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THE GENETICS OF SCRAPIE IN SARDA BREED SHEEP

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The Genetics of Scrapie in Sarda breed sheep

Introduction

Transmissible Spongiform Encephalopathies (TSE)

General concepts

The family of transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases which have fatal *exitus,* no reliable preclinical screening tests and effective treatments. TSE's family includes Scrapie in sheep, goats and muflon, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer, as well as Creutzfeldt-Jakob disease (CJD) and Kuru in man (Aguzzi, 2006). Other prion diseases are reported in Figure 1.

These diseases display specific histopathological features such as neuronal and neuropil vacuolation, astrogliosis and amyloidosis. However, severity and distribution of these changes can vary greatly both between and within species, i.e. amyloidosis is much more rare in BSE (Wells and Wilesmith, 1995) than in ovine scrapie, although its occurrence varies between different groups of sheep (Ligios et al., 2004).

Scrapie is the prototypic of the TSEs which has been clinically recognized for centuries (Brown, 1998) while Kuru, which was discovered in primitive people devoted to ritualistic cannibalism in Papua New Guinea, and CJD, a classical human prion disease, were first described in the 20th century (Creutzfeldt, 1920; Jakob, 1921). However, there had been no devastating public concerns until BSE emerged in the United Kingdom in the 1980s and spread to other European countries.

The massive BSE epidemic in the 1980s and 1990s resulted in huge economic, social, and political trouble as BSE was annually found in hundreds of thousands of cattle.

In the mid-1990s, subsequent to the peak of the BSE outbreak, the transmission of BSE to humans, who had probably consumed contaminated food, further exacerbated the panicky situation in Europe and beyond. It became evident that BSE had crossed the species barrier and created a new variety of human prion disease called variant CJD (vCJD) (Will et al., 1996). BSE-contaminated food for animals also caused feline spongiform encephalopathy (FSE) in both domestic and large captive cats, as well as exotic ungulate encephalopathy (EUE) in a number of ungulate species in zoos (Wells et al., 2004). These have led to bans on the import and export of beef and bovine-derived products, which have resulted in frequent trade conflicts since then until the present day. As a consequence, worldwide concerns about the prevalence of prion disease are not restricted solely to BSE. Prions from other sources, in particular, cervids with CWD, may pose a similar risk to humans (Belay et al., 2004). Independent of BSE, CWD is widespread among free-ranging and captive deer and elk populations in North America and is also found in elk in Korea imported from Canada (Sohn et al., 2002; Williams, 2005). CWD transmits laterally at a highly efficient rate which has never been observed in any other prion diseases. Although there is no compelling evidence to suggest that CWD transmits to humans, the ease by which lateral transmission of CWD occurs has led to concerns about episodes similar to the BSE outbreak.

In addition, the fact that in humans CJD can be transmitted via iatrogenic routes has similarly lead to public health concerns. Hundreds of patients have been infected by CJD during surgical procedures using prion-contaminated instruments, organ and tissue transplantations as well as hormone therapies (Will et al., 2004). More recently, a few cases of CJD transmission via blood transfusion have been reported (Llewelyn et al., 2004). These alarming reports raised another layer of concern about human public

health since blood had previously been believed to be an inefficient reservoir for prion transmission, and thus prion contamination of blood supply had never been suspected.

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Figure 1: The prion disease in humans and animals

Prion disease[a]	Host	Mechanism of transmission	Year recognized
Kuru	Human (Fore people)	Infectious; Exposure to contaminated human tissues during cannibalistic rituals	1957
sCJD	Human	Spontaneous; Somatic mutations or spontaneous conversion of PrP^{C} to PrP^{Sc}	1920
fCJD	Human	Genetic; Heritable mutations in the PRNP gene	1924
iCJD	Human	Infectious; Exposure to prion-infected surgical equipment, or tissue transplants; blood transfusion; human growth hormone therapy	1974
vCJD	Human	Infectious; Exposure to BSE-infected food including meat	1996
GSS	Human	Genetic; Heritable mutations in the PRNP gene	1936
FFI	Human	Genetic; Heritable mutations in the PRNP gene	1986
sFI	Human	Unknown; Spontaneous; Somatic mutations or spontaneous conversion of PrP^{C} to PrP^{Sc}	1997
Scrapie	Sheep, Goat	Infectious; Ingestion or contact with scrapie-infected animals, tissues and secretions derived from the infected animals, or contaminated environment; possible oral exposure	1732
TME	Mink	Infectious; Ingestion of prion-contaminated feed	1947
BSE	Cattle	Infectious; Ingestion of prion-contaminated feed	1986
FSE	Cat, Ocelot, Asiatic golden cat, Tiger, Lion, Puma, Cheetah	Infectious; Ingestion of BSE-contaminated feed	1990
CWD	Deer, Elk, Moose	Unknown; Infectious; Spontaneous; Contact with or ingestion of prions	1967
EUE	Kudu, Oryx, Nyala, Eland, Gemsbok	Infectious; Foodborne exposure to BSE-infected tissue	1986

