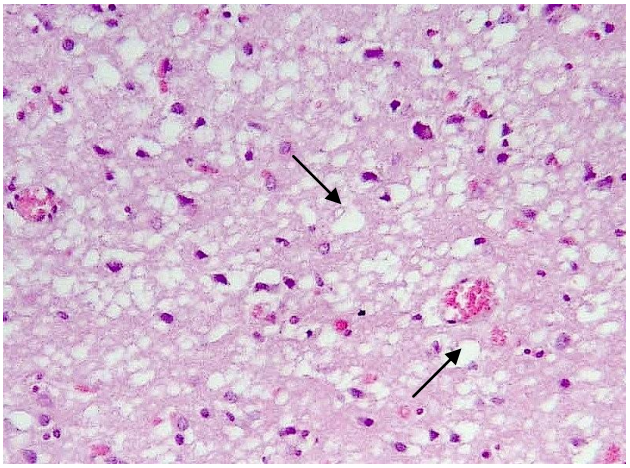
Animal TSEs include scrapie of sheep and goats (Hunter *et al*., 2000), a disease already reported in the eighteenth century (Schneider *et al*., 2008) and the first TSE described (by McGowan in 1922). Other animal TSEs include: chronic wasting disease (CWD) of deer and elk (Williams, 2005), the transmissible mink encephalopathy (TME) (Libersky *et al*., 2009) and bovine spongiform encephalopathy (BSE) (Wells *et al*., 1991) which reached an epidemic diffusion during the end of the last century and transmitted to humans, as variant CJD (vCJD), by ingestion of contaminated food (Will *et al*., 1996).

Despite intensive studies conducted during the last 50 years, many aspects of TSE diseases are still obscure such as the nature of the etiological agent, the molecular mechanism of prion propagation and replication, the neurodegeneration caused by PrP^{TSE} accumulation in the brain, and the wide phenotypic spectrum observed even among the same forms of TSEs disease.

Table 1. *1*: Spectrum of human prion diseases (modified from Aguzzi *et al.*, 2004).

Form	Prion disease	Etiology
Sporadic	Sporadic Creutzfeldt-Jakob disease (sCJD) Sporadic Fatal Insomnia	Unknown
Inherited	Genetic CJD Fatal Familial Insomnia (FFI) Gerstmann-Sträussler-Scheinker disease (GSS)	*PRNP* mutation
Infective	Iatrogenic CJD	Surgical procedure
	Variant CJD	Cattle to human via BSE-contaminated food; Human to human via blood transfusion
	Kuru	Ritualistic cannibalism

Figure 1. *1*. Histological alterations observed in a CJD patient's brain. (a) spongiform degeneration; (b) PrP^{TSE} deposition; (c) gliosis.



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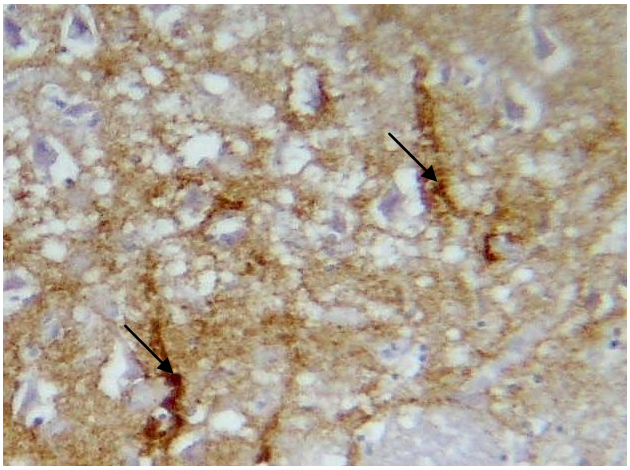

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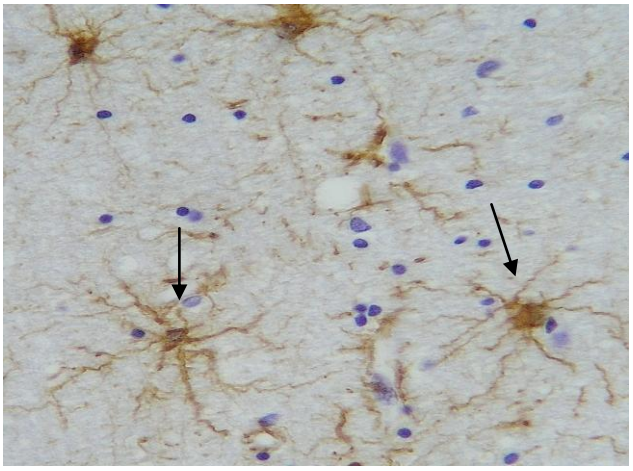


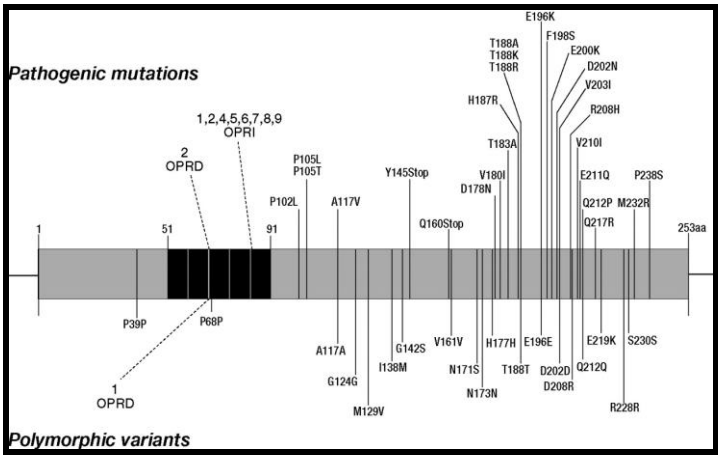
C

1. *2* THE GENE *PRNP* AND THE PROTEIN PrP

PrP^{TSE} is the pathological isoform of the host-encoded cellular prion protein PrP^{C}, a glycosyl-phosphatidylinositol (GPI) membrane-anchored glycoprotein of about 33-35 kDa. The protein is encoded by the *PRNP* gene, placed on the short arm of chromosome 20 in humans (Makrinou *et al.*, 2002).

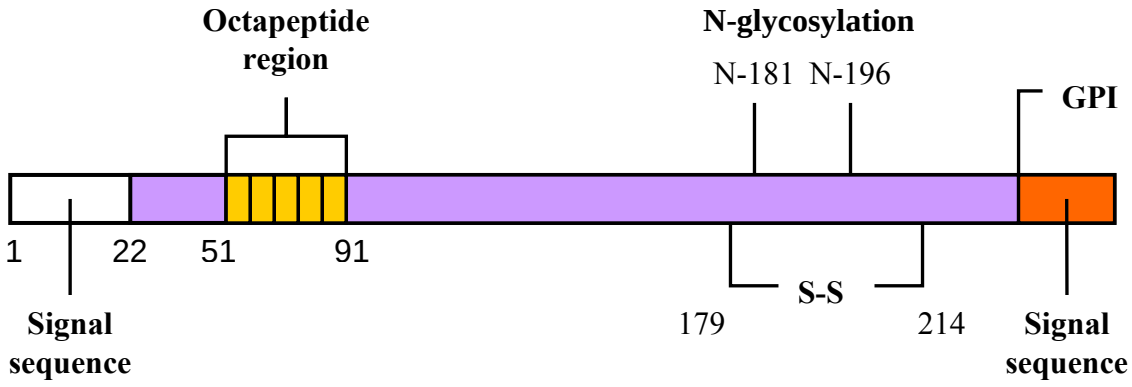
More than thirty point mutations and several insertions encoding additional copies of an octapeptide, regularly present in five copies on *PRNP*, are linked to the genetic forms of human TSEs (Kovács *et al*., 2005). Moreover, although different polymorphisms of *PRNP* have been discovered, only the methionine/valine (M/V) polymorphism at codon 129 has shown a critical influence on clinical, pathological and biochemical phenotype of human TSE diseases. It has also a role in the susceptibility to the disease (Pocchiari *et al.*, 2004) (figure 1. *2*): CJD mainly affects homozygous subjects, especially for methionine, while 129 heterozygosity seems to have a protective effect (Puopolo *et al*., 2003). Indeed 80% of patients with sCJD and 100% of patients with vCJD were homozygous for the methionine at codon 129 (Ironside *et al*., 2004; Alperovitch *et al*., 1999).

Figure 1. *2*. Mutations and polymorphisms of the prion protein associated with human TSEs (from Wadsworth *et al*., 2003).



Human PrP^C consists of 253 amino acids (aa) and its tridimensional structure, inferred by nuclear magnetic resonance (NMR) studies (Zahn *et al*., 2000) on recombinant, non glycosylated forms, consists of a long, unstructured, N-terminal domain of about 100 aa that starts with a signal peptide (aa 1-22) that targets the protein to the cell surface (Turk *et al*., 1988), followed by five repeats of a copper binding octapeptide (Viles *et al*., 1999). The C-terminal domain, highly conserved over many different species, consists of two short antiparallel beta-sheets and three long alpha-helices with a disulfide bridge (cysteine 179 and 214) between helix 2 and 3 (Haire *et al*., 2004) and a GPI anchor (aa 230) that binds the protein to the *raft domains* on the cell surface (Naslavsky *et al*., 1997). PrP^C undergoes facultative N-glycosylation at two sites (aa 181 and 196), resulting in unglycosylated, monoglycosylated, and diglycosylated forms (figure 1. *3*) (Endo *et al*., 1989; Bolton *et al*., 1985). This basic structure was confirmed by studies on a wide range of mammalian species (Cappai *et al*., 2004; Riesner, 2003).

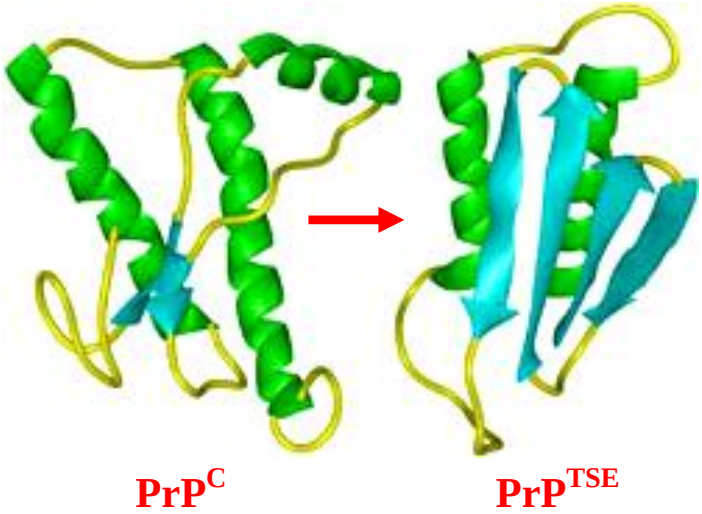
Figure 1. *3*. Human cellular prion protein.



PrP^C is expressed in a wide range of tissues including CNS (mainly in neurons and glia), blood cells, lymphatic tissue, intestine, muscles, lungs, spleen and heart (Shmakov *et al.*, 2000). The physiological role of PrP^C is still under debate. Several functions have been attributed to this protein ranging from signal transduction, antioxidant activity, cell membrane signaling, cell adhesion, synaptic transmission, to regulation of immune system and immune response as well as pro-apoptotic or anti-apoptotic activity (Sauer *et al*., 2003; Gauczynski *et al*., 2001; White *et al*., 1999). The presence of PrP^C is not essential for survival (Wopfner *et al*., 1999; Manson *et al*., 1994; Büeler *et al.*, 1992), but it is necessary for replication of the TSEs infectious agent and neurotoxicity to occur (Collinge *et al*., 2007; Mallucci *et al*., 2005). The close relationship between infection and expression of PrP^C in the host is clearly highlighted in knock-out mice for PrP gene (genotype $prnp^{0/0}$), which are resistant to the experimental infection with scrapie (Büeler *et al*., 1993).

PrP^C and PrP^{TSE} share the same amino acidic sequence and undergo to the same post-translational modifications (Harris *et al*., 2003), but differ profoundly in their conformation, that is accounted for their different biochemical and biophysical properties (Pan *et al*., 1993; Safar *et al*., 1993; Caughey *et al*., 1991). PrP^C is monomeric, proteinase-sensitive and soluble in non-ionic detergents, whereas PrP^{TSE} is insoluble, hydrophobic, and partially resistant to proteinase and tends to aggregate. Indeed proteinase K (PK) digestion of PrP^{TSE} originates a proteinase-resistant core with molecular weight of 27-30 kDa, which retains the full infectivity (Prusiner *et al*., 1984; Bolton *et al*., 1982). The insolubility of PrP^{TSE} in physiological solutions and its aggregated state hamper the definition of its tridimensional structure by high-resolution techniques. Low-resolution optical spectroscopic measurements revealed that PrP^{TSE} contains mostly beta-sheet structures (figure 1. *4*) (Caughey *et al*., 2001; Pan *et al*., 1993), suggesting that the differences between the two isoforms result from their distinct conformations.

Figure 1. ***4.*** Tridimensional structure of mature PrP^{C} obtained by NMR and presumable tridimensional structure of PrP^{TSE}.



1. *3* THE ETIOLOGICAL AGENT OF TSEs

One of the unsolved issues of prion diseases is the definition of the nature of the etiological agent responsible for the pathology. Originally it was defined as a "slow unconventional virus" (Gajdusek, 1977) because of its viral size, peculiar biological properties (i.e. high resistance to inactivation and apparent absence of a nucleic acid), transmissibility, and the long incubation time that characterizes the disease. However, the failure to identify a TSE-specific nucleic acid despite uncountable efforts together with the identification of an insoluble and proteinase-resistant protein (PrP^{TSE}) associated with infectious brain material and the highly unusual resistance to treatments that inactivate viruses or naked nucleic acids (e.g. heating, gamma radiations, nuclease) (Taylor, 2000; Alper, 1993), led to the genesis of the "protein only hypothesis" (Griffith, 1967). This atypical agent was defined by Prusiner as "prion" (**pr**oteinaceous **i**nfectious **on**ly particle) (Prusiner, 1982), to distinguish it from other infectious agent like virus or viroids. Prion particles are credited to be devoid of nucleic acid and to propagate in the host by directing the refolding of the

physiological PrP^{C} protein into the PrP^{TSE} conformation through an autocatalytic process which is still matter for speculation (Morris *et al*., 2009).

