



UNIVERSITÀ DEGLI STUDI DI MILANO
FACOLTÀ DI MEDICINA VETERINARIA

Department of Veterinary Pathology, Hygiene and Public Health
Doctoral Course in Biotechnology Applied
to Veterinary and Animal Sciences

**TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES
(TSEs): EXPERIMENTAL APPROACHES TO
PATHOGENESIS, THERAPY AND PREVENTION IN
ANIMAL MODELS**

Doctoral Thesis

Candidate Name:
Erica Corda
Student Nr R08039

Supervisor: Prof. Paola Dall'Ara

Academic Year 2010 - 2011

Contents

1 - INTRODUCTION	1
2- TSEs IN HISTORY	2
3 - CAUSATIVE AGENT	14
3.1 - History	14
3.2 - Cellular Prion Protein.....	15
3.3 - Post-translational changes of the prion protein	18
3.4 - Protein misfolding	19
3.4.1 - Lipid rafts.....	20
3.5 - Species barrier	22
4 - TSEs PATHOGENESIS IN RUMINANTS.....	23
4.1 - Scrapie and BSE in ruminants.....	27
5 - NEURODEGENERATION IN PRION DISEASES	32
6 - DIAGNOSIS.....	33
6.1 - Histology and immunohistochemistry	34
6.2 - Western blot	34
6.2.1 - TSE types in cattle and small ruminants	35
6.3 - Experimental infection tests.....	37
6.3.1 - Incubation period and lesion profile.....	38
6.3.1.1 - BSE and scrapie	39
6.3.2 - Immunohistochemistry	40
6.4 - Rapid tests for post-mortem diagnosis of TSE	41
6.5 - Protein misfolding cyclic amplification	42
7 - THERAPY.....	43
8 - TSEs AND ALZHEIMER'S DISEASE	50
9 - EUROPEAN LEGISLATION ON TSEs	58
10 - EXPERIMENTAL STUDIES	76
11 - THERAPEUTIC EFFECT OF CHF5074, A NEW γ -SECRETASE MODULATOR, IN A MOUSE MODEL OF SCRAPIE	77
11.1 – MATERIALS AND METHODS.....	78
11.1.1 - Animals and Treatments	78
11.1.2 - Western blot analysis	81
11.1.3 - Histopathological and immunohistochemical analysis.....	82
11.1.4 - Statistical analysis	85
11.2 - RESULTS.....	86
11.2.1 - Survival of the animals	86
11.2.2 - Western blot analysis	87
11.2.3 - Histopathological and immunohistochemical analysis.....	89
11.3 - DISCUSSION	94
12 - ABILITY TO DETECT BSE AFTER CHALLENGE OF WILD TYPE MICE WITH BSE AND SCRAPIE MIXTURES	99
12.1 - MATERIALS AND METHODS.....	101
12.1.1 - Ethical statement.....	101
12.1.2 - Inoculum preparation.....	102
12.1.3 - Animal procedures	102
12.1.4 - Histology	103
12.1.5 - Western blot	105
12.1.6 - Immunohistochemical labelling of PrP ^{Sc}	105
12.1.7 - Statistical analysis	106

12.2 - RESULTS.....	106
12.2.1 - Attack Rate (AR) and Incubation Period (IP) analysis.....	106
12.2.2 - Lesion Profile (LP) analysis.....	109
12.2.3 - Western blot analysis.....	111
12.2.4 - Immunohistochemical analysis.....	112
12.3 - DISCUSSION.....	118
13 - SEARCHING FOR MEMBRANE LIPID ROLE IN REGULATORY MECHANISMS OF PRION DISEASES: EVALUATION OF DIETARY APPROACHES IN MURINE MODEL OF SCRAPIE.....	124
13.1 - DIETS.....	126
13.1.1 - Diet enriched with complex gangliosides.....	126
13.1.2 - Low cholesterol diet.....	127
13.2 - EXPERIMENTAL PROJECT.....	128
14 - ABBREVIATIONS.....	131
15 – BIBLIOGRAPHY.....	134

1 - INTRODUCTION

Prion diseases are perhaps the most mysterious and peculiar diseases in nature. These diseases do not rely on the general dogmas of modern biology, seen in other infectious diseases caused by conventional pathogens, such as viruses and bacteria. On the contrary, their infectious agent is an unconventional proteinaceous pathogen, termed prion, that lacks functional nucleic acids. Prion diseases are also known as Transmissible Spongiform Encephalopathies (TSEs), because the diseases are transmissible from one host to another and manifest a spongiform appearance as result of the destruction of brain tissue during a long incubation period (Prusiner, 1982; Prusiner, 1998). Prion diseases include Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (BSE, “mad cow disease”) in cattle, scrapie in sheep and chronic wasting disease in deer and elks. As demonstrated in the BSE outbreak and its transmission to humans, the onset of diseases is not limited to a certain species but can be transmissible from one host species to another. Such a striking nature of prion has generated huge concerns in public health and attracted serious attention in the scientific communities.

To date, the potential transmission of prions to human has not been alleviated and TSEs still have no reliable preclinical screening tests and effective treatments. To comprehend the threat of prion diseases and to develop diagnostics and therapeutics, it is important to understand how prions emerge, replicate and transmit (Ryou et al., 2007)

2- TSEs IN HISTORY

In the political and economic turmoil of the 18th century Europe, England gradually established itself as the dominant trading power. In that setting, TSEs appear for the first time in official documents. In 1755 in fact, a discussion took place in the British parliament about the economic effects of a fatal and spreading disease in sheep and about the need for government to do something about it, because it was having serious repercussions on the economy of the Country, at that time leader in the wool trade (Journal of the House of Commons, 1755; Brown et al., 1998)

That pathology was the scrapie (name derived from the English verb “to scrape”), whose clinical symptoms were described in a German newspaper in 1759: “Some sheep also suffer from scrapie, which can be identified by the fact that affected animals lie down, bite at their feet and legs, rub their backs against posts, fail to thrive, stop feeding and finally become lame. They drag themselves along, gradually become emaciated and die”. The same article reported that scrapie was a contagious diseases in sheep but not in humans and suggested to dispose of them quickly and slaughter them away from the manorial lands (Brown et al., 1998).

Although in 1772 scrapie was considered to be a widespread disease in England for forty years, no evidence had ever been found (Schneider et al., 2008).

Where and how it appeared for the first time it is still a mystery, even though commonly it is believed that the export of Merino sheep from Spain has played a key role in its spread (Brown et al., 1998).

Around the middle of the 19th century, veterinarians in England, France and Germany initiated the scientific study of scrapie, including systematic neuropathological examinations, and made efforts to identify an infectious pathogen. In particular, Besnoit and his colleagues in the Toulouse school of veterinary medicine recognised neuronal vacuolation as a characteristic feature (Besnoit et al., 1898; Brown et al., 1998). In 1936 other two French doctors, Cuillé and Chelle, pointed out the intraspecies transmissibility of scrapie, succeeding in transmitting it to two healthy sheep by intraocular inoculation of brain or spinal cord tissue from an affected animal. In subsequent experiments, they also transmitted scrapie by using intracerebral, epidural and subcutaneous routes of infection (Cuillé et al., 1936; Brown et al., 1998).

Tragically in the same period in England the transmissible nature of the scrapie agent was confirmed beyond any doubt, by an outbreak of scrapie in several hundred sheep that had been immunised against louping ill with a vaccine prepared from tissue from the brain, spinal cord and spleen of sheep that were belatedly discovered to have been exposed to natural scrapie infection (Gordon, 1946; Brown et al., 1998).

Throughout the 1940s and 1950s the accelerating pace of veterinary research yielded many new discoveries about the behaviour of the causative agent: its distributions through the whole body after experimental and natural infection, its physical association with cell membranes, its susceptibility to host genetic factors and its extraordinary resistance to standard methods of inactivation (Brown et al., 1998). In 1961 Chandler managed to adapt it to laboratory mice, eliciting a collective sigh of relief for experimentalists, who had until then

obliged to work exclusively with sheep and goats, the only animals naturally susceptible (Chandler, 1961; Brown et al., 1998).

The first description of a human TSE was made in 1920 by two German neuropathologists, who gave their names to that new pathology: the Creutzfeldt-Jakob Disease (CJD). It was characterised by a progressive and fatal neurodegeneration that, unlike scrapie, occurred sporadically (with a frequency of about one case per million individuals per year worldwide) with a genetic predisposition (Norrby, 2011).

Sixteen years later a familiar form of human TSE was reported by Gerstman and his colleagues: the Gerstman-Straussler-Scheinker (GSS) Syndrome (Gerstman et al., 1936; Collins et al., 2001)

In the 1947 in Wisconsin and Minnesota (USA) was recognised for the first time the Transmissible Mink Encephalopathy (TME) (Marsh et al., 1991; Marsh et al., 1992; Sigurdson et al., 2003). It is a rare fatal neurological disease, which appears sporadically in farmed mink and, after an incubation period estimated at 7-12 months, induces behavioural changes including aggressiveness, hyperesthesia and progressive ataxia, drowsiness, weakness, until the inevitable death. TME has been largely confined to USA, although incidents have also occurred in Canada, Finland, Germany and Russia (Marsh et al. 1993; Sigurdson et al., 2003)

Its origins have not yet been established, although it is common to think that its onset is linked to the use of food contaminated with the causative agent of scrapie.

Controversly, the last reported outbreak of TME, appeared in Stetsonville, Wisconsin, occurred in minks that were never been fed with meat of sheep

but that had downer cattle as a primary source of food, leading to speculations on a potentially unrecognised BSE-like (see below) disease of American cattle, never confirmed (Marsh et al., 1993; Sigurdson et al., 2003). In 1959 Hadlow, an American veterinarian, had an important insight, which led to have a broader look and aware on many diseases until then considered individually. He was the first to suggest a link between animal and human TSEs, speculating that scrapie could be an animal pathology similar to kuru (Hadlow, 1959), fatal disease spread among the Fore tribe of Aborigines, in Papua New Guinea, described by Gajdusek and Zigas in 1957.

Kuru mainly affected women and children, causing motor dyskinesias, ataxia, facial spasms, swallowing difficulty, tremors and muscle jerks (myoclonus) (Brown et al., 1998).

The histological analysis of samples of brain tissue taken *post-mortem* from people affected by kuru (Klatso et al., 1959) allowed to observe significant similarities with the degenerative lesions seen in patients who died from CJD and, as noted by Hadlow (Hadlow, 1959), in sheep with scrapie.

The ritual cannibalism practiced by the Fore people was found to be the route of transmission of kuru. In fact, they used to prepare the bodies of the deceased relatives for the funeral meals and the central nervous system, containing the largest concentration of the infectious agent, was consumed mainly by women and children.

Since 1960, the year of the abolition of cannibalism, no new born has contracted kuru, and the latest victim died in the 21st century, after an incubation period longer than 40 years (Collinge et al., 2006; Norrby, 2011).

Gajdusek himself managed to prove the infectivity of kuru. He and his collaborators indeed were successful in transmitting first kuru (Gajdusek et al., 1966) and then CJD (Gibbs et al., 1968) by intracerebral inoculation to chimpanzees. For his important scientific discoveries, Gajdusek obtained the Nobel Prize in Medicine in 1976.

In Colorado (USA) in 1967 a TSE able to affect not only farmed animals but also free-ranging wild-life ones was described: the Chronic Wasting Disease (CWD). It is a disease of Rocky Mountain Elk, Mule deer, Black-Tailed Deer, White-Tailed Deer and Moose. Because Red Deer are genetically very similar to Rocky Mountain Elk, it is likely that they are also susceptible to CWD. Early signs in clinically affected deer and elk are extremely subtle and include weight loss, behavioural alterations, a lowered head and drooping ears. As clinical disease progresses, more noticeable signs like flaccid hypotonic facial muscles, excessive salivation, regurgitation of ruminal fluid, ruminal atony and polyuria and polydipsia arise. Individuals may develop aspiration pneumonia in late stage disease. Itching and loss of fleece, the characteristic signs of the end stage of scrapie, are not observed (Williams et al., 1980; Sigurdson et al., 2003; Williams, 2005). Until now, CWD has been reported only in North America, where its prevalence and geographical distribution appears to be increasing. Cases of CWD have also been reported in the Republic of Korea, although these are probably linked to elk exported from Canada (Defra). The origin of CWD is unknown and the disease transmits in the absence of contaminated feed. The precise mechanism of transmission is unclear. It is possible that the infectious agent is shed in the saliva, faeces or urine or as a result of decomposition of infected carcasses and transferred to other cervids

grazing the contaminated areas. It is also possible that some maternal transmission occurs (Sigurdson et al., 2003; Defra). Experimental studies seem to exclude the possibility for the infective agent to jump the species barrier and affect humans or cattle (Sigurdson et al., 2003).

TSEs have been detected also in exotic ruminants in United Kingdom (UK) zoos since 1986. These include antelopes (Eland, Gemsbok, Arabian and Scimitar oryx, Nyala and Kudu), Ankole cattle and Bison. With hindsight the 1986 case in Nyala was diagnosed before the first case of BSE (see below) was identified. The TSE cases in exotic ruminants had a younger onset age and a shorter clinical duration compared to that in cattle with BSE. All the cases appear to be linked to the BSE epidemic via the consumption of feed contaminated with the BSE agent. The epidemic has declined as a result of tight controls on feeding mammalian meat and bone meal to susceptible animals, particularly from August 1996 (Sigurdson et al., 2003; Defra).

The same fate befell to the Feline Spongiform Encephalopathy, described in 1990 in the United Kingdom. It has been reported in cats and other felines in captivity (Cheetah, Lion, Asian leopard cat, Ocelot, Puma and Tiger) and is believed to have a common origin with the TSEs in the exotic ruminants. The disease is characterised by progressive nervous signs, including ataxia, hyper-reactivity and behavioural changes and is fatal (Sigurdson et al. 2003; Defra).

In October 1986, the first case of the Fatal Familial Insomnia (FFI) was recorded in Italy. The patient affected showed progressive insomnia, fever, tremors and other motor disorders, until coma and death nine months after the onset of symptoms. As the name suggests, the FFI has a familial

occurrence, linked to a dominant autosomal genetic mutation (Lugaresi et al., 1986).

The first confirmed cases of BSE were recognised in Great Britain in November 1986, following referral of the brain of two cows with unusual progressive neurological signs from two veterinary investigation centres in Southern England to the Pathology Department of the Central Veterinary Laboratory in Weybridge (now Animal Health Veterinary Laboratory Agency – AHVLA). The disease was recognised as a spongiform encephalopathy because of the neuropathological similarity to that seen in the scrapie-affected sheep (Wells et al., 1987; Smith et al., 2003).

This new disease of cattle was characterised by a slow but progressive and always fatal clinical course, which resulted the affected animals in mental, sensory, postural and motor disorders, as well as weight loss and lower efficiency in dairy cows (Cranwell et al. 1988; Wilesmith et al., 1988). The clinical signs were very variable, however, often subtle and certainly not pathognomonic (Braun et al. 1997; Caveau et al. 2004; Konold et al., 2006)

Since 1986 many other cases of BSE were identified, especially in Great Britain, and, after the introduction in January 2001 in the European Union of the mandatory active surveillance (January 1999 in Switzerland and spring 2000 in France), BSE was found also in many other countries, where the mere passive surveillance had not been enough (Ducrot et al., 2008) (Figure 1 and Table 1).

Figure 1. Number of BSE cases in the European Union between 1989 and 2005. Source European Commission, 2002 to 2006. Reports on the monitoring and testing of ruminants for the presence of transmissible spongiform encephalopathy (TSE) in the EU (Ducrot et al., 2008).

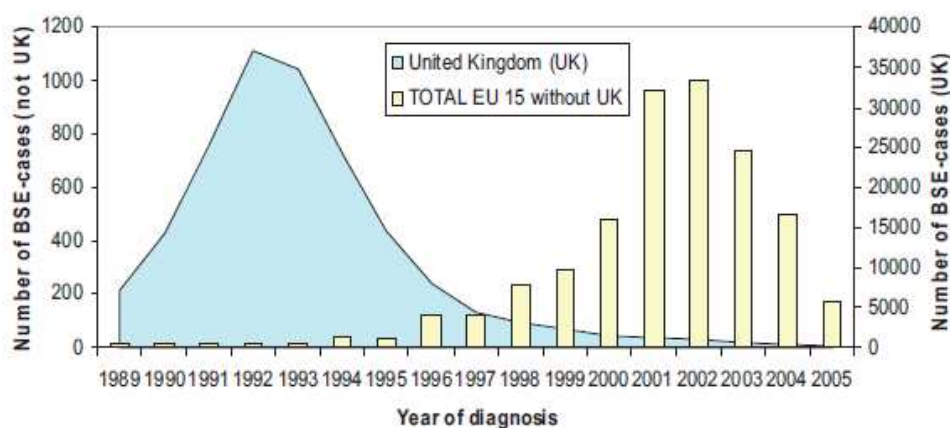


Table 1. Sequence of first report of BSE in native-born cattle (Smith et al., 2003)

1986	UK
1989	Ireland
1990	Portugal
1991	Switzerland
1997	Belgium, Luxembourg, The Netherlands
1998	Lichtenstein
2000	Denmark, Germany Spain
2001	Austria, Czech Republic, Finland, Greece, Italy, Japan, Slovakia, Slovenia
2002	Israel, Poland
2003	Canada

There are retrospective evidences of the occurrence of the BSE clinical disease in April 1985 (Wilesmith et al., 1988) but it is likely that the first cases may have occurred in the early 1980s or even in 1970s (Horn et al., 2001; Smith et al., 2003).

The origin of BSE still remains unclear. The two main reliable theories suggest that it may be a cattle-adapted form of scrapie that crossed the species barrier from sheep to cattle (the scrapie hypothesis) or a sporadic cattle disease that first arose as a spontaneous disease in an individual cow (the sporadic bovine mutation hypothesis) (Wilesmith et al., 1988, Baylis et al., 2002, Smith et al., 2003). Even if research has not been able to solve this question, epidemiological studies managed to identify the transmission route of infection, allowing to take adequate measure to prevent it. These studies revealed that the use of meat-and-bone-meal (MBM) as a high protein supplement feed incorporated into concentrated rations was a linking feature on the farms from which cases had been reported. In the 1970s and 1980s the source materials for these end-products was from all species of farm animals, including fallen stock (animals dead on farm), abattoir and butcher's waste and condemned materials. The starting materials were cooked by various methods to evaporate water to steam and the tallow (fat) was separated by centrifugation or pressing, leaving a solid material, called greaves, from which MBM was derived by grinding. It was hypothesised that once the causative agent of BSE had been introduced into the rendering process it would be recycled to infect other cattle through MBM.

MBM had been used in animal feed for decades and the rendering processes in use at that time were not probably completely effective at inactivating either the BSE agent or a range of scrapie agent, but in the late 1970s and early 1980s in the European Union (1981 in the United Kingdom) the process used to extract tallow by rendering industry changed, presumably increasing the titre of TSE agents in MBM and consequently increasing the exposure of

cattle. It is likely that epidemic started in the UK because the ratio of sheep to cattle tissues going into rendering was higher there and because in the 1970s was the first country to start feeding MBM to young calves, particularly susceptible to infection with the BSE agent (Wilesmith et al., 1988; Wilesmith et al., 1991; Wilesmith et al., 1992).

In the summer of 1988, two years after the identification of the first cases of BSE, Great Britain decreed the prohibition of use of meat from cattle and small ruminants to feed ruminants, the only animals considered susceptible. The effects of the ban were not immediate, probably due to the long incubation period of BSE (on average 5 years), but since 1993 the incidence of BSE in British cattle began to decline (Heim et al., 2000; Ducrot et al., 2008). The feed ban had a major impact on the epidemic, but it was not as effective as had been hoped and cases of disease continued to occur in animals born after the feed control measures had been introduced. Epidemiological studies on these “born after the ban” (BAB) cases revealed that the greatest incidence was in eastern England, corresponding to the area with the greatest prevalence of pig farms. Further investigations led to the hypothesis that BAB cases of BSE might be attributable to accidental cross-contamination of ruminant diets with MBM intended for pigs and poultry (Hoinville et al., 1995; Smith et al., 2003). The discovery that less than 1 g of infected cow brain could experimentally transmit BSE to further cows by the oral route lent weight to this hypothesis, as only a relatively small amount of such contamination would be required to induce disease (Smith et al., 2003; Wells et al., 2007; Ducrot et al. 2008).

Even the ban, introduced in 1990, on the use of specific bovine tissues (specified risk material - SRM) containing the most infectious material from cattle carcasses (spinal cord, tonsils, thymus, spleen and intestine) in the MBM for monogastric has not been found to completely remove the feed-borne risk of exposure. The lack of complete effectiveness of the SRM ban is likely to be due to the difficulties of complete removal of some tissues and the possibility of residual risk in production plants from earlier periods (Smith et al. 2003; Ducrot et al., 2008).

The European Union (EU), which took longer to recognise its real involvement in the problem, banned the use of mammalian MBM for ruminants only in 1994 (Ducrot et al., 2008).

In 1996 the first article on the zoonotic aspect of BSE was published (Will et al., 1996) and consequently a total ban was placed on feeding mammalian protein to any farmed animals. The feed ban that was introduced in the UK in 1996 was essentially the same as the feed ban that was introduced across the EU on January 1st 2001, five years later (Smith et al., 2003; Ducrot et al., 2008).

Even though until then it was considered that the same species barrier had preserved humans from scrapie would be effective also against BSE, clinically suspect or confirmed cases of BSE were banned from the human food chain in the UK in 1988 and in the EU in 1990 (Smith et al., 2003). In 1989 in the UK a specified bovine offal (SBO) ban was instituted. This prohibited bovine brain, spinal cord, tonsil thymus spleen and intestines from entering the human food chain (Smith et al., 2003; Norrby, 2011).

These measures were not enough to prevent the occurrence in 1995 of the first case of CJD putatively derived from the dietary exposure to the causative agent of BSE (Will et al., 1996). The histopathological pattern and the agent strain found in the affected man appeared to be consistent with the BSE ones (Bruce et al., 1997; Hill et al., 1997). This new form of CJD was referred as variant CJD (vCJD). It occurred in younger patients compared to those with the sporadic form of CJD and its epidemic peaked in 2000/2001 (Table 2) (Will et al., 1996; NCJDRSU).

Table 2. Deaths from definite and probable vCJD from the first cases in 1995 up to 2011 ($n = 175$) (* Data updated at 7th November 2011). 120 in 175 deaths are from confirmed vCJD, whilst 54 in 175 are deaths from probable vCJD, since the neuropathological confirmations are pending. There is 1 probable vCJD case still alive (NCJDRSU).

Year	Deaths from definite and probable vCJD
1995	3
1996	10
1997	10
1998	18
1999	15
2000	28
2001	20
2002	17
2003	18
2004	9
2005	5
2006	5
2007	5
2008	2
2009	3
2010	3
2011*	4

3 - CAUSATIVE AGENT

3.1 - History

Despite scrapie, the oldest and most widespread spongiform encephalopathy, was known for over than 200 years, its causative agent was identified only at the end of the 20th century by an American neurologist, Stanley B. Prusiner

(Prusiner, 1982; Prusiner, 1998), whose studies were awarded the Nobel Prize in medicine in 1997.

During the 20th century different definitions of the causative agent of the TSEs and many theories on its nature were put forward, and it was classified amongst the Unconventional Infectious Agent.

In 1966 Alper and colleagues suggested that nucleic acid was not needed for replication (Alper et al., 1966; Alper et al., 1967) and the following year the mathematician J. S. Griffith was the first to formulate a “prion only” theory of scrapie pathogenesis. In 1982, Prusiner was able to extract and purify the causative agent from infected tissues, noting with surprise that this was nothing more than a constitutive protein, the prion protein (PrP), encoded by a host gene and not by a foreign invader. He coined the term “prion”, which was (and is) meant to denote a “proteinaceous infectious particle”, to emphasize the requirement of a protein for infection. However, at that time, it was not sure whether that particle contained nucleic acid or not (Prusiner, 1982; Bolton et al., 1982).

3.2 - Cellular Prion Protein

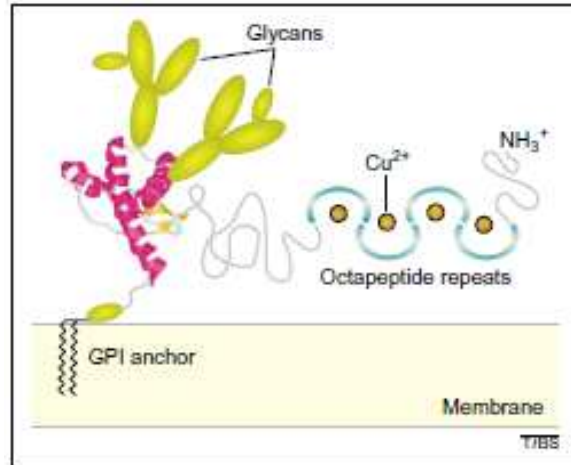
The cellular prion protein (PrP^C) is a constitutive protein encoded by the *Prnp* gene, highly conserved among mammals (Aguzzi et al., 2004); its paralogs have been revealed in birds (Nuvolone et al., 2009), reptiles (Simonic et al., 2000), fish (Rivera-Milla et al., 2003) and amphibians (Strumbo et al., 2001). At a cellular level, PrP^C is synthesized in the cytoplasm, transported through the endoplasmic reticulum and Golgi apparatus and displayed on the plasma

membrane, which binds by a glycosylphosphatidylinositol (GPI) anchor (Ryoux et al., 2007).

Its broad and diverse expression pattern, with expression in skeletal muscle, kidney, heart, pancreas, secondary lymphoid organs and central nervous system (CNS), would point presumably to a general, conserved and broad function (Ford et al., 2002; Aguzzi et al., 2004). Within the CNS and peripheral nervous system (PNS) PrP^C expression levels can be detected in synaptic membranes of neurons as well as in oligodendrocytes, Schwann cells and astrocytes. In the periphery PrP^C expression is reported on lymphocytes and importantly at high levels on stromal cell of the immune system: the follicular dendritic cell (FDC), located in organs of the lymphoreticular system (LRS) (Heikenwalder et al., 2007).

The PrP^C glycoprotein can be either un-, mono- or diglycosylated. Its three dimensional structure preserves common features in different species: mouse, human, cattle and Syrian hamster. It consists of a long, flexible N-terminal tail (residues 23-125), which contains a sequence of 8 amino acids repeated 5 times. The globular C-terminal domain (residues 126-131) is folded to form three α -helices and a short two-stranded anti-parallel β -sheet that flanks the first α -helix; it is stabilised by a disulfide bond linking α -helices two and three (Riek et al., 1997; Ronga et al., 2006; Moore et al., 2009; Heikenwalder et al., 2007) (Figure 2).

Figure 2. Model of PrP^C structural domains. The folded C-terminal portion of PrP^C contains the short β -sheet strands and the α -helices. The remainder of the molecule appears to be flexibly disordered (Caughey et al., 2001)



PrP^C has also been named PrP^{sen}, because of its sensibility to proteinase K (pK) digestion (Caughey et al., 2001).

Despite several possible roles of PrP^C have been proposed, no one has been confirmed (Ryou et al., 2007). PrP^C appears to play a role in synaptic plasticity (Maglio et al., 2004), lymphocyte activation (Cashman et al., 1990; Ryou et al., 2007), response to oxidative stress (Milhavet et al., 2002), signal transduction (Mouillet-Richard et al., 2000) and metabolic functions related to copper-binding properties (Brown et al., 1997; Watt et al., 2003; Ryou et al., 2007). PrP^C is also seems to be involved in differentiation and neurogenesis of neuronal stem cells (Steele et al., 2006) as well as long-term renewal in haematopoietic stem cells (Zhang et al., 2006).

However, independent studies with several different PrP null mouse strains have not found any major impairment as well as distinct physiological and

behavioural changes, suggesting that PrP^C might be not essential to the development and survival (Bueler et al., 1992).

Although the function of PrP^C in animal physiology is ambiguous, its involvement to the pathogenesis of prion disease is clearly understood. Expression of PrP^C is a prerequisite to demonstrate a susceptibility of prion diseases in the hosts. Research has demonstrated that is not possible to transmit the disease when prions are inoculated in PrP-deficient mice, while the incubation time of disease become shortened when prions are transmitted to the transgenic mice, over-expressing PrP^C in the brain at least several times higher than wild-type mice (Bueler et al., 1992; Fischer et al., 1996; Ryou et al., 2007).

3.3 - Post-translational changes of the prion protein

The cellular prion protein undergoes post-translational modifications that take place in the plasma membrane within the lipid rafts (see below) (Tellier et al., 2006). PrP^C is cleaved by an α -secretase inside its sequence 106-126 (residues 110/11-112), leading to the production of N1 and C1 fragments. That proteolysis is mediated by two distinct metalloprotease, a constitutive one, ADAM (A Disintegrin And Metalloprotease) 10, and an inducible one, ADAM 17 or TACE (Tumor necrosis factor Alpha-Converting Enzyme), which prevent the prion protein from the toxic conversion (Vincent et al., 2001; Checkler et al., 2002; Walmsley et al., 2003; Tobias et al., 2003; Vincent, 2004; Laffont-Proust et al., 2005; Ronga et al., 2006; Parkin et al., 2007). Then PrP^C moves out of the lipid rafts, in order to be endocytosed via clathrin-coated pits (Sunyach et al., 2003; Schengrund et al., 2010).

In pathological conditions instead, PrP^C in the lipid rafts is cleaved by an unknown protease, that acts 20 amino acids upstream of the ADAM-cleavage site (residues 90-91), generating the N2 peptide and leaving the 106-126 domain intact, considered potentially toxic because of its tendency to fold into β -sheets, making inaccessible the binding sites to the pK and therefore insoluble the protein (Vincent et al., 2001; Checkler et al., 2002; Walmsley et al., 2003; Tobias et al., 2003; Vincent, 2004; Laffont-Proust et al., 2005; Ronga et al., 2006; Parkin et al., 2007).

3.4 - Protein misfolding

The toxic conversion of the prion protein to prion (PrP^{Sc}), causes the onset of the TSEs and its detection is considered pathognomonic for TSEs diagnosis.

The soluble, protease sensitive form of PrP (PrP^C) is refolded and converted into an insoluble protease-resistant form, the prion (PrP^{Sc}). PrP^{Sc} has the same primary sequence as PrP^C but a different secondary structure. Thus, where PrP^C is higher in α -helical content, PrP^{Sc} is higher in β -sheet content. The misfolding may be due to a sporadic or inherited genetic mutation or may be acquired, as a result of ingestion of prions, able to induce an allosteric conformational change in PrP^C.

That process is still poorly understood (Moore et al., 2009), even though it seems likely that toxic conversion may requires the presence of one or more cofactors, able to mediate the interaction between the two isoforms of the prion protein (Colby et al., 2011).

The different three-dimensional conformations of PrP^C and PrP^{Sc} give them different biochemical and biophysical properties (Ryou et al., 2007) (Table 3).

Table 3. Properties comparison between PrP^C and PrP^{Sc} (Modified from Ryou et al., 2007).

Properties	PrP^C	PrP^{Sc}
Protein	Normal, cellular protein	Disease-associated protein
Infectivity	No infectious	Infectious; pathogenic
Folding	Dominated by alpha-helices	Beta-sheet abundant conformation
Solubility	Easily soluble (hydrophilic)	Insoluble (hydrophobic)
Protease digestion	Sensitive	Partially, but highly resistant

Prion indeed has its own characteristics. The pK, which fully digest PrP^C, usually removes only the N-terminal third (~ 6-8 kDa) of PrP^{Sc}. Moreover, PrP^{Sc} is resistant to high temperatures, relative high pressure, formaldehyde treatment and UV-irradiation (Caughey et al., 2001; Heikenwalder et al., 2007 Ryou et al., 2007)

After PrP^C toxic conversion, PrP^{Sc} amplifies and aggregates in amyloid fibrils, which can accumulate inside and outside the cell and are responsible for the formation of amyloid plaques, gliosis, vacuolisations and neuronal death by apoptosis. The prion protein localization in lipids rafts (see below) appears important in allowing its misfolding and the progression of clinical disease (Pinheiro, 2006).

3.4.1 - Lipid rafts

Traditionally the structure of biological membranes has been defined according to the fluid mosaic model (Singer et al., 1972). In this model the different species of lipids form a homogenous mixture in which membrane proteins are uniformly dispersed. More recent data, however, indicate that there is heterogeneity in biological membranes (Engelman et al., 2005), with a

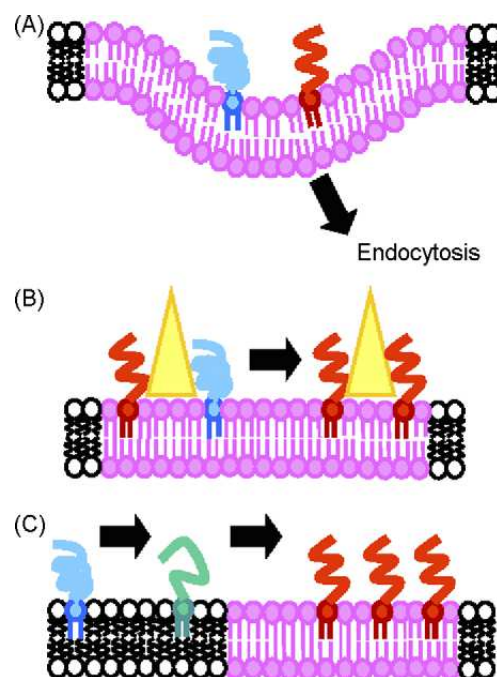
growing body of evidence supporting the existence of specialised lipid domains, including cholesterol- and sphingolipid-rich lipid rafts (Simmons et al., 1997). The main constituent of plasma membrane are phospholipids, sphingolipids and cholesterol. Pure sphingolipids pack tightly together as their saturated acyl chains allow close interaction and form a gel-like phase. This is in contrast to the loose packing of phospholipids, whose kinked unsaturated acyl chains preclude closer interaction. This differential packing ability leads to a phenomenon known as phase separation when the two types of lipid are in a membrane, with sphingolipids forming a gel-like phase and phospholipids a liquid-disordered phase. Cholesterol preferentially interacts with sphingolipids and shift the gel-like phase of pure sphingolipids to a more fluid liquid-ordered phase, which equates to the lipid rafts (Taylor et al., 2007).

Numerous proteins implicated in cellular homeostasis are located within them and it is clear that lipid rafts are important regulators of cellular activity (Garattini, 2007; Ma et al., 2004). They are indeed involved in a range of biological processes, including intracellular trafficking, transmembrane signalling, lipid and protein sorting, vital uptake and regulated proteolysis. In addition, lipid rafts are a site of attack for many pathogens (Taylor et al., 2007).

There is compelling evidence that lipid rafts provide a membrane environment conducive to prion conversion. However, it is currently unclear exactly what is about rafts that favours conversion. Three hypothesis have been suggested. Lipid rafts may provide a mean to target PrP^C and PrP^{Sc} to intracellular compartments that favour conversion. Another potential explanation assumes the presence in that domains of cofactors able to facilitate the prion

misfolding. The third hypothesis suggests that lipid rafts create an environment conducive to toxic conversion destabilizing the α -helix structure within the prion protein (Pinheiro, 2006; Taylor et al., 2007) (Figure 3).

Figure 3: Models for how lipid rafts provide a favourable site for PrP^{Sc} formation. (A) Lipid rafts (pink membrane) provide a platform by which PrP^C (blue) and PrP^{Sc} (red) are targeted to intracellular sites that favour conversion. (B) Rafts contain a co-factor (yellow triangle) that facilitates conversion of PrP^C to PrP^{Sc}. (C) The predominantly α -helical structure of PrP^C is destabilised when PrP^C transits through non-raft regions (white membrane) giving rise to a misfolded intermediate (green). When this misfolded intermediate enters a raft environment it is a better substrate for PrP^{Sc}-mediated conversion (Taylor et al., 2007).



3.5 - Species barrier

To limit the infectivity of PrP^{Sc} there is the so called "species barrier", a phenomenon responsible for the lower efficiency in interspecies than intraspecies transmission of the prion diseases.