Aetiology of TSE

It is increasingly accepted that a novel unconventional infectious agent causes the prion diseases. It is proposed that scrapie is caused by a proteinaceous infectious agent called pathological prion protein (PrP^{Sc}) 27–30 kDa, corresponding to the molecular weight of the proteinase K-resistant core of this protein. This protein is a misfolded form of an endogenously coded prion protein (PrP^{C}), which is found in most cells and is rich in a-helical structure and functionally water-soluble. PrP^{Sc} aggregates to form insoluble fibrils, is unusually stable at high temperatures and is partially resistant to proteinase K digestion (Meyer et al.,1986) (see figure 2). According to the prion hypothesis, PrP^{Sc} can mediate disease by acting as a template for the conversion of its physiological form into copies of itself (Borchelt et al., 1990; Caughey et al., 2001). The host cells are unable to break down PrP^{Sc}, which consequently accumulates to an intolerable level in the central nervous system (CNS), resulting in fatal pathology (Giese et al., 2001). Although some studies indicate that TSE can be transmitted without the formation of detectable PrP^{Sc} (Lasmezas et al., 1997), and that PrP^{Sc} levels do not necessarily correlate with the levels of infectivity (Shaked, 1999), the proteinase-resistant form of PrP is widely acknowledged as a marker of infectivity and used both for diagnostic purposes and as a ‘‘tracking device’’ to investigate pathways followed by the TSE infectious agent as it spreads within the body. It is generally accepted that the agent of a given prion diseases does not cross from one species to another efficiently (Scott et al., 2004). In rare cases, prion disease transmission between different species is possible only after prolonged incubation times. For instance, in a laboratory condition, mouse prions readily infect mice, but not hamsters or transgenic mice expressing hamster PrP (Kimberlin et al., 1987; Scott et al., 1989). Inefficient transmission to a different species is referred to as the species barrier. Usually, a species barrier prevents the prion transmission in a host species infected by heterologous prions. However, the onset of

the disease is not necessarily limited to a definite specie and can be transmissible from one host specie to another by overcoming the species barrier. This was the case of vCJD, in which human beings may have become infected with prions after ingesting meat from cattle infected by BSE (Will R.G., et al. 1996).

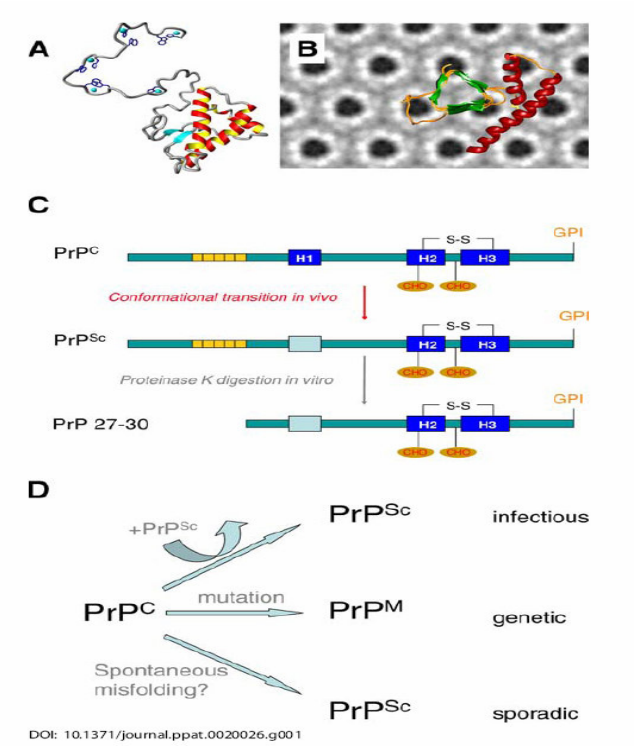

Figure 2: Schematic representation of the various forms of prion protein (Watts et al., 2006)

The prion strains

Most methods used to identify/characterize prion strains have been developed through the urgent need to differentiate BSE from scrapie in sheep. The original definition of a TSE strain was: an agent that induces constant features of spongiform lesions and incubation periods when serially passed through transgenic or wild mice. However, this definition does not cover recent scientific discoveries, as more and more data on strain differences accumulate. These include molecular tests, results of atypical cases, IHC differentiation at cellular level and use of transgenic mice.