The "prion only" hypothesis is a reliable model to explain a number series of observations like the correlation between *PRNP* gene mutations and genetic TSEs (Gabizon *et al*., 1996), the strictly association between infectivity and PrP^{TSE} (Gabizon *et al*., 1988), the finding that PrP knockout mice do not develop the disease (Büeler *et al*., 1993) and the absence of a specific immune response in the infected host (Berg *et al*., 1994; Casaccia *et al*., 1989).

On the other hand, a growing amount of data suggests that TSE infectivity can be separated from PrP^{TSE} (Barron *et al*., 2007; Piccardo *et al*., 2007; Berardi *et al*., 2006; Shacked *et al*., 1999; Lasmèzas *et al*., 1997) and the recent finding of small RNAs possibly associated to the TSE infectious agent (Simoneau *et al*., 2009) is bringing to a renewed interest towards the viral hypothesis.

A third hypothesis lays in the middle: the agent may be a particle called "virino" that is composed by an exogenous nucleic acid enveloped in the host prion protein (Dickinson *et al*., 1988).

1. *4* TSE STRAINS

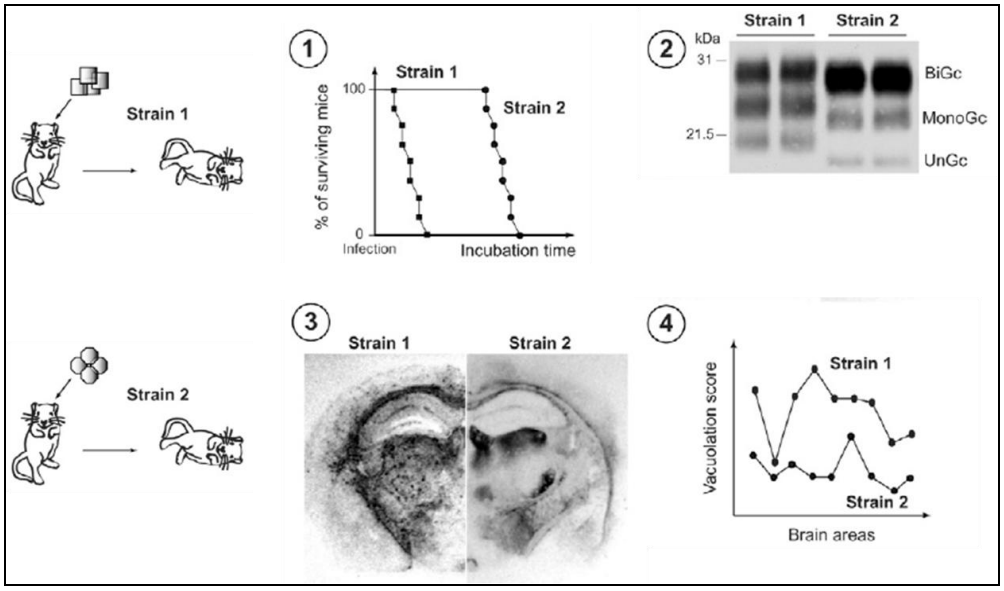
After the seminal observation that scrapie disease gave rise to different clinical syndromes in experimentally affected goats ("scratching" and "drowsy" phenotypes) (Pattison and Millson, 1961), it was only with the development of modern strain characterization techniques in the mouse model that the existence of multiple infectious prion "strains" or "isolates" was detailed and demonstrated beyond any doubt (Bruce *et al*., 1991).

Different strains are traditionally distinguished on the basis of the incubation period, the neuropathological profile and the clinical signs in the recipient host (Bruce, 2003).

Incubation period is defined as the time elapsed from the experimental inoculation to the development of clinical disease and for a given host-infectious strain combination it is a function of the infectious titre and the route of inoculation. For these reasons, although inoculation of different

prion strains in the same host usually results in different and reproducible incubation times, this parameter should be cautiously regarded (Bruce, 1993). A second feature that allows to distinguish the strains is the neuropathological profile or "lesion profile", represented by the distribution and the degree of neurological damages found in the brain of infected animals, according to a standardized procedure for grading the severity of spongiform degeneration in nine brain areas (Fraser and Dickinson, 1973). More recently, some biochemical properties of the pathological prion protein have been added to the list of the traits that characterize prion strains, such as the electrophoretic mobility after PK digestion (Collinge *et al*., 1996; Parchi *et al*., 1996), extent of PK resistance (Bessen *et al*., 1992a), stability towards denaturing agents (e.g. guanidine HCl), position and intensities of infrared bands associated with β-sheet structures, and metal binding capacities (Thomzig *et al*., 2004; Safar *et al*., 2000, 1998; Kuczius and Grouschup, 1999; Wadsworth *et al*., 1999). Moreover, an important factor contributing to the diversity among the strains is the PrP^{TSE} glycosylation: the relative ratios of di-, mono-, and unglycosylated forms of the pathological protein differ in various prion strains (Khalilli-Shirazi *et al*., 2005) (figure 1. *5*).

Figure 1. *5.* Models of prion strains variation. Upon experimental inoculation of susceptible animals with identical genetic background, prion strains exhibit specific traits ('phenotype') such as: (1) incubation time and attack rate, (2) PrP^{TSE} pattern in Western blot, (3) distribution of PrP^{TSE} deposits in the brain, (4) distribution and intensity of vacuolation in standardized brain areas ('lesion profile') (modified from Béringue *et al.*, 2008a).



In some cases these differences are so evident that are used in prion strain typing, as for example to discriminate between sCJD (type 2A) from vCJD (type 2B). Fourier transformed infrared spectroscopy (FTIR) (Aucouturier *et al*., 1999; Caughey *et al*., 1998) and conformation dependent immunoassay (Bellon *et al*., 2003; Safar *et al*., 1998) confirm the hypothesis that different structures acquired by PrP^{TSE} may account for prion strains differences. However, the molecular basis for the biochemical variety of PrP^{TSE} and how this relates to disease features remain unidentified.

Among available experimental host species, the mouse model is widely used for TSE strain primary passage and characterization allowing to identify more than twenty phenotypically distinct strains from different sources: goat and sheep scrapie (Bruce *et al*., 1993), BSE from cattle (Bruce *et al*., 2002; Lasmezas *et al*., 1996), sCJD and GSS from human sources (Muramoto *et al*., 1992; Tateishi *et al*., 1984; Manuelidis *et al*., 1978). Serial passages in the same species with constant biological conditions permit to stabilize and define a prion isolate (see next paragraph).

On some occasions, the experimental passage into mice led to the isolation and separation of different infectious strains coexisting in a single natural host. A representative example is the isolation of "drowsy" (DY) and "hyper" (HY) strains after the injection of the agent responsible for transmissible mink encephalopathy (TME) in the Syrian hamsters (Bartz *et al*., 2000; Bessen *et al*., 1992b). After serial passages (hamster-to-hamster) incubation periods became stable and could be assigned to two groups of different clinical signs: 150 days to the group that presented lethargy (so this strain was called "drowsy") and 60 days to the group characterized by hyperactivity (the strain was then called "hyper"). Differences among the two groups were also concerning the vacuolation distribution and PrP^{TSE} deposition in different brain regions (Bessen *et al*., 1994), as well as electrophoretical mobility of the pathological isoform of the PrP (19 kDa for the unglycosylated band of DY, 21 kDa for that of HY) (Bessen *et al*., 1995). Furthermore, DY and HY had a different resistance to PK digestion, DY being more sensitive than HY (Bessen *et al*., 1992a).

1. *5* EXPERIMENTAL TRANSMISSION STUDIES AND THE SPECIES BARRIER

Transmission of TSE disease from a species to another is often characterized by a prolonged incubation period and an incomplete attack rate (the number of animals affected) as compared with the intra-species transmission. This phenomenon is known as "species barrier" and in some cases can be abrogated through serial passages into the same host, after which a reduction of incubation time and a higher attack rate is observed, reflecting the adaptation, or rather, the selection of the most virulent strain from the original inoculum into the new species (Béringue *et al*., 2008a). Once stabilized into the new host, each strain can be serially propagated *in vivo* with high reproducible incubation period, clinical signs, lesion profile, and biochemical characteristics of PrP^{TSE}, together giving the "strain signature" in that host species.

Early studies suggested that the species barrier lies in the degree of homology of PrP amino acid sequence between the donor and the host species (Scott *et al*., 1989), as PrP sequence identity leads to an enhanced susceptibility to the disease and to a reduced incubation period. Consequently in the years a lot of different models of transgenic mice for sheep (Crozet *et al*., 2001; Vilotte *et al*., 2001; Westaway *et al*., 1994), bovine (Bèringue *et al*., 2006; Buschmann *et al*., 2005; Castilla *et al*., 2003; Scott *et al*., 1999) and human (Beringue *et al*., 2008b; Asano *et al*., 2006; Bishop *et al*., 2006; Korth *et al*., 2003; Taguchi et al., 2003; Asante *et al*., 2002; Kitamoto *et al*., 2002; Telling *et al*., 1994) were created with the purpose to break the barrier and facilitate transmission studies. Usually, transgenic mice with the same PrP primary structure as the donor species are more susceptible to the disease compared to the wild-type mice and can replicate some original strain properties, such as PrP^{TSE} molecular profile.

On the other hand it is important to consider that PrP sequence diversity alone cannot explain the presence of species barrier. This aspect has been brought to the light by the capacity of the BSE agent to transmit to a large panel of different species (exotic ruminants, cats and humans) retaining some strain features such as the PrP^{TSE} molecular profile. Another representative example of transmission efficiency in absence of PrP sequence identity is the experimental transmission of

sporadic and genetic CJD isolates to bank voles (a newly discovered rodent model). Such human strains propagate in these rodents with a low or absent transmission barrier despite the wide divergence between human and vole PrP primary structure (Nonno *et al*., 2006). Moreover wild type mice are more susceptible to vCJD and BSE compared to human or bovine transgenic mice (Bishop *et al*., 2006). Actually a general opinion is that both PrP sequence and the strain properties contribute to the "species barrier" or, more correctly, to the "transmission barrier" (Scott *et al*., 2005).

1. *6* SPORADIC CJD

The sporadic form of CJD (sCJD) accounts for 85% of the total human cases of TSEs with an incidence of 1-2 cases per million people per year worldwide, with an equal incidence in men and women aged between 60-70 years (Ladogana *et al*, 2005). The aetiology of sCJD is still unidentified, since the disease occurs in individuals that do not present mutations on *PRNP* gene, nor there is any evidence of accidental exposure of the patients to an infectious source. It has been speculated that the disease is the consequence of spontaneous conversion of PrP^{C} to PrP^{TSE} as a rare stochastic event (Wadsworth and Collinge, 2007), possibly facilitated by a hypothetical presence of somatic *PRNP* mutations which would explain the late onset of the disease. Alternatively, a change in the biochemical properties of the microenvironment surrounding the protein could be the trigger for PrP pathological conversion (Zanusso *et al*., 2001).

Most cases of sCJD present at onset a rapidly progressive dementia with myoclonus. During the course of the disease the patient can develop extrapyramidal or pyramidal signs, visual or cerebellar disturbance and, at later stages, akinetic mutism. Other rarer forms of sCJD show different clinical presentations such as cerebellar ataxia rather than cognitive impairment (ataxic CJD, 10% of the total sCJD cases), persistent visual disturbances for several weeks before the appearance of other clinical signs (Heidenhain form of sCJD). In the majority of cases, the median duration of the

disease is around 3-6 months. A longer duration of 12 months is observed in 15% of cases, while in about 5% of patients the disease may last for more than 2 years.

Different clinical phenotypes may be accompanied by specific neuropathological lesion profiles built on the basis of the severity and distribution of spongiosis, neuronal loss, gliosis, and PrP^{TSE} deposition (Appleby *et al*., 2009).

The vast majority of sporadic CJD patients can be categorized according to their genotype at the polymorphic codon 129 of *PRNP* gene and according to the electrophoretic mobility of the unglycosylated PK-resistant core of PrP^{TSE} (PrP^{TSE} type 1 and PrP^{TSE} type 2) (Parchi *et al*., 1999, 1996). Unglycosylated PrP^{TSE} type 1 has a molecular size of about 21 kDa with an N-terminus corresponding to the main PK cleavage site at amino acidic residue 82, while unglycosylated PrP^{TSE} type 2 has a molecular size of 19 kDa with an N-terminus at amino acidic residue 97 (figure 1. *6*).

The combination of the two parameters mentioned above allows to classify the sCJD cases in six main subgroups (MM1, MV1, VV1, MM2, MV2, VV2) corresponding to different clinical-pathological phenotypes (table 1. *2*).

Figure 1. *6*. PrP^{TSE} types in sporadic CJD (modified from Gambetti *et al*., 2003).

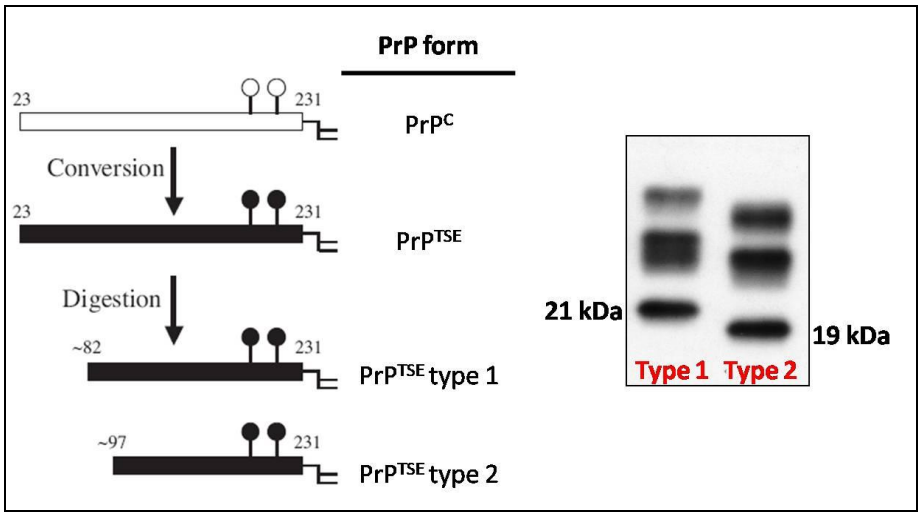


Table 1. *2*. Molecular and phenotypic features of the six subtypes of sCJD (modified from Parchi *et al.*, 1999).

sCJD subgroup	% of Cases	Onset (yr)	Duration (mo)	Clinical Features	Neuropathological Features
MM1 MV1	67 3	65.5 62.1	3.9 4.9	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 vacuoles and perivacuolar PrP staining
VV2	16	61.3	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	9	59.4	17.1	Ataxia in addition to progressive dementia, no typical EEG*, long duration (2 yr) 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	2	52.3	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 with little pathology in other areas; spongiosis may be absent or focal, and PrP^{TSE} is detected in lower amount than in the other variants
MM2-cortical	2	64.3	15.7	Progressive dementia, no typical EEG*	Large confluent vacuoles with perivacuolar PrP staining in all cortical layers; cerebellum is relatively spared
VV1	1	39.3	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

* Typical EEG: electroencephalogram showing periodic sharp-waves complexes typical of sCJD

Polymorphism M/V at position 129 influences not only the susceptibility and the phenotypic features of the disease (Pocchiari *et al*., 2004), but also the production of PrP^{TSE} types: MM homozygosity is more frequently associated with type 1 PrP^{TSE}, while VV or MV favour the production of PrP^{TSE} of type 2 (Parchi *et al*., 2000). From subsequent characterization studies of sCJD it was found that about 30% of cases accumulate both type 1 and type 2 in the brain, either in the same or in distinct anatomical areas (Notari *et al*., 2007; Polymenidou *et al*., 2005; Puoti *et al*., 1999).