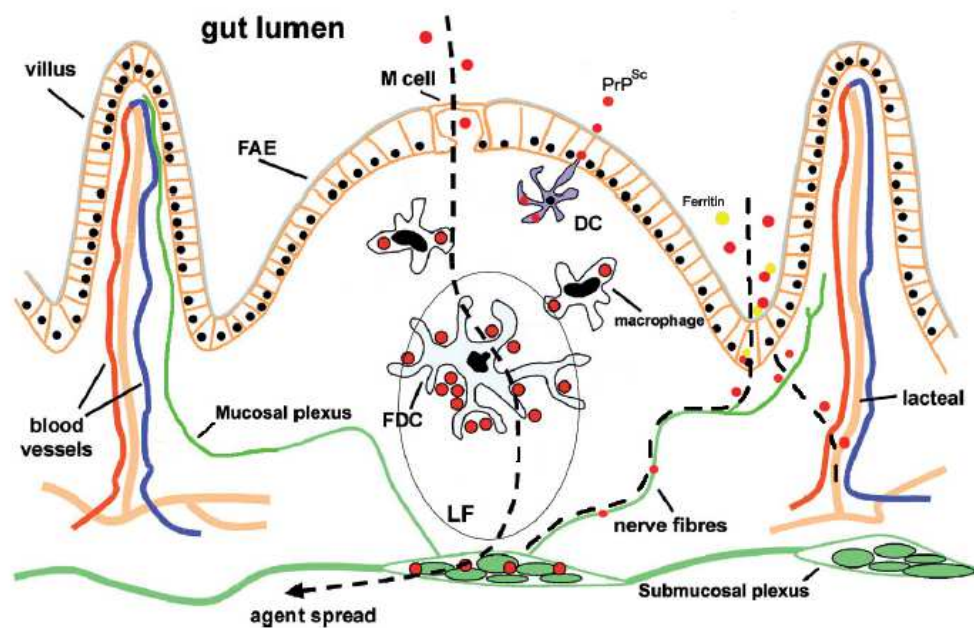
To cross the species barrier there must be a sufficient homology in the primary and secondary structures of the middle third of the prion protein (residues 108-189) between exogenous PrP^{Sc} from one species and endogenous PrP^C of another species. In the rare cases where prion transmission is possible between different species, it occurs only after prolonged incubation times (Caughey et al., 2001; Ryou et al., 2007; Moore et al., 2009). The shortest incubation times (the interval between inoculation and clinical signs of disease) are achieved with intracerebral inoculation of prions with an amino acid sequence identical to that of the host animal. When the donor prion originates from a species different from the host animal and thus the sequences differ between infecting PrP^{Sc} and PrP^C, the incubation time can be prolonged and vary substantially between individual animals inoculated; often, many of the inoculated animals do not develop disease. Variation in PrP sequences indeed exist not only between species but also between individuals within the same species, greatly affecting the susceptibility to prion infection (Pattison et al., 1965; Colby et al., 2011).

4 - TSEs PATHOGENESIS IN RUMINANTS

The pathogenesis of TSEs in sheep and cattle is very similar. The causative agent is the prion and the infection usually occurs by the oral route. In scrapie the infectious agent is present in the environment and taken up orally during grazing on scrapie contaminated pastures. In the case of BSE in cattle, infection has occurred through feeding concentrates with MBM derived from BSE infected cattle (Van Kuelen et al., 2008).

It is still obscure how TSE agents cross the mucosal barrier after they are ingested, but here are three possible ways (Figure 4).

Figure 4. Schematic representation of the possible ways of intestinal epithelial crossing and subsequent invasion of the enteric nervous system. After ingestion, PrP^{Sc} can be taken up by M-cells (1) or dendritic cells (2) and transported to the lymphoid follicles (LF) of the Peyer's patches underlying the follicle-associated epithelium (FAE). Within the lymphoid follicles PrP^{Sc} accumulates on follicular dendritic cells and is taken up by follicular macrophages. This accumulation of PrP^{Sc} could facilitate infection of the neighbouring submucosal plexus of the enteric nervous system. Alternatively, PrP^{Sc} could be taken up as a complex with ferritin and transcytosed by intestinal epithelial cells through the ferritin pathway (3). Once the epithelium is crossed, the mucosal plexus of the enteric nervous system can get infected followed by neural spread to the submucosal plexus (Van Kuelen et al., 2008).



The first is through the membranous epithelial cells (M-cells), a cell type present in the follicle-associated epithelium of the gut and tonsil which specializes in transport of macromolecules and particles across the epithelium (Heppner et al., 2001). It has been shown for prions, viruses and bacteria that M-cells can be used by pathogens to cross the mucosal barrier and gain

access to underlying tissues (Neutra et al., 1996). However, transport of TSE agent across the gut epithelium could also occur independent of M-cell transport. Digestive enzymes can break down the infectious agent into smaller molecules of PrP^{Sc} or even into the protease resistant core of PrP^{Sc}. Such smaller fragments can then form complexes with other proteins like ferritin and get endocytosed in vesicular structures by a ferritin dependent mechanism (Mishra et al., 2004). A third possible route could be through direct uptake by dendritic cells that can open up the tight junctions between epithelial cells and capture antigens by inserting their dendritic processes into the gut lumen (Rescigno et al., 2001 a; Rescigno et al., 2001 b). This had been demonstrated for bacteria but has yet to be shown for TSE agent (Van Keulen et al., 2008).

After crossing the mucosal barrier, infectivity and PrP^{Sc} first accumulates in the gut-associated lymphoid tissues (GALT) of the tonsil and the Peyer's patches in the intestines (Aguzzi et al., 2003). This initial accumulation in the GALT strongly favours the hypothesis of transport of TSE agents through M-cells, because antigens that have been transcytosed by M-cells are actively transported from the basal side of the M-cells to the underlying GALT by either dendritic cells or macrophages.

Then the neuro-invasion occurs and prion reaches the enteric nervous system (ENS) of the gut (Terry et al., 2003; Van Keulen et al., 2008). The ENS is the intramural nervous system of the gut and is composed of two major networks or plexuses, which contain the neuronal cell bodies (also called ganglion cells) and their processes. These two major plexus are the submucosal or Meissner's plexus, located in the submucosa, and the myenteric or

Auerbach's plexus, located between the circular and longitudinal muscle layers (extending from the esophagus to the rectum). In addition to these two major plexus there are minor plexus in the serosa, circular muscle layer and in the mucosa, that contain no or few ganglion cells but consist mainly of small nerve fibres. Although the ENS is capable of functioning independently, it is modulated by the CNS by means of the parasympathetic and sympathetic efferent nerves of the autonomic nervous system that are connected to the nervous system. The cell bodies of the preganglionic parasympathetic neurons are located in the dorsal motor nucleus of the vagus (DMNV) in the medulla oblongata while the preganglionic sympathetic neurons lie within the intermediolateral column (IMLC) in the spinal cord. After infection of the ENS, TSE agent ascends through these parasympathetic and sympathetic efferent neuronal pathways to the brain and (via the ganglion mesentericum craniale/coeliacum) to the spinal cord. Portal of entry of TSE agents in the brain is thus the DMNV in the medulla oblongata at the level of the obex and the IMLC in the thoracic segments of the spinal cord. From these sites in the CNS, infection spreads in both an ascending and descending directions to finally involve the entire neuroaxis (Van Kuelen et al., 2000; Hoffmann et al., 2007; Van Keulen et al., 2008).

In cattle PrP^{Sc} accumulates only in the GALT tissues of the Peyer's patches in the ileum and in the tonsil of experimentally infected animals and its replication in these lymphoid tissues is minimal to absent. From here, except for the neuro-invasion, prion does not spread neither through the rest of the body nor into the environment, making cattle dead-end host for BSE (Van Keulen et al., 2008).

Contrary to BSE in cattle, prion in sheep naturally colonizes the GALT tissues of the Peyer's patches and the tonsils, where it replicates and spreads not only via nervous route but also by lymphatic and haematogenic dissemination. Despite the fact that infectivity has never been found in any secretions or excretions of scrapie infected sheep, it does not exclude possible low levels of infectivity in milk, saliva, faeces or urine, since PrP^{Sc} has been found in ectopic lymphoid tissue in the mammary gland (Ligios et al., 2005), in the renal papillae and in the salivary glands (Vascellari et al., 2007) of scrapie-infected sheep. The environmental contamination, that occurs even through the infected placentas shedding after lambing, and the direct contact between animals make scrapie an endemic disease, meaning that its causative agent transmits under natural conditions (Baylis et al., 2002; Van Keulen et al., 2008).

4.1 - Scrapie and BSE in ruminants

Scrapie, a common disease among sheep and goat, is the oldest TSE known. It is characterised by a long incubation period, behavioural and neurological disorders and inevitable death. It has been found in many countries and it is endemic in the UK, while it has been eradicated in New Zealand and Australia, thanks to rigorous control and preventive measures.

Affected animals are adults, the incubation period ranges from 2 to 5 years and the clinical course of the natural disease ranges from 8 to 24 weeks in sheep and from 2 to 24 weeks in goats (Hunter, 2003; Scanziani et al., 2006; CFSPH, 2007).

Clinical signs often begin with unusual social behaviour and extreme nervous reactions to stimuli such as human contact. The general condition of the affected animal deteriorates, sometimes accompanied by a change in the fleece colour and often it is this latter feature that is first noticed by the farmer or shepherd. Ataxia is common and pruritus can result from the animal scratching an apparently intense itch against fence posts or by biting the affected area, for example around the base of the tail and occasionally the whole of the side of the body can be denuded of wool. In the final stages of the disease, although the appetite may appear normal, the animals lose the ability to feed themselves and the condition degenerate. Scrapie does not seem to alter reproductive ability until muscle wasting interferes with the ability to move. Lambs can, therefore, be born successfully to mothers in the clinical phase of the disease and rams remain fertile and active even when affected by ataxic signs (Hunter, 2003).

BSE owes its fame to the epidemic which broke out in the UK since the mid-eighties and to its zoonotic feature, absent in scrapie (Detwiler, 1992; Brown et al., 1998). The most cases were diagnosed in cattle aged between 4 and 6 years (with a very wide fork: from 20 months to 18 years). Dairy cattle were found to be more affected than beef breeds, probably because of differences in dietary management. The latter are suckled, often for long periods and are not usually fed concentrate rations during their initial period of growth and sometimes not at all or at a low rate. By contrast, calves from dairy cows are removed from their dam soon after birth and they are initially reared on milk substitutes and soon weaned on to a diet of hay and concentrates that often contained MBM (Smith et al., 2003; TSE, 2007).

Prodromal and early clinical signs of BSE are very unspecific and they may take weeks to months to progress to overt clinical disease. Animals lose weight and decrease milk production while maintaining the appetite. They also show behavioural changes, such as anxiety, nervousness, hyperexcitability, aggressiveness, sensory hyperaesthesia, especially with over-reaction against light and noise, postural and locomotion changes, such as stiff gait and ataxia, muscle spasms and tremors, particularly in the hind limbs (Scanziani et al., 2006; TSE, 2007).

Research demonstrated that cattle parenterally inoculated with the scrapie agent developed the disease, producing BSE-like signs, especially locomotor abnormalities; it is of importance that just few of them developed the nervous form, with aggressiveness and over-reaction, which is generally associated with BSE (Cutlip et al., 1994; Konold et al., 2006; TSE, 2007). However, the natural infection seems unlikely, since cattle are resistant to scrapie when infected orally (Cutlip et al., 2001; TSE, 2007).

On the contrary, oral transmission of BSE to sheep and goats is possible and challenged animals develop a disease with clinical signs that are indistinguishable from those caused by scrapie (Foster et al., 1993; Bellworthy et al., 2008). Symptoms are intense pruritus, leading to loss of fleece, ataxia, weight loss and behavioural changes; these signs become progressively more severe over the clinical course of the disease and culminate in death (Baron et al., 2000; Baylis et al., 2002)..

Small ruminants exposure to the causative agent of BSE is a realistic chance, since they have been fed with the infected MBM (Wilesmith et al., 1991; Smith et al., 2003). Anyway, the amount of infectious material consumed would have

been much less than that consumed by cattle. A sheep eats, on average, only 1-2% of the volume of feed concentrate eaten by a bovine and the amount of MBM in sheep concentrate was at most equal to, and probably much less than, that present in the cattle equivalent. Sheep, then, were much less exposed to BSE than were cattle (Kao et al., 2002; Baylis et al., 2002). However, the species barrier that would have prevented the BSE infection of small ruminants, making the exposure to high titers of PrP^{Sc} required for the occurrence of interspecies transmission, is not effective. In fact, even though a 1000 times higher infective dose of cow-derived BSE causative agent is required to kill a mouse than a cow (Wells et al., 1998), BSE species barrier from cattle to genetically susceptible sheep appears to be very small or even non-existent. An experimental study showed indeed that low infectious doses (0-5 g) orally administered to sheep are enough to transmit BSE (Foster et al., 1993). The absence of the scrapie barrier is one of the evidences supporting the hypothesis that BSE is a cattle-adapted form of scrapie (Baylis et al., 2002).

The gene responsible for control of susceptibility and resistance to TSEs is the *Prnp* and, in sheep, the most important amino acids are at numbers 136, 154, 171. At codon 136, the amino acid specified can be either valine (V) or alanine (A), at codon 154 it is arginine (R) or histidine (H) and, at codon 171, arginine (R), glutamine (Q) or histidine (H). Of the twelve possible alleles derivable from these polymorphisms, only five are commonly seen: A₁₃₆R₁₅₄R₁₇₁ (hereafter ARR), ARQ, VRQ, AHQ and ARH (Belt et al., 1995). A sheep contains two such alleles and the combination (i.e. ARR/VRQ) is called PrP genotype. Sheep with the ARR/ARR genotype are highly or completely

resistant to classical scrapie, while sheep with the VRQ/VRQ genotype are most susceptible. The ARQ/ARQ genotype is the highly susceptible to BSE in sheep (Baylis et al., 2002; Hunter et al., 2003; CFSPH, 2007) (Table 4).

Table 4. Prion protein polymorphism and the five commonly found PrP gene alleles in sheep (Hunter et al., 2003).

Codon number	Amino acid	Allele
136	Valine (V)	VRQ
	Alanine (A)	ARQ
		ARR
		AHQ
		ARH
154	Arginine (R)	VRQ
		ARQ
		ARR
		ARH
	Histidine (H)	AHQ
171	Glutamine (Q)	VRQ
		ARQ
		AHQ
	Arginine (R)	ARR
	Histidine (H)	ARH

Retrospective studies have recently identified two cases of natural BSE in field goats, in France (Eliot et al., 2005) and in the UK (Spiropoulos et al., 2011). PrP^{Sc} in small ruminants affected with BSE has been found not only in the CNS, but also widely distributed in the lymphoid tissues (lymph nodes, spleen, tonsil and Peyer's patches) and the PNS. This distribution contrasts dramatically with that seen in cattle BSE and is very similar to the distribution seen in natural scrapie, where the broad tissue tropism is considered the main factor responsible for the sheep-to-sheep transmission (Foster et al., 2001;

Baylis et al., 2002). The similar wide distribution in peripheral tissue would retain BSE infectivity within a flock also after the elimination of the initial source of infection. Recent evidences confirm that BSE can be transmitted naturally between sheep (Bellworthy et al., 2005).

5 - NEURODEGENERATION IN PRION

DISEASES

The inevitable consequence of prion infection is neurodegeneration.

The extraneuronal accumulation of PrP^{Sc} in the CNS lead to microglia activation, which respond to the neurotoxic PrP 106-126 peptide by producing pro-inflammatory cytokines, interleukin 1 β and interleukin-6 (Perry et al., 2002; Kovacs et al., 2008; Hughes et al., 2010). Microglia represent the largest population of phagocytes in the CNS and have a principal role in immune defence and inflammatory responses there (Hughes et al., 2010). Microglia are referred to as activated as judged by their altered morphology and the upregulation of cell surface antigens, including major histocompatibility complex class II, and a number of cytosolic and endosomal antigens. The microglia activation occurs early in prion disease and indeed is one of the few pathological markers that is associated with the early onset of behavioural dysfunction (Perry et al., 2002). Even though there are apparent robust evidence not only of microglial but also of astroglial activation in TSEs, the failure to clear the abundant amyloid deposition suggest that phagocytosis is somehow deficient in these diseases (Hughes et al., 2010) and inversely may contribute to neuronal death and formation of spongiform change

(Rezaie et al., 2001; Kovacs et al., 2008). Spongiosis is the main neuropathological feature associated to TSEs observed in histological analysis of affected brains and is characterised by small (< 10µm) round or oval vacuolization of the neuropil. These vacuoles must be distinguished from vacuoles commonly seen in late stages of various neurodegenerative disorders and from other irregular vacuoles caused by tissue rarefaction or edema. The exact mechanism of spongiform change in prion diseases is still not clear, but it is likely a result of abnormal membrane permeability and increased water content within neuronal processes or it may be also result from autophagy (Kovacs et al., 2008). Neurodegeneration in prion diseases eventually lead to neuronal death by the apoptotic pathway (Kovacs et al., 2008).

6 - DIAGNOSIS

Historically, techniques used to diagnose TSEs were designed to detect, in appropriate tissue samples, lesions characteristic of TSE or the transmissible agent itself (Grassi et al., 2008).

Both BSE and scrapie occur as different types and strains. Types are recognised basing on PrP^{Sc}-banding pattern in Western blot, while strains of TSEs are defined by their phenotypic behaviour after cloning the isolate in rodents, although the original isolate phenotype frequently is strain indicative (Jacobs et al., 2011).

6.1 - Histology and immunohistochemistry

Histological detection of lesions typical of TSE (spongiosis, astrogliosis, amyloid plaques), essentially in the CNS, is the reference method for confirming a clinical diagnosis. It is very specific, since it allows direct observation of the signs of the disease, but it is less sensitive than other techniques (Gavier-Widen et al., 2005; Grassi et al., 2008). The sensitivity of microscopic observation can be increased by immunohistochemical techniques that use antibodies specific to PrP to detect accumulation of PrP^{Sc} in amyloid deposits (van Kuelen et al., 1995; van Kuelen et al., 1996). This technique efficiency depends greatly on sample preparation and on the nature of the antibodies used (Grassi et al., 2008).

Although these methods are ill-suited to rapid, routine analysis, they are excellent for confirmation (Grassi et al., 2008). They are also effective for the analysis of samples of lymphoid tissues (tonsils, Peyer's patches, lymph nodes) and can be utilised, for example, in preclinical diagnosis of scrapie in sheep (van Kuelen et al., 1996; Andréoletti et al., 2000) and CWD (Spraker et al., 2002). These diseases are characterised by marked replication of the prion in the lymphoid organs during the presymptomatic phase.

6.2 - Western blot

Western blot (WB) has been used to detect PrP^{Sc} in tissue extracts for 25 years (Bendheim et al., 1986).

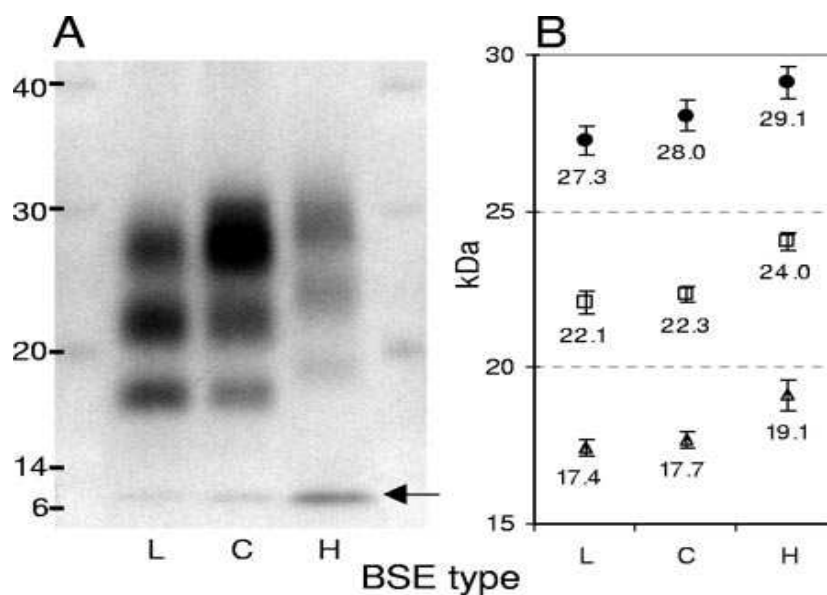
Since all samples always contain PrP^C, the protease-sensitive prion protein, they are systematically treated with pK. After denaturation of the tissue extract by heating with sodium dodecyl sulfate (SDS), it is analysed by

polyacrylamide gel electrophoresis (PAGE) and the denatured protein is transferred to a solid support and detected with an enzyme-labelled antibody. The specificity of WB stems, among other things, from the fact that proteolysis with pK characteristically alters the molecular weight of the PrP^{Sc}, because of the partial degradation of the N-terminal part of the protein. As a consequence, in addition to the residual signal observed, the gel bands shift in a manner typical of PrP^{Sc} (Grassi et al., 2008). The resistant part PrP^{Sc} can be observed by WB usually in the 17-30 kDa region as a triple band, representing the di-, mono- and unglycosylated PrP isoform (Jacobs et al., 2011).

6.2.1 - TSE types in cattle and small ruminants

In cattle C-type, L-type and H-type BSE have been recognised (Jacobs et al., 2011). The definition of L-type and H-type BSE is according to the higher and lower position of the unglycosylated PrP^{Sc} band in WB, respectively, compared to the position of the band in classical BSE (C-type) isolates (Jacobs et al., 2007) (Figure 5).

Figure 5. Results of WB analysis of L-, C-, and H-type BSE isolates with antibody L42. (A) Lanes with L-, C-, and H-type material with tissue equivalents of 0.5, 0.2, and 0.2 mg, respectively, placed between molecular mass marker lanes. (B) Apparent molecular masses of diglycosylated (circles), monoglycosylated (squares), and unglycosylated (triangles) bands with standard deviations for L-type (*n* 11), C-type (*n* 9), and H-type (*n* 6) isolates. Arrow = 7-kDa band (Jacobs et al., 2007).



In small ruminants four TSE types have been recognised based on PrP^{Sc}-banding patterns: classical scrapie, Nor98 scrapie (a form of atypical scrapie), BSE and CH1641 scrapie. The unglycosylated band of classical scrapie has a molecular weight of 18-19 kDa. Nor98 scrapie is characterised by a main PrP^{Sc} band at 7-8 kDa (Jacobs et al., 2011). The unusual scrapie isolate CH1641 and PrP^{Sc} that causes BSE (C/L-type) have unglycosylated bands with similar molecular weights (16-17 kDa), which are lower than the molecular weight of the PrP^{Sc} that causes typical scrapie. Furthermore, CH1641 prions have a protein fragment produced by the C-terminally cleavage of PrP^{Sc}, designed as PrP^{Sc} #2 (molecular weight, 14 kDa), which

permits to differentiate between CH1641 scrapie and BSE (Hope et al., 1999; Baron et al., 2008; Yokoyama et al., 2010).

6.3 - Experimental infection tests

The most sensitive and specific method of diagnosing TSE is unquestionably experimental infection in laboratory animals, classically rodents and in most cases of murine species. Animals are injected, usually by intracerebral route, with a homogenate prepared from the potentially infected tissue and are watched for the appearance of clinical signs. After their death, disease development is confirmed using classic techniques: histology, immunohistochemistry and WB (Grassi et al., 2008).

The mouse bioassay with wild-type strains (C57Bl, RIII and VM) is the gold standard model for assessing the biological properties of prions and the discrimination of TSE strains (Bruce et al., 2002; Beck et al., 2010 b).

Recently, the availability of transgenic mice has broadened the border of research.

PrP knockout mouse model, in which the physiological protein is genetically ablated, led to the awareness that the PrP^C expression is indispensable to the developing of TSEs and was utilised to investigate the physiological role of PrP^C (Groschup et al., 2008).

Moreover, the availability of transgenic mice that overexpress the same PrP as that of the donor species has significantly increased the efficiency of experimental transmission and shortened incubation period (Grassi et al., 2008; Simmons et al., 2008). Mutant PrP expression mouse models have contributed enormously to a better understanding of the nature of prions,

allowing to study the molecular aspects of species barrier effects, the cell specificity of the prion propagation, the role of the PrP glycosylation, the mechanisms of the prion spread and the neuropathological roles of PrP^C and of its abnormal isoform PrP^{Sc}. Transgenic mouse models have also been used for mapping of PrP regions involved in or required for the PrP conversion and prion replication as well as for modelling of familial forms of human prion diseases (Groschup et al., 2008).

However experimental infection tests are too labour-intensive and time-consuming for use in routine high throughput screening (Grassi et al., 2008).

6.3.1 - Incubation period and lesion profile

The agents causing TSEs exhibit clear evidence of strain variation. Studies of mouse-passaged TSE isolates have established that the phenotype of the disease depends on an interaction between the strain of TSE agent and genetic factors in the host (Dickinson et al., 1971; Bruce et al., 1991; Bruce et al., 2002). These observations led, many years ago, to the development of formal TSE strain typing methods, that depend primarily on the measurement of incubation periods (Dickinson et al., 1968) and construction of “lesion profiles” in panels of defined mouse strains. The lesion profile is a semiquantitative representation of severity and distribution of vacuolar degeneration in the brains (Fraser et al., 1968). Each panel of mice must be formed by at least five clinically and pathologically positive animals, to compensate individual variations (Beck et al., 2010 b). Using these methods, numerous distinct laboratory strains of TSE agents have been identified, with

signatures that remain stable over many serial mouse-to-mouse passages (Bruce et al., 1991; Bruce et al., 2002).

The mouse lines used were the wild-type ones, such as RIII and C57Bl, which share the same *Prnp* amino acid sequence (*Prnp^a*), and VM, which differ at codons 108 and 109 (*Prnp^b*) (Westway et al., 1987). This difference in genotype affects both the incubation period and lesion profile of specific isolates (Bruce et al., 1991; Bruce et al., 2002; Beck et al., 2010 b).

6.3.1.1 - BSE and scrapie

After primary transmission of BSE or scrapie, RIII mice had shorter incubation periods than C57Bl ones (Bruce et al., 2002).

The BSE lesion profile has a great stability, both after interspecies or intraspecies transmission (Bruce et al., 1994).

On the primary isolation in mice, BSE gives rise to a characteristic lesion profile, characterised by peaks in vacuolation score in the dorsal medulla, the hypothalamus and the septum. On subsequent subpassages it differs in two stable strains, 301C and 301V, depending on the PrP genotype of the host (*Prnp^a* o *Prnp^b*) (Bruce et al., 2002; Beck et al., 2010 a).

The BSE “signature” has been found in the lesion profile produced by the causative agent of vCJD, revealing the zoonotic potentiality of BSE (Bruce et al., 1997; Hill et al., 1997), and has been recognised also in other animal species, providing evidence that these species have been infected accidentally with BSE (Bruce et al., 1994).

On the contrary, on primary isolation scrapie generates lesion profiles that are highly variable from each other and distinct from the BSE profile (Beck et al., 2010 a). The most common ones are 87A and ME7, in mice having *Prnp^a* and

87V and 111A in case of *Prnp^b* (Bruce et al., 2002). Natural scrapie infection is caused by multiple prion strains and the resultant phenotype depends on which prions are dominantly propagated in a given host. The reason why multiple prion strains emerged during inter- and intraspecies transmission, even amongst animals with the same *Prnp*, is still unclear (Yokoyama et al., 2010).

One possible explanation for the different behaviours of these two TSE agents may be that the BSE agent has emerged recently and is able to cross species barriers without any alteration of its biologic properties. In contrast, scrapie is highly adapted to sheep and different strains of the agent may have evolved over time and adapted to several host-specific factors, the most important being the PrP genotype (Beck et al., 2010 a), which presents a high variability in sheep (Begara-McGorum et al., 2002; Bruce et al., 2002; Hunter et al., 2003).

6.3.2 - Immunohistochemistry

Lesion profiles have historically provided the most informative method of strain characterization through the bioassay. However, since different strains may give rise to unique patterns and types of PrP^{Sc} deposition in the mouse brain, attention has more recently turned to the use of immunohistochemistry for identification and characterization of strains. Early indicators suggest that this method could offer greater sensitivity and the capability of identifying agent strains at the level of individual mice compared with lesion profiling, which requires an average profile plotted from at least five clinically and

pathologically positive mice, because of variation in individual mouse profiles (Brown et al., 2003; Beck et al., 2010 b).

6.4 - Rapid tests for post-mortem diagnosis of TSE

None of the abovementioned methods are really suited to high-throughput screening of TSEs and cannot be automated. After the 1996 mad cow crisis and the fear of possible transmission to humans, it became clear that there was a need to develop new simpler and faster diagnostic tests for large-scale epidemiological studies and more accurate assessment for the characteristics of the epizootic or for routine testing to warrant safety of animal meat, for instance, of all cattle before they enter the food chain or industrial circuits. A new generation of so-called “rapid” diagnostic tests emerged, all based on the immunological detection of PrP^{Sc}, the only identified reliable marker of TSE. Again, due to the absence of antibodies that specifically recognise PrP^{Sc}, it was necessary to resort to indirect approaches to distinguish between the two isomers of PrP on the basis of their biochemical properties, such as the well-known resistance to pK degradation.

The first three rapid tests approved by European Commission in May 1999 were product respectively by Prionics (Zurich, Switzerland), CEA (Saclay, France) and Enfer Technology Ld (Newbridge, Ireland) (Grassi et al., 2008).

The Prionics test uses an industrial format of Western blot that enables large-scale analysis (Schaller et al., 1999) and was the first rapid test used in large scale epidemiological studies, first in Switzerland (Oesch et al., 2000) and then in France (Calavas et al., 2001).

The CEA and Enfer Technology Ld tests instead are based on an immunoenzyme approach (enzyme-linked immunosorbent assay, ELISA). In both cases PrP^{Sc} is initially selectively purified using pK, centrifuged and denatured. Then in the CEA test (now marketed by Bio-Rad, Hercules, CA, USA) it is measured by a two-site (so called sandwich) immunoassay that uses two monoclonal antibodies (Grassi et al., 2000), while in the Enfer Technology Ld test the solubilised and denatured PrP^{Sc} is detected using a polyclonal antibody directed against a peptide sequence characteristic of PrP (Grassi et al., 2008). All these tests were found to have 100% sensitivity and specificity.

In 2002 and 2004 new rapid tests were approved for the post-mortem diagnosis of TSEs in ruminants and most of them work in an ELISA format. Today, virtually all testing of cattle and small ruminants is done with the tests from Bio-Rad, Prionics, IDEXX and Enfer Technology Ld. All positive results recorded using the rapid-tests are confirmed in national reference laboratories, essentially using histopathology, immunohistochemistry and Western blot (Grassi et al., 2008).

6.5 - Protein misfolding cyclic amplification

To facilitate preclinical detections of prions in peripheral tissues, notably blood, it was developed an original approach in which PrP^{Sc} in sample is amplified by means of protein misfolding cyclic amplification (PMCA). In this approach, which seeks to mimic pathological processes and is akin to polymerase chain reaction used to amplify DNA (but without addition of exogenous polymerase enzyme), PrP^{Sc} is incubated in the presence of PrP^C

to allow expansion of aggregates of PrP^{Sc} which are then dispersed by sonication to generate smaller units and to encourage the formation of new aggregates. The quantity of PrP^{Sc} formed depends on the number of expansion/sonication cycles performed (Saborio et al., 2001; Soto et al., 2005). Amplification is achieved by using as a source of PrP^C a brain extract from the same species as that which produced the PrP^{Sc} to be amplified. This method is effective in various mammalian species, including mice, sheep, goats and cattle, cervids and humans (Soto et al., 2005; Jones et al., 2007; Kurt et al., 2007). PMCA has a great potential and is certainly the most promising approach from the view-point of developing a blood test (Grassi et al., 2008).

7 - THERAPY

On molecular level, prion disease therapeutics can be targeted to PrP^C, PrP^{Sc} or to the process of conversion between the two prion protein isoforms. Targeting PrP^{Sc}, the disease-associated isoform, may appear to be the most logical approach, but such targeting may have no effect on disease progression, or even enhance or prolong disease if PrP^{Sc} is a non-pathological end-point of the pathogenic conversion process, or if the rate of PrP^{Sc} deposition is critical to disease progression (Trevitt et al., 2006). Recent evidence indeed suggest that small quantities of the abnormal form of the prion protein may not always necessarily be pathogenic and that there might be silent prions lying dormant in healthy brains (Yuan et al., 2006; Kovacs et al., 2008). Alternatively, targeting PrP^C has the potential to remove the

substrate for the pathogenesis and is applicable regardless of the disease aetiology (Trevitt et al., 2006).

Several compounds acting at these levels have been studied, but none of them seems to have a fully resolving activity.

The sulphated polyanions and the sulphated glycans are polyanionic compounds that manage to delay the onset of the clinical signs in animals experimentally challenged with the prion protein, but to be effective they must be administered before or immediately after the infection.

Amongst the sulphated polyanions the most studied one is the Congo Red (CR), a stain used in histology to detect the amyloid deposits. Some studies *in vitro* and *in vivo* demonstrated its efficacy in preventing prion accumulation, but because of its carcinogenic and teratogenic effects it is not considered a safe drug (Caughey et al., 1992; Villa et al., 2003; Dormont et al., 2003; Poli et al., 2004).

Glycosaminoglycans (GAGs) are the polysaccharide side-chains of proteoglycans (PGs), which are component of the extracellular matrix and are involved in cell-adhesion, migration and proliferation. GAGs share a common architecture, being linear polymers of repeating disaccharide units, including an amino-sugar and at least one negatively charged group (sulphate or carboxylate). Some of them are sulphated glycans, as heparin sulphate, dermatan sulphate, keratan sulphate and chondroitin sulphate. Experimental studies demonstrated that they have a direct effect on the conversion of PrP and that, depending on the circumstances, they may be either cofactors or inhibitors of conversion. In cells they act as a competitive inhibitors of endogenous GAGs, putative cofactors of the toxic misfolding of the prion

protein, while in cell-free system sulphated glycans are themselves cofactors for conversion. The sulphation of these molecules is crucial to their involvement with prion conversion, whether as inhibitors or facilitators (Caughey et al., 1993; Wong et al., 2001; Dormont et al., 2003; Trevitt et al., 2006).

Tetrapyrrolic compounds are known effectors of protein conformational change, with structural similarities to CR (being both aromatic and sulphated) but with improved toxicological and solubility profiles. *In vitro* they were found to decrease PrP^{Sc} levels and to prevent prion propagation. *In vivo* they managed to delay the onset of prion disease after intraperitoneal (i.p.) infection, especially following multiple treatments post inoculation, suggesting that their mode of action is in peripheral tissues at the initial stage of infection. No significant effect was seen either after intracerebral (i.c.) infection or administration of the treatment in a late stage of disease (Priola et al., 2000; Priola et al., 2003).

The anti-prionic activity was assessed also in some antibiotics.

Amphotericin B is a polyene macrolide antibiotic with an antifungal activity. It is derived from *Streptomyces nodosus*, which acts by intercalation into and disruption of the cell membrane. It was found to significantly prolong the incubation time of prion disease but not to prevent it (Amyx et al., 1984, Dormont et al., 2003; Trevitt et al., 2006).

Tetracycline and doxycycline are tetracyclic antibiotics. Their efficacy against experimental scrapie was demonstrated after incubation of the scrapie inoculum with drug prior to i.c. infection, which results in a delayed onset of disease by reducing the titre of the initial inoculum. They have a

decontamination activity rather than a therapeutic one (Forloni et al., 2002). Moreover, they were reported to prevent PrP 106-126 peptide-mediated cytotoxicity in primary cell culture (Tagliavini et al., 2000) and to reduce protease resistant PrP formation in the PMCA replication assay (Barret et al., 2003).

Clioquinol, a blood-brain barrier permeable ion-chelating antibiotic, seems to be able to reduce the cytotoxic effects induced by copper and to promote the solubilization of protein aggregates, reducing the conformational stability of PrP^{Sc} (Pollera et al., 2005).