In recent years, the biochemical characteristics of disease-associated forms of the host prion protein, PrP, have provided further criteria for distinguishing between TSE strains. Formal strain typing protocols in mice, based on incubation periods and neuropathology, have been extensively as research tools. It is now established that these methods can also be used to type the TSE strain present in a naturally infected host.

However, it is still not clear what the basis of scrapie strain variation is at molecular level. This question is key to the debate concerning the molecular nature of these agents. TSEs in ordinary non-transgenic mice are characterised by long asymptomatic incubation periods, lasting between about 4 months and the full life-span of the mouse (over 2 years). Following this asymptomatic phase, progressive neurological signs are seen, usually over a period of a few weeks. Despite the length of the interval between exposure to infection and the clinical phase, if all experimental conditions are kept constant the incubation period is remarkably predictable. However, different TSE strains tested in the same mouse strain give markedly different incubation periods. The incubation period is also strongly influenced by genetic factors in the mouse. In mice, only two alleles of the PrP gene have been recognised encoding proteins that differ by two amino acids. When mice are infected with a single TSE strain, the PrP genotype can make a difference of hundreds of days to the incubation period. This effect is related to the rate of progression of the disease, rather than to differing susceptibilities to infection. TSE strains also show dramatic and reproducible differences in the type, severity and distribution of pathological changes they produce in the brains of infected mice (Fraser, 1993).

In routine histological sections scoring the intensity of neuropil vacuolation in a selected set of neuroanatomic locations and representing the scores versus location in a linear graph, data result in a curve called the ‘lesion profile’. The severity of lesions may vary among animals, reflecting the stage in the course of the disease; advanced

cases have the most severe vacuolation. However, the shape of the curve, remains consistent for a particular agent/host combination. Lesion profiling is conducted on the definitive hosts (e.g., cattle, sheep, goats) (Ligios et al., 2002, Wells et al., 1996) or on experimentally infected animals, such as mice, for the application described previously.

Diagnosis in cases with no lesions and mild or equivocal changes should be confirmed by using more sensitive methods, i.e. IHC or WB analysis.

The targeting of neuropathology can be demonstrated clearly in sections immunostained with PrP-specific antisera. With most TSE strains, pathological accumulations of PrP can readily be demonstrated in the brain, in the form of diffuse deposits in areas of vacuolation and, more focally, as amyloid plaques. As with vacuolation, there are clear and reproducible differences between TSE strains in the distribution and severity of these changes. Some TSE strains target PrP pathology precisely to particular groups of neurons, leaving the surrounding brain substance unaffected. Other strains produce a more generalised pathology, albeit with a preference for particular brain areas. Some TSE strains produce many amyloid plaques while others produce few or none. These observations suggest that a fundamental difference between TSE strains is their ability to recognise and replicate in different neuronal populations (Bruce et al., 2003).

Molecular characterization of the scrapie strains used two criteria: the difference of molecular weight (MW) and of immunoreactivity. The MW consists of the difference between the MW of the unglycosylated band of the test sample and the molecular weight of the unglycosylated band of control (Nonno et al., 2003). The difference in immunoreactivity is the difference between signals with particular monoclonal antibodies (MAbs) such as P4 and Bar 233.

Scrapie

Classical scrapie: general concepts

For more than 250 years, scrapie has been observed in European sheep flocks and has spread to many other countries in the world (Detwiler et al., 2003), causing economical losses, even if it has never regarded as a risk to human health. On the contrary, there is strong evidence that BSE causes vCJD in humans (Bruce et al., 1997; Hill et al., 1997).