As occurs with any other biological phenomenon, such a rigorous classification can not comprise the whole variability of clinical and pathological CJD phenotypes. However it remains a precious

tool to recognize atypical and rare forms of human TSEs that may represent emerging infectious strains with an unknown risk for humans.

1. *7* sCJD TRANSMISSION AND TRANSGENIC HUMANIZED MICE

Experimental transmission studies of human TSE diseases were first carried on non-human primates, such as chimpanzees, squirrel monkeys and cynomolgus macaques as they are evolutionary closer to humans than any other animal model and subsequently showed to have a similar PrP homology with the human counterpart (Williams *et al*., 2007; Herzog *et al*., 2005 Brown *et al*., 1994). In spite of their reliability, these studies are expensive, time consuming and burdened with ethical concerns, and are currently used only to produce data that are urgently needed to face public health problems.

Most types of sCJD did not transmit efficiently to wild-type laboratory mice (Nonno *et al*., 2006; Bruce *et al*., 1997), hence to overcome the human to mouse species barrier several lines of transgenic mice expressing the human prion protein have been developed (table1. *3*).

Table 1. *3.* Transgenic lines expressing human PrP.

Tg line (genotype)	Transgenic method (GT/RGI[a])	Expression level[b]	Reference
Tg152 (MM)	RGI	4-8	Telling et al., 1995
Tg110 (VV)	RGI	1	Telling et al., 1994
Tg440 (MM)	RGI	2	Telling et al., 1995
Tg650 (MM)	RGI	6	Beringue et al., 2008b
HuMM (MM)	GT	1	Bishop et al., 2006
HuVV (VV)	GT	1	Bishop et al., 2006
HuMV (MV)	GT	1	Bishop et al., 2006
Tg35 (MM)	RGI	1-2	Asante et al., 2002
Tg45 (MM)	RGI	4	Asante et al., 2002
Ki-Hu129M/M (MM)	GT	1	Kitamoto et al., 2002
Ki-Hu129M/V (MV)	GT	1	Kitamoto et al., 2002
Ki-Hu129V/V (VV)	GT	1	Kitamoto et al., 2002

a) GT: gene targeting; RGI: random genomic insertion
b) compared to PrP expression in the original host species

Most of such humanized transgenic lines were engineered on endogenous PrP null background in order to avoid any interfering effect of the resident murine PrP gene. Transgenic mice have contributed enormously to the abrogation of the species barrier creating new valuable models characterized by short incubation periods and high attack rates in transmission experiments, therefore allowing a better investigation of the molecular basis of human prion strains (Grouschup and Buschmann, 2008; Telling, 2008). The insertion of human *PRNP* gene in the murine genome

was performed by two different techniques. The random genomic insertion (RGI), is based on the microinjection of the DNA of interest into a fertilized mouse oocyte; this means that it is neither possible to control the insertion site nor to control the number of copies of the transgene integrated, thus leading to an overexpression of the human PrP in the murine background. Furthermore, the number of copies integrated is not directly proportional to the expression levels of PrP, thus highlighting the effects that the point of insertion may have on the expression of the newly integrated transgene. These models of transgenic mice may be useful to investigate the effect of PrP expression levels on the susceptibility to the disease and may represent a valuable resource as a rapid mouse bioassay mode (some of the lines developed clinical signs in less than 100 days post-inoculation) but they cannot model the natural pathogenesis of TSEs.

As a further drawback it is not possible a comparison between different mouse lines since any of it has different level of PrP expression.

The alternative gene targeting technique (GT) (Manson and Tuzi, 2001), is based on the homologous recombination and allows to replace the murine *prnp* gene with the corresponding gene derived from another species. In the case of humanized mice the human *PRNP* is present in a single copy in the same site where was located the *prnp*, therefore under the natural expression modifiers that determine physiological expression levels of the human PrP (Bishop *et al*., 2006). Moreover this system allows a direct comparison between the different mouse lines. The transgenic mice used to perform the studies described in this thesis, produced by means of GT technique, express the human *PRNP* with three alternative genotype at the polymorphic codon 129 (MM, MV, VV), the most recognised factor of susceptibility and pathological phenotype in sCJD.

2. AIM OF THE STUDY

Sporadic CJD, the most common form of human TSE diseases, presents distinctive phenotypes, that could be discriminated by clinical signs at onset, duration of disease, type and distribution of neuropathological lesions (spongiosis, gliosis, and neuronal loss), and by the type and the pattern of deposition of the pathological prion protein PrP^{TSE}, an hallmark of TSE diseases. Parchi and collaborators (Parchi *et al.*, 1996) have shown that the polymorphic codon 129 of the *PRNP* gene and the PrP^{TSE} type (1 or 2) that accumulates in the patient's brain are the major determinants of disease phenotype. Thus, they classified all cases of sCJD into six subtypes (MM1, MM2, VV1, VV2, MV1, and MV2).

Transmission studies of the six sCJD subtypes into transgenic mice expressing the human prion protein suggested that partly the variety of the clinical-pathological phenotypes of sCJD might be related to the presence of distinct human strains of prion agent.

There are, however, rare cases of sCJD that do not fulfils any of the above criteria, suggesting that other minor infectious strains might be responsible of these rare variants. It is still controversial whether TSE strains are influenced by yet unknown endogenous or exogenous factors, but the understanding of the biological characteristics of these strains will improve our knowledge of the pathogenesis of the disease, and hopefully, to find an appropriate therapy for these otherwise fatal disorders.

Aim of my PhD work was to determine whether in 3 atypical Italian sCJD cases identified through the national surveillance of CJD and related disorders at the Istituto Superiore di Sanità, Rome, it was possible to identify novel human prion strains. This objective was pursued by inoculating brain extracts of each patient into gene-targeted transgenic mice carrying the human *PRNP* gene with the three possible genotypes (MM or VV or MV) at the polymorphic codon 129. Survival times, attack rates, lesion profiles, and molecular analysis of the PrP^{TSE} type recovered from mice brains for each genotype/inoculum combination will be compared with data from the transmission of the "classical" sCJD subtypes to clarify the basis of these atypical cases.

3. METHODS

3. *1* ANIMALS

3. *1.1* Transgenic mice

The transgenic model (HuTg) used in transmission experiments is represented by three transgenic lines expressing human PrP homozygous for methionine (HuMM), valine (HuVV) or heterozygous (HuMV) at the polymorphic position 129 in a 129/Ola background (Bishop *et al*., 2006). The murine *prnp* gene was entirely replaced by human *PRNP* gene by using gene targeting technique (Bishop *et al*., 2006; Manson and Tuzi, 2001) that allows the production of human PrP in the same tissues and at the same levels as that wild-type PrP. The three transgenic lines have identical genotype, except for the polymorphic codon 129. Thus the differences in the transmission properties can be directly attributable to the codon 129.

Initial breeding stocks (HuMM and HuVV mice), kindly supplied by Prof. Jean Manson (The Roslin Institute, Neuropathogenesis Division, University of Edinburgh), were used to establish successful colonies of human transgenic mice. The heterozygous HuMV line was generated by crossing the two homozygous lines. The experimental breeding procedures were carried in the animal facility of the Department of Cell Biology and Neuroscience - Istituto Superiore di Sanità, Roma in collaboration with qualified and authorized staff.

3. *1.2* Confirmation of the genotype of the transgenic mice

The following method was used to check both the genotype of the original breeding stocks and that of the mice used for the transmission experiments.

Genomic DNA was isolated from the mouse tail following standard laboratory procedures. Briefly, tailsnips were cut and incubated overnight at 53°C in 0.5 ml of tail solution (50mM Tris-HCl, pH 8, 100mM EDTA, 1% SDS, 100mM NaCl) supplemented with 500 μg of Proteinase K (PCR grade, Roche). DNA was extracted using phenol/chloroform, precipitated in isopropanol and dissolved in TE buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA) and stored at -20°C until use.

DNA was amplified for mouse *prnp* orf with primers Mouse PrpF and R (figure 3.*1*) to verify the absence of endogenous gene. Positive and negative controls were included.

PCR was done using 200-500 ng of DNA in 50 µl volume with standard buffers (*Taq* PCR Master Mix kit, Qiagen). PCR was run through 40 cycles at 94°C for 30 seconds, at 58°C for 30 seconds, at 72°C for 30 seconds. PCR products were run on 1.5% agarose gel and visualized by ethidium bromide under UV light.

Transgene integration in mice was analyzed by PCR using human primers for *PRNP* (figure 3. *1*) and the polymorphism at codon 129 was detected by direct sequencing of PCR products. PCR was done using 200-500 ng of DNA in 50 µl volume with standard buffers (*Taq* PCR Master Mix kit, Qiagen). The first step was 95°C for 15 minutes, continued by 40 cycles of 94°C for 40 sec, 62°C for 40 sec, and 72°C for 40 sec and the final elongation step at 72°C for 10 minutes. The PCR positive products were run on 1.5% agarose gel, visualized by ethidium bromide under UV light and isolated using a QIAquick PCR Purification kit (QIAGEN) according to the standard protocol provided by QIAGEN. The purified PCR products were sequenced by the Sanger dideoxynucleotide chain termination method (Slatko *et al*., 2001) with the GenomeLab DTCS-Quick Start Kit (Beckman). Sequences were run for 25 cycles at 96°C for 30 minutes, 56°C for 15 minutes, and 60°C for 4 minutes. Purified sequences were then electrophoresed on the Beckmann Coulter CEQ 8000 capillary sequencer and analyzed using the sequence navigator software provided by the manufacturer. All PCR and sequencing cycles were performed using the Gene Amp PCR system 9700 (Perkin Elmer). All tests were performed in duplicate.

Figure 3.*1*

Human sequence of the construct inserted in the mouse gene:

ATG GCG AAC CTT GGC TAC TGG CTG CTG GCC CTC TTT GTG ACT ATG TGG
ACT GAT GTC GGC CTC TGC AAA AAG CGG CCA AAG CCT GGA GGG TGG AAC
ACC GGT GGA AGC CGG TAT CCC ggg cag **ggc agc cct gga ggc aac cgc** tac
cca cct cag ggc ggt ggt ggc tgg ggg cag cct cat ggt ggt ggc tgg
ggg cag cct cat ggt ggt ggc tgg ggg cag ccc cat ggt ggt ggc tgg
gga cag cct cat ggt ggt ggc tgg ggt caa gga ggt ggc acc cac agt
cag tgg aac aag ccg agt aag cca aaa acc aac atg aag cac atg gct
ggt gct gca gca gct ggg gca gtg gtg ggg ggc ctt ggc ggc tac
(G/A)tg ctg gga agt gcc atg agc agg ccc atc ata cat ttc ggc agt
gac tat gag gac cgt tac tat cgt gaa aac atg cac cgt tac ccc aac
caa gtg tac tac agg ccc atg gat gag tac agc aac cag aac aac ttt
gtg cac gac tgc gtc aat atc aca atc aag cag cac acg gtc acc aca
acc acc aag ggg gag aac ttc acc gag acc gac gtt **aag atg atg gag cgc**
gtg gtt gag cag atg tgt atc acc cag tac gag agg gaa tct cag gcc tat
tac cag aga gga tcg agc atg gtc ctc ttc tcc tct cca cct gtg atc
ctc ctg atc tct ttc ctc atc ttc ctg ata gtg gga tga ggaaggCCT

Bold: primers for human *PRNP* screening by PCR
CAPITALS: mouse sequence
Lower case: human sequence
Blue case: M129V polymorphism
Red case: primers used for PCR products sequencing

Primers used for PCR and sequencing:

Hum F1 5'-gca gcc ctg gag gca acc gc-3'
Hum R1 5'-aac cac gcg ctc cat cat ctt-3'

Hum F 5'-cta ccc acc tca ggg cgg tgg tgg c-3'
Hum R 5'-tgg ttg ctg tac tca tcc at-3'

MousePrpF 5' tgt ggc agg ggc tgc g-3'
MousePrpR 5'-gct gga tct tct ccc g-3'

Bold: primers for human *PRNP* screening by PCR
Red case: primers used for PCR products sequencing.
Green case: primers used for mouse *prnp* PCR.

3. *2* INOCULATION OF THE TRANSGENIC MICE

3. *2.1* Sporadic CJD patients

Patients were recruited through the National CJD surveillance unit of the Istituto Superiore di Sanità, that received the permission from the ethical committee to collect tissue samples from patients referred to the unit and use them for research purposes. Patient CJD diagnoses were confirmed by histopathology, immunohistochemistry and Western blot (Cardone *et al.*, 1999). Each of the three transgenic mice lines were inoculated with the sCJD cases described below:

- A typical CJD case (referred in the thesis as **MM1**), characterized by a onset at 71 years old and a duration of 4 months. The patient had a pseudoperiodic EEG, typical for sporadic CJD, and manifested dementia, myoclonus and pyramidal, extrapyramidal, cerebellar and visual signs. After the death the brain was removed and half hemisphere was fixed in formaline for the histological investigations and the other half was frozen to perform biochemical, genetic and transmission studies. The brain presented intense spongiosis. Western blot analysis revealed presence of type 1 PrP^{TSE}. Genetic analysis on *PRNP* displayed methionine homozygosity at polymorphic codon 129 and no presence of disease-associated mutations. The present case was used as control, because of it is the most common form of sporadic CJD.
- An atypical case (referred in the thesis as **MVx**) (Zanusso *et al*., 2007), represented by a patient who exhibited behavioural and personality changes followed by rapidly evolving dementia. The duration was of 14 months. After the death the brain was removed and half hemisphere was fixed in formaline for the histological investigations and the other half was frozen to perform biochemical, genetic and transmission studies. Post-mortem neuropathological examination of the brain showed an atypical CJD phenotype characterized by intracellular prion protein deposition and the presence of axonal swellings filled with amyloid fibrils. Biochemical analysis of the pathological prion protein disclosed

a previously unrecognized PrP^{TSE} tertiary structure lacking diglycosylated species. *PRNP* analysis revealed Met/Val heterozigosity at codon 129 and no pathological mutation.