These compounds have not been the only drugs for which the anti-prionic activity was tested.

Quinacrine, a tricyclic compound used as anti-malarial drug, was found to be a very efficient inhibitor of PrP^{Sc} propagation in infected cells (Korth et al., 2001). Despite that, quinacrine treatment showed no effect on the incubation time of i.c.-infected animals, in either wild-type mice treated orally (Collins et al., 2002) or in tg7 mice treated by intra-ventricular infusion. In fact a high dose of intra-ventricular quinacrine causes a decrease in scrapie incubation time (Doh-ura et al., 2004) and a further study reported that a i.p. quinacrine treatment of BSE-infected mice results in an increase in splenic PrP^{Sc} deposition at 30 days after infection (Barret et al., 2003).

Chlorpromazine is another tricyclic compound and, as quinacrine, is a drug licensed in human for other indications, able to cross the blood brain barrier (Dormont et al., 2003). It is less effective than quinacrine in cell culture (Korth et al., 2001) but was reported to increase incubation time in mice after i.c. but not i.p. infection. These results were published using the compound name

aminasine and have not been substantiated to date (Roikhel et al., 1984; Trevitt et al., 2006).

The statins (lovastatin and squalastatin) are inhibitors of two enzymes of the cholesterol synthetic pathway (near the beginning and end of the pathway, respectively) and cause a decrease in cellular levels of cholesterol. The accumulation of PrP^{Sc} in infected cell lines is prevented by treatment of the cells with lovastatin or squalastatin. In both cases, this effect is abrogated by the addition of cholesterol, suggesting an important role for cholesterol-sensitive processes in PrP^{Sc} formation. It was postulated that the effect of the statins is mediated by reducing the amount of PrP^C available for conversion to PrP^{Sc}, by either inaccessibility on the surface, reduced export to plasma membrane or increased partitioning to internal compartments (Taraboulos et al., 1995; Bate et al., 2004).

Another compound which was investigated is the curry spice curcumin (diferuloylmethane), a planar aromatic small molecule analogous to CR. It is a non-toxic antioxidant with antiinflammatory properties (Menonet et al., 2007; Riemer et al., 2008), which has improved blood-brain barrier permeability compared with CR, though is still limited (Trevitt et al., 2006). It was reported to inhibit aggregation and promote disaggregation of β -amyloid (Yang et al., 2005; Garcia-Alloza et al., 2007;). It is also a potent inhibitor of prion replication in cell culture assays (Caughey et al., 2003) while *in vivo* is able to prolong the survival time in a murine prion model (Riemer et al., 2008).

There are varying reports about the efficiency of immunosuppression as a treatment for prion disease.

Prednisone and arachis oil are able to delay disease onset following administration around the time of i.p. infection, but not following administration late in the disease course or after i.c. scrapie-infection (Outram et al., 1974; Outram et al., 1975). Non-steroidal anti-inflammatory drugs were found both in neuroblastoma cell lines and primary neuronal cultures to be protective against cytotoxicity caused specifically by a prion peptide or by partially purified PrP^{Sc} preparation from scrapie sick mouse brains (Bate et al., 2002). Treatment of scrapie-infected mice with either interferon or interferon stimulators had no effect on the disease progression (Allen et al., 1977; Gresser et al., 1983; Trevitt et al., 2006).

Although there is no obvious humoral immune response stimulated in TSEs, active and passive immunisations have been tested as putative preventive or therapeutic approaches.

Initial attempts to produce anti-prion antibodies included immunization with purified prions or scrapie-associated fibrils (SAFs) with a greater response observed in prion knockout mice compared to wild-type mice. Then the use of appropriate adjuvants permitted to use recombinant cellular prion protein as immunogen in both knockout and wild-type mice (Trevitt et al., 2006). Several groups reported moderate beneficial effects, as prolongation of incubation time, of active immunization of mice or hamsters with prion protein or peptides prior to infection with scrapie isolates (Sigurdsson et al., 2002; Magri et al., 2005).

An increase in disease incubation time was reported also after passive immunization (Trevitt et al., 2006).

Another anti-prion therapeutic approach is based on the use of β -sheet breaker peptides, which consist in sequences from the target protein into which extra prolin residues are inserted. Prolin in an imino acid unable to take the conformation required by an ordered β -sheet structure and its presence in a sequence of amino acids residues with otherwise high β -propensity prevents the formation of β -sheet by that peptide (Trevitt et al., 2006). The design of a β -sheet breaker peptide to specifically interact to prion protein conversion (Soto et al., 2000) was spurred by the successful employment of β -sheet breaker peptides in models of AD and by the encouraging results obtained by Chabry and colleagues (1999). In fact, using prion protein peptides of both mouse and hamster sequences (residues 109-141, of which 119-136 are identical in mouse and hamster protein), they investigated the species specificity of conversion and report that both mouse and hamster peptides can inhibit toxic conversion of proteins from either species. PrP 116-136 is also effective in decreasing PrP^{Sc} *in vitro*, whereas other peptides, including PrP 119-128, did not show this effect (Chabry et al., 1999).

Although most of the studied compounds proved to be promising both *in vitro* and in animal models, none of them was later turned into a useful drug, because of toxicity or unfavourable pharmacokinetic properties.

To date, the only approach which is effective for the prevention of symptomatic neurological disease in animals with established prion neuropathology is the knockout of neuronal PrP^C in transgenic animals (Malucci et al., 2003). Although transgenic techniques to ablate the prion gene have no direct clinical application at present, RNA interference offers a corresponding strategy for PrP^C knockdown *in vivo* (Malucci et al., 2005).

RNA interference (RNAi) is the tool by which post-transcriptional gene silencing mechanisms in the cell can be initiated. The introduction of small double-stranded RNA to a cell leads to the specific degradation of mRNA homologous to the introduced RNA molecules. RNAi is thus a means by which specific genes can be turned off without the requirement for genomic DNA manipulation (Trevitt et al., 2006). Studies *in vitro* showed that RNAi is able to suppress both exogenous and endogenous *Prnp* expression (Tilly et al., 2003) and reduce transiently the level of PrP^{Sc} in scrapie-infected cell cultures (Daude et al., 2003).

Research allowed to better understand etiology and pathogenesis of prion diseases, even though it has not yet been identified a compound capable of providing therapeutic decisive action.

8 - TSEs AND ALZHEIMER'S DISEASE

Protein misfolding and amyloid deposition in the CNS are mutual pathogenic characteristics of TSEs and Alzheimer's disease (AD). In prion diseases proteinaceous deposits are formed by the insoluble, partially protease resistant abnormal prion protein isoform (PrP^{Sc}). AD is characterised by the formation of extracellular insoluble plaques, consisting predominantly of amyloid- β (A β) peptide of 40-42 amino acids, due to an abnormal cleavage of the amyloid precursor protein (APP) (Checkler et al., 2002; Parkin et al., 2007).

Both APP and PrP^C are cell surface proteins residing in lipid rafts of the cell membrane and bear potential toxic sequences (A β and PrP 106-126, respectively), with the propensity to form β -sheet-rich insoluble and protease-

resistant fibrils, which undergo a series of proteolytic attacks that are triggered by identical proteolytic activities and that are similarly regulated (Checkler et al., 2002; Ronga et al., 2006; Hooper et al., 2008).

Physiologically, both APP and PrP^C undergo a cleavage mediated by α -secretases (ADAM 10 and ADAM 17) inside their toxic core (Figure 6), while in pathologic conditions they both experience alternative cleavages that maintain their toxic domains intact. PrP^{Sc} seems to derive from a unique cleavage of PrP^C sustained by an unknown activity. A β is produced from APP by sequential proteolytic cleavage of β - and γ -secretase (Figure 7).

Figure 6. Physiological processing of amyloid precursor protein (APP) and cellular prion protein (PrPC). This scheme illustrates the ‘normal’ metabolic pathway for APP (right) and PrPC (left). Two distinct disintegrins, ADAM 10 and TACE (tumor-necrosis-factor- α converting enzyme, also known as ADAM 17) cleave both proteins inside their toxic sequences (red and orange segments). The products are N1 and C1 fragments, and secreted APP (sAPP α) and P10, from PrPC and APP, respectively. Interestingly, TACE-mediated processing is strongly upregulated by protein kinase C (PKC) agonists, whereas ADAM 10 is responsible mainly for the constitutive pathway. Concomitant action of both enzymes results in (1) disruption of A β and PrPC 106-126 containing fragments, thereby preventing their deleterious effects, and (2) secretion of the trophic and neuroprotective product sAPP α . The physiological effects of the N1 fragment have yet to be elucidated (Checler et al., 2002).

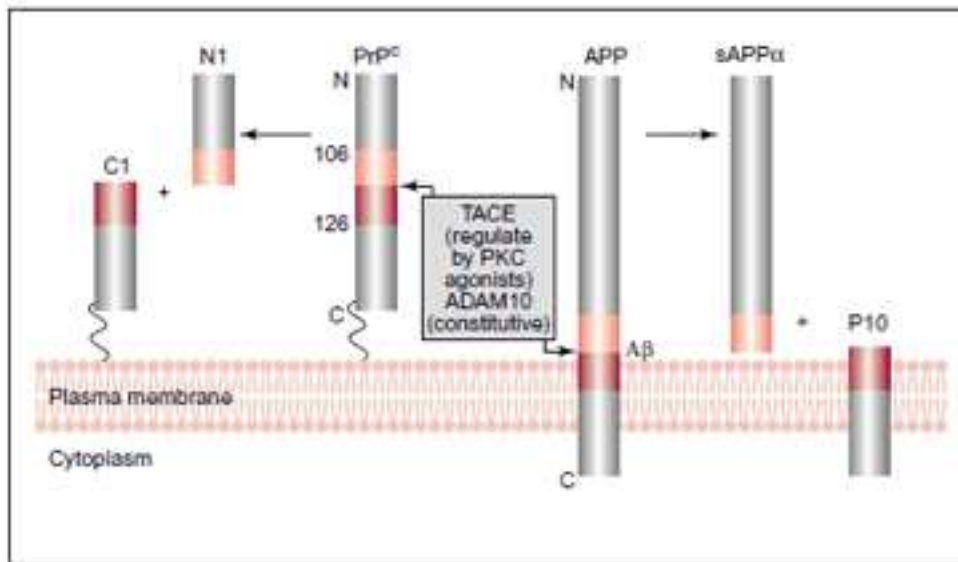
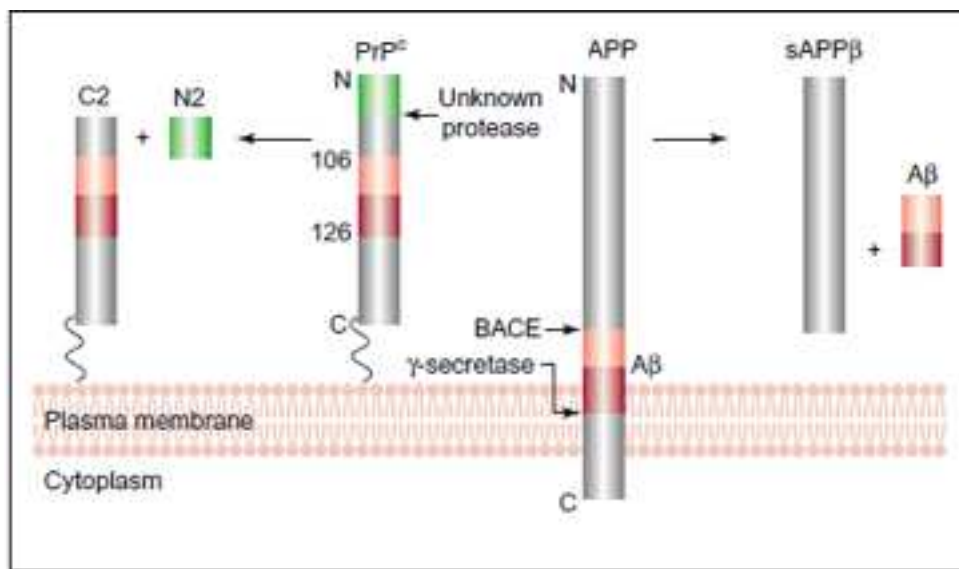


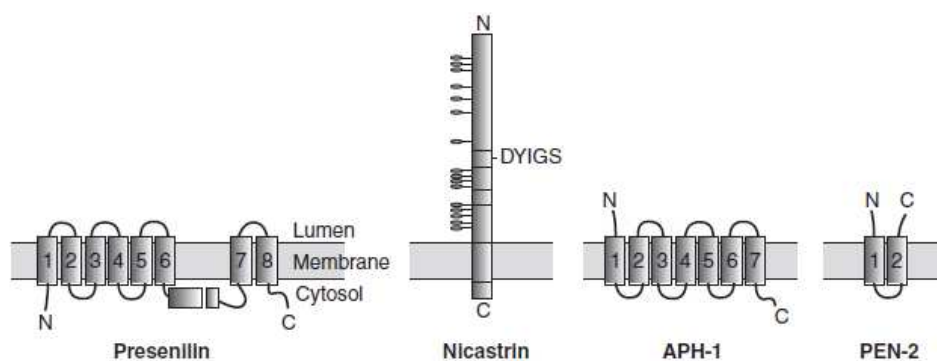
Figure 7: Amyloidogenic and pathogenic processing of APP and PrP^C, respectively. The schematic representation of β APP amyloidogenic and PrP^C pathogenic pathways discriminates between the two-step generation of A β (by BACE and γ -secretase) and the unique cleavage of PrP^C by an unknown activity. Moreover, it is important to note that, although A β is produced in normal conditions, the C2 fragment is detected only in affected brains and never occurs in healthy patients. However, in both cases (1) A β and the PrP^C 106-126 containing peptide C2 are generated, and (2) the secretion of the trophic and neuroprotective product sAPP α is abolished. The amyloidogenic and/or toxic potency of the N2 and C2 fragments have yet to be elucidated (Checler et al., 2002).



The β -secretase involved is BACE (β -site APP-cleaving enzyme) 1.

The γ -secretase is a complex of four different membrane proteins: presenilin (PS), believed to contain the catalytic core of γ -secretase, nicastrin, anterior pharynx 1 (APH-1), and PS enhancer 2 (PEN-2) (Evin et al., 2006) (Figure 8).

Figure 8. The γ -secretase complex comprises four integral membrane proteins. Presenilin (PS1 or PS2) is predicted to contain eight or nine transmembrane domains (the eight transmembrane structure is represented) and two critical aspartate residues in transmembrane domains 6 and 7 that are proposed to form the catalytic site of the enzymatic complex. Presenilin becomes proteolytically processed into an N-terminal fragment with six membrane-spanning domains, and a C-terminal fragment with two transmembrane domains, before becoming inserted into active complexes of high molecular weight. Nicastrin comprises a single transmembrane domain and becomes highly glycosylated during maturation of the γ -secretase complex. The conserved motif DYIGS is important for the recognition of γ -secretase substrates. APH-1 (named after anterior pharynx defects caused by this gene mutation in the worm) is predicted to contain seven transmembrane domains. Alternative forms of APH-1 have been described: APH-1a and APH-1b, which are the products of homologous genes. APH-1a can be expressed as two isoforms that result from alternative splicing of the C-terminal region. Nicastrin and APH-1 can associate as a subcomplex. PEN-2 (presenilin enhancer) contains two transmembrane domains, and is required for the endoproteolysis of presenilin (Evin et al., 2006).



This enzyme mediates the ultimate step in the release of $A\beta$, cleaving APP within the membrane. It has no sequence specificity, since it generates $A\beta$ peptides of different lengths, but it is conformation specific since it selectively binds and cleaves α -helical substrates (Checkler et al., 2002; Evin et al., 2006). γ -secretase complex cleaves many substrates other than APP, such as Notch, essential for normal encephalic functions, and a subset of cell-

surface receptors and proteins involved in embryonic development, haematopoiesis, cell adhesion and cell/cell contacts (Evin et al., 2006).

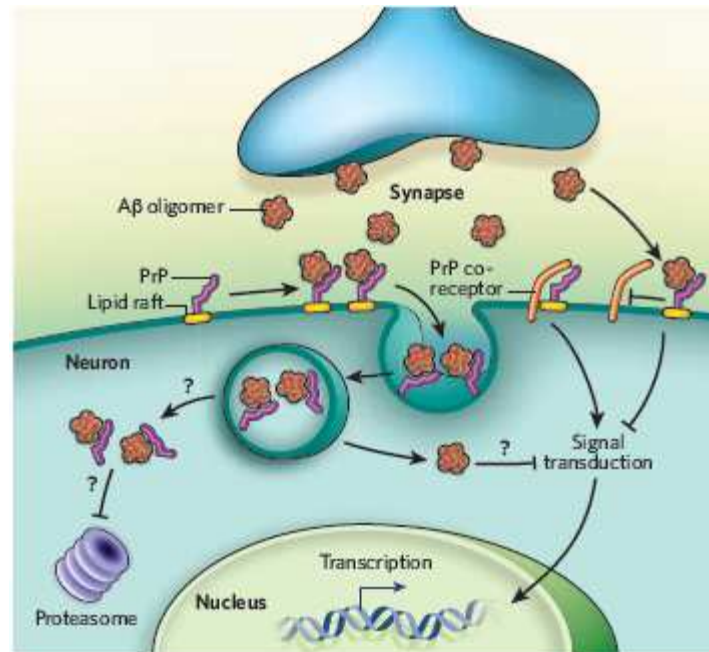
There are several similarities between A β and PrP. They both have high-affinity metal-binding sites, which favour the binding of transition metals such as copper and zinc, allowing important regulatory functions (Barnham et al., 2006). In close proximity to these metal-binding sites they have a methionine residue (Met35 of A β e Met129 of human PrP) that is reportedly essential for several activities of both of them. Met35 is thought to modulate A β neurotoxicity through its crucial role in modulating A β redox chemistry and A β aggregation. Met129 modulates disease susceptibility in human prion disease. Most cases of sporadic CJD occur in homozygotes at codon 129 of the human *Prnp*, where either methionine or valine might be encoded. Moreover to date all patients with vCJD have been methionine homozygotes, further reinforcing the importance of this residue (Collinge, 2001; Barnham et al., 2006). Of potential importance is that the crucial methionine residue is located in the putative transmembrane regions of both A β and PrP. A prominent feature of these transmembrane regions is the presence of several repeats of the GxxxG transmembrane-localization motif. The GxxxG motif is one example of how the primary sequence regulates peptide and protein structure, in this instance by affecting elix-to-elix packing. This packing is a key element in defining the tertiary structure of many membrane-bound proteins. Both A β and PrP contain three repeats of the GxxxG motif: the repeat is present in A β between residues 25 and 37 (GSNKGAIIGLMVG), while the repeat pattern is similar in PrP between residues 119-131 (GAVVGGLGGYMLG). The crucial methionine of both A β and PrP is located

in the middle of the last GxxxG repeat (GxMxG). The presence of GxxxG repeat motif indicates that PrP, A β and/or its parent molecule (APP) are probably involved in transmembrane helix-helix interactions, including the possible assembly into ion channel-like structure (Barnham et al., 2006).

TSEs and AD share a number of clinical, pathological and biochemical characteristics. The codon 129 polymorphism of the human *Prnp* was reported to affect the number of A β deposits in cerebral aging and methionine homozygosis at codon 129 was suggested as risk-factor not only for vCJD but also for early-onset AD (Zimmermann et al., 1999; Ironside et al., 2004; Riemenschneider et al., 2004; Del Bo et al., 2006; Baier et al., 2008).

It was demonstrated that PrP^C expression, when localized in lipid rafts, inhibits BACE 1-mediated APP cleavage, markedly decreasing the production of A β . PrP^C N-terminus interacts, through glycosaminoglycans (GAGs), with one or more of the heparin-binding sites on BACE 1 and this interaction then restricts access of BACE 1 to its substrate, APP (Parkin et al., 2007; Hooper et al., 2008). Controversially, PrP^C localised in lipid rafts seems to be a main receptor for A β oligomers, which bind to amino acid residues 95-110, mediating their deleterious effects on synaptic plasticity and function (Laurén et al., 2009; Cisse et al., 2009) (Figure 9).

Figure 9. Laurén and colleagues (2009) show that A β oligomers interact with the membrane-bound prion protein PrP^C. This interaction may in turn disrupt interaction between PrP^C and a co-receptor, impairing the neuron's signal-transduction pathways required for synaptic plasticity. Alternatively, internalization of PrP^C may allow A β oligomers to reach intracellular compartments, where they might interfere with cellular functions such as protein degradation by the proteasome complex, and PrP^C-dependent gene transcription (Cisse et al., 2009).



A β -positive senile plaques in AD patients were found to contain deposits of PrP^C (Voigtlander et al., 2001; Baier et al., 2008). Recently an *in vivo* study has shown that memory impairment in transgenic mouse models of AD mice requires PrP^C (Gimbel et al., 2010). Interestingly, the α -secretase enzyme, which precludes A β production by cleaving the A β precursor protein APP within the A β domain, also cleaves PrP^C between residues 111 and 112, thus releasing from the membrane the portion of PrP^C to which A β would otherwise bind (Cisse et al., 2009). Also the misfolded PrP^{Sc} seems to be involved in the fibrillogenesis of A β and consequently in the acceleration of disease progression (Baier et al., 2008).

9 - EUROPEAN LEGISLATION ON TSEs

After the recognition of the first case of BSE in 1986 in the UK, the EU has introduced several laws first to curb the epidemic of the mad cow disease and then aimed to TSEs eradication in Europe and to protect animal and human health.

Following there is the chronological list of Community legislation on TSEs (Table 5) (European Commission, Food and Feed Safety).

Table 5. Chronological list of Community legislation on TSEs (European Commission, Food and Feed Safety).

Year	Legal text	Contents
1989	89/469/EEC: Commission Decision of 28 July 1989 concerning certain protection measures relating to bovine spongiform encephalopathy in the United Kingdom (Official Journal L 225 , 3.2.1989, P. 51)	Restrictions on the dispatch of certain live cattle from the UK
1990	90/59/EEC: Commission Decision of 7 February 1990 amending Decision 89/469/EEC concerning certain protection measures relating to bovine spongiform encephalopathy in the United Kingdom (OJ L 41, 15.2.1990, p. 24)	Amendment of Decision 89/469/EEC - Dispatch limitation to only calves under 6 months old
	90/134 EEC: Commission Decision of 6 March 1990 amending for the second time Council Directive 82/894/EEC on the notification of animal diseases within the Community and temporarily amending the frequency of notification for bovine spongiform encephalopathy (OJ L 76, 22.3.1990, p. 23)	Compulsory notification of BSE
	90/200 EEC: Commission Decision of 9 April 1990 concerning additional requirements for some tissues and organs with respect to Bovine Spongiform Encephalopathy (BSE) (OJ L 105, 25.4.1990, p. 24)	Restrictions on the dispatch of certain bovine tissues and organs from the UK
	90/261/EEC: Commission Decision of of 8 June 1990 amending Decision 89/469/EEC concerning certain protection measures relating to bovine spongiform encephalopathy (BSE) in the United Kingdom and Decision 90/200/EEC concerning additional requirements for some tissues and organs with respect to bovine spongiform encephalopathy (OJ L 146, 9.6.1990, p. 29)	Amendment of Decisions 89/469/EEC and 90/200/EEC - Guarantees on identification of animals and certification for beef dispatch

Year	Legal text	Contents
1991	91/89/EEC: Commission Decision of 5 February 1991 making financial provision for a project relating to the inactivation of the agents of scrapie and bovine spongiform encephalopathy (OJ L 49, 22.2.1991, p. 31)	Financial provision for a project relating to the inactivation of the agents of scrapie and BSE
1992	92/290/EEC: Commission Decision of 14 May 1992 concerning certain protection measures relating to bovine embryos in respect of bovine spongiform encephalopathy (BSE) in the United Kingdom (OJ L 49, 22.2.1991, p. 31)	Restrictions on the dispatch of bovine embryos from the UK
	92/450/EEC: Commission Decision of 30 July 1992 amending for the third time Council Directive 82/894/EEC on the notification of animal diseases within the Community and temporarily amending the frequency of notification for bovine spongiform encephalopathy(OJ L 248, 28.8.1992, p. 77)	Compulsory notification of BSE (Repeals and replaces D 90/134/EEC)
1994	94/381/EC: Commission Decision of 27 June 1994 concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein (OJ L 172, 7.7.1994, p. 23)	Ban on the use of proteins derived from mammalian tissues for feeding ruminants
	94/382/EC: Commission Decision of 27 June 1994 on the approval of alternative heat treatment systems for processing animal waste of ruminant origin, with a view to the inactivation of spongiform encephalopathy (OJ L 172, 7.7.1994, p. 25)	Rendering systems for processing ruminant waste into MBM (inactivation of BSE agents)
	94/474/EC: Commission Decision of 27 July 1994 concerning certain protection measures relating to bovine spongiform encephalopathy and repealing Decisions 89/469/EEC and 90/200/EEC (OJ L 194, 29.7.1994, p. 96)	Restrictions on the dispatch from the UK of live cattle and certain ruminant products - Destruction of specified bovine offal (Repeals D 89/469/EC and 90/200/EC)
	94/794/EC: Commission Decision of 14 December 1994 concerning certain protection measures relating to bovine spongiform encephalopathy and repealing Decisions 89/469/EEC and 90/200/EEC (OJ L 325, 17.12.1994, p. 60)	Amendment of D 94/474/EC - Certification for beef dispatch
1995	95/29/EC: Commission Decision of 13 February 1995 amending Decision 94/382/EC on the approval of alternative heat treatment systems for processing animal waste of ruminant origin, with a view to the inactivation of spongiform encephalopathy agents (OJ L 38, 18.2.1995, p. 17)	Amendment of D 94/382/EC - Batch rendering systems
	95/60/EC: Commission Decision of 6 March 1995 amending Decision 94/381/EC concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein (OJ L 55, 11.3.1995, p. 43)	Amendment of D 94/381/EC - Derogation to the feed ban

Year	Legal text	Contents
	95/287/EC: Commission Decision of 18 July 1995 amending Decision 94/474/EEC concerning certain protection measures relating to bovine spongiform encephalopathy and repealing Decisions 89/469/EEC and 90/200/EEC (OJ L 181, 1.8.1995, p. 40)	Amendment of D 94/474/EC - Certification for beef dispatch
1996	96/239/EC: Commission Decision of 27 March 1996 on emergency measures to protect against bovine spongiform encephalopathy (OJ L 78, 28.3.1996, p. 47)	Total ban on dispatch of live cattle and all cattle products from the UK (UK embargo)
	96/362/EC: Commission Decision of 11 June 1996 amending Decision 96/239/EC on emergency measures to protect against bovine spongiform encephalopathy (OJ L 139, 12.6.1996, p. 17)	Amendment of D 96/239/EC - Conditional lifting of the ban for certain cattle products
	96/381/EC: Commission Decision of 20 June 1996 approving the measures to be implemented as regards bovine spongiform encephalopathy in Portugal (OJ L 149, 22.6.1996, p. 25)	Eradication programme for BSE in Portugal
	96/385/EC: Commission Decision of 24 June 1996 approving the plan for the control and eradication of bovine spongiform encephalopathy in the United Kingdom (OJ L 151, 26.6.1996, p. 39)	Eradication programme for BSE in the UK
	96/449/EC: Commission Decision of 18 July 1996 on the approval of alternative heat treatment systems for processing animal waste with a view to the inactivation of spongiform encephalopathy agents (OJ L 184, 24.7.1996, p. 43)	Pressure cooking system for processing mammalian waste into MBM (inactivation of TSE agents)
1997	97/18/EC: Commission Decision of 16 December 1996 approving the measures to be implemented as regards bovine spongiform encephalopathy in France (OJ L 6, 10.1.1997, p. 43)	Eradication programme for BSE in France
	97/312/EC: Commission Decision of 12 May 1997 approving the measures to be implemented as regards bovine spongiform encephalopathy in Ireland (OJ L 133, 24.5.1997, p. 38)	Eradication programme for BSE in Ireland
	97/534/EC: Commission Decision of 30 July 1997 on the prohibition of the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 216, 8.8.1997, p. 95)	Prohibition of the use of SRM (mainly brain, eyes and spinal cord)
	97/735/EC: Commission Decision of 21 October 1997 concerning certain protection measures with regard to trade in certain types of mammalian animal waste (OJ L 294, 28.10.1997, p. 7)	Restrictions on trade in MBM

Year	Legal text	Contents
	<p>Commission Directive 97/65/EC of 26 November 1997 adapting, for the third time, to technical progress Council Directive 90/679/EEC on the protection of workers from risks related to exposure to biological agents at work (OJ L 335, 6.12.1997, p. 17)</p>	<p>Protection of workers from risks related to exposure to Spongiform Encephalopathy agents at work</p>
	<p>97/866/EC: Commission Decision of 16 December 1997 amending Decision 97/534/EC on the prohibition of the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 351, 23.12.1997, p. 69)</p>	<p>Postponement to 1/4/1998 of the date of application of D 97/534/EC (SRM)</p>
	<p>97/870/EC: Commission Decision of 16 December 1997 amending Decision 96/385/EC approving the plan for the control and eradication of bovine spongiform encephalopathy in the United Kingdom (OJ L 353, 24.12.1997, p. 45)</p>	<p>Amendment of D 96/385/EC - Clarification of the definition of high-risk cattle in the UK</p>
1998	<p>98/12/EC: Commission Decision of 15 December 1997 amending for the fourth time Council Directive 82/894/EEC on the notification of animal diseases within the Community and temporarily amending the frequency of notification for bovine spongiform encephalopathy (OJ L 4, 8.1.1998, p. 63)</p>	<p>Compulsory notification of BSE (Repeals and replaces D 92/450/EEC)</p>
	<p>98/256/EC: Council Decision of 16 March 1998 concerning emergency measures to protect against bovine spongiform encephalopathy, amending Decision 94/474/EC and repealing Decision 96/239/EC (OJ L 113, 15.4.1998, p. 32)</p>	<p>Amendment of the UK embargo - Reinforcement of controls and first steps towards lifting the ban under Export Certified Herds Scheme of Northern Ireland (Repeals D 96/239/EC)</p>
	<p>98/248/EC: Council Decision of 31 March 1998 amending Commission Decision 97/534/EC on the prohibition of the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 102, 2.4.1998, p. 26)</p>	<p>Postponement to 1/1/1999 of the date of application of D 97/534/EC (SRM)</p>
	<p>98/272/EC: Commission Decision of 23 April 1998 on epidemio-surveillance for transmissible spongiform encephalopathies and amending Decision 94/474/EC (OJ L 122, 24.4.1998, p. 59)</p>	<p>Epidemio-surveillance for all animal TSEs</p>
	<p>98/351/EC: Commission Decision of 29 May 1998 setting the date on which dispatch from Northern Ireland of bovine products under the Export Certified Herds Scheme may commence by virtue of Article 6(5) of Council Decision 98/256/EC (OJ L 157, 30.5.1998, p. 110)</p>	<p>Date on which dispatch from Northern Ireland may commence (1/6/1998)</p>

Year	Legal text	Contents
	98/477/EC: Commission Recommendation of 22 July 1998 concerning information necessary to support applications for the evaluation of the epidemiological status of countries with respect to transmissible spongiform encephalopathies (OJ L 212 , 30/07/1998 p. 58)	Information necessary to support applications for the evaluation of TSE status
	98/564/EC: Commission Decision of 7 October 1998 amending Council Decision 98/256/EC as regards certain emergency measures to protect against bovine spongiform encephalopathy (OJ L 273, 9.10.1998, p. 37)	Amendment of D 98/256/EC - Dispatch of BSE samples for scientific research
	98/653/EC: Commission Decision of 18 November 1998 concerning emergency measures made necessary by the occurrence of bovine spongiform encephalopathy in Portugal (OJ L 311, 20.11.1998, p. 23)	Total ban on dispatch of live cattle and all cattle products from Portugal (Portugal embargo)
	98/692/EC: Commission Decision of 25 November 1998 amending Commission Decision 98/256/EC as regards certain emergency measures to protect against bovine spongiform encephalopathy (OJ L 328, 4.12.1998, p. 28)	Amendment of the UK embargo - Principles of the second step towards lifting the ban under the Date-based Export Scheme applicable in the entire UK
	98/745/EC: Council Decision of 17 December 1998 amending Commission Decision 97/534/EC on the prohibition of the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 358, 31.12.1998, p. 113)	Postponement to 31/1/1999 of the date of application of D 97/534/EC (SRM)
1999	1999/129/EC: Commission Decision of 29 January 1999 amending for the second time Decision 94/381/EC concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein (OJ L 41, 16.2.1999, p. 14)	Amendment of D 94/381/EC - Hydrolysed proteins
	1999/514/EC: Commission Decision of 23 July 1999 setting the date on which dispatch from the United Kingdom of bovine products under the date-based export scheme may commence by virtue of Article 6(5) of Council Decision 98/256/EC (OJ L 195, 28.7.1999, p. 42)	Date on which dispatch from the UK of certain bovine products may commence (1/8/1999)
	1999/517/EC: Commission Decision of 28 July 1999 amending Decision 98/653/EC concerning emergency measures made necessary by the occurrence of bovine spongiform encephalopathy in Portugal (OJ L 197, 29.7.1999, p. 45)	Amendment of D 98/653/EC - Extension of the ban against Portugal

Year	Legal text	Contents
	<p>1999/534/EC: Council Decision of 19 July 1999 on measures applying to the processing of certain animal waste to protect against transmissible spongiform encephalopathies and amending Commission Decision 97/735/EC (OJ L 204, 4.8.1999, p. 37)</p>	<p>Conditions for the production of MBM and tallow (Repeals D 96/449/EC)</p>
	<p>1999/713/EC: Commission Decision of 21 October 1999 amending Decision 98/653/EC concerning emergency measures made necessary by the occurrence of bovine spongiform encephalopathy in Portugal (OJ L 281, 4.11.1999, p. 90)</p>	<p>Amendment of D 98/653/EC – Dispatch of MBM for incineration and fighting bulls</p>
	<p>1999/881/EC: Council Decision of 14 December 1999 amending Commission Decision 97/534/EC on the prohibition of the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 331, 23.12.1999, p. 78)</p>	<p>Postponement to 30 June 2000 of the date of application of D 97/534/EC (SRM)</p>
2000	<p>2000/104/EC: Commission Decision of 31 January 2000 amending Decision 98/653/EC concerning emergency measures made necessary by the occurrence of bovine spongiform encephalopathy in Portugal (OJ L 29, 4.2.2000, p. 36)</p>	<p>Amendment of D 98/653/EC - Extension of the ban against Portugal</p>
	<p>2000/345/EC: Commission Decision of 22 May 2000 setting the date on which dispatch from Portugal to Germany of certain products for the purpose of incineration may commence by virtue of Article 3(6) of Decision 98/653/EC (OJ L 121, 23.5.2000, p. 9)</p>	<p>Starting date for the dispatch from Portugal of MBM for the purpose of incineration</p>
	<p>2000/371/EC: Commission Decision of 6 June 2000 setting the date on which dispatch of fighting bulls from Portugal to France may commence by virtue of Article 3(7) of Decision 98/653/EC (OJ L 134, 7.6.2000, p. 34)</p>	<p>Starting date for the dispatch from Portugal of fighting bulls to France</p>
	<p>2000/372/EC: Commission Decision of 6 June 2000 setting the date on which dispatch of fighting bulls from Portugal to Spain may commence by virtue of Article 3(7) of Decision 98/653/EC (OJ L 134, 7.6.2000, p. 35)</p>	<p>Starting date for the dispatch from Portugal of fighting bulls to Spain</p>
	<p>2000/374/EC: Commission Decision of 5 June 2000 amending Decision 98/272/EC on epidemio-surveillance for transmissible spongiform encephalopathies (OJ L 135, 8.6.2000, p. 27)</p>	<p>Amendment of D 98/272/EC – Introduction of rapid post-mortem test in monitoring for BSE</p>