In the last decade, scrapie has become a public concern because of its relationship with the origin of BSE, which may have originated from scrapie-infected sheep that were fed to cattle through concentrates containing meat and bone meal, allowing this disease eventually to cross the species barrier (Wilesmith et al., 1988; Wilesmith et al., 1991). However the possible origin from a sporadic unknown cattle disease is also still considered. In the light of this hypothesis, for those countries or regions in which ovine scrapie is epidemic, conventional elimination efforts (culling of symptomatic sheep or of complete flocks) have become fundamentally important.

In sheep, susceptibility or resistance to scrapie is strongly modulated by the polymorphisms at codons 136, 154 and 171 of the prion protein gene region (*PRNP*), which codifies for the C-terminal structured domain of the PrP (Hunter et al., 1992).

On the basis of this knowledge, current European Union regulations aim to eradicate ovine scrapie from the member states by selecting for the PrP haplotype ARR regarded to be most resistant to scrapie, and to eliminate the other haplotypes, mainly VRQ, which is suspected to be most susceptible. This is implemented within scrapie-affected flocks by genotyping and the subsequent culling of susceptible sheep (Commission Regulation 1492/2004) and within sheep populations by breeding programs (Commission Decision 2003/100/EC).

Within scrapie-affected sheep flocks, particularly those in which the disease is associated with the ARQ allele, some sheep that possess alleles for susceptibility to scrapie may not develop disease within the commercial life span of the animal (O'Rourke et al., 1997; Jeffrey et al., 2001).

Furthermore, sheep in countries free from scrapie (Australia and New Zeland), also possess alleles associated with high susceptibility to scrapie (Hunter et al., 1997). Thus, possession of a susceptible PrP genotype alone is insufficient to induce scrapie. In some circumstances, particularly where large numbers of sheep become diseased within a single flock, all animals of the most susceptible genotype will develop scrapie, but some sheep of a less susceptible genotype may remain healthy.

Whether scrapie infection persists in scrapie-resistant or partly resistant genotypes and whether sheep with these genotypes can then act as carriers of infection is not known. Anecdotal accounts of the sudden emergence of scrapie in otherwise scrapie-free flocks have been available for many years. There are probably many different explanations for such accounts. However, it is currently unknown whether partly susceptible or scrapie-resistant sheep may be infected without developing clinical disease and act as potential carriers of infection.

Prevalence of scrapie

In any country, it is difficult to estimate the true prevalence of scrapie due to the long incubation period, the lack of a practical ante mortem screening test, the finite economic worth of individual sheep and the apprehension concerning admission of the presence of scrapie by flock owners. In the general EU sheep population, on the basis of the EU monitoring plan in 2006 (EC, 2006), TSE prevalence is currently estimated to be 0.1% (0.06% in healthy slaughtered sheep, 0.17% in those at risk).

Transmission of infection and environmental contamination

Routes of transmission of the scrapie agent within and between infected flocks is still under discussion. In 1936, Cuillé and Chelle succeeded, beyond any doubt, in demonstrating that scrapie is transmissible. After an observation period of 18 months before inoculations to demonstrate the absence of scrapie, the authors inoculated sheep and goat intraocularly, epidurally, subcutaneously and intracerebrally with homogenates from infected brain and spinal cord. Many of their experimental animals died after inoculation from unrelated diseases (mostly inflammations). The surviving animals, both sheep and goats, had scrapie. However, while the incubation periods for sheep were between 11 and 22 months, goats stayed apparently healthy for a duration of 25–26 months. The authors found no differences in the presentation of natural and experimentally infected animals. In 1946, Gordon et al. (1946) described another accidental experiment concerning the infectivity of scrapie.

During the period from 1931 to 1934, trials to test a vaccine against looping-ill were conducted. Sheep were infected with looping-ill virus by intracerebral inoculation. Three batches of 10% saline suspension were made from the brains, spinal cords and spleens of treated sheep, respectively, sufficient to immunize more than 44,000 sheep against the disease. Formalin was added to these suspensions to inactivate the looping-ill virus. During 1935 and 1936 the vaccine proved its effectiveness against looping-ill. In autumn 1937, the first cases of scrapie appeared in a group of vaccinated animals where scrapie had not previously been observed. By reconstructing the vaccination schedule, batch number two could be identified as the one which must have transmitted this unexpected disease.

This was the first iatrogenic transmission of one TSE and demonstrated that:

1. scrapie is definitely transmissible.

2. the scrapie agent is found in the brain, the spinal cord and/or the spleen of diseased animals (since vaccine was produced using these organs).

3. the scrapie agent is resistant to treatment with formalin.