- A case (referred in the thesis as **MV1/2**) defined atypical because characterized by an early onset (38 years old), a long duration (36 months), psychiatric symptoms and sleep disturbances. After the death the brain was removed and half hemisphere was fixed in formaline for the histological investigations and the other half was frozen to perform biochemical, genetic and transmission studies. Neuropathological studies showed diffuse spongiosis and kuru-like plaques. Biochemical analysis of the pathological prion protein in the brain revealed the presence of both PrP^{TSE} types (type 1 and type 2). The part of the brain that was inoculated for transmission studies in the transgenic mice contains only type 2 PrP^{TSE}, in order to avoid having a confounding factor in the inoculum (co-presence of type 1 and type 2). Genetic investigation on *PRNP* showed Met/Val heterozigosity at codon 129 and no disease-linked mutations.
- An atypical case (referred in the thesis as **MV2At**) characterized by an early onset (44 years old), a very long duration (62 months) and psychiatric symptoms. After death, the brain was removed and half hemisphere was fixed in formaline for the histological investigations and the other half was frozen to perform biochemical, genetic and transmission studies. Neuropathological analysis showed a diffuse spongiosis and deposition of kuru-plaques like in the cerebellum and Western blot displayed type 2 PrP^{TSE}. Sequencing of *PRNP* gene revealed Met/Val heterozygosity at codon 129 and no pathological mutations.

3. *2.2* Preparation of inocula from human brains

Human brain samples were collected from areas showing pathology and PrP^{TSE} accumulation. Brain tissues were homogenized at 10% (w/v) concentration in sterile phosphate buffered saline (PBS) (138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, KH_2PO_4 1.4 mM, pH 7.6) using new, dedicated glass/Teflon Potter tissue grinders. The 10% homogenate was aliquoted into 5 ml glass vials,

sealed, and stored at -80ºC (Pocchiari *et al*., 1989). On the day of inoculation the homogenate was thawed, vortexed to homogeneity and then diluted to 1% (w/v) in sterile PBS. Preparation and dilution of inocula were carried out in a Class 2 microbiological Safety Cabinet, located in a Category 3 hazard risk room.

3. *2.3* Inoculation of mice

The animals were housed in compliance with Directive N. 86/609/EEC on the protection of animals used for experimental and other scientific purposes and the Legislative Decree of 27 January, N. 116/12 (*Gazzetta Ufficiale-Suppl. Ord.*- n. 40, 18 febbraio 1992). Before the inoculation procedure, mice were anesthetized by intraperitoneal administration of 0.1 ml of a solution prepared by mixing 1 ml Ketamine-50, 0.15 ml Xylazine, 9.75 ml of saline (NaCl 0.9%). The inoculum (20 µl of 1% human brain homogenate) was injected into the left cerebral hemisphere by using an insulin syringe with a 26G needle. The animals were sacrificed by CO_2 asphyxia. Experimental inoculation procedures were carried out in the animal facility of the Department of Cell Biology and Neuroscience - Istituto Superiore di Sanità, Roma in collaboration with qualified and authorized staff.

3. *3* EXAMINATION OF INOCULATED MICE

3. *3.1* Clinical signs

The animals were observed two days per week from 100 days after the inoculation to record the onset of the clinical signs which manifest as motor dysfunctions (ataxia), rigidity of the tail, hunched back and weight loss (Carp *et al*., 1984) and were culled at the terminal stage of the disease.

3. *3.2* Survival time

For each combination inoculum/genotype we recorded the survival time, corresponding to the time between the inoculation and the culling, for animals clinically sick, or to the time intercurrent between inoculation and spontaneous death, for animals that did not show clinical signs.

Survival time of each group of animals is expressed as the average of the survival times of PrP^{TSE} positive mice only. The survival curves were built according Kaplain-Meier method by considering PrP^{TSE} positivity. All PrP^{TSE} negative cases entered in the analyses as censored. Comparison between survival curves were carried out by the log-rank test.

3. *3.3* Histological and immunohistochemical examination

At postmortem, each brain was divided in two parts by a sagittal paramedian cut. One half was immediately frozen and stored at -80°C for Western blot analysis, while the other half was immersed and fixed in 4% buffered formaldehyde for neuropathological analyses.

Fixed brains were cut in five sections (2 mm thick) at standard coronal levels, transferred into embedding cassettes, immersed in 98% formic acid for 1 hour to reduce infectivity and washed for 2 hours in running water. The samples were then dehydrated in graded alcohols (70% o.n. - 95% o.n. - 100% for 2 h), clarified in xylene for 30 min and finally embedded in paraffin wax.

Five-µm-thick sections were cut on rotative microtome, collected on glass slides for histological analyses or on charged slides (Superfrost® Plus slides, Menzel-Glaser) for immunohistochemistry and let dry at 37°C overnight in an oven.

For histology, the slides were dewaxed in xylene (2 x 20 mins), rehydrated in decreasing graded alcohols (100% 2 x 20min, 95% - 70% - distilled water 5 min each), stained with haematoxylin and eosin (H&E), coded and examined blind with a Leica microscope for pathological assessment.

For the construction of the lesion profile, vacuolar changes were scored (table 3. *1*) in nine grey-matter areas of the brain (table 3. *2*) according to the scoring system introduced by Fraser and

Dickinson (Fraser and Dickinson, 1968). Vacuolation scores are derived from at least four individual mice per group, and are reported as means ± standard error of the mean.

Table 3. *1.* Definition of vacuolation scores.

Vacuolation score	Description
0	No vacuoles
1	A few vacuoles widely and unevenly scattered
2	A few vacuoles evenly scattered
3	Moderate numbers of vacuoles, evenly scattered
4	Many vacuoles with some confluence
5	Dense vacuolation with most of microscopic field confluent, lace-like appearance

Table 3. *2.* List of the brain regions of HuTg mice and corresponding numbers used to build up the "lesion profile" curves.

Brain regions	Corresponding numbers in the lesion profile figures
Dorsal medulla	1
Cerebellar cortex	2
Superior colliculus	3
Hypothalamus	4
Thalamus	5
Hippocampus	6
Septum	7
Posterior cerebral cortex	8
Anterior cerebral cortex	9

For immunohistochemistry, slides were dewaxed, rehydrated and immunostained for the presence of PrP^{TSE} using the mouse monoclonal antibody (mAb) SAF84 (SPI-BIO, Massy, France). The sections were immersed for 10 min in methanol containing 3% H_2O_2 to block endogenous peroxidase activity, washed in distilled water for 10 min and subjected to antigen retrieval by autoclaving at 121°C for 30 minutes in distilled water. The slides were cooled at room temperature, treated with 98% formic acid for 1 min to enhance staining, rinsed, and incubated with 4 M guanidine thiocyanate for 30 min at 4°C in a humid camber. After a one hour incubation in PBS containing 3% Normal Goat Serum (NGS), the slides were then incubated overnight at 4°C with mouse mAb SAF84 (1.5 μg/ml) in PBS/NGS 3%. Subsequent antibody detection involved incubation with a biotinylated goat anti mouse secondary antibody for one hour (1:200 dilution, Vector Laboratories) at room temperature, followed by incubation with the avidin-biotin-peroxidase complex (Vectastain ABC-Elite kit, Vector Laboratories) according to the manufacturer's instructions. The samples were stained with 3'- 3'diaminobenzidine (DAB, Sigma) or 3-amino-9-ethylcarbazole substrate (AEC Plus; Dako, Glostrup, Denmark) as chromogen to visualize the reaction product and then counterstained with hematoxylin. Positive (sections of previously immunostained brains) and negative (sections stained with the omission of the primary antibody from the incubation solution and sections from uninfected brains) control sections were included in each run.

3. *3.4* Preparation of the brain tissue for the Western blot analysis

The protocol described below was kindly provided by Doctor Matthew Bishop (National CJD Surveillance Unit, Western General Hospital, Edinburgh).

Each frozen half brain was thawed, transferred in a 2 ml safelock sterile tube and weighed. A homogenate of the tissue was prepared to a final concentration of 10% (w/v) in sterile saline (NaCl 0.9%) by using a disposable plastic micropestle. An aliquot of 250 µl of the 10% homogenate was transferred to a 1.5 ml safelock tube and 25 µl of extraction buffer (5% Nonidet P-40, 5% Sodium

deoxycholate, 200 mM Tris-HCl pH 7.4) were added. After mixing by vortex, the sample was centrifuged at 2000 rpm for 5 minutes at 4 ºC in a microfuge.

An aliquot of 100 µl of supernatant was taken and treated with proteinase K (PK, Sigma) to a final concentration of 50 µg/ml. This step enables to detect only the pathological form and not the cellular form of PrP (completely digested after this treatment). The digestion was carried out at 37ºC for 1 hour with constant agitation (1000 rpm of Thermomixer), then was stopped by adding 2 µl of 25 X Complete Inhibitors Cocktail (Roche).

The samples were centrifuged at 14000 rpm for 60 minutes at 4 ºC in a microfuge. The resulting pellet was resuspended in 20 µl of LDS Sample buffer 2X prepared from NuPAGE LDS Sample buffer 4 X Invitrogen (106 mM Tris-HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM Serva Blue G250, 0.175 mM Phenol red) plus DTT 0.5 M, boiled at 99 ºC for 10 minutes and stored at – 20 ºC.

Tissue manipulation was carried out in a Class 2 microbiological Safety Cabinet in a Category 3 hazard risk room.

3. *3.5* PrP^{TSE} detection by Immunoblot

Denatured samples were loaded onto a 1 mm thick NuPAGE SDS Electrophoresis polyacrylamide gel (pH 7.0) with an acrylamide concentration of 12% (Invitrogen) and run for 2 h at 100 V constant voltage.

Once proteins were separated, they were electrotransferred onto a nitrocellulose membrane by a semi-dry cell (Semi-dry transfer unit, Hoefer Semi-phore) in transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% methanol) for 1 hour at constant amperage of 125 mA. The membrane was then incubated for 1 hour at 37 °C in blocking buffer: 5% milk powder (Non-Fat Dry Milk, Bio Rad) diluted in TBS / 0.05% Tween-20 (TBST) with constant agitation. This step is necessary to cover non-specific binding sites on the membrane.

After two quick washes in TBST, the membrane was incubated with the anti-PrP 3F4 primary antibody diluted 1:1000 in 5% milk / TBST for overnight at 4°C with constant agitation. 3F4 monoclonal antibody binds residues 109-112 of PrP (Lund *et al*., 2007; Kanyo *et al*., 1999; Bolton *et* al., 1991; Rogers *et al*., 1991) and was chosen for its high specificity and affinity for the human PrP (Kascsak *et al*., 1987). After 5 washes of 5 minutes each in TBST, the membrane was incubated for 2 hours at 37°C with constant agitation with anti-mouse IgG peroxidase-linked secondary antibody (Amersham) diluted 1:5000 in 5% milk / TBST. PrP bands were finally revealed by incubation for 1 minute in the dark with a solution containing luminol (Western Blotting Luminol Reagent, Santa Cruz Biotechnology) and exposure of the membrane to light sensitive film (Amersham Hyperfilm).

4. RESULTS

4. *1* ATTACK RATE

The presence of spongiosis in H&E stained brain sections or the detection of the pathological form of PrP (PrP^{TSE}) in the brain (by immunohistochemistry or Western blot) were the essential parameters to define the transmission of the disease. The attack rates (percentage of diseased brains) for each combination inoculum/genotype are summarized in table 4. *1*.

MM1, MV1/2 and MV2At transmitted efficiently to all three lines with high percentages of attack rate. MV2At displayed the highest efficiency, with an attack rate of 100% in all three lines.

MVx case transmitted only to HuVV mice with a low attack rate value.

Table. 4. *1*. Attack rate.

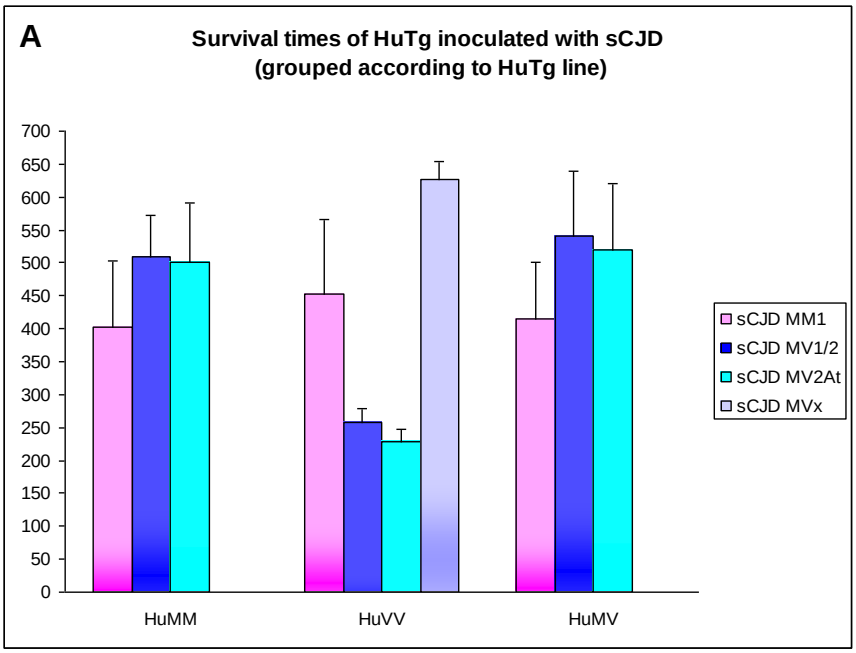
Inoculum from human sporadic CJD	Mouse genotype	PrP^{TSE} positive/tested	Attack rate (% positive)
MM1	MM	18/19	95%
	VV	12/15	80%
	MV	18/18	100%
MVx	MM	*0/14*	*0%*
	VV	4/18	22%
	MV	*0/14*	*0%*
MV1/2	MM	13/16	81%
	VV	20/20	100%
	MV	17/19	89%
MV2At	MM	16/16	100%
	VV	20/20	100%
	MV	17/17	100%

Italic red, clinical but not PrP^{TSE} positive.