Year	Legal text	Contents
	2000/418/EC: Commission Decision of 29 June 2000 regulating the use of material presenting risks as regards transmissible spongiform encephalopathies and amending Decision 94/474/EC (OJ L 158, 30.6.2000, p. 76)	Prohibition of the use of SRM (Repeals D 97/534/EC)
	2000/764/EC: Commission Decision of 29 November 2000 on the testing of bovine animals for the presence of bovine spongiform encephalopathy and amending Decision 98/272/EC on epidemio-surveillance for transmissible spongiform encephalopathies (OJ L 305, 6.12.2000, p. 35)	Amendment of D 98/272/EC – Reinforcement of the surveillance
	2000/766/EC: Council Decision of 4 December 2000 concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein (OJ L 306, 7.12.2000, p. 32)	Temporary ban on use of MBM
2001	2001/2/EC: Commission Decision of 27 December 2000 amending Decision 2000/418/EC regulating the use of material presenting risks as regards transmissible spongiform encephalopathies (OJ L 001 , 4.1.2001 p. 21)	Amendment of D 2000/418/EC – Extension of the list of SRM (bovine intestines)
	2001/8/EC: Commission Decision of 29 December 2000 amending Decision 2000/764/EC on the testing of bovine animals for the presence of bovine spongiform encephalopathy and updating Annex IV of Decision 98/272/EC on epidemio-surveillance for transmissible spongiform encephalopathies (OJ L 2, 5.1.2001, p. 28)	Amendment of D 2000/764/EC and update of D 98/272/EC
	2001/9/EC: Commission Decision of 29 December 2000 concerning control measures required for the implementation of Council Decision 2000/766/EC concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein (OJ L 2, 5.1.2001, p. 32)	Conditions for feeding certain animal proteins
	2001/25/EC: Commission Decision of 27 December 2000 prohibiting the use of certain animal by-products in animal feed (OJ L 6, 11.1.2001, p. 16)	Prohibition of the use of dead animals in the production of animal feed
	2001/165/EC: Commission Decision of 27 February 2001 amending as regards hydrolysed proteins Decision 2001/9/EC concerning control measures required for the implementation of Council Decision 2000/766/EC concerning certain protection measures with regard to transmissible spongiform encephalopathies and the feeding of animal protein (OJ L 58, 28.2.2001, p. 43)	Amendment of D 2001/9/EC – Hydrolysed proteins

Year	Legal text	Contents
	<p>2001/233/EC: Commission Decision of 14 March 2001 amending Decision 2000/418/EC as regards mechanically recovered meat and bovine vertebral column (OJ L 84, 23.3.2001, p. 59)</p>	<p>Amendment of D 2000/418/EC – Extension of the list of SRM (vertebral column)</p>
	<p>2001/270/EC: Commission Decision of 29 March 2001 amending Decision 2000/418/EC as regards imports from third countries (OJ L 94, 4.4.2001, p. 29)</p>	<p>Amendment of D 2000/418/EC – Imports from third countries</p>
	<p>2001/376/EC: Commission Decision of 18 April 2001 concerning measures made necessary by the occurrence of bovine spongiform encephalopathy in Portugal and implementing a date-based export scheme (OJ L 132, 15.5.2001, p. 17)</p>	<p>Principles for lifting the Portuguese ban under a Date-based Export Scheme (Repeals D 98/653/EC)</p>
	<p>2001/384/EC: Commission Decision of 3 May 2001 amending Decision 2000/418/EC as regards imports from Brazil and Singapore (OJ L 137, 19.5.2001, p. 29)</p>	<p>Amendment of D 2000/418/EC – Extension of the list of derogating third countries</p>
	<p>Regulation (EC) 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 147, 31.5.2001, p. 1)</p>	<p>Harmonized EU rules on prevention, control and eradication of certain TSE</p>
	<p>Commission Regulation (EC) No 1248/2001 of 22 June 2001 amending Annexes III, X and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance and testing of transmissible spongiform encephalopathies (OJ L 173, 27.6.2001, p. 12)</p>	<p>Amendment of R 999/2001 – Surveillance and testing</p>
	<p>Commission Regulation (EC) No 1326/2001 of 29 June 2001 laying down transitional measures to permit the changeover to the Regulation of the European Parliament and of the Council (EC) No 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies, and amending Annexes VII and XI to that Regulation (OJ L 177, 30.6.2001, p. 60)</p>	<p>Amendment of R 999/2001 – Transitional measures</p>
	<p>2001/577/EC: Commission Decision of 25 July 2001 setting the date on which dispatch from Portugal of bovine products under the Date-Based Export Scheme may commence by virtue of Article 22(2) of Decision 2001/376/EC (OJ L 203, 28.7.2001, p. 27)</p>	<p>Date on which dispatch from Portugal of certain bovine products may commence (1/8/2001)</p>

Year	Legal text	Contents
2002	<p>Commission Regulation (EC) No 270/2002 of 14 February 2002 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards specified risk material and epidemio-surveillance for transmissible spongiform encephalopathies and amending Regulation (EC) No 1326/2001 as regards animal feeding and the placing on the market of ovine and caprine animals and products thereof (OJ L 45, 15.2.2002, p. 4)</p>	<p>Amendments of R 999/2001 – SRM, surveillance, animal feeding and placing on the market of ovine and caprine animals and products thereof</p>
	<p>2002/248/EC: Commission Decision of 27 March 2002 amending Council Decision 2000/766/EC and Commission Decision 2001/9/EC with regard to transmissible spongiform encephalopathies and the feeding of animal proteins (OJ L 84, 28.3.2002, p. 71)</p>	<p>Amending Council D 2000/766/EC and Commission D 2001/9/EC with regard to the feeding of animal proteins</p>
	<p>2002/670/EC: Commission Decision of 20 August 2002 amending Council Decision 98/256/EC concerning emergency measures to protect against bovine spongiform encephalopathy (OJ L 228, 24.8.2002, p. 22)</p>	<p>Amendment off D 98/256/EC – Adaptation of some date-based export scheme conditions</p>
	<p>Commission Regulation (EC) No 1494/2002 of 21 August 2002 amending Annexes III, VII and XI to Regulation (EC) No 999/2001 of the European Parliament and the Council as regards monitoring of bovine spongiform encephalopathy, eradication of transmissible spongiform encephalopathy, removal of specified risk materials and rules for importation of live animals and products of animal origin (OJ L 225, 22.8.2002, p. 3)</p>	<p>Amendment of R 999/2001 – continuing of BSE testing in fallen stock. Deletion of the restrictions on trade of bovine embryos. Clarifications of some SRM rules</p>
	<p>2002/1003/EC: Commission Decision of 18 December 2002 laying down minimum requirements for a survey of prion protein genotypes of sheep breeds (OJ L 349, 24.12.2002, p. 105)</p>	<p>Survey of prion protein genotypes of sheep breeds</p>
2003	<p>Commission Regulation (EC) No 260/2003 of 12 February 2003 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the eradication of transmissible spongiform encephalopathies in ovine and caprine animals and rules for the trade in live ovine and caprine animals and bovine embryos (OJ L 37, 13.2.2003, p. 7)</p>	<p>Amendment of R 999/2001 – monitoring in ovine and caprine animals and rules for the trade in live ovine and caprine animals and bovine embryos</p>
	<p>2003/100/EC Commission Decision of 13 February 2003 laying down minimum requirements for the establishment of breeding programmes for resistance to transmissible spongiform encephalopathies in sheep (OJ L 41, 14.2.2003, p. 41)</p>	<p>Laying down minimum requirements for the establishment of breeding programmes for resistance to transmissible spongiform encephalopathies in sheep</p>

Year	Legal text	Contents
	<p>Commission Regulation (EC) No 650/2003 of 10 April 2003 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the import of live ovine and caprine animals (OJ L 95, 11.4.2003, p. 15)</p>	<p>Amendment of R 999/2001 – import conditions of live ovine and caprine animals</p>
	<p>Regulation (EC) No 1128/2003 of the European Parliament and of the Council of 16 June 2003 amending Regulation (EC) No 999/2001 as regards the extension of the period for transitional measures (OJ L 160, 28.6.2003, p. 1)</p>	<p>Amendment of R 999/2001 – extension of the period for transitional measures</p>
	<p>Commission Regulation (EC) No 1053/2003 of 19 June 2003 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards rapid tests (OJ L 152, 20.6.2003, p. 8)</p>	<p>Amendment of R 999/2001 – extension of the list of rapid tests approved for TSE monitoring with two new tests – procedure for possible modification to the test or the test protocol</p>
	<p>Commission Regulation (EC) No 1139/2003 of 27 June 2003 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards monitoring programmes and specified risk material (OJ L 160, 28.6.2003, p. 22)</p>	<p>Amendment of R 999/2001 – targeted testing should be carried out on animals destroyed following confirmation of TSE in ovine and caprine animals – amendments to the list of specified risk material in bovine, ovine and caprine animals – special provisions when harvesting the bovine tongue and head meat</p>
	<p>Commission Regulation (EC) No 1234/2003 of 10 July 2003 amending Annexes I, IV and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council and Regulation (EC) No 1326/2001 as regards transmissible spongiform encephalopathies and animal feeding (OJ L 173, 11.7.2003, p. 6)</p>	<p>Amendment of R 999/2001 – introducing the current provisions of the feed ban into Regulation (EC) No 999/2001 without a fixed time schedule, thus ending the transitional nature of the feed ban</p>
	<p>Commission Regulation (EC) No 1809/2003 of 15 October 2003 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards rules for importation of live bovine animals and products of bovine, ovine and caprine origin from Costa Rica and New Caledonia (OJ L 265, 16.10.2003, p. 10)</p>	<p>Amendment of R 999/2001- deleting Costa Rica and including New Caledonia in the list of countries exempted from certain TSE-related trade conditions for live bovine animals and products of bovine, ovine and caprine origin</p>

Year	Legal text	Contents
	<p>Commission Regulation (EC) No 1874/2003 of 24 October 2003 approving the national scrapie control programmes of certain Member States, and defining additional guarantees, and granting derogations concerning breeding programmes for TSE resistance in sheep pursuant to Decision 2003/100/EC (OJ L 275, 25.10.2003, p. 12)</p>	<p>Derogations for certain Member States concerning breeding programmes for TSE resistance in sheep</p>
	<p>Commission Regulation (EC) No 1915/2003 of 30 October 2003 amending Annexes VII, VIII and IX to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the trade and import of ovine and caprine animals and the measures following the confirmation of transmissible spongiform encephalopathies in bovine, ovine and caprine animals (OJ L 283, 31.10.2003, p. 29)</p>	<p>Amendment of R 999/2001 - modification of the measures following the confirmation of transmissible spongiform encephalopathies in bovine, ovine and caprine animals – amendments to the trade and imports rules of ovine and caprine animals</p>
	<p>Commission Regulation (EC) No 2245/2003 of 19 December 2003 amending Annex III to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards monitoring of transmissible spongiform encephalopathies in ovine and caprine animals (OJ L 333, 20.12.2003, p. 28)</p>	<p>Amendment of R 999/2001 – modification to the ovine and caprine surveillance programme</p>
2004	<p>Commission Regulation (EC) No 836/2004 of 28 April 2004 laying down the transitional measures to be applied by Cyprus with regard to scrapie (OJ L 127, 29.4.2004, p. 48)</p>	<p>Laying down the transitional measures to be applied by Cyprus with regard to Scrapie</p>
	<p>Commission Regulation (EU) No 876/2004 of 29 April 2004 amending Annex VIII to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards trade in ovine and caprine animals for breeding (OJ L 162, 30.4.2004, p. 52)</p>	<p>Amendment of R 999/2001 – trade in bovine and caprine animals for breeding</p>
	<p>Commission Regulation (EU) No 1471/2004 of 18 August 2004 amending Annex XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the import of cervid products from Canada and the United States (OJ L 271, 19.8.2004, p. 24)</p>	<p>Amendment of R 999/2001 – introducing requirements for the import of cervid products from Canada and the United States</p>
	<p>Commission Regulation (EU) No 1472/2004 of 18 August 2004 amending Regulation (EC) No 1874/2003 approving the national scrapie control programmes of certain Member States, and defining additional guarantees, and granting derogations concerning breeding programmes for TSE resistance in sheep pursuant to Decision 2003/100/EC (OJ L 271, 19.8.2004, p. 26)</p>	<p>Amendment of R 1874/2003 - Derogations for certain Member States concerning breeding programmes for TSE resistance in sheep</p>

Year	Legal text	Contents
	<p>Commission Regulation (EU) No 1492/2004 of 23 August 2004 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards eradication measures for transmissible spongiform encephalopathies in bovine, ovine and caprine animals, the trade and importation of semen and embryos of ovine and caprine animals and specified risk material (OJ L 274, 24.8.2004, p. 3)</p>	<p>Amendment of R 999/2001 – modification of eradication measures for bovine, ovine and caprine animals, trade and import conditions for semen and embryos of ovine and caprine animals and the list of specified risk materials</p>
	<p>2004/653/EC: Commission Decision of 16 September 2004 amending Commission Decision 2001/376/EC as regards the dispatch of meat-and-bone meal of mammalian origin and related products from Portugal (OJ L 298, 23.9.2004, p. 25)</p>	<p>Amendment of Commission Decision 2001/376/EC as regards the dispatch of meat-and-bone meal of mammalian origin and related products from Portugal</p>
	<p>Commission Regulation (EU) 1993/2004 of 19 November 2004 amending Regulation (EC) 999/2001 of the European Parliament and of the Council as regards Portugal (OJ L 344, 20.11.2004, p. 12)</p>	<p>Lifting of the embargo on Portugal – Amendment of R 999/2001 as regards SRM and repeal of Commission Decision 2001/376/EC and related measures</p>
2005	<p>Commission Regulation (EC) No 36/2005 of 12 January 2005 amending Annexes III and X to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance for transmissible spongiform encephalopathies in bovine, ovine and caprine (J L 10, 13.1.2005, p. 9)</p>	<p>Amendment of R 999/2001 – Systematic discriminatory tests (differentiation BSE-scrapie) for all confirmed TSE cases in small ruminants</p>
	<p>Commission Regulation (EC) 214/2005 of 9 February 2005 amending Annex III to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards monitoring of transmissible spongiform encephalopathies in caprine animals (OJ L 37, 10.2.2005, p. 9)</p>	<p>Amendment of R 999/2001 – Increased TSE monitoring in goats</p>
	<p>Commission Regulation (EU) No 260/2005 of 16 February 2005 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards rapid tests (OJ L 46, 17.2.2005, p. 31)</p>	<p>Amendment of R 999/2001 - Approval of new rapid tests</p>
	<p>Regulation (EC) No 932/2005 of the European Parliament and of the Council of 8 June 2005 amending Regulation (EC) No 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies as regards the extension of the period for transitional measures (OJ L 163, 23.6.2005, p. 1)</p>	<p>Amendment of R 999/2001 – Extension of the period for transitional measures</p>

Year	Legal text	Contents
	<p>2005/598/EC: Commission Decision of 2 August 2005 prohibiting the placing on the market of products derived from bovine animals born or reared within the United Kingdom before 1 August 1996 for any purpose and exempting such animals from certain control and eradication measures laid down in Regulation (EC) No 999/2001 (OJ L 204, 5.8.2005, p. 22)</p>	<p>Prohibiting the placing on the market of products derived from bovine animals born or reared in the UK before 1 August 1996</p>
	<p>Commission Regulation (EC) No 1292/2005 of 5 August 2005 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards animal nutrition (OJ L 205, 6.8.2005, p. 3)</p>	<p>Amendment of R 999/2001 as regards animal nutrition</p>
	<p>Commission Regulation (EC) No 1974/2005 of 2 December 2005 amending Annexes X and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards national reference laboratories and specified risk material (OJ L 317, 3.12.2005, p. 4)</p>	<p>Amendment of R 999/2001 as regards national reference laboratories and Specified Risk Materials – modification of the age limit for vertebral column in bovines</p>
2006	<p>Commission Regulation (EC) No 253/2006 of 14 February 2006 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards rapid tests and measures for the eradication of TSEs in ovine and caprine animals (OJ L 44, 15.2.2006, p. 9)</p>	<p>Amendment of R 999/2001 as regards rapid tests and TSE eradication measures on small ruminants</p>
	<p>Commission Regulation (EC) No 339/2006 of 24 February 2006 amending Annex XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the rules for importation of live bovine animals and products of bovine, ovine and caprine origin (OJ L 55, 25.2.2006, p. 5)</p>	<p>Amendment of R 999/2001- deleting Brazil, Chile, El Salvador, Nicaragua, Namibia, Botswana and Swaziland from the list of countries exempted from certain TSE-related trade conditions for live bovine animals and products of bovine, ovine and caprine origin</p>
	<p>Commission Regulation (EC) No 546/2006 of 31 March 2006 implementing Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards national scrapie control programmes and additional guarantees and derogating from certain requirements of Decision 2003/100/EC and repealing Regulation (EC) No 1874/2003 (OJ L 94, 1.4.2006, p. 28)</p>	<p>Implementing R 999/2001 as regards national scrapie control programmes and additional guarantees and derogating from certain requirements as regards breeding programmes in certain Member States</p>

Year	Legal text	Contents
	<p>Commission Regulation (EC) No 657/2006 of 10 April 2006 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the United Kingdom and repealing Council Decision 98/256/EC and Decisions 98/351/EC and 1999/514/EC (OJ L 116, 29.4.2006, p. 9)</p>	<p>Lifting of the embargo on the United Kingdom – Amendment of R 999/2001 as regards SRM, BSE monitoring and repeal of Commission Decision 98/256/EC and related measures</p>
	<p>Commission Regulation (EC) No 688/2006 of 4 May 2006 amending Annexes III and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the monitoring of transmissible spongiform encephalopathies and specified risk material of bovine animals in Sweden (OJ L 120, 5.5.2006, p. 10)</p>	<p>Amendment of R 999/2001 as regards BSE monitoring in Sweden</p>
	<p>Commission Regulation (EU) No 1041/2006 of 7 July 2006 amending Annex III to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards monitoring of transmissible spongiform encephalopathies in ovine animals (OJ L 187, 8.7.2006, p. 10)</p>	<p>Amendment of R 999/2001 as regards TSE monitoring in sheep. Increased number of tests</p>
	<p>Regulation (EC) No 1923/2006 of the European Parliament and of the Council of 18 December 2006 amending Regulation (EC) No 999/2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 404, 30.12.2006, p. 1)</p>	<p>Amendment of R 999/2001 as regards categorisation of countries, specified risk materials, TSE surveillance, Import conditions</p>
2007	<p>2007/182/EC: Commission Decision of 19 March 2007 on a survey for chronic wasting disease in cervids (OJ L 84, 24.3.2007, p. 37)</p>	<p>Laying down rules for a survey for Chronic wasting disease in cervids</p>
	<p>2007/315/EC: Commission Decision of 30 April 2007 laying down specific measures to be applied by Cyprus with regard to scrapie (OJ L 118, 8.5.2007, p. 23)</p>	<p>Specific measures for Cyprus regarding scrapie</p>
	<p>Commission Regulation (EC) No 722/2007 of 25 June 2007 amending Annexes II, V, VI, VIII, IX and XI to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 164, 26.6.2007, p. 7)</p>	<p>Amendment of R 999/2001. End of transitional measures from 1 July 2007</p>
	<p>Commission Regulation (EC) No 727/2007 of 26 June 2007 amending Annexes I, III, VII and X to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 165, 27.6.2007, p. 8)</p>	<p>Amendment of R 999/2001. modification of eradication measures for ovine and caprine animals and monitoring in ovine and caprine animals</p>

Year	Legal text	Contents
	2007/453/EC: Commission Decision of 29 June 2007 establishing the BSE status of Member States or third countries or regions thereof according to their BSE risk (OJ L 172, 30.6.2007, p. 84)	Establishing the BSE status of Member States and certain third countries
	2007/667/EC: Commission Decision of 15 October 2007 authorising the use of at risk bovine animals until the end of their productive lives in Germany following official confirmation of the presence of BSE (OJ L 271, 16.10.2007, p. 16)	BSE cohort culling in Germany
	Commission Regulation (EC) No 1275/2007 of 29 October 2007 amending Annex IX to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 284, 30.10.2007, p. 8)	Amendment of R 999/2001. Import conditions for products of animal origin from bovine, ovine and caprine animals
	Commission Regulation (EC) No 1428/2007 of 4 December 2007 amending Annex VII to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 317, 5.12.2007, p. 61)	Amendment of R 999/2001. Derogation: possibility to delay the destruction of animals in TSE affected flocks for 5 breeding years
2008	Commission Regulation (EC) No 21/2008 of 11 January 2008 amending Annex X to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the lists of rapid tests (OJ L 9, 12.1.2008, p. 3)	Amendment of R 999/2001. List of rapid tests
	Commission Regulation (EC) No 315/2008 of 4 April 2008 amending Annex X to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the lists of rapid tests (OJ L 94, 5.4.2008, p. 3)	Amendment of R 999/2001. List of rapid tests
	Commission Regulation (EC) No 357/2008 of 22 April 2008 amending Annex V to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 111, 23.4.2008, p. 3)	Amendment of R 999/2001. Modification of the age limit for vertebral column in bovines
	Commission Regulation (EC) No 571/2008 of 19 June 2008 amending Annex III to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards the criteria for revision of the annual monitoring programmes concerning BSE (OJ L 161, 20.6.2008, p. 4)	Amendment of R 999/2001. Criteria for the revision of annual BSE monitoring programmes

Year	Legal text	Contents
	<p>Commission Regulation (EC) No 746/2008 of 17 June 2008 amending Annex VII to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 202, 31.7.2008, p. 11)</p>	<p>Amendment of R 999/2001. modification of eradication measures for ovine and caprine animals</p>
:	<p>2008/661/EC: Commission Decision of 1 August 2008 amending Decision 2007/182/EC on a survey for chronic wasting disease in cervids (OJ L 215, 12.8.2008, p. 8)</p>	<p>Extending the survey for Chronic wasting disease in cervids</p>
	<p>Commission Regulation (EC) No 956/2008 of 29 September 2008 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 260, 30.9.2008, p. 8)</p>	<p>Amendment of R 999/2001. Authorisation for the use of fishmeal in milk replacers intended for feeding to young animals of ruminant species</p>
	<p>Commission Decision (EC) 2008/829 of 30 October 2008 amending the Annex to Decision 2007/453/EC establishing the BSE status of Member States or third countries or regions thereof according to their BSE risk (OJ L 294, 1.11.2008, p. 14)</p>	<p>Member States and third countries or regions thereof are classified into three risk categories as regards BSE (negligible risk, controlled risk and undetermined risk) according to OIE standards</p>
	<p>Commission Decision (EC) 2008/908 of 28 November 2008 authorising certain Member States to revise their annual BSE monitoring programme (OJ L 327, 5.12.2008, p. 24)</p>	<p>Certain Member States may revise their annual BSE monitoring programme</p>
2009	<p>Commission Regulation (EC) No 220/2009 of 11 March 2009 amending Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies, as regards the implementing powers conferred on the Commission (OJ L 87, 31.3.2009, p. 155)</p>	<p>Amendment of R 999/2001. Modification of the implementing powers conferred on the Commission</p>
	<p>Commission Regulation (EC) No 103/2009 of 3 February 2009 amending Annexes VII and IX to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 34, 4.2.2009, p. 11)</p>	<p>Amendment of R 999/2001. Modification of eradication measures for ovine and caprine animals</p>

Year	Legal text	Contents
	<p>Commission Regulation (EC) No 162/2009 of 26 February 2009 amending Annexes III and X to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 55, 27.2.2009, p. 11)</p>	<p>Amendment of R 999/2001. Modification of certain approved laboratory methods for the diagnostic of TSEs</p>
	<p>Commission Regulation (EC) No 163/2009 of 26 February 2009 amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (OJ L 55, 27.2.2009, p. 17)</p>	<p>Amendment of R 999/2001. Modification of certain feed ban provisions</p>

In 2005, in order to clarify all previous legislation on TSEs, the European Commission presented "The TSE Roadmap" (The TSE Roadmap, 2005) and in 2010 the Commission communicated to Parliament its views and intentions for the future in "The TSE Road Map 2 - A strategy Paper on Transmissible Spongiform Encephalopathies for 2010 - 2015". In the Communication, the European Commission outlines its vision on further changes to the TSE eradication measures and the TSE surveillance regime.

On 6 July 2011, the European Parliament (EP) adopted a resolution on the implementation and outlook for EU legislation on TSEs and on related feed and food controls.

The resolution states that despite the fall in BSE cases in the EU, surveillance must remain vigilant and any change to BSE safety rules must maintain high animal and public health standards. However, the ban on feeding animal protein to non-ruminants, such as pigs, could gradually be lifted if more safeguards are implemented. The resolution indicates that the European Commission is shortly expected to review changes to current EU laws, which

could include new rules on removing specific risk materials from animal feed, a gradual relaxation of the animal protein feed ban, changes to cohort culling policy and a higher age limit for BSE testing (EP Resolution, 6 July 2011).

10 - EXPERIMENTAL STUDIES

The Microbiology and Immunology Section of the Department of Veterinary Pathology, Hygiene and Public Health of the School of Veterinary Medicine at the University of Milan has been involved for long in the study of TSEs (Pollera et al., 2003; Poli et al., 2004; Magri et al., 2005; Bareggi et al., 2009; Bondiolotti et al., 2010). Just in this context and field I carried out my doctoral studies, being involved in three experimental projects aimed to clarify different aspects of prion diseases. In all of them we used wild-type mouse bioassays, which are the gold standard for assessing the biological properties of prions (Bruce et al., 2002; Beck et al., 2010 b).

In the first study our goal was to assess the therapeutic and/or preventive activity on TSEs of the chronic administration of a new γ -secretase modulator. The second research investigated the ability to identify BSE in presence of scrapie. The third project was aimed to study the effects induced by chronic administration of lipid enriched/depleted specific diets on the pathogenesis of prion diseases.

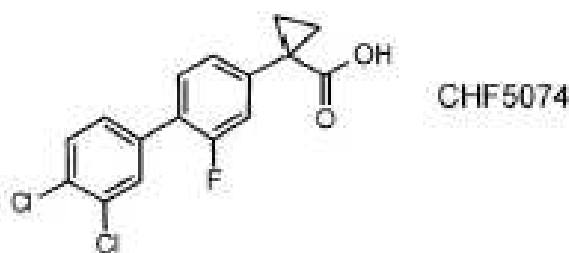
11 - THERAPEUTIC EFFECT OF CHF5074, A NEW γ -SECRETASE MODULATOR, IN A MOUSE MODEL OF SCRAPIE

The laboratory analysis of this experimental study were performed at the Italian Reference Laboratory for TSEs (CEA), Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy, during my training period there (November 1st, 2009 – December 6th, 2009).

The similarities between TSEs and AD suggest that drugs effective for the treatment of AD can be successfully used for the treatment of TSEs too.

Some non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to allosterically modulate the activity of γ -secretase. 1-(3',4'-Dichloro-2-fluoro[1,1'-biphenyl]-4-yl)-cyclopropanecarboxylic Acid (CHF5074) (Figure 10) is a new NSAID γ -secretase modulator, that is proposed as a potential therapeutic agent in AD, because of its promising effects and its lack of toxicity.

Figure 10. Structural formula of CHF5074 (Imbimbo et al., 2007 a).



The chronic oral administration of CHF5074 in two different transgenic murine models of AD reduces the deposition of A β in the brain, both in terms of area occupied by plaques and number of plaques, and attenuates the spatial memory impairment (Imbimbo et al., 2007 a, b; Imbimbo et al., 2009; Imbimbo et al., 2010 a). Moreover it seems to have a good bioavailability, since it is orally well absorbed (50 %) and it is slowly eliminated from the blood (half-life = 20 h) (Peretto et al., 2005). In contrast with other NSAIDs, CHF5074 has no toxic effects, because it is endowed with selective A β ₄₂-lowering activity but devoid of COX and NOTCH inhibitory activity, and so it is suitable for chronic use (Imbimbo et al., 2007 b; Imbimbo et al., 2009).

The aim of the present study is to assess the therapeutic and/or preventive activity of CHF5074 on murine model experimentally infected with the causative agent of prion diseases.

11.1 – MATERIALS AND METHODS

11.1.1 - Animals and Treatments

Ninety-one CD1 female mice aged 3-4 weeks and initially weighing 10-12 g were under survey. Outbred CD1 mouse strain was used to mimic the conditions of a previous study with a γ -secretase inhibitor (Spilman et al., 2008). Mice were maintained in a controlled environment (22 \pm 1 $^{\circ}$ C, 55 \pm 5% relative humidity, 12-h light/dark cycles) and fed *ad libitum*.

Mice were neither be handled nor disturbed for a week, to allow them to settle in.

Then animals were randomly divided into two groups according to the route of infection (intracerebrally, i.c., or intraperitoneally, i.p.) with the mouse-adapted Rocky Mountains Laboratory (RML) scrapie. A 10% (weight/volume) homogenate of RML-infected CD1 brain was diluted in sterile saline to a final concentration of 1%, and 25 µl or 50 µl of the suspension were injected i.c. or i.p., respectively.

The i.c. inoculation occurred always on the right side of the skull, at the thalamic level. After analgesia with lidocaine 2 %, each animal was lying on his left side and the skin of his skull was stretched: the landmark for the injection was located 3 mm dorsal to the middle of an imaginary line drawn between the lateral canthus of the eye and the ear. The insulin syringe used had a cylinder spacer on its needle, which ensured the constant depth of the injection, performed perpendicularly to the skull.

The i.p. inoculation was made into the caudal left (to avoid the cecum on the right) abdominal quadrant. Each mouse was restrained manually and held with the head and body tilted downward. The injection was performed with an insulin syringe: the tip of the needle was inserted through the skin and just past the abdominal wall, avoiding to perforate abdominal organs.

The day of scrapie inoculation was considered as Day 1 of the study. Animals in the infected groups (i.c. or i.p.) were then split into two subgroups, each consisting of 15 CHF5074-treated or vehicle-treated mice (i.c. infected CHF5074-treated, i.c. infected vehicle-treated; i.p. infected CHF5074-treated; i.p. infected vehicle-treated). Similarly, uninfected animals were divided into two subgroups of 8 animals each, then CHF5074-treated and inoculated i.c. or i.p. respectively with the same volume of a 1% brain homogenate from

uninfected CD1 mice (i.c. uninfected CHF5074-treated; i.p. uninfected CHF5074-treated). Furthermore, a negative group was created, uninfected and vehicle-treated. CHF5074-treated animals received an oral diet medicated with CHF5074 (375 ppm, corresponding to approximately 60 mg/kg/day) while vehicle-treated animals were given a standard diet. CHF5074 was manufactured by Minakem (Beuvry-la-Forêt, France) and CHF5074-medicated diet (4RF21 certificate – PF1610 – mice and rats) by Mucedola (Settimo Milanese, Italy). CHF5074-medicated and standard diets were started 14 days before scrapie strain inoculation (Table 6).

Table 6. Mice were randomly divided into groups depending on the route of inoculum and on the type of treatment.

Groups	Route of inoculum	Treatment	Number of animals
i.c. Infected vehicle-treated	intracerebral	standard diet	15
i.c. Infected CHF5074-treated	intracerebral	CHF5074	15
i.p. Infected vehicle-treated	intraperitoneal	standard diet	15
i.p. Infected CHF5074-treated	intraperitoneal	CHF5074	15
i.c. Uninfected CHF5074-treated	intracerebral	CHF5074	8
i.p. Uninfected CHF5074-treated	intraperitoneal	CHF5074	8
Uninfected vehicle-treated	/	standard diet	15

Mice were observed daily to monitor for onset and development of neurological signs, such as kyphotic posture, ataxia, proprioceptive deficits, lethargy and frozen posture. All mice were euthanized with CO₂ when severe symptoms were apparent as previously described (Thackry et al., 2002; Meeker et al., 2005).

At necropsy brains (cerebral hemispheres, brain stem and cerebellum) were removed. Each brain was then divided longitudinally; one part was fixed in

10% formalin for histopathological and immunohistochemical analysis, and the other was stored at -20 °C for Western Blot analysis.

11.1.2 - Western blot analysis

Ten percent (w/v) homogenates of each frozen brain were prepared in lysis buffer (10% N-lauroylsarcosine diluted in Tris Buffer Saline -TBS- pH 7.4). After incubation for 20-30 minutes at room temperature, they were clarified by centrifugation at 22000x g for 20 minutes at 10 °C (Ultracentrifuge Optima TLX, Rotor TLA 55, Beckman Coulter, Fullerton, CA). A rate of 1 mL was removed from each supernatant and digested with pK (40 µg/mL; Product N° FS-M-112; Fisher Molecular Biology, Trevose, USA) for 1 hour at 37 °C with continuous shaking. After digestion, 10 µL of the proteinase K inhibitor phenylmethyl sulfonyl fluoride (PMSF 100 mM; Product N° A0999; Applichem, Germany) were added. The samples were then centrifuged at 215000x g for 1 hour at 10 °C (Ultracentrifuge Optima TLX, Rotor TLA 110, Beckman Coulter, Fullerton, CA). The pellets obtained were dissolved in 50 µL of Laemmli buffer. After boiling for 5-10 minutes at 99 °C, 10 µL of each extract (10 mg of tissue) were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis on a 12 % handmade minigel (acrylamide to bisacrylamide ratio of 37.5:1) and then transferred onto PVDF membranes (Product N° IPVH20200; Immobilon P, Millipore, Billerica, MA). Blots were blocked with TBS-BSA 5% and incubated at 4 °C overnight with the monoclonal antibody SAF 70 (0,5 µg/ml; Product N° A023206; Spi Bio, Cayman Chemical, Ann Arbor, MI), recognising human PrP residues 142-160. The immunodetection was carried out with an alkaline phosphatase-conjugated goat anti-mouse IgG

(Product N° 12000-09; Prionics AG, Switzerland) revealed by a chemiluminescent substrate (Product N° 170-5012; ImmunoStar, Bio-Rad Laboratories, Hercules, CA) and then visualised onto Hyperfilm ECL (Product N° 28906837; GE-Healthcare, St. Giles, UK) or by a UVI Prochemi (Uvitec, Cambridge, UK) analysis system. PrP^{Sc} signals of each sample were quantified using UVI Bandmap software (Uvitec, Cambridge, UK), comparing them with the PrP^{Sc} signals of a classical scrapie sample, used as reference.

11.1.3 - Histopathological and immunohistochemical

analysis

Following fixation, brains were coronally cut into five sections (medulla, pons and cerebellum, mid-brain, diencephalon, telencephalon) (Fraser et al., 1968). These samples were processed (Table 7) and embedded in paraffin wax according to standard histopathological procedures.

Table 7. Mouse processing procedure.

Step	Reagent	Time
1	95 % Alcohol	2 h
2	95 % Alcohol	2 h
3	95 % Alcohol	2 h
4	95 % Alcohol	3 h
5	Absolute Alcohol	30 min
6	Absolute Alcohol	30 min
7	Absolute Alcohol + Xylene (1:1)	30 min
8	Xylene	1 h 30 min
9	Xylene	1 h 30 min
10	Xylene + Wax (4:1)	30 min
11	Wax	1 h
12	Wax	1 h

The 3 μ m-thick sections obtained from each hemisphere were placed on slides with positive electrostatic charge and left for 24 hours at 37 °C. An hematoxylin-eosin staining was performed for each brain section (Table 8).

Table 8. Haematoxylin-eosin staining procedure.

Step	Reagent	Time
1	Xylene	3 min
2	Absolute Alcohol	3 min
3	Mayer's Haematoxylin	17 min
4	Tap water	5 min
5	0,1 % Acid Alcohol	1 min
6	Tap water	5 min
7	1,5 % Lithium Carbonate	30 sec
8	Purified Water	2 min
9	Eosin	31 sec
10	Tap water	5 min
11	Absolute Alcohol	1 min
12	Absolute Alcohol	3 min
13	Xylene	3 min

Slides for immunohistochemical analysis were dewaxed and rehydrated by routine methods and then immersed in 98% formic acid for 15 minutes. After washing in water, the sections were autoclaved for 30 minutes at 121 °C in citrate buffer (pH 6.1) to unmask antigenic sites. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide diluted in methanol for 20 minutes at room temperature and samples were left overnight in distilled water at 2-8 °C. To block non-specific tissue antigens, the sections were incubated with 2% horse blocking serum (pH 7.4) for 20 minutes at room temperature and then incubated for 1 hour at room temperature with the mouse monoclonal antibody ICSM 35 diluted 1:1000 (Product N°0130-03501, D-Gen Ltd. London UK). ICSM 35 monoclonal antibodies have been shown to efficiently immunoprecipitate native PrP^{Sc} and recognised epitopes between residues 93-102 (Khalili-Shirazi et al., 2005). After rinsing in TBST, a biotinylated goat anti-mouse secondary antibody (1:200 dilution, Vectastain ABC kit PK-4002, Vector Laboratories) was applied to the tissue sections for 30 minutes at room temperature, followed by the avidin-biotin-peroxidase complex (Vectastain ABC kit PK-4002, Vector Laboratories), according to the manufacturer's protocol. After rinsing in TBST, PrP^{Sc} immunoreactivity was visualised using 3,3'-diaminobenzidine (Dako Liquid DAB + Substrate Chromogen System Cod. K3468, Dako) as a chromogen, blocked with distilled water. The sections were then counterstained with Mayer's haematoxylin (Table 9).

Table 9. Counterstain in Mayer's haematoxylin procedure.

Step	Reagent	Time
1	Tap Water	5 min
2	Mayer's Haematoxylin	1 min 10 sec
3	Tap Water	7 min
4	Absolute Alcohol	3 min
5	Absolute Alcohol	3 min
6	Absolute Alcohol	3 min
7	Xylene	3 min
8	Xylene	3 min
9	Xylene	3 min

Spongiosis and PrP^{Sc} deposition in different encephalic areas (medulla, cerebellum, mid-brain, hypothalamus, thalamus, hippocampus, para terminal body, frontal cortex and parietal cortex) were evaluated by light microscopy and an intensity grade was assigned to vacuolation and to different patterns detected: absent (0), slight (1), moderate (2), marked (3), very marked (4).

11.1.4 - Statistical analysis

The survival analysis was performed using the Log-Rank test. To evaluate the differences in the PrP^{Sc} among the groups, the results of quantification performed by Western blot analysis were analysed by ANOVA, after checking the assumption of normality and homogeneity of variances. Data obtained by histological and immunohistochemical analysis were analysed by Student t test for unpaired data with equal variances.

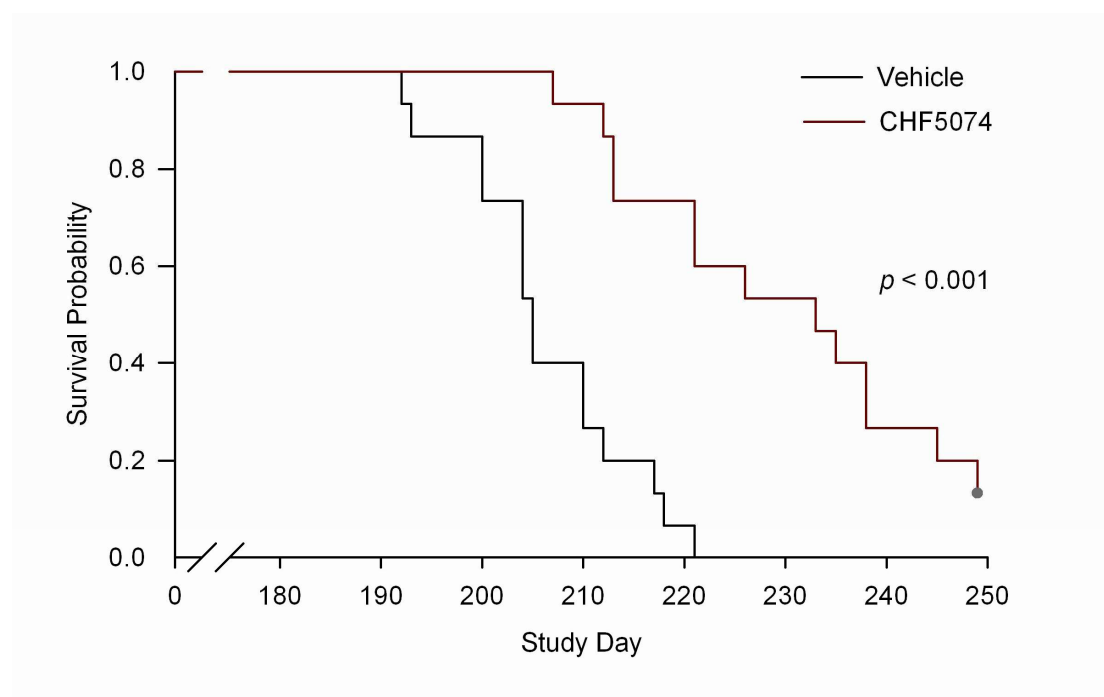
11.2 - RESULTS

11.2.1 - Survival of the animals

Uninfected treated and control animals did not show any clinical signs of disease. Early clinical signs of prion disease induced by RML strain of scrapie set out in control mice appearing at about Day 130 in the i.c.-infected animals and at Day 180 in i.p.-infected animals. Mice initially displayed ruffled coats, assumed kyphotic posture and a tendency to display a straight tail. These early signs of prion disease were followed by ragged or wobbly gait, ataxia and proprioceptive deficits, as evidenced by clasped feet when raised by tail. Then, they became extremely listless, lethargic and cachectic and appeared to adopt a frozen posture. All mice of the infected groups, except for two animals in the i.p.-infected CHF5074-treated group, reached terminal phase of the disease. Sacrifice of the i.c.-infected mice in terminal phase started at Day 143 and ended at Days 177 and 178 in control and CHF5074-treated animals, respectively. Sacrifices of the i.p.-infected mice reaching clinical end-point took place between Day 192 and Day 221 for the control group, and between Day 207 and Day 249 for the treated group. The two CHF5074-treated mice showing no abnormal clinical signs were steadily fed with the medicated diet until Day 318 and then put on standard diet for 44 additional, days, to verify if the absence of the drug consumption would have allowed the appearance of scrapie-related symptoms. Again, no clinical signs of disease were observed. At the end of this period, these two animals were sacrificed. Regarding i.c.-infected mice, no differences in median survival times were observed between CHF5074-treated (161 days) and control (161 days) mice. On the contrary, a

significant difference emerged between the median survival times of i.p.-infected controls (205 days) and CHF5074-treated (226 days) animals ($p < 0.001$ Log-Rank test) (Figure 11).

Figure 11. Survival curve of intraperitoneally infected mice. Log-Rank test indicates a significant difference ($p < 0.001$) between the median survival time of vehicle- and CHF5074-treated animals (205 versus 226 days, respectively).



11.2.2 - Western blot analysis

All mice sacrificed at clinical end stage of disease showed presence of PrP^{Sc} in their brains, characterised by three bands corresponding to di-, mono- and un-glycosylated forms with a molecular weight between 30 and 20 kDa. Representative Western blot profiles of PrP^{Sc} extracted from the brains of i.c.- and i.p.-infected mice are shown in Figure 12 and the corresponding quantification of densitometric analysis is given in Figure 13.

Figure 12. Representative Western blot profiles of PrP^{Sc} extracted from the brain of intracerebrally (panel A) and intraperitoneally (panel B) infected mice. Lanes A1-A3 show three vehicle-treated mice, lanes B1-B4 deal with four CHF5074-treated mice, and lane C- considers a control non-infected mouse. PrP^{Sc} signals of each sample were quantified comparing them with the PrP^{Sc} signals of an ovine with classical scrapie (lane C+), used as reference. M.w. indicates molecular mass markers. Membrane was probed with monoclonal antibody SAF 70.

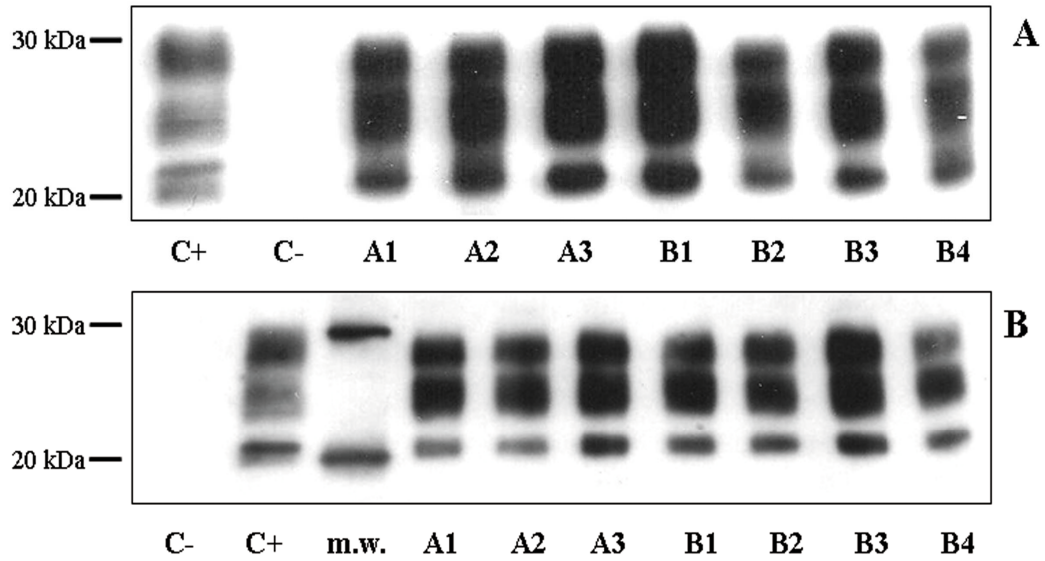
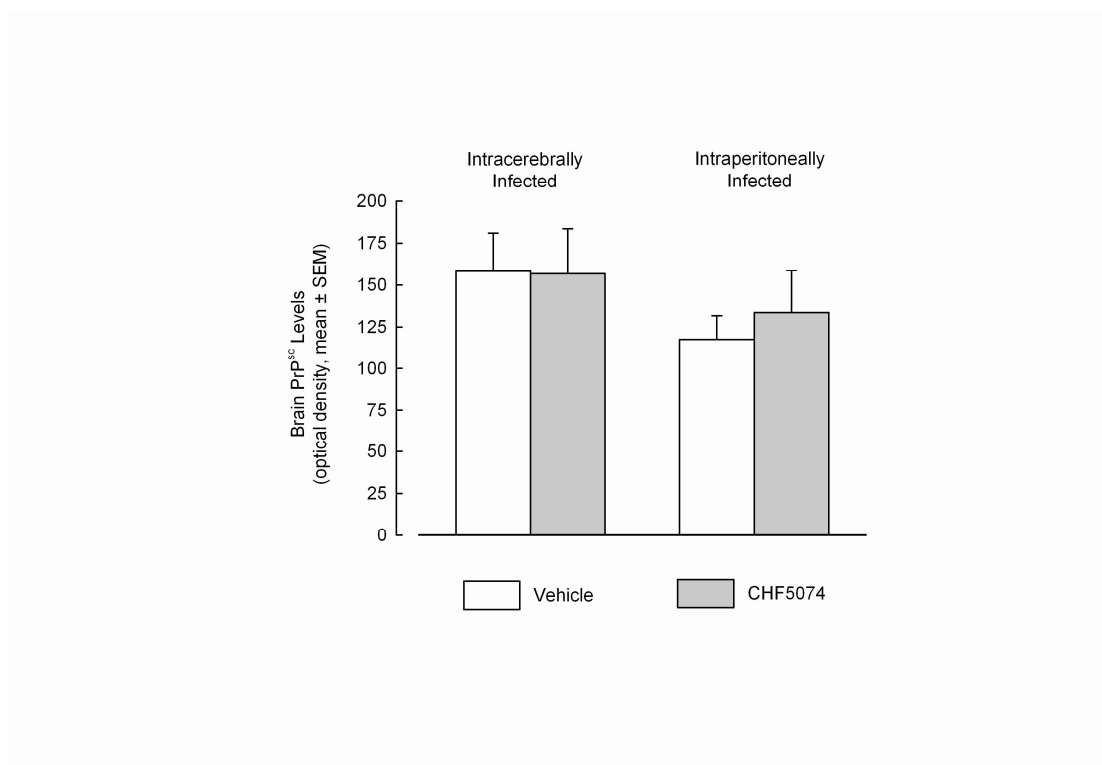


Figure 13. Quantification of PrP^{Sc} signals obtained from Western blot analyses of brains of intracerebrally (panel A) and intraperitoneally (panel B) infected mice treated with vehicle or CHF5074. Columns indicate the mean of optical densities. Error bars represent the standard error of the means.



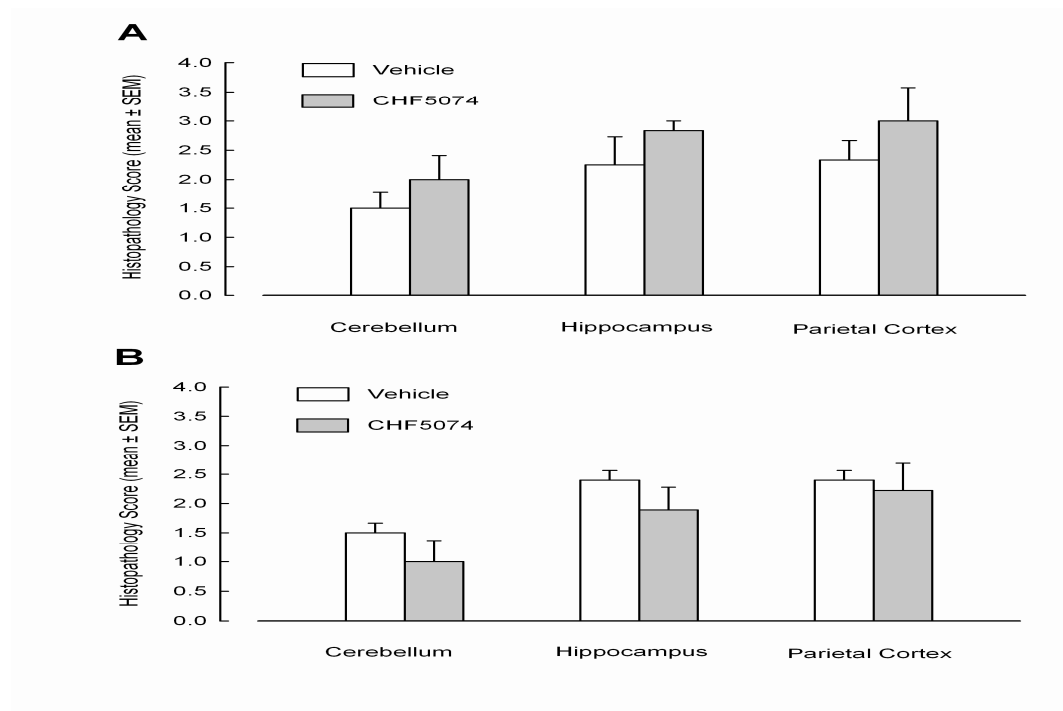
No significant differences appeared between vehicle- and CHF5074-treated groups in brain PrP^{Sc} levels both in i.c- and i.p.-infected animals. Western blot analysis performed on the brains of mice sacrificed without clinical signs of disease, including the two i.p.-infected mice treated with CHF5074, did not reveal the presence of PrP^{Sc}.

11.2.3 - Histopathological and immunohistochemical analysis

Hematoxylin-eosin staining allowed to detect and score spongiosis in nervous tissue and to create a lesion profile of the different encephalic areas. Figure

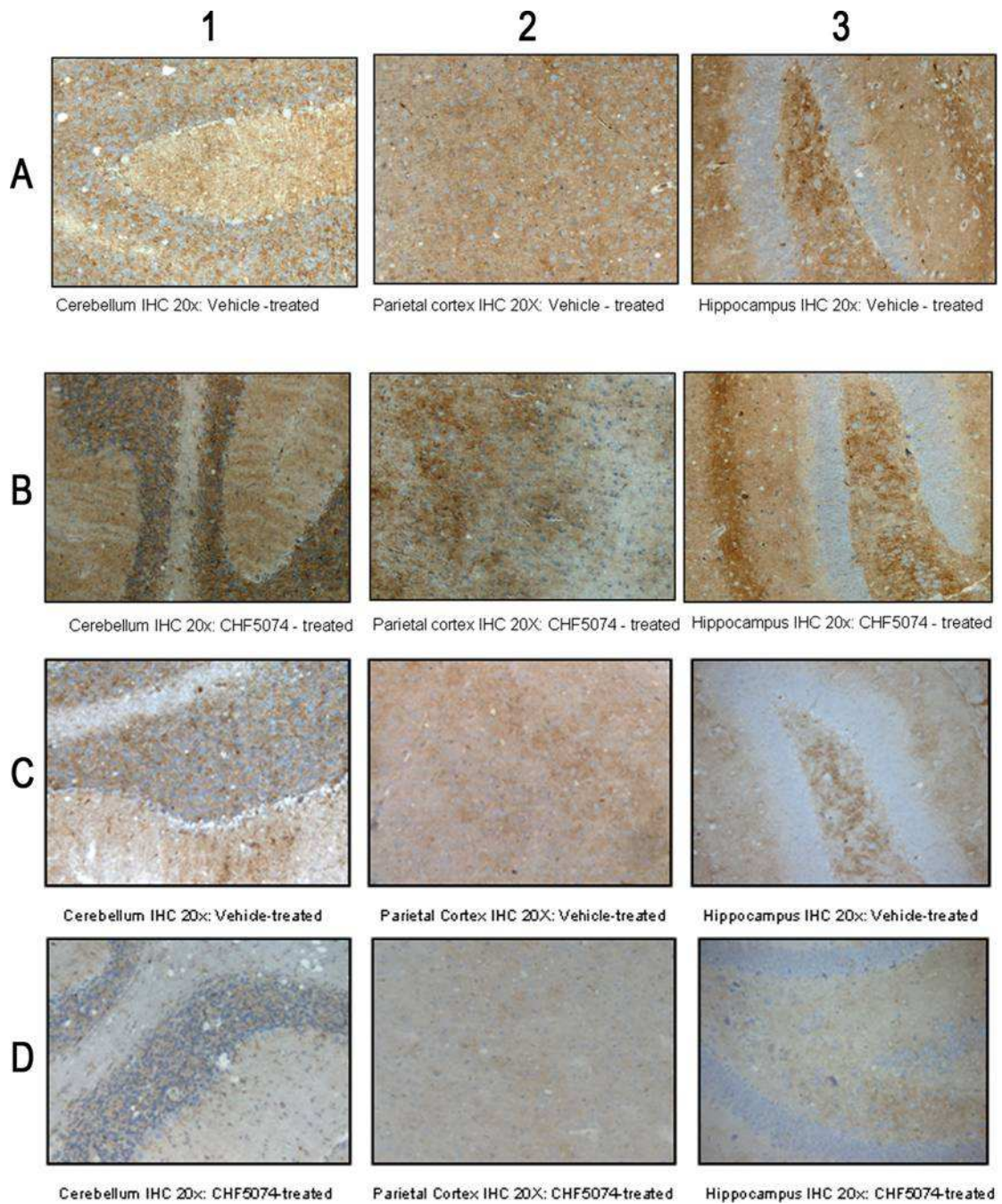
14 illustrates mean spongiosis severity score in different brain areas of the i.c. and i.p. infected mice. No significant differences were found between vehicle- and CHF5074-treated groups.

Figure 14. Severity of spongiosis in cerebellum, hippocampus and parietal cortex in hematoxylin-eosin stained samples of intracerebrally (panel A) and intraperitoneally (panel B) infected mice treated with vehicle or CHF5074. Columns indicate the mean of spongiosis severity for each group. Error bars represent the standard error of the means.



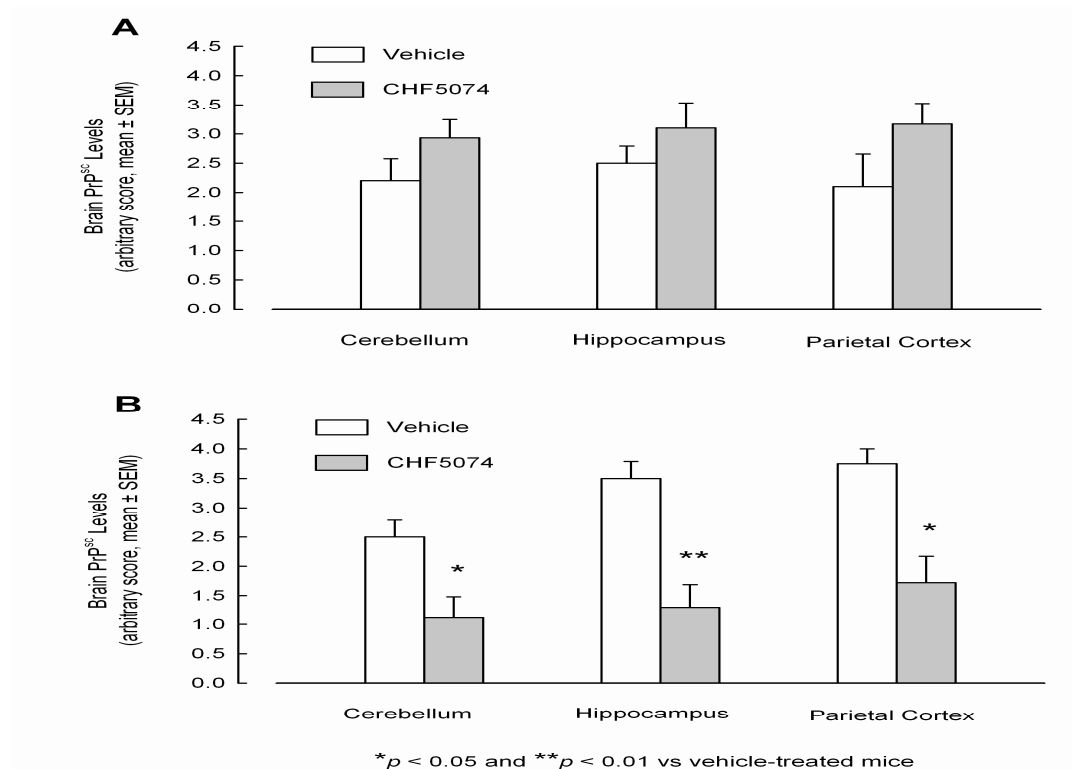
Immunohistochemical analysis confirmed presence of PrP^{Sc} in all the samples from symptomatic animals. Representative images of the i.c.- and i.p.-infected mice are presented in Figure 15.

Figure 15. Representative immunohistochemical staining with monoclonal antibody ICSM 35. Panel A: coronal brain sections of intracerebrally infected mice treated with vehicle (A1: Cerebellum IHC 20x; A2: Parietal Cortex IHC 20x; A3: Hippocampus IHC 20x); Panel B: coronal brain sections of intracerebrally infected mice treated with CHF5074 (B1: Cerebellum IHC 20x; B2: Parietal Cortex IHC 20x; B3: Hippocampus IHC 20x); Panel C: coronal brain sections of intraperitoneally infected mice treated with vehicle (C1: Cerebellum IHC 20x; C2: Parietal Cortex IHC 20x; C3: Hippocampus IHC 20x); Panel D: coronal brain sections of intraperitoneally infected mice treated with CHF5074 (D1: Cerebellum IHC 20x; D2: Parietal Cortex IHC 20x; D3: Hippocampus IHC 20x).



Quantification of PrP^{Sc} deposition in i.c. infected mice did not differ between vehicle- and CHF5074-treated mice (Figure 16A). Conversely, within i.p. infected mice, CHF5074-treated animals showed a significant lower amount of PrP^{Sc} in their cerebellum ($p < 0.05$ Student test for unpaired samples), hippocampus ($p < 0.01$ Student test for unpaired samples) and parietal cortex ($p < 0.05$ Student test for unpaired samples) than vehicle-treated animals (Figure 16B).

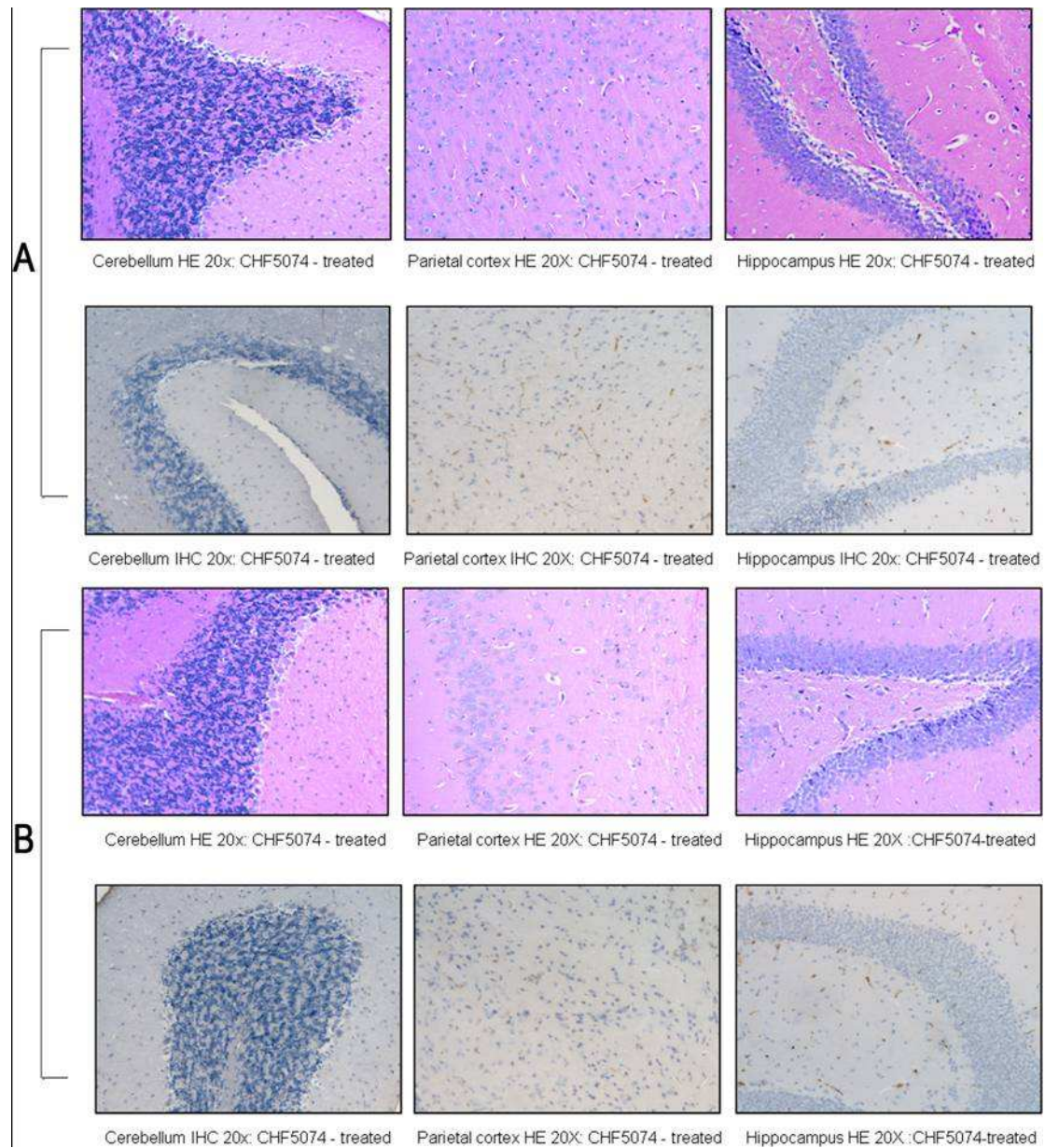
Figure 16. Mean quantification scores of PrP^{Sc} deposition in cerebellum, hippocampus and parietal cortex of intracerebrally (A) and intraperitoneally (B) infected mice treated with vehicle or CHF5074. Columns indicate mean severity score of PrP^{Sc} staining by immunohistochemistry (ICSM 35 monoclonal antibodies). Error bars represent the standard error of the means.



Histological and immunohistochemical analysis performed on the brains of the two i.p. infected animals treated with CHF5074 without neurological signs, disclosed the presence neither of PrP^{Sc} nor of neuropathological lesions

associated to prion diseases. Histopathology and immunohistochemistry images of these two animals are reported in Figure 17.

Figure 17. Histopathological (HE) and immunohistochemical (IHC) images of two intraperitoneally-infected mice (Panel A: animal H12; Panel B: animal H13) treated with CHF5074 which did not develop neurological signs and survived.



11.3 - DISCUSSION

Chronic oral administration of CHF5074 with the diet (about 60 mg/kg/day) for up to 8 months was well tolerated by both scrapie-infected and non-infected CD1 mice. Body weight and clinical signs of CHF5074-treated animals were similar to those of control mice. Good tolerability of CHF5074 after prolonged oral administration has also been observed in previous studies employing other mice strains (Imbimbo et al., 2010 a).

Two intraperitoneally-infected mice treated with a CHF5074-medicated diet did not develop any prion disease. We can assume that their inoculations failed, even though 2 inefficient inoculations out of 60 are not much likely to occur in the same group, i.e. CHF5074-treated, intraperitoneally-infected animals, just by chance ($p = 0.059$, Fisher's exact test). These animals remained asymptomatic even after CHF5074-treatment was over, and showed no neuropathological or immunohistochemical abnormalities (Figure 17). Anyway, in both these cases the median survival time of i.p.-infected CHF5074-treated animals remains significantly higher than the median survival time of i.p.-infected control mice (i.p. < 0.001 Log Rank test).

Onset timing and clinical sign pattern observed in other infected animals are compatible with those described in mice infected by RML scrapie strain (Thackry et al., 2002; Meeker et al., 2005). Histology showed spongiosis, a neuropathological feature typical of TSEs, while immunohistochemical and Western blot analysis confirmed the presence of PrP^{Sc} in the brain of these animals without significant regional differences.

The significant difference related to survival time displayed by CHF5074-treated and control mice in the i.p. infected group was not observed in the i.c.

infected animals. This suggests that CHF5074 concentrations at brain level were not high enough to counteract the substantive infection load achieved after intracerebral scrapie inoculation. Plasma CHF5074 concentrations measured in two i.p.-infected mice were around 270 μM (263 e 282 μM , respectively). We did not measure drug brain levels in these animals that, based on previous studies, were expected to be about 2-3% of the corresponding plasma levels (i.e., 5-8 μM) (Imbimbo et al., 2007; Imbimbo et al., 2009). This means that the delayed disease onset due to CHF5074 treatment should occur during prion propagation from periphery to brain. Histological and immunohistochemical analysis of the brains of i.c. infected mice did not show significant differences between vehicle- and CHF5074-treated animals. Conversely, immunohistochemical analysis of i.p. infected mice indicated noticeably lower PrP^{Sc} deposits in different brain areas (cerebellum, hippocampus and parietal cortex) of CHF5074-treated animals compared to controls. However, Western blot analysis of a limited brain area did not confirm meaningful differences in PrP^{Sc} accumulation between treatment groups. This apparent discrepancy may be due to the fact that immunohistochemistry of paraffin-embedded tissues can highlight PrP^{Sc} deposits at cellular level and their distribution in different brain areas, whereas Western blot assay detects PrP^{Sc} at molecular level but can not localize the protein as sample preparation requires a homogenization step. Anyway, the ability of CHF5074 in reducing PrP^{Sc} brain deposits needs to be confirmed in further experiments, using antibodies recognising the same PrP residues in both immunohistochemistry and Western blot and performing immunobiochemical analysis on different brain areas of the same sample.

Histological analysis of the brains of i.p. infected mice did not encounter significant differences between treatment groups, maybe because spongiosis is not directly proportional to PrP^{Sc} deposition in the final stages of disease (Malucci et al., 2003).

The protective effect of CHF5074 in this murine model of prion disease must be further detected. Prion diseases are characterised by accumulation of large amounts of protease-resistant aggregates of an altered isoform (PrP^{Sc}) of prion protein (PrP^C). PrP^C normal isoform is a copper binding glycoprotein expressed on the surface some cell types, mainly on neurons. Though this protein has a relatively short half-life of about an hour, when converted into the protease-resistant isoform (PrP^{Sc}) it accumulates in the extracellular space and can remain there for much longer. Its conversion process is still to be thoroughly understood; anyway, it is believed to involve a seeding mechanism in which small aggregates are formed over a long period. Aggregates that can then rapidly initiate the conversion of more protein, resulting in high levels of PrP^{Sc} in brain. Accumulation of these aggregates there seems to be responsible for the pathology of prion disease (Sakudo et al., 2009). Considerable evidence suggests that PrP^{Sc} is directly toxic to neurons and initiates an apoptotic process that seemingly requires the involvement of oxidative stress and microglia activation (Rezaie et al., 2001). Studies in transgenic mice models of AD have shown that CHF5074 is able to attenuate brain microglia activation associated to A β deposits (Imbimbo et al., 2009; Imbimbo et al., 2010 a). Thus, in scrapie-infected mice CHF5074 may counteract PrP^{Sc} toxicity mediated by microglia activation. Alternatively, it may alter astrocyte migration, a property demonstrated both *in vitro* (Lichtenstein et

al., 2010) and *in vivo* models of AD (Imbimbo et al., 2010 a). Indeed, scrapie-infected mice show marked reactive astroglyosis around neuropathological lesions (Meeker et al., 2005). It has been shown that transgenically attenuated astroglyosis in scrapie infected mice led to a reduced accumulation of the misfolded isoform of the prion protein PrP^{Sc}, a delayed disease onset and significantly prolonged survival times (Schultz et al., 2004). Therefore, CHF5074-induced beneficial effects in scrapie-infected mice are likely to be due to its modulatory effects on reactive astrocytes.

Alternative processes can also be considered. In prion diseases, cellular expression of PrP^C is required for neuronal death (Brown et al., 1994; Brown et al., 1996), and mice knocked out for the prion protein are resistant to infection with prion disease (Büeler et al., 1993; Prusiner et al., 1993). Similarly, PrP^C is required for impairment of synaptic plasticity induced by A β oligomers in hippocampal slices (Laurén et al., 2009), and familial AD transgenic mice knocked-out for PrP^C showed brain A β accumulation without developing memory deficit (Gimbel et al., 2010). It has been demonstrated that CHF5074 attenuated impairment of synaptic plasticity and associated memory impairment in young, plaque-free Tg2576 mice without affecting brain levels of A β oligomers (Balducci et al., 2011). Hypothetically, CHF5074 may work in AD transgenic mice by binding and inactivating PrP^C on the surface of neuronal membrane (Balducci et al., 2011). Similarly, CHF5074 may delay prion disease onset acting as antagonist of cell-surface receptors for PrP^{Sc} on neurons of scrapie-infected mice.

Therapeutic activity of CHF5074 in the present murine model of prion diseases might also be explained considering γ -secretase as the unknown

protease involved in the pathologic cleavage of PrP^C. A hypothesis that seems almost unlikely, however, since γ -secretase exerts its proteolytic activity within the plasma membrane, whereas the pathologic breakdown of the prion protein is located at the 90-91 N-terminal site (Vincent et al., 2001; Evin et al., 2006). The N-terminal portion is an octapeptide repeat region binding copper and zinc and usually reputed not to interact with the membrane, being PrP^C a cell surface glycosyl-phosphatidylinositol (GPI)-anchored protein. Nevertheless, several *in vitro* studies indicated that the GPI-anchor is not obligatory for the raft association of PrP, which can be mediated by a determinant within the N-terminal region of the ectodomain (residues 23-90) (Sanghera et al., 2002; Walmsley et al., 2003; Taylor et al., 2007). Furthermore, *in vivo* studies showed that the N-terminal region strongly influenced susceptibility to and incubation times of prion disease (Fischer et al., 1996; Moore et al., 2009). The interaction of γ -secretase with the N-terminal region may then be possible and plausible. An unexpected but not isolated event, being the involvement of disintegrin (ADAM) in the physiological catabolism of prion protein likewise unusual; substrates of disintegrins are indeed generally transmembrane proteins, as APP (Checler et al., 2002).