4. *2* SURVIVAL TIME

Transgenic mice inoculated with brain homogenates of sporadic CJD patients were difficult to evaluate for the onset of clinical signs because these were rather not specific, (i.e., hunching of the back, hypo-activity, lowering of the hind back, and ruffled fur), therefore, we regarded the time between inoculation and death as a more reliable end-point. The mean survival times for each inoculum/genotype combination are summarized in figure 4. *1* and in table 4. *2*.

Figure 4. *1*. Survival times of HuTg mice injected with different sporadic CJD cases.



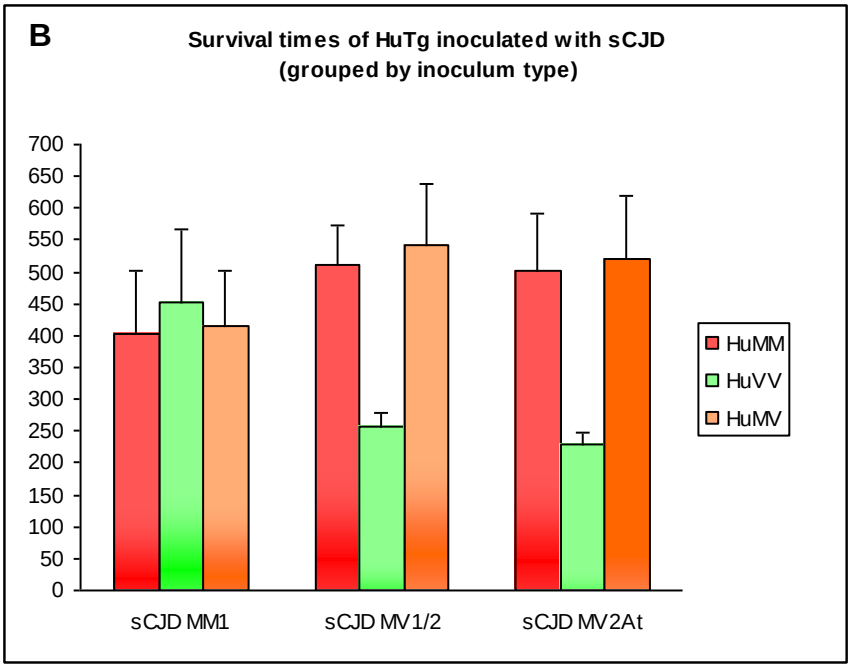


Table 4. *2.* Survival times in days (mean ± SD) for each combination inoculum/genotype.			
	TRANSGENIC LINES		
sCJD INOCULUM	**HuMM**	**HuVV**	**HuMV**
MM1	402.7 ± 99.33	453.0 ± 112.6	415.1 ± 86.2
MV1/2	510.2 ± 62.2	258.1 ± 21.1	540.8 ± 98.7
MV2At	501.9 ± 88.2	229.1 ± 17.6	519.0 ± 100.8
MVx	*511.9 ± 106.9*[#]	626.0 ± 28.6	*453.6 ± 144.4*[#]
[#] Survival times of clinical positive but PrP^{TSE} negative mice (no PrP^{TSE} positive mice found)			

The Kaplan-Meier survival curves drawn for each inoculum are in figure 4. *2*, showing the survival differences between the 129 genotypes.

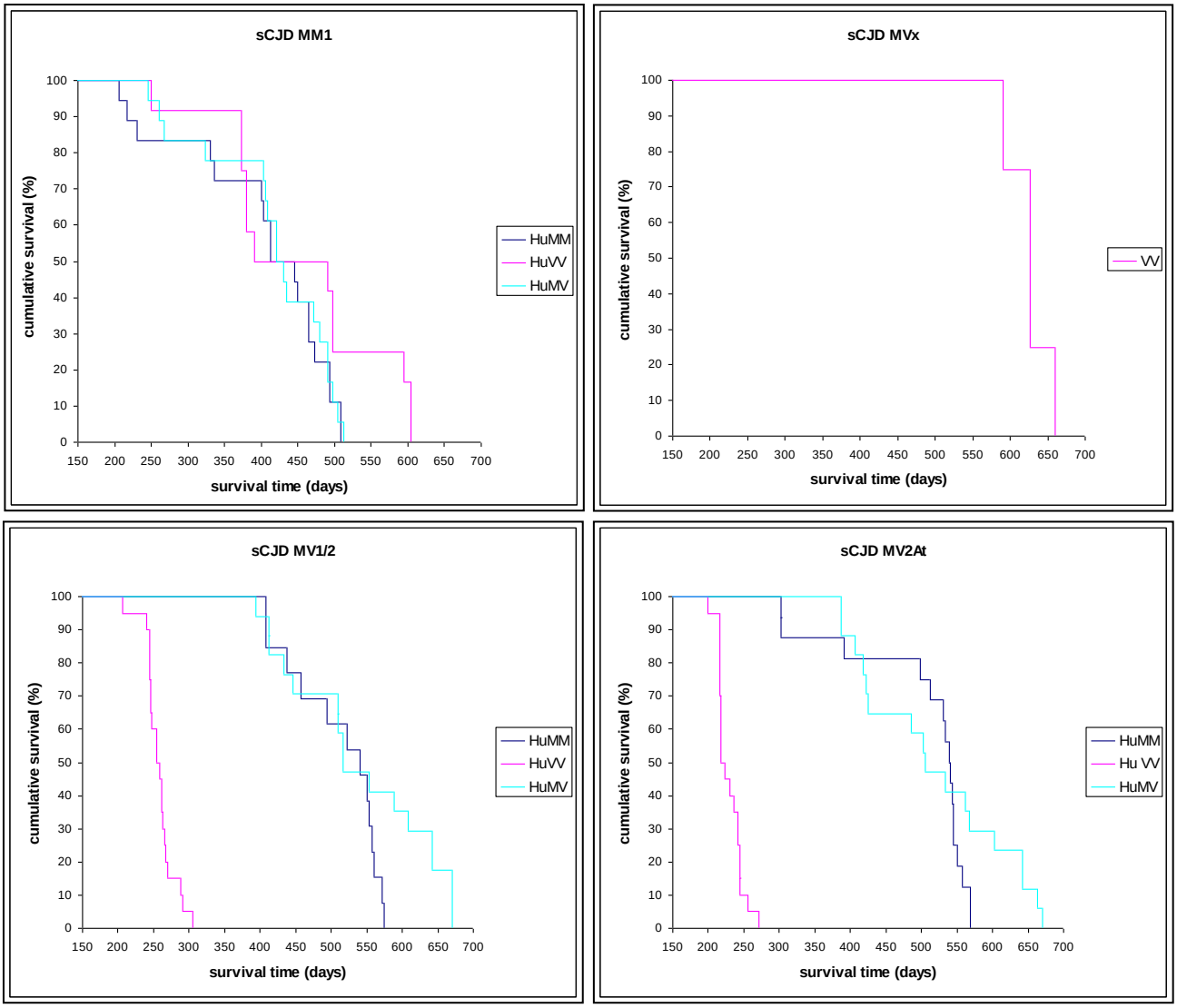
MM1 case successfully transmitted to the three lines with similar survival times and overlapping survival curves. Indeed no statistically significant differences among survival curves are present between different HuTg mice (p=0.13).

MVx case transmitted only to HuVV mice with very long survival times. No survival data in the other different lines are available for comparison.

MV1/2 successfully transmitted to all three HuTg lines, with significantly shorter survival times in HuVV as compared with HuMM and HuMV. In line with this observation, the survival curve for HuVV mice shows a very steep decay and is significantly different (p= 0.00) from those of HuMM and HuMV mice, which result statistically indistinguishable.

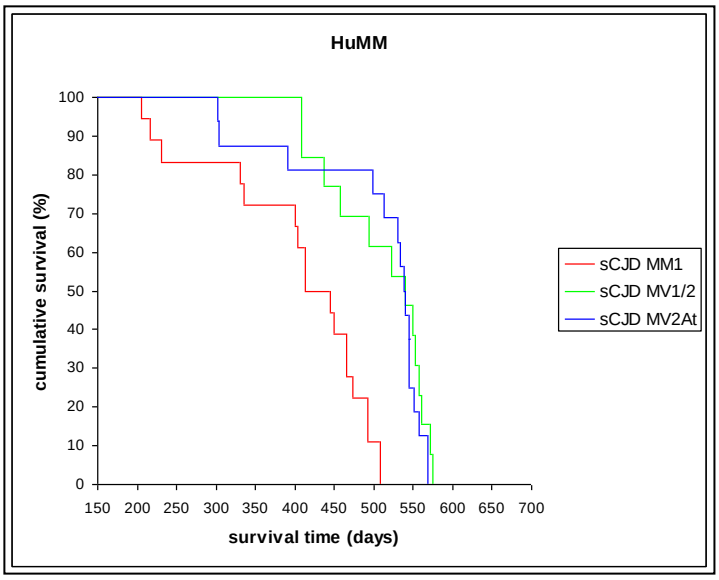
MV2At transmission gave the same results as the MV1/2 inoculum in the three HuTg mouse lines with shorter survival time in the HuVV line with respect to HuMM and HuMV lines. Statistical analyses show a significantly (p= 0.00) shift of HuVV survival curve with regard to those of HuMM and HuMV, which do not differ between them.

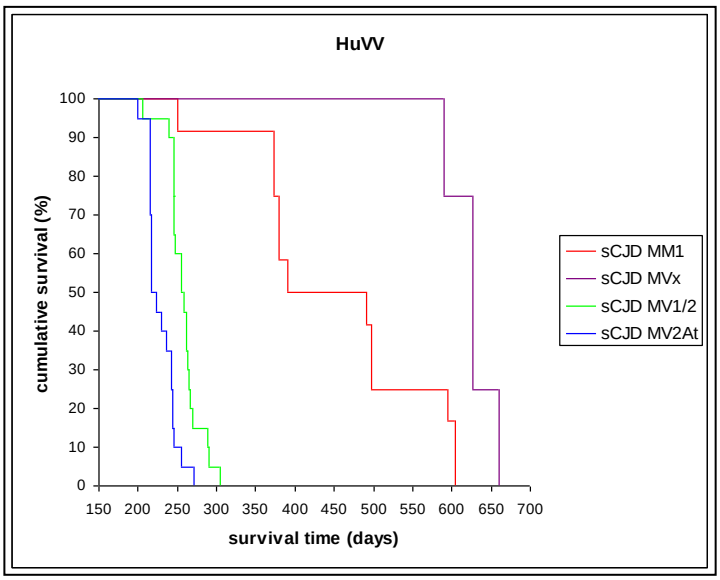
Figure 4. *2.* Kaplan-Meier survival curves grouped by inoculum type.

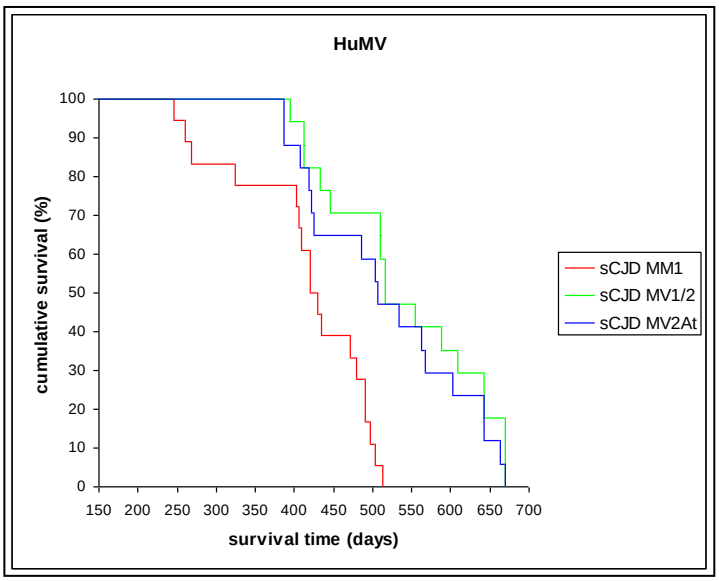


The Kaplan-Meier survival curves of each HuTg line inoculated with different inocula are depicted in figure 4. *3*. These curves allow to visualize the effect of the inoculum type in the same 129 genotype: in the HuMM line and HuMV line, the MM1 inoculum was faster than the MV1/2 and MV2At inocula whereas in the HuVV line, an opposite trend was observed with a relatively fast transmission in animals injected with MV1/2 and MV2At inocula, followed by MM1 and MVx which showed a very long survival time.

Figure 4. 3. Kaplan-Meier survival curves grouped by HuTg mouse line.







4. *3* TOPOGRAPHY OF SPONGIOSIS AND PrP^{TSE} DEPOSITION

To characterize the phenotype of affected mice, we analyzed the pattern of vacuolar degeneration in nine grey matter brain areas, which are represented by the lesion profile (figure 4. *4*).

The MM1 case presented a pattern of spongiosis that resulted remarkably similar among the three human transgenic lines, with slightly more intense spongiform changes in HuMV mice in all the areas, except in the superior colliculus (area 3).

MV1/2 showed similar profiles of spongiform degeneration in HuMV and HuVV mice, with more severe changes in HuVV mice, confirming that this transgenic genotype is more susceptible to this inoculum. In HuMM mice the thalamus (area 5) was the area mainly affected similarly to HuMV mice, the other line with methionine (in heterozigosity) at codon 129.

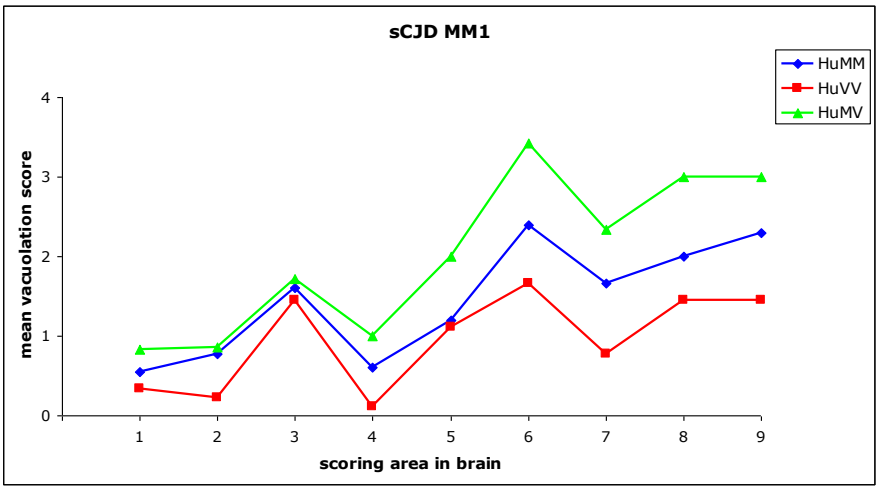
MV2At case produced more intense spongiform changes in HuVV and HuMM involving all brain areas except the cerebellar cortex (area 2). A much lower degree of spongiosis could be appreciated in HuMV were the thalamus (area 5) was the most severely affected area similarly to what observed in HuMM mice, the other methionine 129 containing genotype.

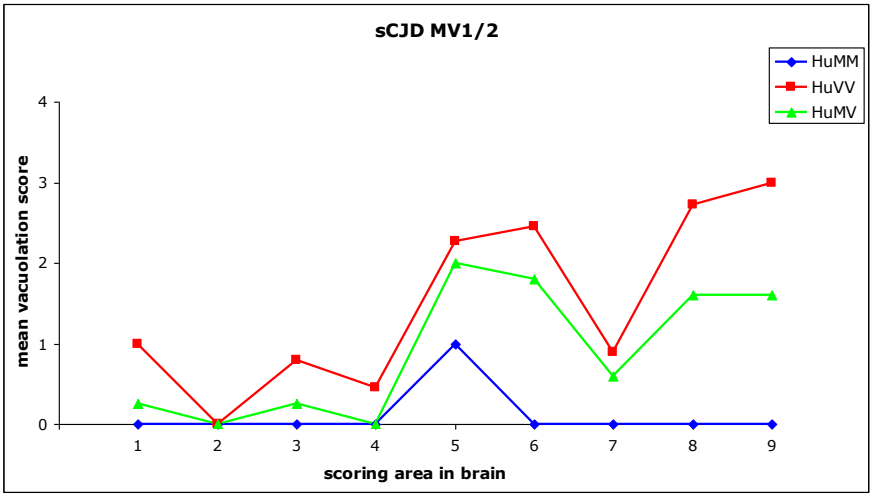
While both the MV1/2 and the MV2At sCJD cases associated with vacuolation in the deep layer of the cerebral cortices, the MM1 determined vacuolation of superficial cortical layers in all infected animals (fig. 4. *5*).

In most of the animals belonging to the three genotypes, large vacuoles can be observed in several white matter areas (i.e. striatum, internal capsule, cerebellar white matter and medulla). The same vacuolation is however present in age-matched control animals, suggesting that such phenomenon may not be related to the disease but rather to an aging process (fig. 4. *6*).

All mice belonging to the three lines were negative at the neurophatological analysis both for the presence of spongiosis and for PrP^{TSE} deposition once inoculated with the MVx case.

Figure 4. *4*. Lesion profiles grouped by inoculum type.





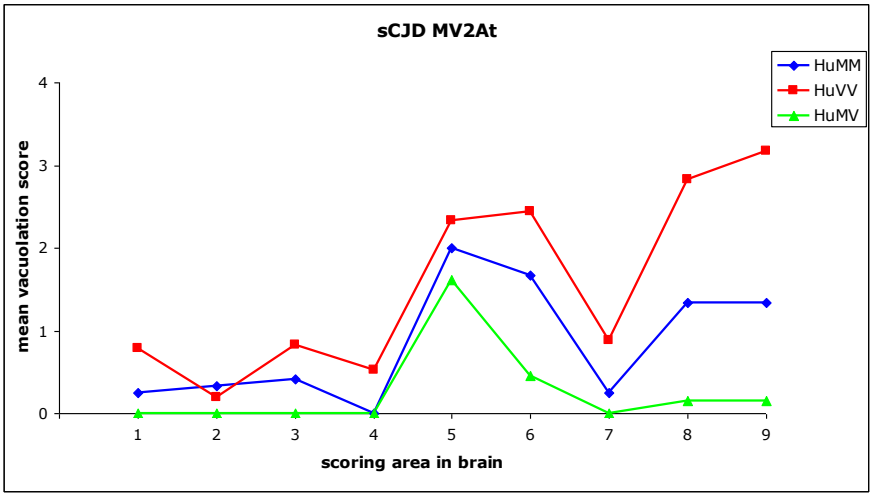


Figure 4. *5*. Vacuolation in the cerebral cortex of HuMM mice infected with MM1 sCJD (a,b) and with MV2At sCJD (c,d). Vacuoles are present in the superficial layers of the cortex in mice infected with MM1 sCJD (arrows in b) and in the deep layers of the cortex adjacent to the corpus callosum in mice infected with MV2At sCJD (arrows in d). b and d are higher magnifications of the boxed areas in a and c. CC: Corpus Callosum.

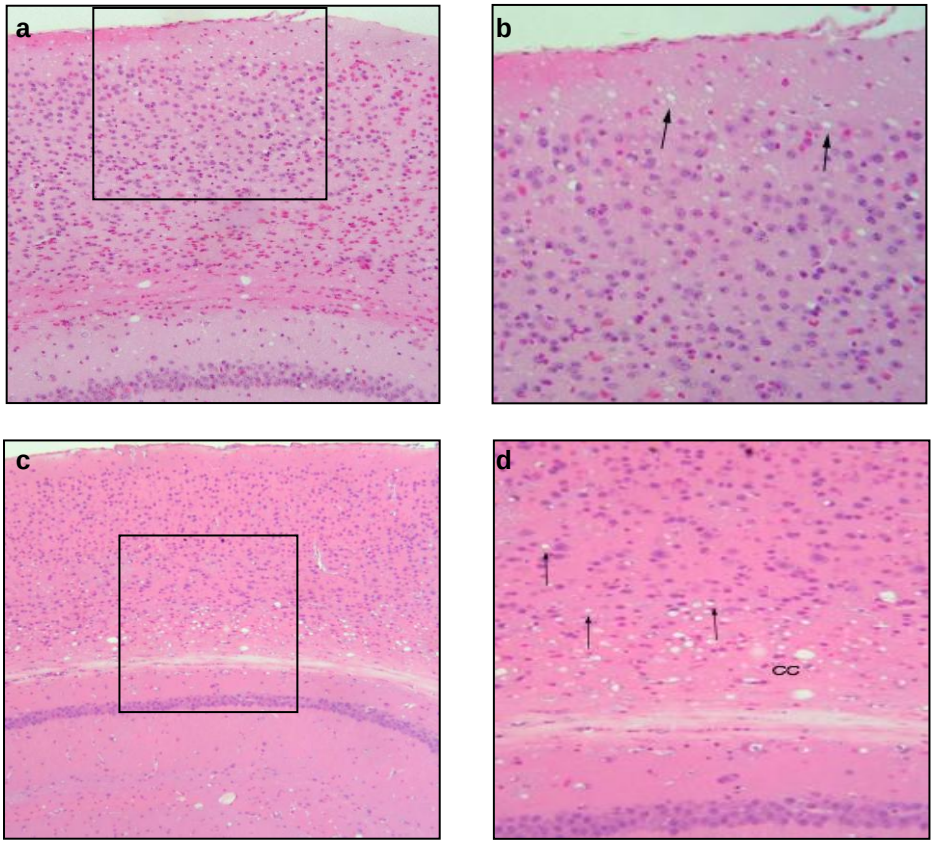
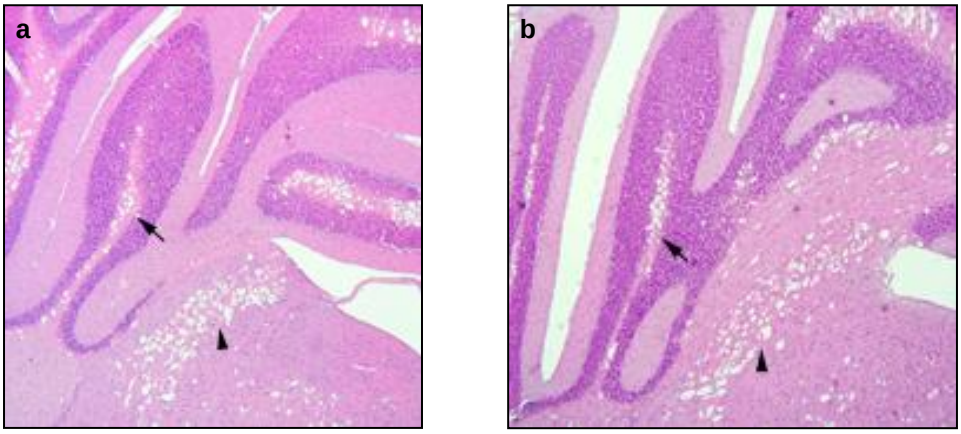


Figure 4. *6*. White matter vacuolation in affected (a) and healthy (b) animals in cerebellar cortex (arrows) and in vestibular nuclei (arrowheads).

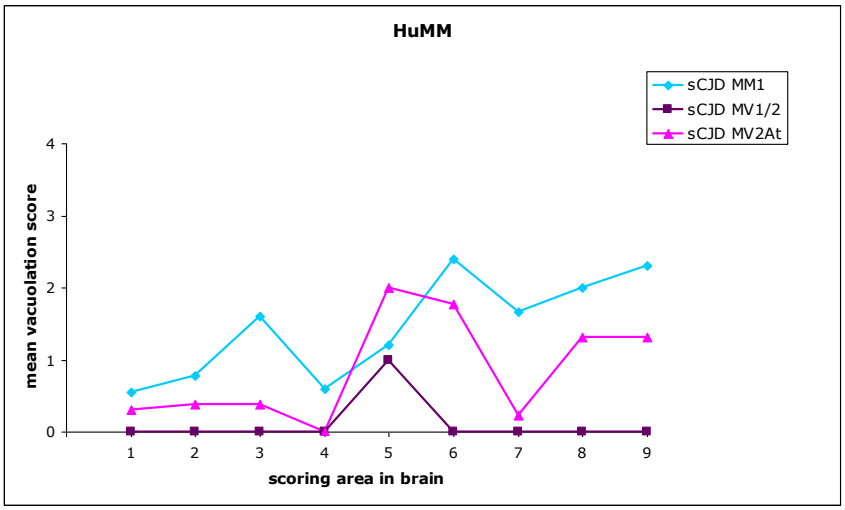


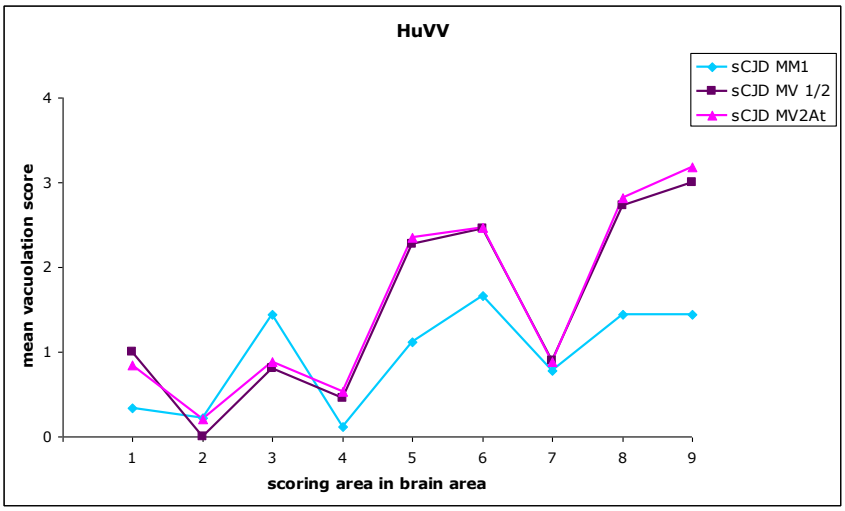
When neuropathological data are analysed on the basis of the recipient genotype (figure 4. *7*) it can be seen that in HuMM mice the three inocula determined different lesion profiles, highlighting the role of the strain on lesion distribution in the brain, despite the same 129 polymorphism in the host. Both MV cases (MV1/2 and MV2At) mainly targeted the thalamus (area 5), with the MV2At inoculum inducing a higher severity of the lesions in the hippocampus (area 6) and cerebral cortices (areas 8 and 9). HuMM mice infected with the MM1 sCJD case had a completely distinct lesions profile, characterized by a more severe vacuolation in the superior colliculus (area 3) and the septum (area 7), and a lower spongiform change in the thalamus (area 5).

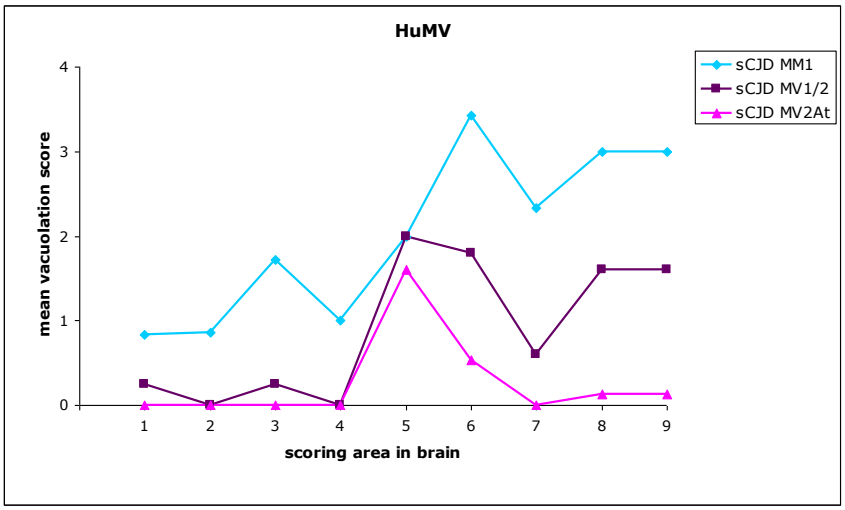
In HuVV mice, the two MV cases (MV1/2 and MV2At) produced an identical pattern of vacuolar changes, with the higher spongiform change in the thalamus (area 5), hippocampus (area 6) and cerebral cortices (8 and 9 areas). This pattern was clearly different from that observed with the MM1 sCJD case, where the level of vacuolation was less severe in all the nine areas except for the superior colliculus (area 3) and the septum (area 7).

In HuMV mice, the MV1/2 and the MV2At sCJD cases had similar vacuolation scores in the posterior and central areas of the brain but lower severity of lesions in the hippocampus (area 6), septum (area 7) and cerebral cortices (areas 8 and 9) of animals injected with the MV2At sCJD inoculum. Again, mice injected with MM1 sCJD showed a completely different lesion profile characterized by the presence of lesions in all the nine areas examined.