In conclusion we can state that chronic oral administration of CHF5074 appears to be a promising treatment not only of AD but also of TSEs, even though further studies are definitely needed to confirm its therapeutic potential in animal models of prion disease as well as to acquire an in-depth understanding of the mechanism underlying its protective effects.

12 - ABILITY TO DETECT BSE AFTER CHALLENGE OF WILD TYPE MICE WITH BSE AND SCRAPIE MIXTURES

This experimental study was held at and in collaboration with the Animal Health Veterinary Laboratory Agency (AHVLA), Weybridge, Surrey, UK, during my internship there (October 1st, 2010 – April 30th, 2011).

The sensitivity of small ruminants to BSE is known (Foster et al., 1993; Bellworthy et al., 2008), as well as their exposure to its infectious agent through the intake of contaminated MBM (Wilesmith et al., 1991; Smith et al., 2003).

Even though an extensive survey of the UK flock has shown no evidence of classical BSE phenotype (Stack et al., 2006), its presence can not be excluded with certainty (see below). Bellworthy (2008) indeed pointed out that the long incubation period of BSE could exceed the commercial lifespan of animals, especially if scrapie-affected.

As in small ruminants scrapie can not be distinguished from BSE on the basis of clinical signs (Foster et al., 1993; Baron et al., 2000; Baylis et al., 2002; Bellworthy et al., 2008), many experimental studies have been successfully aimed to the development of immunoblot and immunohistochemical tests able to discriminate between BSE and scrapie (Baron et al., 2000; Jeffrey et al., 2001; Stack et al., 2002; Jeffrey et al., 2006; Beck et al., 2010 a; Jacobs et al., 2011). Application of these discriminatory tests in national surveillance

projects and retrospective studies to identify BSE in small ruminants revealed two caprine BSE cases, one in France (Eloit et al., 2005) and one in UK (Spiropoulos et al., 2011).

Although the discriminatory tests have been validated rigorously on the basis that a small ruminant may be affected by either BSE or scrapie, their performance on samples where BSE and scrapie prions co-exist has not been tested, despite evidence that coinfection of multiple TSE strains in one animal is plausible (Bruce et al., 2002; Yokoyama et al., 2010, Mazza et al., 2010).

Therefore the possibility that sheep and goats in commercial holdings might have been affected by BSE which remains undetected due to the simultaneous presence of scrapie cannot be excluded unequivocally.

In contrast to the pathogenesis of BSE in cattle, where the PrP^{Sc} distribution is limited to the nervous system, except for a short period after oral challenge where it can be identified in the Peyer's patches and in the myenteric plexus of the distal ileum (Terry et al., 2003; Hoffman et al., 2007), the peripheral pathogenesis of BSE in sheep is similar to that of scrapie with extensive distribution of the infectious agent in peripheral tissues (Foster et al., 2001; Baylis et al., 2002).

This widespread distribution of the infectious agent is considered to be the main factor responsible for the sheep-to-sheep transmission of scrapie (Baylis et al., 2000) and similarly BSE infectivity in a flock could be sustained even after the original source of infection is eliminated. Recent evidence seems to confirm this hypothesis, suggesting that the sheep BSE propagation within a flock is a realistic chance (Bellworth et al., 2005).

This possibility ought to be carefully considered, since the peripheral wide distribution of BSE prions in sheep suggests that the measures that were responsible for the successful eradication of BSE from the cattle population would appear inadequate to eliminate BSE in sheep and makes virtually impossible to ensure absence of BSE infectivity to consumers after consumption of sheep meat products (Baylis et al., 2000).

It becomes evident that there is a clear need to detect BSE in small ruminants not only when it is the only infectious prion but also when it co-exists with scrapie. Under such conditions discrimination between BSE and scrapie may not be straightforward, as according to the only experimental coinfection study where mice were challenged with mixtures of mouse adapted BSE and scrapie. The different electrophoretic migration of PrP^{Sc} were not enough to distinguish the two TSEs strains, because one might mask the other and new intermediate banding patterns might be observed (Baron et al., 2001).

In this second part of my study we investigated the ability of wild-type mouse bioassay to identify BSE in the presence of scrapie during primary isolation after administration of mixtures of BSE and scrapie using histopathology, immunohistochemistry and Western blot.

12.1 - MATERIALS AND METHODS

12.1.1 - Ethical statement

All animal procedures were performed in compliance with the Animal (Experimental Procedures) Act 1986 under licence from the UK Home Office, and were approved by the local ethics committee.

12.1.2 - Inoculum preparation

Initially a classical scrapie and an ovine BSE homogenate were prepared. The classical scrapie inoculum, hereafter referred to as 100% scrapie, was made using a pool of brains collected from natural confirmed cases of classical scrapie representing the commonest PrP genotypes, i.e. ARQ/ARQ, VRQ/VRQ and ARQ/VRQ. The ovine BSE inoculum, hereafter referred to as 100% BSE, was prepared from a pool of sheep brains which had been challenged experimentally and subsequently succumbed to BSE. The brains were homogenised using a handheld blender and suspended in 9 volumes of normal saline. Subsequently they were filtered through a single layer of a sterile gauze to remove large pieces of tissue, tested for microbiological sterility and treated with antibiotics if necessary. Aliquots of these two inocula were combined to produce mixtures of BSE and scrapie at the following relative ratios: “1 BSE : 99 scrapie” (hereafter referred to as 1% BSE), “2 BSE : 98 scrapie” (hereafter referred to as 2% BSE), “10 BSE : 90 scrapie” (hereafter referred to as 10% BSE) and “50 BSE : 50 scrapie” (hereafter referred to as 50% BSE). All homogenates were stored at -80 °C until they were used for inoculations.

12.1.3 - Animal procedures

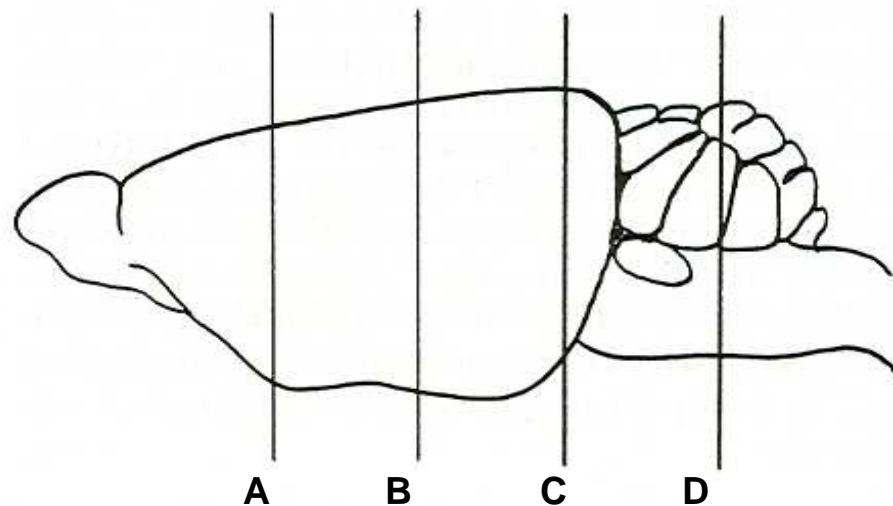
Each inoculum was administered in three panels of wild type mice namely C57Bl6, RIII and VM. Each panel consisted of 20 mice and each mouse received 20 µl of homogenate intracerebrally and 100 µl intraperitoneally. Inoculations were performed under general anaesthesia using an insulin syringe fitted with a 25 gauge needle. Mice were monitored for signs of clinical

disease and euthanased either at specified clinical end points or based on animal welfare justification. After euthanasia each animal was subjected to a postmortem examination. The brain was removed and placed in 10% formalin and allowed to fix at room temperature for at least three days.

12.1.4 - Histology

After fixation was completed the brains were cut at four different coronal levels (Figure 18), embedded in wax following standard procedures, sectioned, mounted on slides and stained with haematoxylin and eosin for histopathological assessment (TSE EU Community Reference Laboratory).

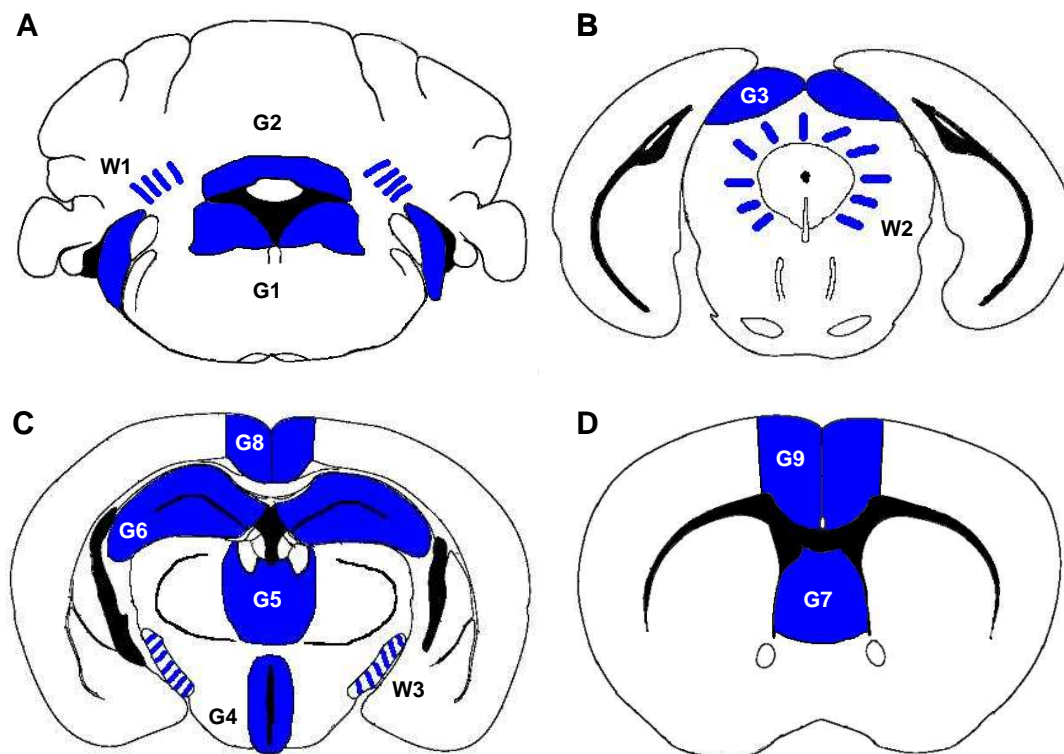
Figure 18. Mouse brain. The lines indicate the four neuroanatomical levels where transversal cuts are performed (A = frontal level; B = thalamic level; C = mid-brain level; D = medullar level).



Based on the existence of convincing TSE specific vacuolation, each sample was diagnosed as TSE positive, negative or inconclusive. On TSE positive

samples the lesion intensity in specific neuroanatomical areas (Figure 19) was further assessed semi-quantitatively and assigned a score on a scale 0-5. The lesion scores from clinically and histopathologically positive samples were plotted against the respective brain areas to produce lesion profiles as described previously (Outram et al., 1973; Green et al., 2005).

Figure 19. Mouse brain sections and mandatory areas. Areas are made up of grey matter (G areas) or white matter (W areas). Medullar section (A): G1 = dorsal medulla nuclei, including the cochlear and vestibular nuclei mid-brain level; G2 = cerebral cortex of the folia adjacent to the fourth ventricle; W1 = cerebellar white matter. Mid-brain section (B): G3 = dorsal medulla nuclei; W2 white matter in the mesencephalic tegmentum. Thalamic section (C): G4 = hypothalamus; G5 = central thalamus; G6 = hippocampus; G8 = cerebral cortex; W2 = white matter of the pyramidal tract. Frontal section (D): G7 = septal nuclei of the paraterminal body; G9 = cerebral cortex.



12.1.5 - Western blot

Western blot analysis were performed on samples from both inocula and wild-type mice.

A 5% brain homogenate (200 μ l) [in a 0.32 M Sucrose buffer containing 0.5% (w/v) DOC (deoxycholic acid sodium salt) and 0.5% (w/v) NP40 (Nonidet-P 40)] were incubated with 50 μ g/ml pK for 60 minutes at 55 $^{\circ}$ C to completely digest all PrP^C. The reaction was stopped by addition of 10 mM PMSF (phenylmethylsulfonylfluoride). After incubating with 10% sodium N-lauroyl sarcosinate and 10mM Tris-HCl, final concentrations (room temperature, 15 min), proteins were purified by centrifugation (540,000x g, 45 min) over a 10% sucrose cushion (250 μ l). Pellets were solubilised in Laemmli buffer and proteins were separated on 16% Bis/Tris gels, electrotransferred to membrane and labelled with specific anti-prion antibody. Immunoreactivity was visualised following incubation with chemiluminescence substrate CDP-Star (Tropix, Bedford, USA). PrP^{Sc} in inocula was detected using Sha31 and P4 while for mouse brains we used Sha31 and 12B2, as antibody P4 apparently did not react with murine PrP (data not shown).

12.1.6 - Immunohistochemical labelling of PrP^{Sc}

Samples from clinically and histopathologically positive mice were further analysed with immunohistochemistry as described at http://vla.defra.gov.uk/science/docs/sci_tse_rl_prp_ihc.pdf.

Briefly, PrP^{Sc} was detected using RB486, a rabbit polyclonal antibody raised against a bovine PrP epitope spanning amino acids 221 to 223, and a secondary biotinylated antibody. Antigen antibodies were developed using a

commercial kit (ABC, Vector Laboratories) and diaminobenzidine (DAB) as a chromogen. Slides were counterstained in Meyer's haematoxylin.

12.1.7 - Statistical analysis

Cluster analysis was performed using Statistica (Version 10). For all other statistical analyses the STATA (Version 10) or the Genstat (Version 5) programmes were used.

12.2 - RESULTS

12.2.1 - Attack Rate (AR) and Incubation Period (IP)

analysis

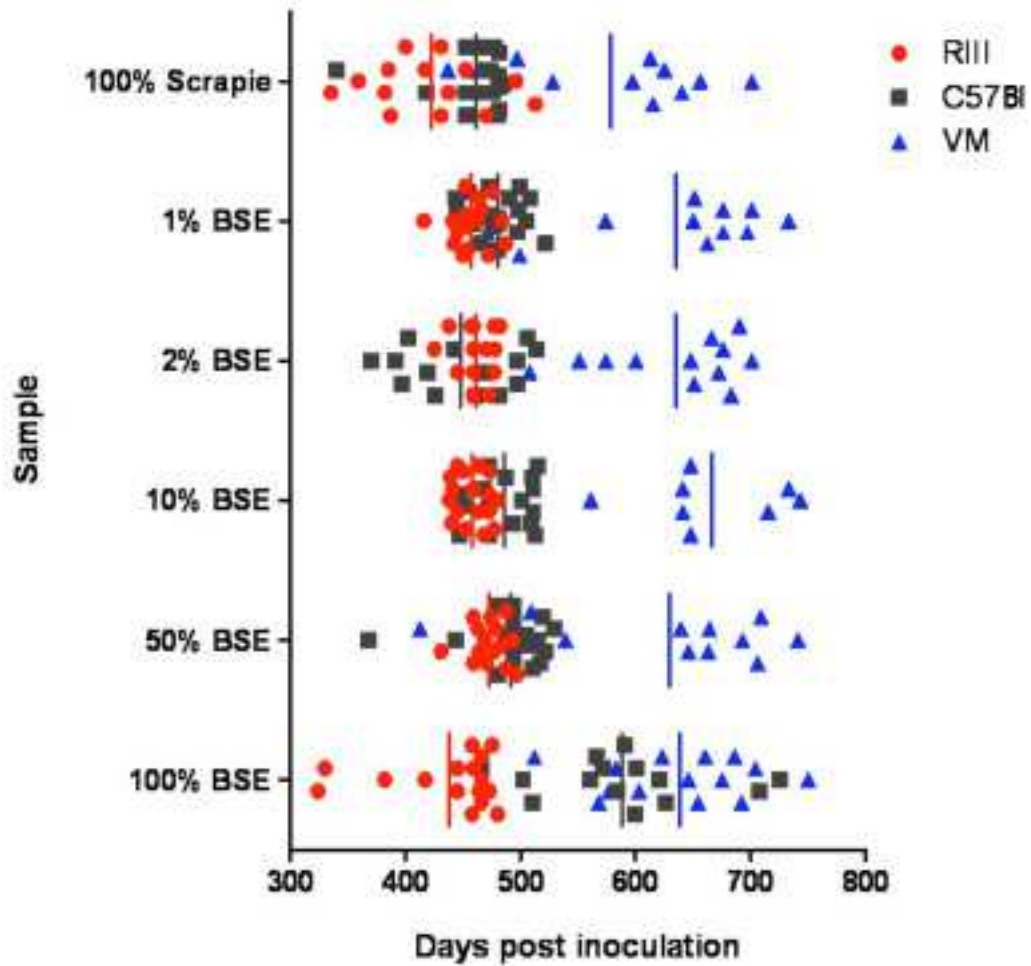
The AR expresses the ratio of TSE positive mice over the number of mice inoculated with classical scrapie, BSE or their mixtures (n = 20 per group). The number of TSE positive mice diagnosed using immunohistochemistry, irrespective to their clinical or histopathological status, are presented in Table 10. All inocula showed high AR in each of the three mouse lines used. Significantly, both the 100% scrapie and 100% BSE showed as high attack rates as observed with other inocula of the same TSEs in the laboratory of the AHVLA of Weybridge, UK, implying that each source represented a high infectivity titre. Similarly high attack rates were also recorded for all mixtures.

Table 10. Animals positive to immunohistochemistry after classical scrapie and/or BSE challenge.

Inoculum	Mouse line		
	RIII	C57BI	VM
100% scrapie	16	15	20
1% BSE	19	19	19
2% BSE	16	17	20
10% BSE	19	18	19
50% BSE	16	19	20
100% BSE	16	15	20

The IP data are shown in Figure 20. Generally there is wide overlap between all inocula to allow any discrimination even between the controls, 100% scrapie and 100% BSE. The only notable observation was the prolonged IP of the 100% BSE source in C57BI mice compared to all other isolates. The variance of IP in this group of mice was also increased comparatively to the other C57BI groups.

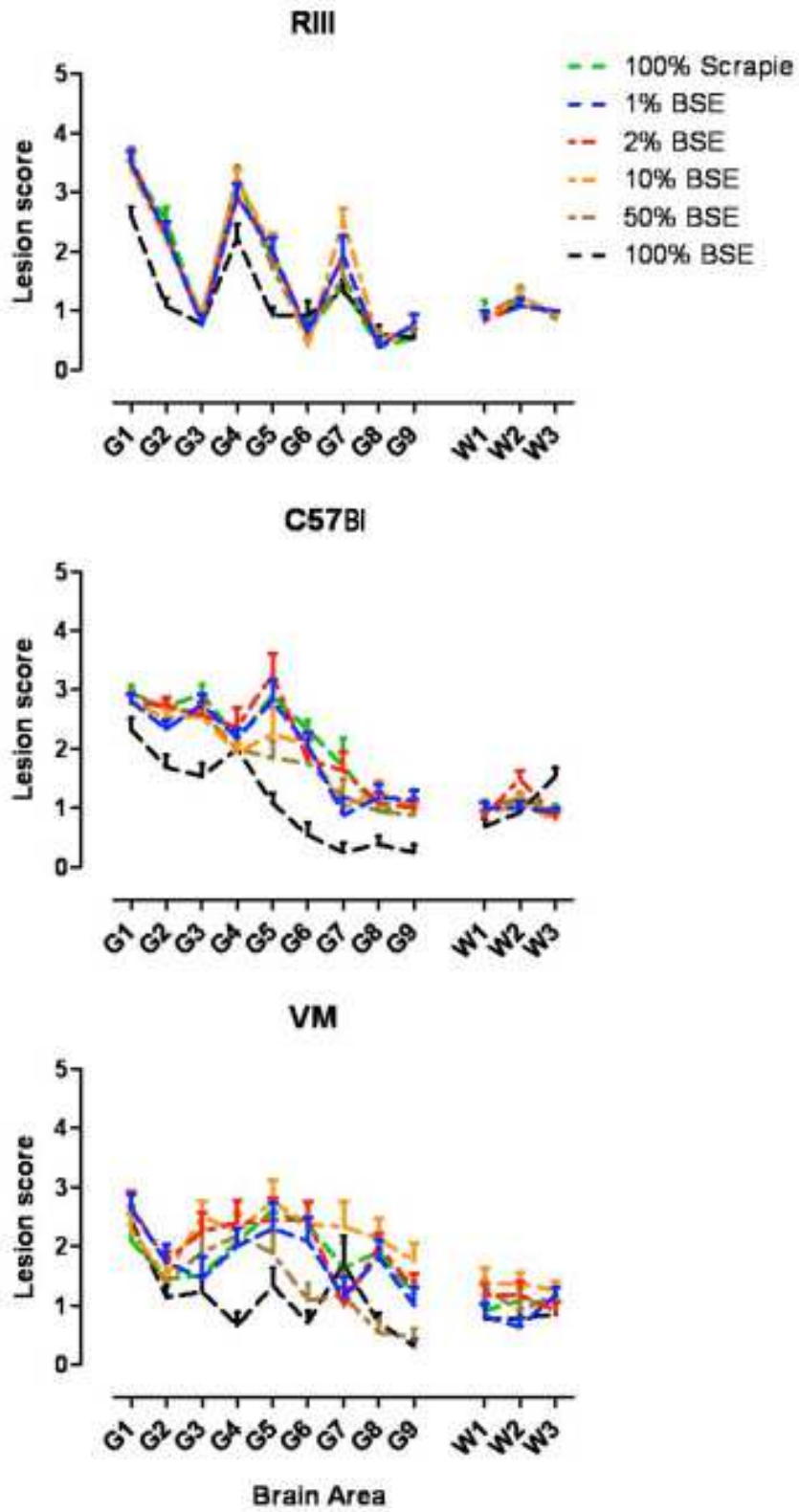
Figure 20. Graphical representation of incubation period data. Each symbol corresponds to a clinically and pathologically TSE positive mouse. Vertical bars indicate mean values.



12.2.2 - Lesion Profile (LP) analysis

Due to the high infectivity of all inocula there were at least five clinically and pathologically positive mice in each mouse group. Therefore it was feasible to construct LP from each mouse group (Figure 21). In C57Bl and VM mice the LP of 100% BSE was different from that generated by the 100% scrapie source. The LP that were generated from the various mixtures seemed to align better with the 100% scrapie control rather than the 100% BSE isolate.

Figure 21. Lesion profile analysis arising from primary isolation of scrapie, BSE and scrapie/BSE mixtures from distinct clusters of wild-type mice.



In the RIII mice the LP from mixtures also appear to be more akin to the LP of the 100% scrapie source. However in the RIII mice the difference between the LP generated by 100% BSE and 100% scrapie sources was not as profound as it was in the C57Bl and VM mice. In RIII mice both the 100% BSE and 100% scrapie produced LP which showed similar contours and the differences between them was only quantitative. This property of the RIII has been reported extensively elsewhere and the scrapie associated profile has been designated 1-4-7 scrapie to denote its similarity with BSE induced LP (Beck et al., 2010 a) which consistently produces profiles with peaks in these areas (Green et al., 2005). Interestingly this profile has only been derived from ARQ/ARQ classical scrapie sources (Beck et al., 2010 a; Beck et al., 2010 b) whilst in the current study a pool containing an assortment of the commonest ovine PrP genotypes affected by classical scrapie was used.

12.2.3 - Western blot analysis

Application of Western blot on the inocula showed that all the mixtures essentially behaved as scrapie, showing a higher molecular weight unglycosylated band compared to BSE and a binding affinity for antibody P4.

Application of Western blot on brains of C57Bl and RIII mice also showed that animals challenged with mixtures were indistinguishable from those challenged with the scrapie control, showing a high molecular weight unglycosylated band compared to the BSE control. In addition, affinity to antibody 12B2 was retained even in the high mix inocula (10% and 50% BSE). No attempt was made to apply Western blot in VM mice as in this

mouse line BSE cannot be distinguished from scrapie using this technique (Grouschup et al., 2000).

12.2.4 - Immunohistochemical analysis

Slides were divided into three groups, according to the three mouse lines used: C57Bl, RIII and VM.

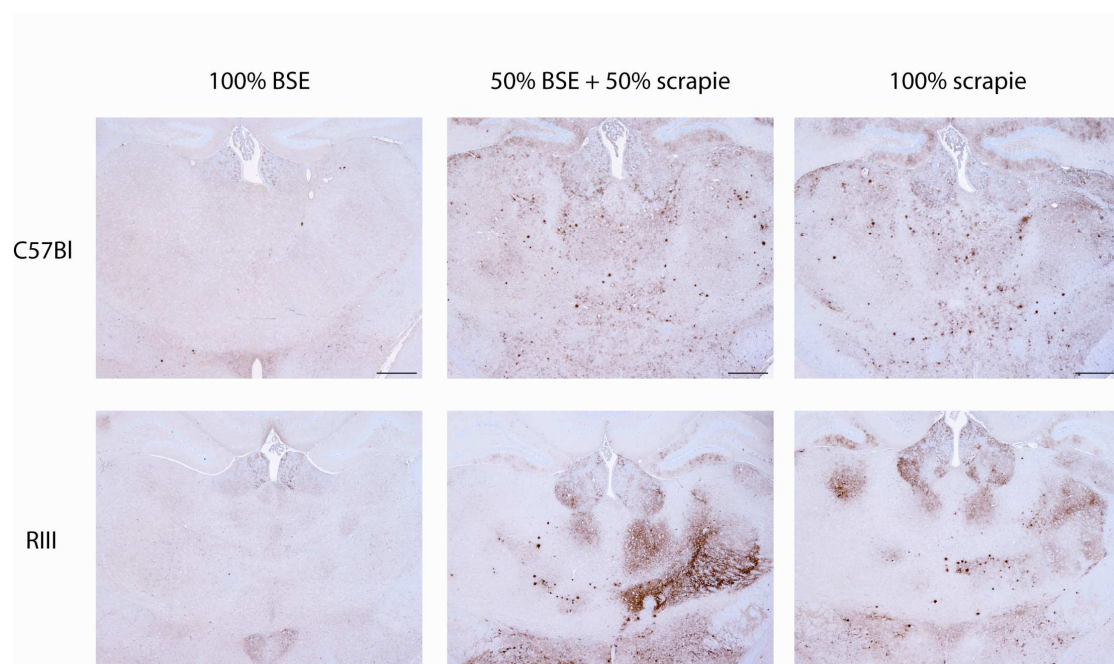
All slides were interpreted by a single assessor. During the first phase the 100% BSE and 100% scrapie samples were compared unblinded for the reader to gain the ability to identify the hallmarks of the PrP^{Sc} patterns in mice challenged with either pure BSE or scrapie. The remaining slides which derived from mice inoculated with 1%, 2%, 10% and 50% BSE were all mixed randomly. Subsequently the reader assessed them blind and allocated them in their perspective groups based on the PrP^{Sc} markers they exhibited. This exercise was undertaken to identify potential immunohistochemical markers that could be used to identify BSE in a scrapie mixture.

The BSE infection generated in both C57Bl and RIII mice a mild PrP^{Sc} deposition, diffuse throughout all the brain in RIII mice and weaker in the cranial sections in C57Bl ones. It was characterised by diffuse granular type, intraglial and widespread ordered aggregates of various dimensions. This pattern was usually denser in locus coeruleus, choclear nuclei, cerebellar peduncoli, raphe and red nuclei. In the last ones and in the medulla intraneuronal type was also observed. At the thalamic level RIII mice often had denser PrP^{Sc} deposition in the dorsolateral nuclei of the hypothalamus, while C57Bl ones seldom displayed perineuronal type and plaques in the zona incerta.

The scrapie strain which came out from the pool of inoculum in these two mouse lines was the 87A. Unlike BSE, it produced in both C57Bl and RIII mice a strong PrP^{Sc} deposition in all the brain, made up by intense spread granular type, ordered and disordered aggregates, intragial, some plaques and, in defined areas, perineuronal and intraneuronal types too. In the cortex of C57Bl mice PrP^{Sc} deposition was localised in the molecular and pyramidal layers, forming a characteristic double band staining. The scrapie pattern in RIII mice usually appeared lateralised in the thalamic level, mainly in the right side.

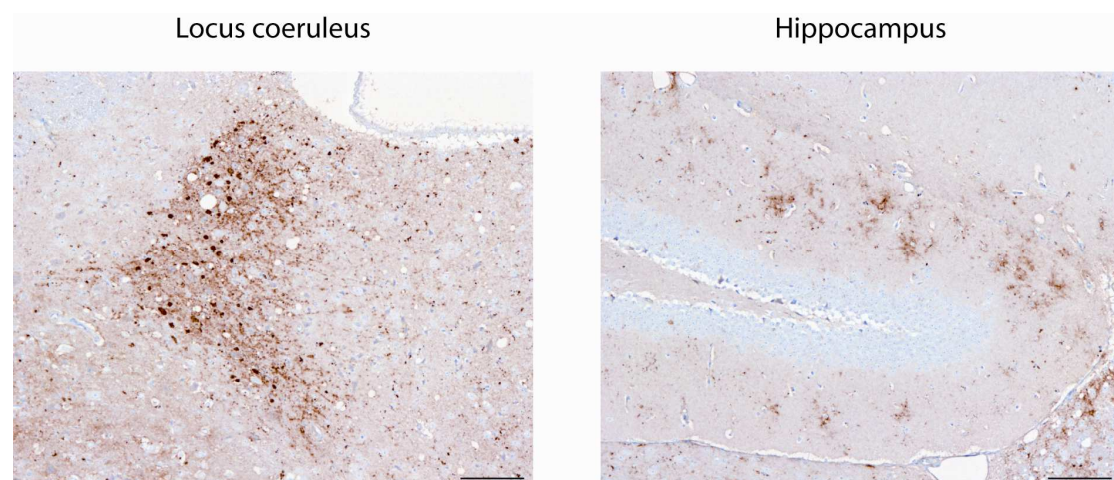
In both of these mouse lines during BSE and scrapie coinfection the stronger scrapie phenotype was dominant on the mild BSE one. Thus no peculiar marker able to suggest the presence of BSE in scrapie infected animals has been found (Figure 22).

Figure 22. Comparison of PrP^{Sc} deposition patterns in the thalamic level of C57Bl and RIII mice following infection with different percentages of BSE and scrapie (100% BSE, 50% BSE + 50% scrapie, 100% scrapie). Scale bars = 500 µm.



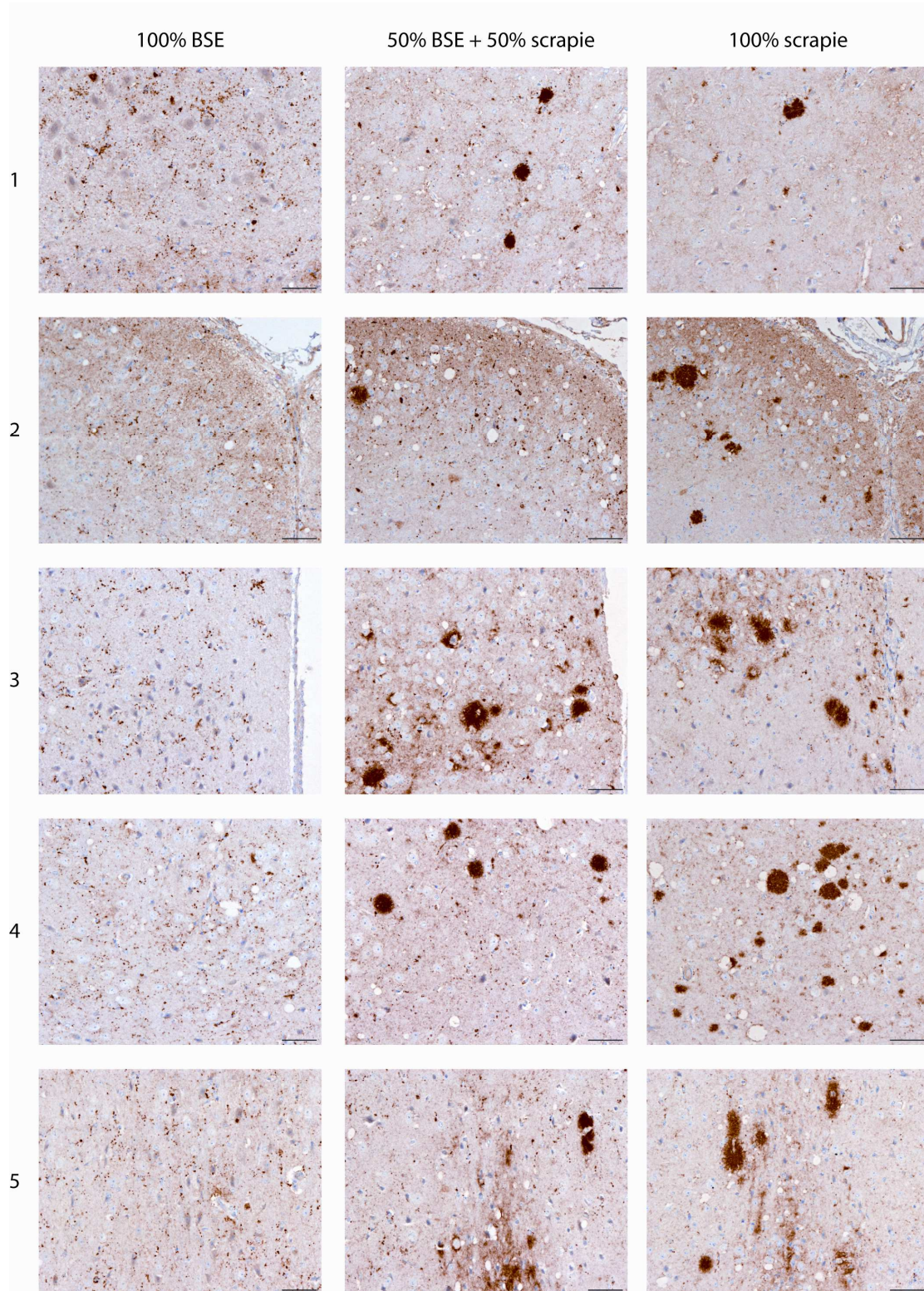
In VM mice the scrapie strain which emerged from the pool of inoculum was the 87V. Both 100% BSE and 100% scrapie infections generated granular and intraglial PrP^{Sc} pattern. Some intraneuronal deposits were also found in medulla and red nuclei. Multifocal small dense aggregates were observed in BSE infected animals, while larger ones were occasionally present in thalamic and frontal levels. During scrapie infection big dense aggregates were mixed with some plaques in all the brain. Rare plaques were occasionally observed in the CA1 area of the hippocampus in BSE infected VM mice. Moreover BSE induced in this strain of mice very characteristic PsP^{Sc} patterns: speckled deposition in locus coeruleus, stellate type in hippocampus (Figure 23) and diffuse strong punctate pattern. The latter was observed also during scrapie 87V infection, but was milder, much more localised in few particular areas and beads were smaller.

Figure 23. Unique immunohistochemical patterns are observed in BSE infected VM mice. Ovoid dense and spotted deposits, termed “speckled”, frequently appear in locus coeruleus. Radiating star-like deposits of PsP^{Sc} within the neuropil characterise the BSE pattern in the hippocampus. Scale bars = 100 µm.



In contrast with speckled and stellate types which were not observed during BSE and scrapie coinfection, the BSE-associated punctate pattern appeared to be present in some mice that had otherwise a scrapie compatible phenotype and so it was chosen as marker of BSE (Figure 24).