Figure 4. *7*. Lesion profiles grouped by HuTg line.







Immunohistochemical analyses revealed a heterogeneous deposition of PrP^{TSE} in different brain areas with all three CJD subtypes (table 4. *3*). After inoculation of MM1 case in HuMM mice, granular deposition can be observed in the ventral thalamic nuclei and only rare and small plaque-like aggregates are present in the hippocampus. Scattered PrP^{TSE} deposits were also found in the septum and in the mesencephalic nuclei in some of the animals analyzed.

In HuVV mice, the MM1 inoculum is characterized by the total absence of plaques and by the presence of little granular PrP^{TSE} deposition in the hippocampus; the thalamic nuclei are involved with few scattered deposits.

In HuMV mice the thalamic nuclear group and the hippocampus are always sites of PrP^{TSE} deposition, with the other brain areas involved only occasionally at the diencephalic (cortical) and mesencephalic level. Plaques are always absents. Animals with longer survival times are characterized by a more abundant PrP^{TSE} deposition.

The MV1/2 case determined in HuMM mice granular PrP^{TSE} deposition in the thalamic nuclei, invariably present in all the animals analyzed; a variable number of plaques are observed along the corpus callosum at the level of the hippocampus and in few mice in the retrosplenial cortex but are absent in all other levels. Some granular deposits can be observed in the mesencephalon and in the medulla. As in HuMM mice, the MV1/2 inoculum in HuVV mice is characterized by a granular deposition in the thalamic nuclei and plaques along the corpus callosum. In some animals the PrP^{TSE} is also found in the motor cortex, in the mesencephalon and in the medulla.

The ventral thalamus is the only area of deposition in HuMV mice inoculated with MV1/2, but in such mice the deposition is mainly represented by plaques whereas the granular deposition is almost absent.

Regarding MV2At case in all the three mice lines, the pattern of PrP deposition is the same as for the MV1/2 inoculum, with granular deposits in the thalamus and plaques along the corpus callosum in HuMM and HuVV mice; and plaques, mainly present in the thalamus, in HuMV mice.

All three inocula (MM1, MV1/2, MV2At) determined in mice the presence of PrP^{TSE} deposition in the ventral nuclear groups of the thalamus (median and lateral nuclei, fig. 4. *8*) and, interestingly, in the lateral nuclei, the PrP^{TSE} deposition parallels the distribution of the vacuoles.

PrP plaques can be observed along the corpus callosum or in the thalamus in all three mice lines infected with the MV1/2 and MV2At inocula, and can be also easily identified with the haematoxylin and eosin staining (fig. 4. *9*). Small plaque-like aggregates can be occasionally found in the hippocampus of MM mice infected with the MM1 sCJD.

Table 4. *3.* PrP^{TSE} distribution and deposition pattern.

Inoculum	**Mouse genotype**	**Brain level / PrP^{TSE} pattern**							
		A		**B**		**C**		**D**	
		granular	*plaques*	*granular*	*plaques*	*granular*	*plaques*	*granular*	*plaques*
	HuMM	+*	-	+	+*	+*	-	-	-
sCJD MM1	HuVV	-	-	+	-	-	-	-	-
	HuMV	+*	-	+	-	+*	-	-	-
	HuMM	-	+*	+	+	+*	-	+*	-
sCJD MV1/2	HuVV	+*	-	+	+	+*	-	+*	-
	HuMV	-	+*	+*	+	-	+*	-	+*
sCJD MV2At	HuMM	-	+*	+	+	+*	-	+*	-
	HuVV	+*	-	+	+	+*	-	+*	-
	HuMV	-	+*	+*	+	-	+*	-	+*

* Observed only in some animals

BRAIN LEVELS

Figure 4. *8*. PrP^{TSE} Immunohistochemistry (red) shows the presence of plaques along the hippocampus (arrowheads) and granular deposition in the ventral and lateral thalamic nuclei (arrows). The deposition in the thalamus parallels the presence of vacuoles (b,c). a,b: immunohistochemistry; c: Haematoxylin/Eosin staining.

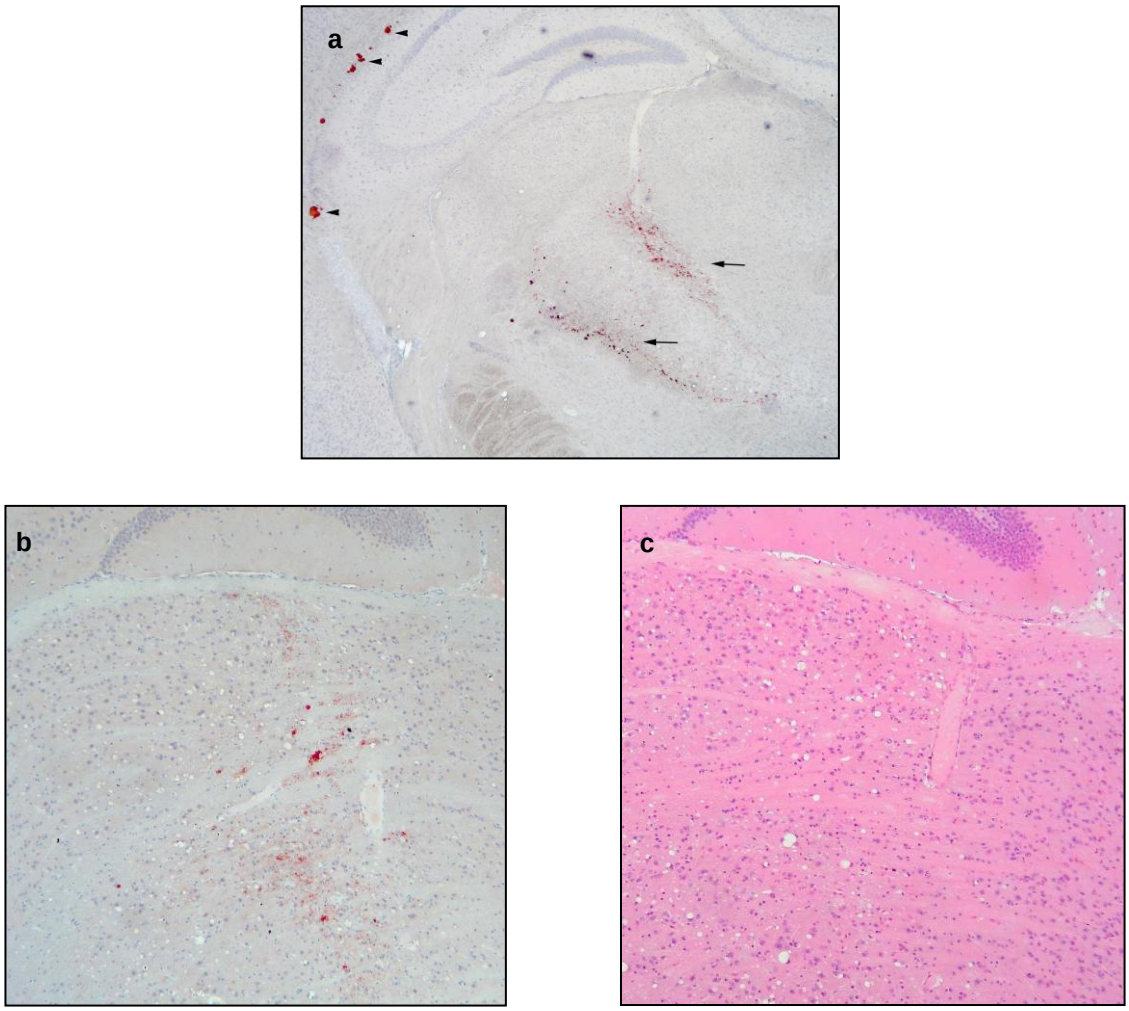
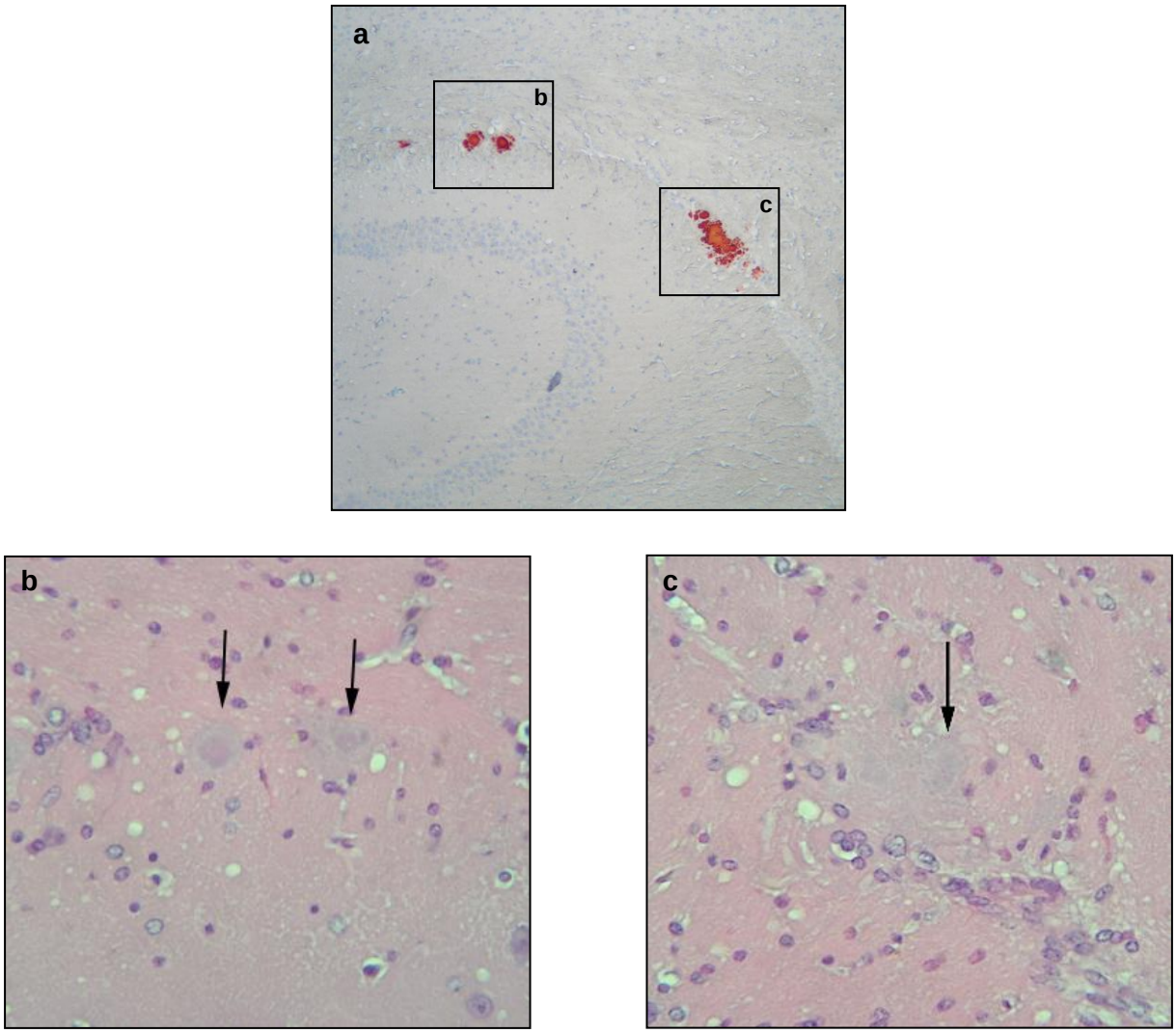


Figure 4. 9. PrP^{TSE} immunohistochemistry in HuMM mice infected with the MV2At sCJD case shows the presence of plaques (a). The same plaques can be easily observed with the Haematoxylin/Eosin staining (b,c).



4. *4* PrP^{TSE} *GLYCOTYPE*

The results of Western blot glycotyping of PrP^{TSE} extracted from the brains of transgenic mice and from the corresponding sCJD cases are shown in figure 4. *10*.

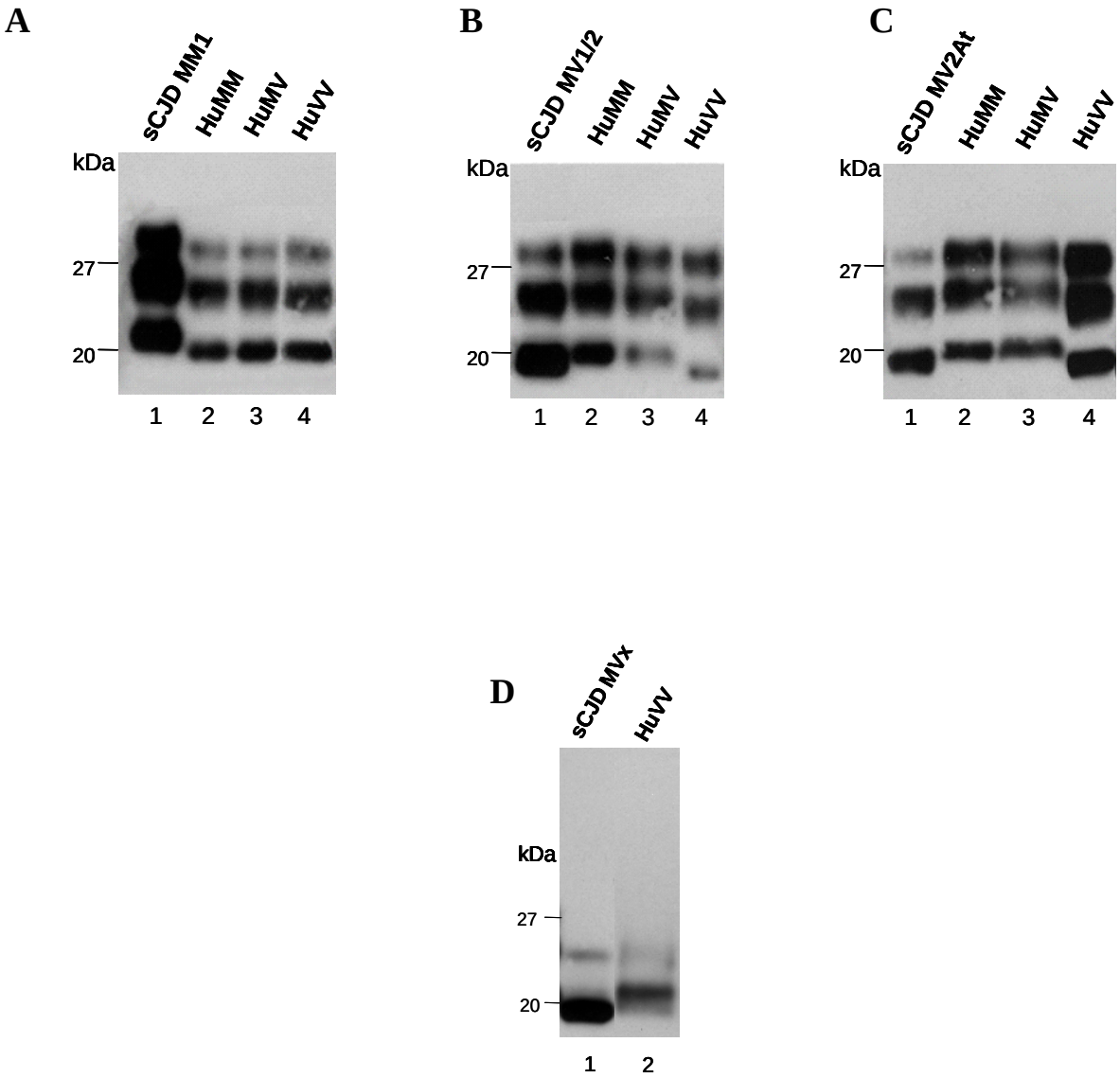
Transmission of the MM1 CJD case (A, lane 1) resulted in type 1 PrP^{TSE} formation in all three recipient mouse lines (A lanes 2, 3, 4). The molecular weight of the PrP^{TSE} in mice is slightly lower than that of the inoculum. The PrP^{TSE} in the three lines manteined the same glycoform ratio as the original inoculum (monoglycosylated > non-glycosylated > diglycosylated).