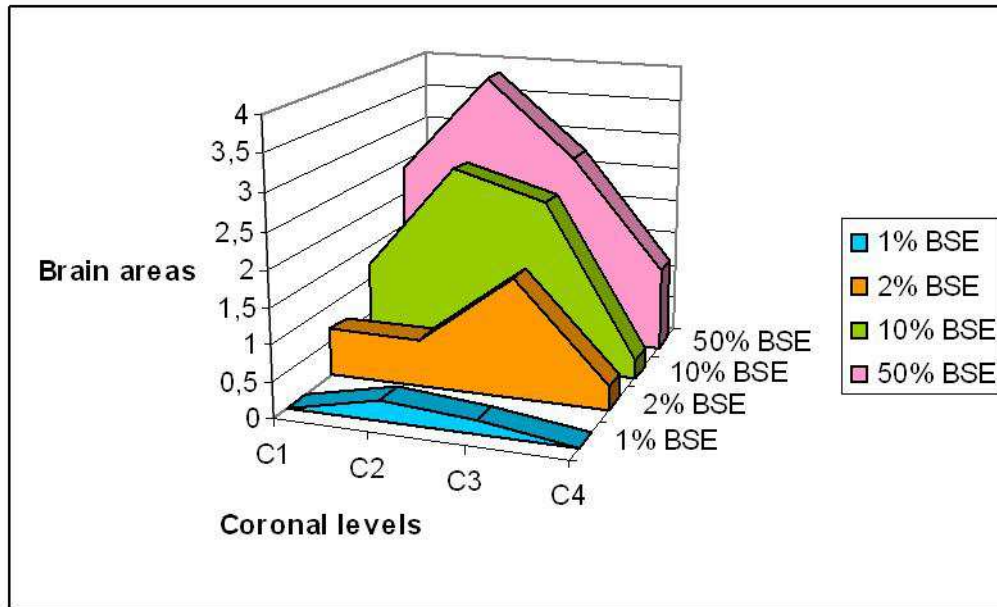
Figure 24. Representative immunohistochemical staining in neuroanatomical brain areas of VM mice infected with different percentages of BSE and scrapie (100% BSE, 50% BSE + 50% scrapie, 100% scrapie). A: medulla; B: superior colliculus; C: hypothalamus; D: thalamus; E: vertical limb of the diagonal band. Scale bars = 50 μ m.



Subsequently, all samples derived from VM mice challenged with scrapie, BSE, or scrapie/BSE mixtures were assorted randomly and assessed blind by the same operator. The presence of BSE-associated punctate deposits in specific neuroanatomical areas allocated in four coronal levels (medulla, mid-brain, thalamic and frontal levels) was noted, considering as baseline the scrapie-like punctate pattern.

At each coronal level the number of neuroanatomical areas affected by BSE-associated punctate deposits was used to predict the presence and percentage of BSE (Figure 25). The test predictions were compared to the actual values and the associations between test and true status were not random ($p < 0.0001$ by Fisher's exact test). The sensitivity, specificity and accuracy of the approach were 78% (62-89), 91% (75-98) and 84% (73-91); values in brackets denote 95% confidence intervals. The higher percentage of BSE in the mixtures was associated with higher scores of BSE-specific punctate deposits and the predictions were more accurate in the high mix (50% and 10%) inocula whilst the low mix inocula (2% and 1%) proved more difficult to predict accurately.

Figure 25. Mean BSE-associated punctate pattern distributions in four coronal brain sections of VM mice coinfecting with BSE and scrapie. The coronal brain sections reveal: medullar level (C1), mid-brain level (C2), thalamic level (C3), frontal level (C4). Animals are grouped on the base of the predicted percentage of BSE they received during coinfection.



12.3 - DISCUSSION

Despite the significant progress and development of tests that can discriminate BSE from scrapie in a single infection scenario, not enough studies that address the performance of the discriminatory tests in cases where BSE and scrapie exist in the same animal, sheep or goat, have been performed. Ideally, the best possible experimental materials should derive from sheep coinfecting experimentally with natural scrapie and BSE sources via natural routes of inoculation, under conditions that would reflect as close as possible naturally occurring coinfection. To the best of our knowledge, however, materials from such experiments are not widely available. Therefore we used the best possible alternative: we produced BSE and scrapie mixtures

in vitro and use them as an alternative to coinfecting tissue. A similar approach has been followed in the only other study where distinguishing BSE from scrapie under coinfection conditions was attempted (Baron et al., 2001). In that report the BSE and scrapie sources were mouse adapted sources whilst in the current study we used ruminant tissues to replicate closer a scenario where potentially coinfecting ruminant tissues may be used to challenge rodents. Collectively these data suggest that mixtures of scrapie with BSE, either of ruminant or mouse adapted sources, behave essentially like scrapie as none of the BSE features is retained.

Based on AR, IP, LP and Western blot our data suggest that all three mouse lines that were challenged with BSE/scrapie mixtures exhibited a classical scrapie phenotype and it was not possible to identify any BSE attributes in the BSE/scrapie mixtures. AR and IP data are considered to be crude parameters based on average values derived from a group of animals and as a result they cannot always be used to identify with confidence significant differences even between different TSE strains. In the current study the BSE derived LP was different from that of scrapie particularly in the C57Bl and VM mouse lines. In RIII mice, however, LP generated by the BSE and scrapie controls were quite similar. Interestingly the LP that was produced by the scrapie control has only been generated in the past by ARQ/ARQ classical scrapie sources (Beck et al., 2010 a; Beck et al., 2010 b).

Each mouse that was challenged with either a BSE/scrapie mixture or 100% scrapie control in the current study exhibited a stable classical scrapie IHC pattern, which is usually isolated from ARQ/ARQ classical scrapie sources (Beck et al., 2010 a; Beck et al., 2010 b). In *Prnp^a* mice this IHC pattern was

indistinguishable from the one observed in mice challenged with 87A, whilst in *Prnp^b* mice it was indistinguishable from the pattern generated by 87V (Beck et al., 2010 b). As in classical scrapie a link between ovine PrP genotype and strain has been reported (Thackray et al., 2008; Beck et al., 2010 a; Beck et al., 2010 b; Thackray et al., 2011), the current finding is intriguing since only 16% of the scrapie cases that contributed to the 100% scrapie source were ARQ/ARQ whilst the majority of the genotypes were either ARQ/VRQ (58%) or VRQ/VRQ (20%). A possible explanation could be that a classical scrapie strain associated with the ARQ/ARQ genotype was preferentially propagated in all three mouse lines at the expense of other strains. This is interesting since in our experience it is not unusual to identify individual mice with IHC features of different classical scrapie strains on primary passage of single isolates or isolate different strains after serial passage particularly when ARQ/ARQ cases are bioassayed in C57Bl or RIII mice (Beck et al., 2010 b; Thackray et al., 2011). These observations suggest that pooling different classical scrapie strains derived from different PrP genotypes *in vitro* may alter their stability or their propagation properties.

The Western blots from C57Bl and RIII mice that were challenged with BSE/scrapie mixtures showed that even with the highest BSE fraction (50%) the classical scrapie characteristics dominated and any BSE signal was undetectable within the resolution limits of this method.

Using immunohistochemistry it was not possible to identify any BSE characteristics in either C57Bl or RIII mice that were challenged with BSE/scrapie mixtures. As with the western blots, these mice were indistinguishable from the 100% scrapie control.

Collectively the above data indicate that in the C57Bl and RIII mice challenged with BSE/scrapie mixtures only classical scrapie phenotypic traits were identified. This finding could reflect either a preferential propagation of classical scrapie at the expense of BSE in these mouse lines or that although BSE was also propagated it had a recessive phenotype. The last suggests that propagation of a mixture of two strains is feasible, although in the case of C57Bl and RIII mice only one of the two strains may be identifiable. Propagation of a mixture of strains is also supported by the data from the VM mice where, although the overall phenotype of classical scrapie prevailed, a subtle but distinct BSE feature was observed in mice challenged with BSE/scrapie mixtures. That feature was more prominent in the mice that were exposed to high content of BSE (10% or 50%) compared to those that received a low content of BSE (1% or 2%).

The similarity of results obtained from C57Bl and RIII mice is probably justified by the sharing of the same PrP sequence, that influences the TSE-strains features, while the PrP sequence in VM mice differs at codons 108 and 109 (Westway et al., 1987; Bruce et al., 1991).

Although we were able to identify a BSE-associated trait that could potentially be used to detect BSE in the presence of scrapie using VM mice on primary isolation, this study shows that overall classical scrapie can dominate the phenotype of the disease even if BSE prions propagate in the background.

It must be noted that the observations of this study cannot be generalised without exercising due caution. Firstly the infectious titre of each of the two sources that were used to produce the mixtures was not evaluated, therefore the mixtures did not reflect a titre ratios but simply the volumetric ratios.

In addition, the titres are the function of the strain-host system used (BSE is usually titrated in RIII mice whilst scrapie is usually titrated in C57 mice) and cross reading between different strain-host systems is not informative. Therefore it is questionable if titre matching provides an optimal approach to coinfection experiments. However in the current study both sources showed very high attack rates, indicating that none of the source inocula had unusually low titre.

Interactions between different classical scrapie strains and BSE is another parameter that must be considered. In the current study all mice showed IHC patterns that were associated with strains related to the ARQ/ARQ genotype, 87A in *Prnp^a* mice and 87V in *Prnp^b* mice (Beck et al., 2010 b; Thackray et al., 2011). However, it is impossible to predict how other classical scrapie strains, such as ME7, would interact with BSE.

Moreover it must be noted that the selected marker is subtle and only an experienced observer could interpret it correctly and only in the high mix BSE inocula (10% or 50%), as in the low mix BSE the levels of the marker dropped appreciably. Therefore using this model low BSE levels on a classical scrapie background may remain undetected. This observation could be extended even in the original host (i.e. small ruminants).

In conclusion, despite all its limits, this is the first study to highlights the greater sensitivity of immunohistochemistry compared to histology in detect BSE during scrapie/BSE coinfections. It is therefore worth to investigate IHC patterns in further mouse lines, in order to find other BSE-markers able to improve the accuracy of the diagnosis of coinfection based on the only immunohistochemical analysis.

Anyway, under these circumstances, a policy of eradicating or minimizing classical scrapie is, scientifically and financially, the most feasible option to prevent BSE from entering the food chain to a level that could present a public health risk.

13 - SEARCHING FOR MEMBRANE LIPID ROLE IN REGULATORY MECHANISMS OF PRION DISEASES: EVALUATION OF DIETARY APPROACHES IN MURINE MODEL OF SCRAPIE

Cell membrane consist of a lipid bilayer, mostly unsaturated, containing phospholipids, cholesterol, sphingolipids, and proteins in dynamic equilibrium. The plasma membrane is not homogeneous, but contains specific lipid microdomains, mostly saturated, rich in cholesterol and glycosphingolipids, like sphingolipids and gangliosides, called lipid rafts (Taylor et al., 2006; Gilch et al, 2006; Garattini et al., 2007; Taylor et al, 2007).

Numerous proteins implicated in cellular homeostasis are located within them and it is clear that rafts are important regulators of cellular activity (Garattini, 2007; Ma et al., 2004). They contain, among the others, ADAM 10 and ADAM 17 metalloproteases, responsible for the physiological cleavage of PrP^C, whose activity is regulated modulating the entry of their substrates into the lipid rafts. Moreover these domains seem to have a significant role in TSEs, being the sites where prion toxic conversion occurs (Gilch et al., 2006; Tellier et al., 2006).

Changes in membrane properties, due to altered lipid composition, affect the organization and interact between lipids and protein therein (Ma et al., 2004). Alterations in cholesterol and/or ganglioside composition of lipid rafts can

significantly affect cell function, being involved in diseases affecting neurodegeneration, such as AD and prion diseases (Schengrund et al., 2010). Cellular membrane lipids can be remodelled by altering dietary fat intake (Ma et al., 2004; Yaqoob, 2009).

TSEs are characterised by a long incubation period and a difficulty to diagnose them *in vivo*. All the drugs which gave promising therapeutic effects demonstrated that, to be effective, had to be administered preventively or close to the infection time and then for long (Trevitt et al., 2006). This represent a crucial limit in the therapeutic approach, since means that the therapy to be effective would start before knowing to be sick. So prevention has a crucial role in counteracting prion diseases. Thus, modulating dietary compounds intake seems to be the most reliable and effective way to make prevention, rather than encourage preventive medications.

Starting from these assumptions, the aim of this part of my study is to know if, changing *in vivo* membrane lipid composition using specific diets, it is possible to modulate expression, localisation and functionality of certain proteins involved in the development of TSEs, and to verify if and how this approach is able to influence the clinical progression of prion diseases. Both of the diets chosen are expected to have an anti-prionic activity.

This experimental project has been awarded of the PRIN grant 2009 (Project of Relevant National Interest, funded by the Italian Ministry of University and Research) and is being performing at the Microbiology and Immunology Section of the Department of Veterinary Pathology, Hygiene and Public Health, at the School of Veterinary Medicine of the University of Milan.

13.1 - DIETS

13.1.1 - Diet enriched with complex gangliosides

Gangliosides are a class of sphingolipid conjugated to sugar and N-acetyl neuroaminic acid (NANA) molecules, that are present in the outer leaflets of all mammalian plasma membranes, where they participate in recognition and signalling activity. The five major gangliosides in the brain occurred in the order GD1a > GT1b > GD1b > GQ1b > GM1 and together accounted for 70% of the total brain gangliosides, while GT1a, GD3 and GM3 contributed to approximately 10% of the total. Gangliosides are enriched in lipid rafts in neuronal cell membranes and play an important role in brain physiology and pathology (Park et al., 2005 a; Yaqoob, 2009; Schengrund et al., 2010).

Sphingolipid depletion inhibits the intracellular trafficking of GPI-anchored proteins, suggesting that lipid-protein interaction directly modulates gene expression and cellular trafficking important for cell development and behaviour.

During prion diseases it has been observed a marked decrease in ganglioside content, with a shift from complex (GM1, GD1a, GD1b, GT1b, GQ1b) to simpler gangliosides (GD3, GM3, GD2) (Naslavsky et al., 1999; Gilch et al., 2006; Piccinini et al., 2010). GM1 has been often associated with brain function and is the ganglioside used most commonly in brain-related research (McJarrow et al., 2009)

Experimental studies demonstrated that animals fed with a ganglioside enriched diet (0.02 % of the diet) have significantly higher ganglioside content in the intestinal mucosa, plasma and brain, compared with control ones (Park et al., 2005 a; McJarrow et al., 2009) and exogenous gangliosides

administrated *per os* seem to be directly incorporated into the microdomains (Park et al., 2005 b).

Moreover, an oral integration of gangliosides results in a significant lowering of microdomain cholesterol levels (see below) (Park et al., 2005 b).

13.1.2 - Low cholesterol diet

The brain consist of ~ 2% cholesterol that comes from primarily *de novo* synthesis. Astrocytes provide cholesterol needed by the CNS and apolipoprotein E (apoE). Cholesterol and apoE are released as a complex that is taken up by neurons via receptor-mediated endocytosis. Cholesterol is essential for normal neuronal development and it appears to play important roles in regulating synaptic plasticity, memory encoding and storage (Pfrieger, 2003; Schengrund et al., 2010).

Research demonstrated that cholesterol content of the brain increased during scrapie infection, raising the possibility that the presence of PrP^{Sc} alters the composition and function of lipid rafts, the integrity of which is necessary for synaptic functions (Kempster et al., 2007). Drugs able to inhibit the synthesis of cholesterol (statins) have proved to have an anti-prionic activity (Bate et al., 2004).

Statins act as reversible competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase, which, as the key rate-controlling enzyme in cholesterol biosynthesis, catalyses the conversion of HMG–CoA to mevalonic acid. Statins are categorized pharmacologically as lipophilic (i.e. lovastatin and simvastatin) or hydrophilic (i.e. pravastatin) compounds,

depending on their solubility in lipid solvents or water; both of them are able to cross the blood brain barrier (Vetrugno et al., 2009).

Statins are known to reduce the formation of the disease-associated isoform of the prion protein (PrP^{Sc}) in neuroblastoma cells *in vitro* (Bate et al., 2004). In murine prion models, statins administration (both at high- and low-dosage, the last usually used to treat hypercholesterolemia in humans) affect the progression of experimental scrapie, delaying disease progression and prolonging survival times (Mok et al., 2006; Kempster et al., 2007; Vetrugno et al., 2009).

The lower formation of PrP^{Sc} after cholesterol depletion induced by statins is related to the reduced expression of PrP^C on the cell membranes, which is presumably due to the disruption of the lipid rafts structure and/or to the prevention of the plasma membrane surface transport of the cellular prion protein. Cholesterol indeed is essential both in lipid rafts stability and in the secretory pathway of PrP^C (Taraboulos et al., 1995; Gilch et al., 2006).

Furthermore, cholesterol and gangliosides content in lipid rafts is tightly related and a depletion of cholesterol was shown to enhance expression of GM1, ganglioside with neuroprotective properties (Park et al., 2005 a; Schengrund et al., 2010).

13.2 - EXPERIMENTAL PROJECT

For this research project we are going to use the CD1 mouse model. Animals have been challenged via intraperitoneal route (i.p.) with the scrapie strain adapted to mice Rocky Mountain Laboratory (RML) and fed with medicated feed (low cholesterol diet or diet enriched with complex gangliosides) since

two weeks before mice inoculation. Four control groups have been formed: the “positive” control one, scrapie-infected and fed with standard feed, and three “negative” control groups, made up of animals challenged with a mouse brain homogenate from an healthy TSE-free animal and fed with the low cholesterol diet, with ganglioside-enriched diet or with the standard feed, respectively. Each of these six groups will consist of 15 elements (Table 11).

Table 11. Mice have been randomly divided into groups depending on the type of diet and scrapie challenged.

Group	Scrapie challenged	Diet	Number of animals
A	yes	standard	15
B	yes	low cholesterol	15
C	yes	ganglioside-enriched	15
D	no	standard	15
E	no	low cholesterol	15
F	no	ganglioside-enriched	15

Feed consumption and animal weights are recorded weekly. Mice are carefully monitored to detect the onset and the progression of scrapie-induced symptoms and will be euthanized using CO₂ at the terminal stage of disease. Animal survival times will be noted and, after necroscopy, their brains will be investigated by histological, immunohistochemical and Western blot analysis. The content of cholesterol and GM1 in lipid domains of the cortex and cerebellum and the localization of ADAM 10, ADAM 17 and prion protein will

be analysed at the Department of Neuropathology at the University of Bicocca in Milan.

14 - ABBREVIATIONS

AD: Alzheimer's Disease

ADAM: A disintegrin and metalloprotease

AHVLA: Animal Health Veterinary Laboratory Agency

APH-1: Anterior Pharynx 1

APP: Amyloid precursor protein

AR: Attack rate

ARR: A₁₃₆R₁₅₄R₁₇₁

A β : Amyloid- β

BAB: Born after ban

BACE: β -site APP-cleaving-enzyme

BSE: Bovine Spongiform Encephalopathy

CHF5074: 1-(3',4'-Dichloro-2-fluoro[1,1'-biphenyl]-4-yl)-
cyclopropanecarboxylic Acid

CJD: Creutzfeldt-Jakob Disease

CNS: Central nervous system

CR: Congo Red

CWD: Chronic Wasting Disease

DAB: Diaminobenzidine

DMNV: Dorsal motor nucleus of the vagus

DOC: deoxycholic acid sodium salt

ENS: Enteric nervous system

FAE: Follicle associated epithelium

FDC: Follicular dendritic cell

GAGs: Glycosaminoglycans

GALT: Gut-associated lymphoid tissues

GPI: glycosylphosphatidylinositol

GSS: Gerstman-Straussler-Scheinker

HE: Histopathological

i.c.: Intracerebral

i.p.: Intraperitoneal

IHC: immunohistochemical

IMLC: Intermediolateral column

IP: incubation period

LF: Lymphoid follicles

LP: lesion profile

LRS: Lymphoreticular system

m.w.: Molecular weight

MBM: Meat and bone meal

M-cells: membranous epithelial cells

Met: Methionine

NP40: Nonidet-P 40

PAGE: polyacrylamide gel electrophoresis

PEN-2: Presenilin enhancer 2

PGs: Proteoglycans

pK: Proteinase K

PKC: Protein kinase C

PMCA: Protein misfolding cyclic amplification

PMSF: phenylmethyl sulfonyl fluoride

PNS: peripheral nervous system

PrP^C: cellular prion protein

PrP^{Sc}: Prion

PrP^{Sen}: Protease-sensitive prion protein

PS: Presenilin

RNAi: Ribonucleic acid interference

SAF: Scrapie associated fibril

sAPP α : Secreted APP

SDS: Sodium dodecyl sulfate

TACE: Tumor necrosis factor α converting enzyme

TME: Transmissible Mink Encephalopathy

TSE: Transmissible Spongiform Encephalopathy

vCJD: variant Creutzfeldt-Jakob Disease

WB: Western blot

15 – BIBLIOGRAPHY

- Aguzzi A, Heppner FL, Heikenwalder M, Prinz M, Mertz K, Seeger H, Glatzer M: Immune system and peripheral nerves in propagation of prions to CNS. *British Medical Bulletin*, 2003; 66:141-159
- Aguzzi A, Polymenidou M: Mammalian prion biology. One century of evolving concepts. *Cell*, 2004; 116:313-327
- Allen LB, Cochran KW: Acceleration of scrapie in mice by target-organ treatment with interferon inducers. *Ann N Y Acad Sci*, 1977; 284:676-681
- Alper T, Cramp WA, Haig DA, Clarke MC: Does the agent of scrapie replicate without nucleic acid? *Nature*, 1967; 214:764-766
- Alper T, Haig DA, Clarke MC: The exceptionally small size of the scrapie agent. *Biochemical and Biophysical Research Communications*, 1966; 22:278-284
- Amyx H, Salazar AM, Gajdusek CD, Gibbs CJ: Chemotherapeutic trials in experimental slow virus diseases. *Neurology*, 1984; 34 (Suppl. 1)
- Andréoletti O, Berthon P, Marc D, Sarradin P, Grosclaude J, van Keulen L, Schelcher F, Elsen JM, Lantier F: Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. *J Gen Virol*, 2000; 81:3115-3126
- Baier M, Apelt J, Riemer C, Gultner S, Schwarz A, Bamme T, Burwinkel M, Schliebd R: Prion infection of mice transgenic for human APPSwe: increased accumulation of cortical formic acid extractable A β (1-42) and rapid scrapie disease development. *Int J Devl Neuroscience*, 2008; 26:821-824
- Balducci C, Mehdawy B, Mare L, Giuliani A, Lorenzini L, Sivilia S, Giardino L, Calzà L, Lanzillotta A, Sarnico I, Pizzi M, Usiello A, Viscomi AR, Ottonello S, Villetti G, Imbimbo BP, Nisticò G, Forloni G, Nisticò R: The γ -secretase modulator CHF5074 restores memory and

- hippocampal synaptic plasticity in plaque-free Tg2576 mice. *J Alzheimers Dis*, 2011 Jan; 24(4):799-816
- Bareggi SR, Braida D, Pollera C, Bondiolotti G, Formentin E, Puricelli M, Poli G, Ponti W, Sala M: Effects of clioquinol on memory impairment and the neurochemical modifications induced by scrapie infection in golden hamsters. *Brain Res*, 2009; 1280:195-200
 - Barnham KJ, Cappai R, Beyreuther K, Masters CL, Hill AF: Delineating common molecular mechanisms in Alzheimer's and prion diseases. *TRENDS in Biochemical Sciences*, 2006; 31(8):465-472
 - Baron T, Bencsik A, Vulin J, Biacabe AG, Morignat E, Verchere J, Betemps D: A C-terminal protease-resistant prion fragment distinguishes ovine "CH1641-like" scrapie from bovine classical and L-Type BSE in ovine transgenic mice. *PLoS Pathog*, 2008; 4:e1000137
 - Baron TG, Biacabe AG: Molecular Analysis of the Abnormal Prion protein during coinfection of mice by bovine spongiform encephalopathy and a scrapie agent. *J Virol*, 2001 Jan; 75(1):107-114
 - Baron TG, Madec JY, Calavas D, Richard Y, Barillet F: Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. *Neurosci Lett*, 2000 Apr 28; 284(3):175-178
 - Barret A, Tagliavini F, Forloni G, Bate C, Salmona M, Colombo L, De Luidi A, Limido L, Suardi S, Rossi G, Auvré F, Adjou KT, Salès N, Williams A, Lasmézas C, Deslys JP: Evaluation of quinacrine treatment for prion diseases. *J Virol*, 2003; 77:8462-8469
 - Bate C, Rutherford S, Gravenor M, Reid S, Williams A: Cyclooxygenase inhibitors protect against prion-induced neurotoxicity in vitro. *Neuroreport*, 2002; 13:1933-1938
 - Bate C, Salmona M, Diomedea L, Williams A: Squalenostatine cures prion-infected neurones and protects against prion neurotoxicity. *J Biol Chem*, 2004; 279:14983-14990
 - Baylis M, Houston F, Goldmann W, Hunter N, McLean AR: The signature of scrapie: differences in the PrP genotype profile of scrapie-

- affected and scrapie-free UK sheep flocks. *Proc R Soc Lond B Biol Sci*, 2000; 267:2029-2035
- Baylis M, Huston F, Kao RR, Mc Lean AR, Hunter N, Gravenor MB: BSE - a wolf in sheep's clothing? *Trends Microbiol*, 2002 Dec; 10(12):563-570
 - Beck KE, Chaplin M, Stack M, Sallis RE, Simonini S, Lockey R, Sporopoulos J: Lesion profiling at primary isolation in RIII mice is insufficient in distinguishing BSE from classical scrapie. *Brain Pathol*, 2010 Mar; 20(2):313-322 (a)
 - Beck KE, Sallis RE, Lockey R, Simmons MM, Spiropoulos J: Ovine PrP genotype is linked with lesion profile and immunohistochemistry patterns after primary transmission of classical scrapie to wild-type mice. *J Neuropathol Exp Neurol*, 2010 May; 69(5):483-497 (b)
 - Begara-McGorum I, Gonzalez L, Simmons M, Hunter N, Houston F, Jeffrey M: Vacuolar lesion profile in sheep scrapie: factors influencing its variation and relationship to disease-specific PrP accumulation. *J Comp Pathol*, 2002 Jul; 127(1):59-56
 - Bellworthy SJ, Dexter G, Stack M, Chaplin M, Hawkins SAC, Simmons MM: Natural transmission of BSE between sheep within an experimental flock. *Veterinary Record*, 2005; 157:206
 - Bellworthy SJ, Dexter G, Stack M, Chaplin M, Hawkins SAC, Simmons MM: Oral transmission of BSE to VRQ/VRQ sheep in an experimental flock. *Veterinary Record*, 2008; 162:130-131
 - Belt PB, Muileman IH, Schreuder BE, Bos-de Ruijter J, Gielkens AL, Smits MA: Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *J Gen Virol*, 1995 Mar; 76:509-517
 - Bendheim PE, Bolton DC: A 54-kDa normal cellular protein may be the precursor of the scrapie agent protease-resistant protein. *Proc Natl Acad Sci USA*, 1986; 83:2214-2218
 - Besnoit C, Morel C: Note sur les lésion nerveuses de la tremblante du mouton. *Rev Vet*, 1898; 23:397-400

- Bolton DC, McKinley MP, Prusiner SB: Identification of a protein that purifies with the scrapie prion. *Science*, 1982; 218:1309-1311
- Bondiolotti G, Rossoni G, Puricelli M, Formentin E, Lucchini B, Poli G, Ponti W, Bareggi SR: Changes in sympathetic activity in prion neuroinvasion. *Neurobiol Dis*, 2010; 37(1):114-117
- Braun U, Kihm U, Posterla N, Schomann M: Clinical examination of cattle upon with suspicion of bovine spongiform encephalopathy (BSE). *Schweiz Arch Tierheilkd*, 1997; 139:35-41
- Brown DA, Bruce ME, Fraser JR: Comparison of the neuropathological characteristics of bovine encephalopathy (BSE) and variant Creutzfeldt-Jakob disease (vCJD) in mice. *Neuropathol Appl Neurobiol*, 2003; 29:262-272
- Brown DR, Clive C, Haswell SJ: Antioxidant activity related to copper binding of native prion protein. *J Neurochem*, 2001; 76:69-76
- Brown DR, Herms J, Kretzschmar HA: Mouse cortical cells lacking cellular PrP survive in culture with a neurotoxic PrP fragment. *Neuroreport*, 1994; 5(16):2057-2060
- Brown DR, Schmidt B, Kretzschmar HA: Role of microglia and host prion protein in neurotoxicity of a prion protein fragment. *Nature*, 1996; 380(6572):345-347
- Brown P, Bradley R: 1755 and all that: a historical primer of transmissible spongiform encephalopathy. *BMJ*, 1998; 317:1688-1692
- Bruce ME, Boyle A, Cousens S, McConnell I, Foster J, Goldmann W, Fraser H: Strain characterization of natural sheep scrapie and comparison with BSE. *Journal of General Virology*, 2002; 83:695-704
- Bruce ME, McConnell I, Fraser H, Dickinson AG: The disease characteristics of different strains of scrapie in Sinc congenic mouse lines: implications for the nature of the agent and host control of pathogenesis. *Journal of General Virology*, 1991; 72:595-603
- Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttle A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ: Transmission to mice indicate that "new variant" CJD is caused by the BSE agent. *Nature*, 1997 Oct 2; 389(6650):498-501

- Bruce ME: Scrapie strain variation and mutation. *British Medical Bulletin*, 1993; 49:822-838
- Büeler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, et al. Mice devoid of PrP are resistant to scrapie. *Cell* 1993; 73(7):1339-1347
- Büeler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C: Normal development and behavior of mice lacking the neuronal cell-surface PrP protein. *Nature*, 1992; 356:577-582
- Calavas D, Ducrot C, Baron T, Morignat E, Vinard JL, Biacabe AG, Madec JY, Bencsik A, Debeer S, Eliazsewicz M: Prevalence of BSE in western France by screening cattle at risk: preliminary results of a pilot study. *Vet Rec*, 2001; 149:55-56
- Cashman NR, Loertscher R, Nalbantoglu J, Shaw I, Kascsak RJ, Bolton DC, Bendheim PE: Cellular isoform of the scrapie agent protein participates in lymphocyte activation. *Cell*, 1990; 61:185-192
- Caughey B, Race RE: Potent inhibition of scrapie-associated PrP accumulation by Congo red. *J Neurochem*, 1992; 59:768–771
- Caughey B, Raymond GJ: Sulfated polyanion inhibition of scrapie-associated PrP accumulation in cultured cells. *J Virol*, 1993; 67:643-650
- Caughey B, Raymond LD, Raymond GJ, Maxson L, Silveira J, Baron GS: Inhibition of protease-resistant prion protein accumulation in vitro by curcumin. *J Virol*, 2003; 77:5499-5502
- Caughey B: Interaction between prion protein isoforms: the kiss of death? *TRENDS in Biochemical Sciences*, 2001; 26(4):235-242
- Cazeau G, Ducrot C, Collin E, Desjouis G, Calavas D: Questionnaire analysis of BSE cases in France detected by active surveillance and the reasons for non-notification. *Vet Rec*, 2004; 154:133-136
- CFSPH (The Center for Food Security & Public Health): <http://www.cfsph.iastate.edu/Factsheets/pdfs/scrapie.pdf> (updated in April 2007)
- Chabry J, Priola SA, Wehrly K, Nishio J, Hope J, Chesebro B: Species independent inhibition of abnormal prion protein (PrP) formation by a

- peptide containing a conserved PrP sequence. *J Virol*, 1999; 73:6245-6250
- Chandler RL: Encephalopathy in mice produced with scrapie brain material. *Lancet*, 1961; i:1378-1379
 - Checler F, Vincent B: Alzheimer's and prion diseases: distinct pathologies, common proteolytic denominators. *Trends Neurosci*, 2002 Dec; 25(12):616-20
 - Cisse M, Mucke L: A prion protein connection. *Nature*, 2009; 457:1090-1091
 - Colby DW, Prusiner SB: Prions. *Cold Spring Harb Perspect Biol*, 2011; 3:a006833
 - Collinge J, Whitfi J, McKintosh E, Beck J, Mead S, Thomas DJ, Alpers MP: Kuru in the 21st century - an acquired human prion disease with very long incubation periods. *Lancet*, 2006; 367:2068-2074
 - Collinge J, Whittington MA, Sidle KCL, Smith CJ, Palmer MS, Clarke AR, Jefferys JG: Prion protein is necessary for normal synaptic function. *Nature*, 1994; 370:295-297
 - Collinge J: Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci*, 2001; 24:519-550
 - Collins S, McLean CA, Masters CL: Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia, and kuru: a review of these less common human transmissible spongiform encephalopathies. *Journal of Clinical Neuroscience*, 2001; 8(5):387-397
 - Collins SJ, Lewis V, Brazier M, Hill AF, Fletcher A, Masters CL: Quinacrine does not prolong survival in a murine Creutzfeldt–Jakob disease model. *Ann Neurol*, 2002; 52:503-506
 - Cranwell MP, Hancock RD, Hindson RD, Hall SA, Daniel NJ, Hopkins AR, Wonnacott B, Vivian ML, Hunt PT: Bovine spongiform encephalopathy. *Letter, Vet Rec*, 1988; 122:190
 - Cuillé J, Chelle PL: La maladie dite "tremblante" du mouton; est elle inoculable? *Compte Rend Acad Sci*, 1936; 203-1552

- Cutlip RC, Miller JM, Hamir AN, Peters J, Robinson MM, Jenny AL, Lehmkuhl HD, Taylor WD, Bisplinghoff FD: Resistance of cattle to scrapie by the oral route. *Can J Vet Res*, 2001, 65:131-132
- Cutlip RC, Miller JM, Race RE, Jenny AL, Katz JB, Lehmkuhl HD, Debey BM, Robinson MM: Intracerebral transmission of scrapie to cattle. *J Infect Dis*, 1994, 169:814-820
- Daude N, Marella M, Chabry J: Specific inhibition of pathological prion protein accumulation by small interfering RNAs. *J Cell Sci*, 2003; 116:2775-2779
- Defra:
<http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/bse/otherses/index.htm>
- Del Bo R, Scarlato M, Ghezzi S, Martinelli-Boneschi F, Fenoglio C, Galimberti G, Galbiati S, Virgilio R, Galimberti D, Ferrarese C, Scarpini E, Bresolin N, Comi GP: Is M129V of PRNP gene associated with Alzheimer's disease? A case-control study and a meta-analysis. *Neurobiol Aging*, 2006; 27(5):770.e1–770.e5
- Detwiler LA: Scrapie. *Rev Sci Tech*, 1992 Jun; 11(2):491-537
- Dickinson AG, Meikle VM H: Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. *Molecular and General Genetics*, 1971; 112:73-79
- Dickinson AG, Meikle VM, Fraser H: Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J Comp Pathol*, 1968 Jul; 78(3):293-299
- Dickinson AG, Meikle VMH, Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. *Molecular and General Genetics*, 1971; 112: 73-79
- Doh-ura K, Ishikawa K, Murakami-Kubo I, Sasaki K, Mohri S, Race R, Iwaki T: Treatment of transmissible spongiform encephalopathy by intraventricular drug infusion in animal models. *J Virol*, 2004; 78:4999-5006
- Dormont D: Approaches to prophylaxis and therapy. *British Medical Bulletin*, 2003; 66:281-292

- Ducrot C, Arnold M, De Koeijer A, Heim D, Calavas D: Review on the epidemiology and dynamics of BSE epidemics. *Vet Res*, 2008; 39:15-32
- Eloit M, Adjou K, Couplier M, Fontaine JJ, Hamel R, Lilin T, Messiaen S: BSE agent signatures in a goat. *Veterinary Record*, 2005; 156: 523-524
- Engelman DM: Membranes are more mosaic than fluid. *Nature*, 2005; 438:578-580
- EP Resolution 6 July 2011: <http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//TEXT+TA+P7-TA-2011-0328+0+DOC+XML+V0//EN>
- European Commission, Food and Feed Safety: http://ec.europa.eu/food/food/biosafety/tse_bse/chronological_list_tse_en.htm
- Evin G, Sernee MF, Masters CL: Inhibition of γ -secretase as a therapeutic intervention for Alzheimer's disease: prospects, limitations and strategies. *CNS Drugs*, 2006; 20(5):351-372
- Fisher M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C: Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J*, 1996; 15:1255-1264
- Ford MJ, Burton Lj, Morris RJ, Hall SM: Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience*, 2002; 113:177-192
- Foster JD, Hope J, Fraser H: Transmission of bovine spongiform encephalopathy to sheep and goats. *Vet Rec*, 1993 Oct 2; 133(14):339-341
- Foster JD, Parnham DW, Hunter N, Bruce M: Distribution of the prion protein in sheep terminally affected with BSE following experimental oral transmission. *J Gen Virol*, 2001 Oct; 82(10):2319-26
- Fraser H, Dickinson AG: The sequential development of the brain lesions of scrapie in three strains of mice. *Journal of Comparative Pathology*, 1968; 78:301-311

- Gajdusek DC, Gibbs CJ Jr, Alpers M: Experimental transmission of a kuru-like syndrome to chimpanzees. *Nature*, 1966; 209:794-796
- Gajdusek DC, Zigas V: Degenerative disease of the central nervous system in New Guinea: epidemic occurrence of "kuru" in the native population. *N Engl J Med*, 1957; 257:974-978
- Garattini S: Long-chain n-3 fatty acids in lipid rafts: implication for anti-inflammatory effects. *J Cardiovasc Med*, 2007 Sep 8; Suppl 1:S30-S33
- Garcia-Alloza M, Borrelli LA, Rozkalne A, Hyman BT, Bacskai BJ: Curcumin labels amyloid pathology in vivo, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. *J Neurochem*, 2007; 102:1095-1104
- Gavier-Widen D, Stack MJ, Baron T, Balachandran A, Simmons MM: Diagnosis of transmissible spongiform encephalopathies in animals: a review. *J Vet Diagn Invest*, 2005; 17:509-527
- Gerstmann J, Sträussler E, Scheinker I. Über eine eigenartige hereditär-familiäre Erkrankung des Zentralnervensystems. Zugleich ein Beitrag zur Frage des vorzeitigen lokalen Alterns. *Z Neurol* 1936; 154: 736-762
- Gibbs CJ Jr, Gajdusek DC, Asher DM, Alpers MP, Beck E, Daniel PM, Matthews WB: Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science*, 1968; 161:388-389
- Gilch S, Kehler C, Schatzl HM: The prion protein requires cholesterol for cell surface localization. *Mol Cell Neurosci*, 2006; 31:346-353
- Gimbel DA, Nygaard HB, Coffey EE, Gunther EC, Laurén J, Gimbel ZA, Strittmatter SM: Memory impairment in transgenic Alzheimer mice requires cellular prion protein. *J Neurosci*, 2010; 30(18):6367-6374
- Gordon WS: Advances in veterinary research. *Vet Rec*, 1946; 58:516-520
- Grassi J, Creminon C, Frobert Y, Fretier P, Turbica I, Rezaei H, Hunsmann G, Comoy E, Deslys JP: Specific determination of the proteinase K-resistant form of the prion protein using two-site

immunometric assays. Application to the post-mortem diagnosis of BSE. *Arch Virol Suppl*, 2000; 16:197-205

- Grassi J, Maillet S, Simon S, Morel N: Progress and limits of TSE diagnostic tools. *Vet Rec*, 2008; 39(4):33
- Green R, Horrocks C, Wilkinson A, Hawkins SA, Ryder SJ: Primary isolation of the bovine spongiform encephalopathy agent in mice: agent definition based on a review of 150 transmissions. *J Comp Pathol*, 2005; 132:117-131
- Gresser I, Maury C, Chandler RL: Failure to modify scrapie in mice by administration of interferon or anti-interferon globulin. *J Gen Virol*, 1983; 64:1387-1389
- Griffith JS: Self-replication and scrapie. *Nature*, 1967; 1043-1044
- Grouschup MH, Buschmann A: Rodent models for prion diseases. *Vet Res*, 2008 Jul-Aug; 39(4):32
- Hadlow WJ: Scrapie and kuru. *Lancet*, 1959; 2:289-290
- Heikenwalder M, Julius C, Aguzzi A: Prions and peripheral nerves: a deadly rendezvous – review. *Journal of Neuroscience Research*, 2007; 85:2714-2725
- Heim D, Wilesmith JW: Surveillance of BSE. *Arch Virol*, 2000; 16:127-133
- Heppner FL, Christ AD, Klein MA, Prinz M, Fried M, Kraehenbuhl JP, Aguzzi A: Transepithelial prion transport by M cells. *Nat Med*, 2001; 7:976-977
- Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J, Doey LJ, Lantos P: The same prion causes vCJD and BSE. *Nature*, 1997 Oct 2; 389(6650):448-450
- Hoffmann C, Ziegler U, Buschmann A, Weber A, Kupfer L, Oelschlegel A, Hammerschmidt B, Groschup MH: Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy. *J Gen Virol*, 2007; 88:1048-1055

- Hoinville LJ, Wilesmith JW, Richards MS: An investigation of risk factors for cases of bovine spongiform encephalopathy born after the introduction of “feed ban”. *Vet Rec*, 1995; 136:312-318
- Hooper NM, Turner AJ: A new take on prions: preventing Alzheimer’s disease. *Trends Biochem Sci*, 2008; 33(4):151-155
- Hope J, Wood SC, Birkett CR, Chong A, Bruce ME, Cairns D, Goldmann W, Hunter N, Bostock CJ: Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. *J Gen Virol*, 1999; 80(Pt 1):1-4
- Horn G, Bobrow M, Bruce M, Goedert M, McLean A, Webster J: Review of the origin of BSE: report to UK government. London: The Stationery Office, 2001
- Hughes MM, Field RH, Perry VH, Murray CL, Cunningham C: Microglia in the degenerating brain are capable of phagocytosis of beads and of apoptotic cells, but do not efficiently remove PrP^{Sc} even upon LPS stimulation. *Glia*, 2010; 58(16):2017-2030
- Hunter N: Scrapie and experimental BSE in sheep. *British Medical Bulletin*, 2003; 66:171-183
- Imbimbo BP, Balducci C, Mare L, Forloni G, Villetti G, Nisticò R: CHF5074, a novel γ -secretase modulator, completely reverses hippocampal long-term potentiation and non-spatial memory deficits in young, plaque-free, human amyloid precursor protein transgenic mice. 13th International Conference on Alzheimer’s Disease. Honolulu. 2010 (b)
- Imbimbo BP, Del Giudice E, Cenacchi V, Volta R, Villetti G, Facchinetti F, Riccardi B, Puccini P, Moretto N, Grassi F, Ottonello S, Leon A: In vitro and in vivo profiling of CHF5022 and CHF5074: two β -amyloid1-42 lowering agents. *Pharmacological Research*, 2007; 55:318-328 (a)
- Imbimbo BP, Del Giudice E, Colavito D, D’Arrigo A, Dalle Carbonare M, Villetti G, Facchinetti F, Volta R, Pietrini V, Baroc MF, Serneels L, De Strooper B, Leon A: L’1-(3’,4’-dichloro-2-fluoro[1,1’-biphenyl]-4-yl)-cyclopropanecarboxylic acid (CHF5074), a novel γ -secretase modulator, reduces brain β -amyloid pathology in a transgenic mouse

- model of Alzheimer's Disease without causing peripheral toxicity. JPET, 2007; 323:822-830 (b)
- Imbimbo BP, Giardino L, Sivilia S, Giuliani A, Gusciglio M, Pietrini V, Del Giudice E, D'Arrigo A, Leon A, Villetti G, Calzà C: CHF5074, a novel γ -secretase modulator, restores hippocampal neurogenesis potential and reverses contextual memory deficit in a transgenic mouse model of Alzheimer's disease. Journal of Alzheimer's Disease, 2010; 20:159-173 (a)
 - Imbimbo BP, Hutter-Paier B, Villetti G, Facchinetti F, Cenacchi V, Volta R, Lanzillotta A, Pizzi M, Windisch M: CHF5074, a novel γ -secretase modulator, attenuates brain β -amyloid pathology and learning deficit in a mouse model of Alzheimer's Disease. British Journal of Pharmacology, 2009; 156:982-993
 - Ironside JW, Head MW: Neuropathology and molecular biology of variant Creutzfeldt-Jakob disease. Curr Top Microbiol Immunol, 2004; 284:133-59
 - Jacobs JG, Langeveld JP, Biacabe AG, Acutis PL, Polak MP, Gavier-Widen D, Buschmann A, Caramelli M, Casalone C, Mazza M, Groschup M, Erkens JH, Davidse A, van Zijderveld FG, Baron T: Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. J Clin Microbiol, 2007 Jun; 45(6):1821-1829
 - Jacobs JG, Sauer M, van Kuelen LJ, Tang Y, Bossers A, Langeveld JP: Differentiation of ruminant transmissible spongiform encephalopathy isolate types, including bovine spongiform encephalopathy and CH1641 scrapie. J Gen Virol, 2011 Jan; 92(1):222-232
 - Jeffrey M, Gonzalez L, Chong Foster AJ, Goldmann W, Hunter N, Martin S: Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunochemical similarities can be resolved by immunohistochemistry. J Comp Path, 2006; 134:17-29

- Jeffrey M, Martin S, Gonzalez L, Ryder SJ, Bellworthy SJ, Jackman R: Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. *J Comp Pathol*, 2001 Nov; 125(4):271-284
- Jones M, Peden A, Prowse C, Groner A, Manson J, Turner M, Ironside JW, MacGregor IR, Head MW: In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrP(Sc). *J Pathol*, 2007; 213:21-26
- *Journal of the House of Commons*, 1755; 27:87
- Kao RR, Gravenor MB, Baylis M, Bostock CJ, Chihota CM, Evans JC, Goldmann W, Smith AJ, McLean AR: The potential size and duration of an epidemic of bovine spongiform encephalopathy in British sheep. *Science*, 2002 Jan 11; 295(5553):332-335
- Kempster S, Bate C, Williams A: Simvastatin treatment prolongs the survival of scrapie-infected mice. *Neuropharmacology and Neurotoxicology*, 2006; 18(5):479-482
- Khalili-Shirazi A, Summers L, Linehan J, Mallinson G, Anstee D, Hawke S, Jackson GS, Collinge J: PrP glycoforms are associated in a strain-specific ratio in native PrPSc. *J Gen Virol*, 2005; 86:2635-2644
- Klatzo I, Gajdusek DC, Zigas V: Pathology of kuru. *Lab Invest*, 1959; 8:799-847
- Konold T, Lee YH, Stack MJ, Horrocks C, Green RB, Chaplin M, Simmons MM, Hawkins SA, Lockey R, Spiropoulos J, Wilesmith JW, Wells GA: Different prion disease phenotypes result from inoculation of cattle with two temporally separated sources of sheep scrapie from Great Britain. *BMC Vet Res*, 2006, 2:31
- Konold T, Sivam SK, Ryan J, Gubbins S, Laven R, Howe MJH: Analysis of clinical signs associated with bovine spongiform encephalopathy in casualty slaughter cattle. *Vet J*, 2006; 171:438-444
- Korth C, May BC, Cohen FE, Prusiner SB. Acridine and phenothiazine derivatives as pharmacotherapeutics for prion disease. *Proc Natl Acad Sci USA*, 2001; 98:9836-9841
- Kovacs GG, Budka H: Prion diseases: from protein to cell pathology. *The American Journal of Pathology*, 2008; 172(3):555-565

- Kurt TD, Perrott MR, Wilusz CJ, Wilusz J, Supattapone S, Telling GC, Zabel MD, Hoover EA: Efficient in vitro amplification of chronic wasting disease PrPres. *J Virol*, 2007; 81:9605-9608
- Laffont-Proust I, Faucheux BA, Hassing R, Sazdovitch V, Simon S, Grassi J, Hauw JJ, Moya KL, Haik S: The N-terminal cleavage of cellular prion protein in the human brain. *FEBS Lett*, 2005 Nov 21; 579(28):6333-6337
- Laurén J, Gimbel DA, Nygaard HB, Gimbel JW, Strittmatter SM: Cellular prion protein mediates impairment of synaptic plasticity by amyloid- β oligomers. *Nature* 2009; 457(7233):1128-1132
- Lichtenstein MP, Carriba P, Baltrons MA, Wojciak-Stothard B, Peterson JR, García A, Galea E: Secretase-independent and RhoGTPase/PAK/ERK-dependent regulation of cytoskeleton dynamics in astrocytes by NSAIDs and derivatives. *J Alzheimers Dis*, 2010; 22(4):1135-1155
- Ligios C, Sigurdson CJ, Santucci C, Carcassola G, Manco G, Basagni M, Maestrale C, Cancedda MG, Madau L, Aguzzi A: PrPSc in mammary glands of sheep affected by scrapie and mastitis. *Nat Med*, 2005; 11:1137-1138
- Lugaresi E, Medori R, Montagna P, Baruzzi A, Cortelli P, Lugaresi A, Zucconi M, Gambetti P: Fatal familial insomnia and dysautonomia with selective degeneration of thalamic nuclei. *N Engl J Med*, 1986; 315(16):997-1003
- Ma DWL, Seo JS, Switzer KC, Fan YY, McMurray D, Lupton JR, Chpkin RS: n-3 PUFA and membrane microdomains: a new frontier in bioactive lipid research. *J Nutr Biochem*, 2004 Nov; 15(11):700-706
- Maglio LE, Perez MF, Martins VR, Brentani RR, Ramirez OA: Hippocampal synaptic plasticity in mice devoid of cellular prion protein. *Mol Brain Res*, 2004; 131:58-64
- Magri G, Clerici M, Dall'Ara P, Biasin M, Caramelli M, Casalone C, Giannino ML, Longhi R, Piacentini L, Della Bella S, Gazzuola P, Martino PA, Della Bella S, Pollera C, Puricelli M, Servida F, Crescio I, Boasso A, Ponti W, Poli G: Decrease in pathology and progression of

- scrapie after immunisation with synthetic prion protein peptides in hamsters. *Vaccine*, 2005; 23(22):2862-2868
- Mallucci G, Collinge J: Rational targeting for prion therapeutics. *Nat Rev Neurosci*, 2005; 6:23-34
 - Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J: Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science*, 2003; 302:871-874
 - Marsh RF, Bessen RA, Lehmann S, Hartsough GR: Epidemiological and experimental studies on a new incident of transmissible mink encephalopathy. *J Gen Virol*, 1991; 72:589-594
 - Marsh RF, Bessen RA: Epidemiologic and experimental studies on transmissible mink encephalopathy. *Dev Biol Stand*, 1993; 80:111-118
 - Marsh RF, Bessen RA: Transmissible mink encephalopathy. *Rev Sci Tech*, 1992; 11:539-550
 - Mazza M, Iulini B, Vaccari G, Acutis PL, Martucci F, Esposito E, Peletto S, Barocci S, Chiappini B, Corona C, Barbieri I, Caramelli M, Agrimi U, Casalone C, Nonno R: Co-existence of classical scrapie and Nor98 in a sheep from an Italian outbreak. *Res Vet Sci*, 2010 Jun; 88(3):478-485
 - McJarrow P, Schnell N, Jumpsen J, Clandinin T: Influence of dietary gangliosides on neonatal brain development. *Nutrition Reviews*, 2009; 67(8):451-463
 - Meeker HC, Ye X, Carp RI: The mouse model for scrapie. Inoculation, clinical scoring and histopathological techniques. *Methods Mol Biol*, 2005; 299:309-23
 - Menon VP, Sudheer AR: Antioxidant and antiinflammatory properties of curcumin. *Adv Exp Med Biol*, 2007; 595:105-125
 - Milhavet O, Lehmann S: Oxidative stress and the prion protein in transmissible spongiform encephalopathies. *Brain Res Brain Res Rev*, 2002; 38:328-339
 - Mishra RS, Basu S, Gu Y, Luo X, Zou WQ, Mishra R, Li R, Chen SG, Gambetti P, Fujioka H, Singh N: Protease-resistant human prion protein and ferritin are cotransported across Caco-2 epithelial cells:

implications for species barrier in prion uptake from the intestine. *J Neurosci*, 2004; 24:11280-11290

- Mok SWF, Karin M, Thelen KM, Riemer C, Bamme T, Gultner S, Lutjohann D, Baier M: Simvastatin prolongs survival times in prion infections of the central nervous system. *Biochemical and Biophysical Research Communications*, 2006; 348:697–702
- Moore AR, Taubner LM, Priola SA: Prion protein misfolding and disease. *Curr Opin Struct Biol*, 2009; 19:1-9
- Mouillet-Richard S, Ermonval M, Chebassier C, Laplanche JL, Lehmann S, Launay JM, Kellermann O: Signal transduction through prion protein. *Science*, 2000; 289:1925-1928
- Naslavsky N, Shmeeda H, Friedlander G, Yanai A, Futerman AH, Barenholz Y, Taraboulos A: Sphingolipid depletion increases formation of the scrapie prion protein in neuroblastoma cells infected with prions. *J Biol Chem*, 1999; 274:20763-20771
- NCJDRSU (The National Creutzfeldt-Jakob Disease Research & Surveillance Unit): <http://www.cjd.ed.ac.uk/>
- Neutra MR, Pringault E, Kraehnstuhl JP: Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu Rev Immunol*, 1996; 14:275-300
- Norrby E: Prions and protein-folding diseases. *J Intern Med*, 2011; 270(1):1-14
- Nuvolone M, Aguzzi A, Heikenwalder M: Cells and prions: a license to replicate. *FEBS Letters*, 2009; 583:2674-2684
- Oesch B, Doherr M, Heim D, Fischer K, Egli S, Bolliger S, Biffiger K, Schaller O, Vendevelde M, Moser M: Application of prionics Western blotting procedure to screen for BSE in cattle regularly slaughtered at Swiss abattoirs. *Arch. Virol Suppl*, 2000; 16:189-195
- Outram GW, Dickinson AG, Fraser H: Reduced susceptibility to scrapie in mice after steroid administration. *Nature*, 1974; 249:855-856
- Outram GW, Dickinson AG, Fraser H: Slow encephalopathies, inflammatory responses and arachis oil. *Lancet*, 1975; 1:198-200

- Outram GW, Fraser H, Wilson DT: Scrapie in mice: some effects on the brain lesion profile of ME7 agent due to genotype of donor, route of injection and genotype of recipient. *Journal of Comparative Pathology*, 1973; 83(1):19-28
- Park EJ, Suh M, Ramanujam K, Steiner K, Begg D, Clandinin MT: Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *Journal of Pediatric Gastroenterology and Nutrition*, 2005; 40:487-495 (a)
- Park EJ, Suh M, Thomson B, Thomson ABR, Ramanujam K, Clandinin MT: Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology*, 2005; 15(10):935-942 (b)
- Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, Manson JC, Baybutt HN, Turner AJ, Hooper NM: Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U.S.A.*, 2007 Jun 26; 104(26):11062-11067
- Peretto I, Radaelli S, Parini C, Zandi M, Raveglia LF, Dondio G et al.: Synthesis and biological activity of flubiprofen analogues as selective inhibitors of β -amyloid1-42 secretion. *J Med Chem*, 2005; 48:5705-5720
- Perry VH, Cunningham C, Boche D: Atypical inflammation in the central nervous system in prion disease. *Curr Opin Neurol*, 2002; 15:349-354
- Pfriegeer FW. Role of cholesterol in synapse formation and function. *BBA Biomembranes*, 2003; 1610:271-280
- Piccinini M, Scandroglio F, Prioni S, Buccinnà B, Loberto N, Aureli M, Chigorno V, Lupino E, DeMarco G, Lomartire A, Rinaudo MT, Sonnino S, Prinetti A: Deragulated sphingolipid metabolism and membrane organization in neurodegenerative disorders. *Mol Neurobiol*, 2010; 41(2-3):314-340
- Pinheiro T: The role of rafts in the fibrillization and aggregation of prions. *Chemistry and Physics of Lipids*, 2006; 141:66-71

- Poli G, Martino PA, Villa S, Carcassola G, Giannino ML, Dall'Ara P, Pollera C, Iussich S, Tranquillo VM, Bareggi S, Mantegazza P, Ponti W: Evaluation of anti-prion activity of congo red and its derivatives in experimentally infected hamsters. *Arzneimittelforschung*, 2004; 54(7):406-415
- Pollera C, Carcassola G, Ponti W, Poli G: Development of in vitro cell cultures for the evaluation of molecules with antiprionic activity. *Vet Res Commun*, 2003; 27 Suppl 1:719-721
- Pollera C, Lucchini B, Formentin E, Bareggi S, Poli G, Ponti W: Evaluation of anti-prionic activity of clioquinol in an in vivo model (*Mesocricetus auratus*). *Vet Res Commun*, 2005; 29(Suppl 2):253-255
- Priola SA, Raines A, Caughey W: Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. *J Infect Dis*, 2003; 188:699-705
- Priola SA, Raines A, Caughey WS: Porphyrin and phthalocyanine antiscrapie compounds. *Science*, 2000; 287:1503–1506
- Prusiner SB, Groth D, Serban A, Koehler R, Foster D, Torchia M, Burton D, Yang SL, DeArmond SJ: Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. *Proc Natl Acad Sci U.S.A.*, 1993; 90(22):10608-10612
- Prusiner SB: Novel proteinaceous infectious particles cause scrapie. *Science*, 1982; 216:136-144
- Prusiner SB: Prions. *Proc Natl Acad Sci USA*, 1998; 95:13363-13383
- Rescigno M, Rotta G, Valzasina B, Ricciardi Castagnoli P: Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology*, 2001; 204:572-581 (a)
- Rescigno M, Urbano M, Valzasina B, Francolini M, Rottta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi Castagnoli P: Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*, 2001; 2:361-367 (b)
- Rezaie P, Lantos PL: Microglia and the pathogenesis of spongiform encephalopathies. *Brain Res Brain Res Rev*, 2001; 35(1):55-72

- Riek R., Hornemann S, Wider G, Glockshuber R, Wuthrich K: NMR characterization of the full-length recombinant murine prion protein mPrP(23-231). *FEBS Lett*, 1997; 413:282-288
- Riemenschneider M, Klopp N, Xiang W, Wagenpfeil S, Vollmert C, Muller U, Forstl H, Illig T, Kretzschmar H, Kurz A: Prion protein codon 129 polymorphism and risk of Alzheimer disease. *Neurology*, 2004; 63(2):364-366
- Riemer C, Burwinkel M, Schwarz A, Gultner S, Mok SW, Heise I, Holtkamp N, Baier M: Evaluation of drugs for treatment of prion infections of the central nervous system. *J Gen Virol*, 2008 Feb; 89(Pt 2):594-597
- Rivera-Milla E, Stuermer CA, Malaga-Trillo E: An evolutionary basis for scrapie disease: identification of a fish prion mRNA. *Trends Genet*, 2003; 19:72-75
- Roikhel VM, Fokina GI, Pogodina VV: Influence of aminasine on experimental scrapie in mice. *Acta Virol*, 1984; 28: 321-324
- Ronga L, Tizzano B, Palladino P, Ragone R, Urso E, Maffia M, Ruvo M, Benedetti E, Rossi F: The prion protein: structural features and related toxic peptides. *Chem Biol Drug Des*, 2006; 68:139-147
- Ryou C: Prions and prion diseases: fundamental and mechanistic details. *J Microbiol Biotechnol*, 2007; 17(7):1059-1070
- Saborio GP, Permanne B, Soto C: Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature*, 2001; 411:810-813
- Sakudo A, Ikuta K: Prion protein functions and dysfunction in prion diseases. *Curr Med Chem*, 2009; 16:380-389
- Sanghera N, Pinheiro TJ. Binding of prion protein to lipid membranes and implications for prion conversion. *J Mol Biol*, 2002; 315(5):1241-1256
- Scanziani E, Stella S, Ghisleni G: Manuale di ispezione e controllo delle carni, II edizione. Epitesto, 2006
- Schaller O, Fatzer R, Stack M, Clark J, Cooley W, Biffiger K, Doherr M, Vandeveld M, Heim D, Oesch B, Moser M: Validation of a western

- immunoblotting procedure for bovine PrP(Sc) detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). *Acta Neuropathol*, 1999; 98:437-443
- Schengrund CL: Lipid rafts: keys to neurodegeneration. *Brain Research Bulletin*, 2010; 82:7-17
 - Schneider K, Fangerau H, Michaelsen B, Raab WHM: The early history of the transmissible spongiform encephalopathies exemplified by scrapie. *Brain Research Bulletin*, 2008; 77:343-355
 - Schultz J, Schwarz A, Neidhold S, Burwinkel M, Riemer C, Simon D, Kopf M, Otto M, Baier M: Role of interleukin-1 in prion disease-associated astrocyte activation. *Am J Pathol*, 2004; 165:671-678
 - Sigurdson CJ, Miller MW: Other animal prion diseases. *Br Med Bull*, 2003; 66:199-212
 - Sigurdsson EM, Brown DR, Daniels M, Kascsak RJ, Kascsak R, Carp R, Meeker HC, Frangione B, Wisniewski T: Immunization delays the onset of prion disease in mice. *Am J Pathol*, 2002; 161:13-17
 - Simmons MM, Spiropoulos J, Hawkins SAC, Bellworthy SJ, Tongue SC: Approaches to investigating transmission of spongiform encephalopathies in domestic animals using BSE as an example. *Vet Res*, 2008; 39:34
 - Simonic T, Duga S, Strumbo B, Asselta R, Ceciliani F, Ronchi S: cDNA cloning of turtle prion protein. *FEBS Lett*, 2000; 469:33-38
 - Simons K, Ikonen E: Functional rafts in cell membranes. *Nature*, 1997; 387:569-572
 - Singer SJ, Nicolson GL: The fluid mosaic model of the structure of cell membranes. *Science*, 1972; 175:720-731
 - Smith PG, Bradley R: Bovine spongiform encephalopathy (BSE) and its epidemiology. *British Medical Bulletin*, 2003; 66:185-198
 - Soto C, Anderes L, Suardi S, Cardone F, Castilla J, Frossard MJ, Peano S, Saa P, Limido L, Carbonatto M, Ironside J, Torres JM, Pocchiari M, Tagliavini F: Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. *FEBS Lett*, 2005; 579:638-642

- Spilman P, Lessard P, Sattavat M, Bush C, Tousseyn T, Huang EJ, Giles K, Golde T, Das P, Fauq A, Prusiner SB, DeArmond SJ: A γ -secretase inhibitor and quinacrine reduce prions and prevent dendritic degeneration in murine brains. *PNAS*, 2008; 29(105):10595-10600
- Spiropoulos J, Lockey R, Sallis RE, Terry LA, Thorne L, Holder TM, Beck KE, Simmons M: Isolation of Prion with BSE Properties from a Farmed Goat. *Emerging Infectious Diseases*, 2011; 17(12): *in press*
- Spraker TR, O'Rourke KI, Balachandran A, Zink RR, Cummings BA, Miller MW, Powers BE: Validation of monoclonal antibody F99/97.6.1 for immunohistochemical staining of brain and tonsil in mule deer (*Odocoileus hemionus*) with chronic wasting disease, *J Vet Diagn Invest*, 2002; 14:3-7
- Stack M, Jeffrey M, Gubbins S, Grimmer S, Gonzalez L, Martin S, Chaplin M, Webb P, Simmons M, Spencer Y, Bellerby P, Hope J, Wilesmith J, Matthews D: Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998-2004. *Journal of General Virology*, 2006; 87:2099-2107
- Stack MJ, Chaplin MJ, Clark J: Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta Neuropathol*, 2002 Sep; 104(3):279-86
- Steele ADJ, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD: Prion protein (PrPC) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. *Proc Natl Acad Sci USA*, 2006; 103:3416-3421
- Strumbo B, Ronchi S, Bolis LC, Simonic T: Molecular cloning of the cDNA coding for *Xenopus laevis* prion protein. *FEBS Lett*, 2001; 508:170-174
- Sunyach C, Jen A, Deng J, Fitzgerald KT, Frobert Y, Grassi J, McCaffrey MW, Morris R: The mechanism of internalization of

glycosylphosphatidylinositol-anchored prion protein. *EMBO J*, 2003; 3591-3601

- Tagliavini F, Forloni G, Colombo L, Rossi G, Girola L, Canciani B, Angeretti N, Peressini E, Awan T, De Gioia L, Ragg E, Bugiani O, Salamona M: Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) in vitro. *J Mol Biol*, 2000; 300:1309-1322
- Taraboulos A, Scott M, Semenov A, Avraham D, Laszlo L, Prusiner SB: Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J Cell Biol*, 1995; 129:121-132
- Taylor DR, Hooper NM. Role of lipid rafts in the processing of the pathogenic prion and Alzheimer's amyloid- β proteins. *Semin Cell Dev Biol*, 2007; 18(5):638-648
- Taylor DR, Hooper NM: The prion protein and lipid rafts (review). *Mol Membr Biol*, 2006 Jan-Feb; 23(1):89-99
- Terry LA, Marsh S, Ryder SJ, Hawkins SA, Wells GA, Spencer YI: Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy. *Vet Rec*, 2003 Mar 29; 152(13):387-92
- Thackray AM, Hopkins L, Lockey R, Spiropoulos J, Bujdoso R: Emergence of multiple prion strains from single isolates of ovine scrapie. *J Gen Virol*, 2011; 92:1482-1491
- Thackray AM, Hopkins L, Spiropoulos J, Bujdoso R: Molecular and transmission characteristics of primary passaged ovine scrapie isolates in conventional and ovine PrP transgenic mice. *J Virol*, 2008; 82:11197-11207
- Thackray AM, Klein MA, Aguzzi A, Bujdoso R: Chronic subclinical prion disease induced by low-dose inoculum. *J Virol*, 2002; 76(5):2510-2517
- The TSE Roadmap, 2005: http://ec.europa.eu/food/food/biosafety/bse/roadmap_en.pdf
- Tilly G, Chapuis J, Vilette D, Laude H, Vilotte JL: Efficient and specific downregulation of prion protein expression by RNAi. *Biochem Biophys Res Commun*, 2003; 305:548-551

- Tobias MJ, Parkin ET, Turner AJ, Hooper NM: ADAMs family members as amyloid precursor protein alpha-secretases. *J Neurosci Res*, 2003; 74:342-352
- Trevitt CR, Collinge J: A systematic review of prion therapeutics in experimental models. *Brain*, 2006; 129:2241-2265
- TSE EU Community Reference Laboratory: Clinical signs of bovine spongiform encephalopathy in cattle, 2007: http://vla.defra.gov.uk/science/docs/sci_bse_res.pdf
- TSE EU Community Reference Laboratory: fixation, tissue processing, histology and immunohistochemistry staining procedures for diagnosis of animal TSE (BSE, scrapie, atypical scrapie). Updated March 2009: http://www.defra.gov.uk/vla/science/docs/sci_tse_rl_prp_ihc.pdf
- van Keulen LJ, Schreuder BE, Meloen RH, Mooij-Harkes G, Vromans ME, Langeveld JP: Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. *J Clin Microbiol*, 1996; 34:1228-1231
- van Keulen LJ, Schreuder BE, Meloen RH, Poelen-van den BM, Mooij-Harkes G, Vromans ME, Langeveld JP: Immunohistochemical detection and localization of prion protein in brain tissue of sheep with natural scrapie. *Vet Pathol*, 1995; 32:299- 308
- Van Keulen LJM, Bossers A, Van Zijderveld F: TSE pathogenesis in cattle and sheep. *Vet Res*, 2008; 39:24
- Van Kuelen LJM, Schreuder BE, Vromans ME, Langeveld JP, Smits MA: Pathogenesis of natural scrapie in sheep. *Arch Virol Suppl*, 2000; 16: 57-71
- Vascellari M, Nonno R, Multinelli F, Bigolaro M, Di Bari MA, Melchiotti E, Marcon S, D'Agostino C, Vaccari G, Conte M, De Grossi L, Rosone F, Giordani F, Agrimi U: PrPSc in salivary glands of scrapie-affected sheep. *J Virol*, 2007; 81:4872-4876
- Vetrugno V, Di Bari MA, Nonno R, Puopolo M, D'Agostino C, Pirisinu L, Pocchiari M, Agrimi U: Oral pravastatin prolongs survival time of scrapie infected mice. *Journal of General Virology*, 2009; 90:1775–1780

- Vincent B, Paitel E, Saftig P, Frobert Y, Hartmann D, De Strooper B, Grassi J, Lopez-Perez E, Checkler F: The disintegrins ADAM 10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein. *J Biol Chem*, 2001 Oct 12; 276(41):37743-37746
- Vincent B: ADAM protective role in Alzheimer's and prion disease? *Curr Alzheimer Res*, 2004 Aug; 1(3):165-74
- Voigtlandre T, Kloppel S, Birner P, Jarius C, Flicker H, Verghese-Nikolakaki S, Sklaviadis T, Guentchev M, Budka H: Marked increase of neuronal prion protein immunoreactivity in Alzheimer's disease and human prion diseases. *Acta Neuropathol*, 2001; 101(5):417-423
- Walmsley AR, Zeng F, Hooper NM: The N-terminal Region of the Prion Protein Ectodomain Contains a Lipid Raft Targeting Determinant. *J Biol Chem*, 2003 Sep; 279(39):37241-37248
- Watt NT, Hooper NM: The prion protein and neuronal zinc homeostasis. *Trends Biochem Sci*, 2003; 28:406-410
- Wells GA, Hawkins SA, Green RB, Austin AR, Dexter I, Spencer YI, Chaplin MJ, Stack MJ, Dawson M: Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update. *Vet Rec*, 1998 Jan 31; 142(5):103-106
- Wells GA, Konold T, Arnold ME, Austin AR, Hawkins SAC, Stack MM, Lee YH, Gavier-Widen D, Dawson M, Wilesmith JW: Bovine spongiform encephalopathy: the effect of oral exposure dose on attack rate and incubation period in cattle. *J Gen Virol*, 2007; 88:1363-1373
- Wells GA, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R: A novel progressive spongiform encephalopathy in cattle. *Vet Rec*, 1987; 121:419-420
- Westway D, Goodman PA, Miranda CA, McKinley MP, Carlson GA, Prusiner SB: Distinct prion proteins in short and long scrapie incubation period mice. *Cell*, 1987; 51: 651-662
- Wilesmith JW, Ryan JB, Atkinson MJ: Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec*, 1991 Mar 2; 128(9):199-203

- Wilesmith JW, Ryan JBM, Hueston WD: Bovine spongiform encephalopathy: case-control studies of calf feeding practices and meat and bone meal inclusion in proprietary concentrates. *Vet Rec Sci*, 1992; 52:325-331
- Wilesmith JW, Wells GAH, Cranwell MP, Ryan JBM: Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec*, 1988; 123:638-644
- Will RG, Ironside JW, Zeider M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG: A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*, 1996 Apr 6; 347(9006):921-5
- Williams ES, Young S: Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wild Dis*, 1980; 16:89-98
- Williams ES: Chronic Wasting Disease (review). *Vet Pathol*, 2005; 42:530-549
- Wong C, Xiong LW, Horiuchi M, Raymond L, Wehrly K, Chesebro B, Caughey B: Sulfated glycans and elevated temperature stimulate PrP^{Sc}-dependent cellfree formation of protease-resistant prion protein. *EMBO J*, 2001; 20:377-386
- Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kaye R, Glabe CG, Frautschy SA, Cole GM: Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. *J Biol Chem*, 2005; 280:5892-5901
- Yaqoob P: The nutritional significance of lipid rafts. *Annu Rev Nutr*, 2009; 29:257-282
- Yokoyama T, Masujin K, Schmerr MJ, Shu Y, Okada H, Iwamaru Y, Imamura M, Matsuura Y, Murayama Y, Mohri S: Intraspecies prion transmission results in selection of sheep scrapie strains. *PloS One*, 2010 Nov 16; 5(11):e15450
- Yuan J, Xiao X, McGeehan J, Dong Z, Cali i, Fujioka H, Kong Q, Kneale G, Gambetti P, Zou WQ: Insoluble aggregates and protease-resistant conformers of prion protein in uninfected human brains. *J Biol Chem*, 2006; 281:34848-34858

- Zhang CC, Steele AD, Lindquist S, Lodish HF: Prion protein is expressed on long-term repopulating hematopoietic stem cells and is important for their self-renewal. *Proc Natl Acad Sci USA*, 2006; 103:2184-2189
- Zimmermann K, Turecek PL, Schwarz HP: Genotyping of the prion protein gene at codon 129. *Acta Neuropathol*, 1999; 97(4):355-358