Transmission of both the MV1/2 and the MV2At cases (B lane 1 and C lane 1) resulted in the presence of type 1 PrP^{TSE} in HuMM and HuMV mice (B, lanes 2 and 3; C lanes 2 and 3), and type 2 PrP^{TSE} in HuVV mice (B, lane 4; C, lane 4). Again, the molecular weight of type 2 PrP^{TSE} in HuVV mice is slightly lower than that of type 2 PrP^{TSE} of inoculum.

The three lines produced a similar pattern of glycoform ratio for both MV1/2 and MV2At inocula, but different from that observed in the human counterparts, with a higher presence of the glycosylated forms in mice.

As described above, the CJD case MVx (D, lane 1) transmitted only to HuVV line, determining in these mice the deposition of a PrP^{TSE} type lacking the diglycosylated band (D, lane 2) similar to those found in the original CJD inoculum. In this case, the molecular weight of the PrP^{TSE} accumulated in mice has a higher molecular weight than the human counterpart. The non-glycosylated is more represented than the mono-glycosylated band in both inoculum and mice.

Figure 4. ***10.*** Detection of PrP^{TSE} in human and mice brains by Immunoblot. A) MM1 case; B) MV1/2 case; C) MV2At case; D) MVx case. PrP^{TSE} was revealed by using the monoclonal anti-PrP antibody 3F4. The standards molecular weights are expressed in kiloDalton (marker: Magic Mark XP Western Protein Standard, Invitrogen).



5. DISCUSSION

The design of this project was developed in order to obtain useful informations on a group of unusual, apparently sporadic, cases of CJD. These cases may be well truly sporadic, or derive from unrecognized interhuman, or rather zoonotic, episodes of transmission, and have an unknown potential to transmit to genetically susceptible individuals.

Unique transgenic mouse lines expressing the three normal genotypes at position 129 of human PrP generated by gene targeting technique allow us to rule out possible misinterpretations about permissive genotype caused by the over-expression of the cellular prion protein commonly encountered with traditional transgenic approaches.

The advantages of gene targeted transgenics, versus wild type mice (e.g. C3H line), for strain characterization, can be easily appreciated by comparing the short survival times and high attack rates presented here on transgenic mice injected with sCJD case MM1 (see summarized data in table 5. *1*) with the long survival time and very low attack rate observed in C3H mice injected with the same inoculum (Nonno *et al*., 2006).

The MV1/2 case was considered atypical because of a long clinical duration, a long phase of psychiatric and sleep disturbances, a relatively young age at onset and the presence of both types (1 and 2) of PrP^{TSE}.

Although it is now recognized that the combination of type 1 and type 2 in the same brain, or even in the same brain area, is a relatively common event, the significance of this finding remains unclear. A possible hypothesis is that these patients are simultaneously infected by two independent prion strains as it often occurs in naturally affected sheep with scrapie. In this study we inoculated HuTg mice with a patient's brain sample containing only type 2 PrP^{TSE} because we wanted to avoid any confusion due to the co-presence of type 1 and type 2 in our inoculum. However, it would now be of great interest to investigate whether a brain sample with mixed PrP^{TSE} types would produce in HuTg mice a pattern (attack rates, survival curves, lesion profiles, and PrP^{TSE} "signature") similar to those observed with the pure type 2 PrP^{TSE} containing inoculum.

Patient MV2At presented very atypical clinical and laboratory features that the diagnosis *in vita* was thought to be either Alzheimer's or Pick's disease. He also had an unusual long clinical duration for a sporadic CJD. Thus, it would have been very difficult to make a correct diagnosis without the post-mortem examination of the brain.

These two atypical cases (both MV1/2 and MV2At) transmitted with high efficiency to all three mouse lines, similarly to MM1 classical case, yet, at variance with the latter inoculum, they yielded an attack rate of 100% in HuVV mice with the shortest survival times of the whole study (table 5. *1*), suggesting that presence of PrP with 129 methionine reduces the susceptibility to the disease. Looking at the survival curves in figure 4. *3*, the MM1 inoculum was faster in HuMM and HuMV than MV1/2 and MV2At inocula, while the opposite occurred in HuVV mice. At variance with MM1, during the transmission of type 2 cases, the 129 genotype affected both the susceptibility to the disease and the type of PrP^{TSE} deposited in the brain of diseased mice. In particular, the presence of at least one methionine at codon 129 promoted production of type 1 PrP^{TSE}, whilst in valine homozygous mice, production of type 2 PrP^{TSE} was favoured (see table 5. *1* and figure 4. *10*). This variability indicates that in type 2 transmissions the interaction between infectious strain and the host is more complex than in type 1 transmission, and that the acquisition of type 2 conformation of PrP^{TSE} is inhibited by the presence of a methionine residue at position 129.

To better understand the mechanisms behind these observations it will be useful to establish the quantitative ratio between the two PrP allotypes in the heterozygous mice inoculated with the different human cases. This would explain if and how the 129 genotype and the infectious strain result in an allotype selective accumulation of PrP^{TSE} with methionine, or valine, at 129 and lead to a different propensity for conversion into a type 1 or type 2 conformation.

The identical biochemical signature and pattern of PrP^{TSE} deposition within all three recipient mouse lines injected with MV1/2 and MV2At, and the similarities between survival curves in HuMM and HuMV (see figure 4. *3*), as well as the lesions profiles in HuVV and HuMV mice (fig. 4. *7*), suggest that this two inocula may contain the same main strain. On the other hand, the slight,

though significant difference, among survival curves in HuVV (see figure 4. *3*) and the differences observed in the lesion profiles in HuMM line infected with this two atypical cases (see figure 4. *7*) indicate the opposite. To better characterize the two strains a second passage (mouse-to-mouse) of each isolate should be performed, with the aim of discriminating possible differences.

Patient MVx had a clinical course of disease that was relatively characteristic for sporadic CJD, but presented a unique pattern of PrP^{TSE} by Western blot, which showed the complete absence of the diglycosylated band of the protein. A frozen brain sample of this patient was also intracerebrally inoculated in bank voles that developed the disease after an unusual long incubation period (about 500 days) with a PrP^{TSE} pattern typical of that observed in animals inoculated with "classical" sporadic CJD patients bearing type 1 PrP^{TSE} (Zanusso *et al*., 2007).

The atypical case MVx had very low transmission efficiency, with few mice (all valine homozygous) resulting PrP^{TSE} positive by Western blotting and very long survival times. Interestingly, neither histological lesions nor immunohistochemical PrP^{TSE} staining were observed in the brain of these animals, suggesting that the PrP^{TSE} did not form relevant aggregates, and do not trigger the classical cascade of events leading to cytotoxicity. This phenomenon could be attributable to a low infection titre in this inoculum, and/or to a reduced capability of this particular biochemical form of PrP^{TSE} to convert the host PrP^{C}. This possibility is strengthened by the observation that the pattern of PrP^{TSE} in mice resembles that of patient's brain with respect to the absence of the diglycosylated band (see figure 4. *10*) suggesting that this infectious strain has the capacity to convert only the mono and non glycosylated forms of PrP^{C}.

This unglycosylated dominant pattern, observed in the sporadic CJD MVx and in the recipient HuVV mice, differs from glycotypes encountered in sporadic CJD and variant CJD, whereas it is similar to that encountered in *PRNP* Thr183Ala and Val180Ile mutations (Grasbon-Frodl *et al*., 2004) that result in allelic removal of the first consensus glycosylation site. However, differently from these mutations, the MVx case and HuVV mice show a normal representation of diglycosylated, monoglycosylated, and unglycosylated PrP^{C} isoforms (Zanusso *et al.*, 2007; Bishop

et al., 2006) ruling out a constitutive glycosylation defect. This confirms that this PrP^{TSE} pattern is dictated by the infectious strain.

For comparison (see table 5. *2*), we use data produced by Bishop in the same line of HuTg mice (in yellow). The inocula taken from the brain of our sporadic CJD patient MV2At was compared with the "classical" sporadic CJD MV2 (from Bishop's panel) whereas our sporadic CJD MV1/2 and CJD MVx were compared with "classical" MV1 and MV2 (from Bishop's panel) since these inocula might possibly behave as "classical" type 1 or type 2.

The attack rates and the type of PrP^{TSE} that the MV1/2 and MV2At sporadic CJD inocula produced in HuTg mice are much similar to those observed after transmission of the "classical" sporadic CJD MV2 patient (see table 5. *2*) confirming the hypothesis that the broad phenotypic variations observed in sporadic CJD MV2 patients likely do not depend on the presence of different infectious strains, but from other, yet unidentified, factors.

Regarding patient MV1/2, it is worth to mention that this inoculum gave a pattern much more similar to the "classical" sporadic CJD MV2, rather than to the CJD MV1, as suggested by the formation of PrP^{TSE} type 2 in HuVV mice (see table 5. *2*). As we mentioned before, it would be interesting to inoculate in HuTg mice a brain sample containing mixed PrP^{TSE} type before drawing any strong conclusion on whether patients with double PrP^{TSE} type harbour one or two different prion strains.

For what concern MVx case the very long survival time recorded in the positive mice and the pattern of PrP^{TSE}, dissimilar to any other PrP^{TSE} observed in HuTg, strongly indicate that this case could represent a new strain of sCJD.

These data highlight the usefulness of this model for the identification and studies of novel strains of human prion disease, relevant for the surveillance of the disease and public health, including the evaluation of potential human-to-human iatrogenic transmission.

Table 5. *1*. Survival time, attack rate and PrP^{TSE} type for each case inoculated in the three transgenic lines.

Inoculum from human sCJD	Mice genotype	Survival time (mean ± SD)	Attack rate (% positive)	PrP^{TSE} type
MM1	MM	402.7 ± 99.33	95%	1
	VV	453.0 ± 112.6	80%	1
	MV	415.1 ± 86.2	100%	1
MV1/2	MM	510.2 ± 62.2	81%	1
	VV	258.1 ± 21.1	100%	2
	MV	540.8 ± 98.7	89%	1
MV2At	MM	501.9 ± 88.2	100%	1
	VV	229.1 ± 17.6	100%	2
	MV	519.0 ± 100.8	100%	1
MVx	MM	*511.9 ± 106.9*	*0%*	-
	VV	626 ± 28.6	22%	as inoculum
	MV	*453.6 ± 144.4*	*0%*	-

Italic red, clinical but not PrP^{TSE} positive

Table 5. *2*. Overall comparisons of different human inocula into HuTg mice carrying the three different genotype at codon 129 of *PRNP* gene.

sCJD	HuMM		HuVV		HuMV	
	Rate	PrP^{TSE}	Rate	PrP^{TSE}	Rate	PrP^{TSE}
MM1	95%	1	80%	1	100%	1
MM1	100%	1	87%	1	100%	1
MV1	94%	1	83%	1	89%	1
MV2	91%	1	100%	2	89%	1
MV1/2	81%	1	100%	2	89%	1
MV2At	100%	1	100%	2	100%	1
MVx	0%	-	22%	x	0%	-

6. CONCLUSIONS

The work described in this thesis validates the usefulness of our model of transgenic mice for the identification and the characterization of human prion strains, and to investigate the influence of the polymorphisms at codon 129 of *PRNP* on the development of the disease.

Remarkably, these mice have the advantage over other "humanized" transgenics (Groschup and Buschmann, 2008) to express the human PrP at physiological levels and to develop the disease only when inoculated with human TSE infected brain tissues but not spontaneously as reported for other transgenics.

In our study, a novel CJD strain originated form the MVx was likely isolated in HuVV mice, and it would be useful to perform a subsequent passage (mice-to-mice) to better characterize this strain, and to investigate whether PrP^{TSE} negative mice replicated infectivity, and eventually contain the same strain that replicated into HuVV mice.

Concerning the other two atypical cases transmitted into our HuTg mice, a second passage would help to stabilize the strain and to have a reference for comparison to the MVx strain.

Another important aspect to be highlighted regards the possibility to use this model of humanized mice to evaluate the potential iatrogenic risk of human -to -human transmission of CJD. Our data suggest that valine homozygous individuals might be susceptible to develop iatrogenic CJD if accidentally contaminated during surgical or dental procedures with prion diseases originated from all subtypes of sporadic CJD, including very atypical cases that would not affect methionine homozygous or heterozyghous subjects.

In addition the data here presented might also explain why accidentally contaminated individuals develop CJD with a broad range of incubations periods (Graziano and Pocchiari, 2009; Brown, 2007). Assuming that HuTg mice represent individuals carrying the three possible genotype at codon 129 on *PRNP* gene, from our data is possible to deduce that, depending upon the strain they have been exposed, each genotype would develop the disease with different incubation periods.

Although the idea that data obtained by inoculation of HuTg mice can be directly applied to what occurs in humans is intriguing, we have to be careful not to over-interpret these data, because it is likely that other factors differently expressed in humans than in mice might influence the susceptibility and the phenotypic features of the disease in the two species.

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