4. the incubation period is 2 years or longer.

Numerous and reliable observations argue strongly for peri-partal transmission of scrapie, as lambs born from ewes incubating scrapie present a higher risk of developing scrapie (Hoinville, 1996; Elsen et al., 1999). Recent studies confirm and extend these previous observations indicating that PrP^{Sc} accumulates in the placentae of ewes incubating scrapie and that the genotype of the fetus modulates this accumulation (Andreoletti et al., 2002; Tuo et al., 2002). By IHC, it has been demonstrated that PrP^{Sc} accumulates mainly in the foetal trophoblast, although some maternal epithelial cells, which form syncytia with foetal trophoblasts, also accumulate PrP^{Sc} (Jeffrey et al., 2007). Despite this cellular location of PrP^{Sc}, there remain no confirmed cases of *in-utero* transmission of infection (Jeffrey et al., 2007).

PrP^{Sc} and/or prion infectivity were demonstrated to accumulate at extraneural and extralymphatic sites (Andreoletti et al., 2004; Ligios et al., 2005; Tuo et al., 2002; Vascellari et al., 2007). However, it is difficult to envisage how tissues that are not exposed to the outer environment might contribute to prion spread among sheep in a flock. Blood was long believed to harbour very little prion infectivity. However, efficient blood-borne means of transmission of TSE in sheep (Hunter et al., 2002), cervid (Mathiason et al., 2006) and primates including men have recently been demonstrated. In addition, in mice, sheep and deer, chronic lymphofollicular inflammation can shift the distribution pattern of PrP^{Sc} or prion infectivity to non-lymphoid organs in the kidney of mouse and deer (Hamir et al., 2006; Seeger et al., 2005), liver and pancreas of mouse (Heikenwalder et al., 2005), and mammary gland of sheep (Ligios et al., 2005).

The presence of PrP^{Sc} in kidney was detected in renal glomerula of cats (Ryder et al., 2001) and cheetah (Lezmi et al., 2003) affected by feline spongiform encepaholpathy by the means of immunohiostochemical analysis. Similarly, the presence of pathological prion protein in sheep kidneys of with ARQ genotype (Sisò et al., 2006 and Ligios et al., 2007) was found. Recently, milk from VRQ/VRQ sheep incubating scrapie has been identified as a source of scrapie agent transmission (Konold et al., 2008). Another study from Lacroux et al. (2008) independently demonstrated that Classical scrapie can be transmitted from susceptible ewes to transgenic mice by milk.

Moreover, the level of prion infectivity in small ruminant milk could become higher during the course of mastitis (Lacroux et al., 2008).

PrP^{Sc} was detected in major (parotid and mandibular) and minor (buccal, labial, and palatine) salivary glands of naturally and experimentally infected sheep (Vascellari et., al 2007).

This highlights the possible role of saliva in the horizontal transmission of scrapie.

However it should be stressed that differences in scrapie transmission linked to the genotypes of the host – the sheep under these studies in the above mentioned researches were of only one given genotype – or to some scrapie isolates cannot be excluded.

Clinical signs

Clinical signs of natural scrapie in sheep can last from 2 weeks to 6 months and often begin with unusual social behaviour and extreme nervous reactions to stimuli. Ataxia is common and pruritis 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 (Hunter et al., 2003).

In the final stages of the disease, although the appetite may appear normal, the animals lose the ability to feed themselves and the condition degenerates. 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 et al., 2003).

BSE can also be transmitted to sheep by inoculation of affected bovine brain homogenate. Clinical signs vary in the different breeds of sheep used by different researchers. In one reported study in NPU Cheviots, after a long incubation period, the animals were affected by a relatively acute illness of short duration, in some cases less than one week. In this case, the main sign was ataxia with little pruritis. In another study carried out in France with indigenous sheep breeds, there was intense pruritis leading to loss of fleece, and ataxia with degenerating condition until death after a clinical course of around 3 months.

Pathogenesis

In scrapie, it is generally believed that the infectious agent is present in the environment and taken up orally from scrapie contaminated pastures (Van Keulen et al., 2008).

The invasion of a host by a TSE agent can be divided into uptake of the agent, accumulation of the agent within lymphoid tissues, which precedes or accompanies neuroinvasion of peripheral nervous tissue (PNS), and finally the invasion of the agent in the central nervous system (CNS) and the clinical course. All the phases from the initial uptake of the agent to neuroinvasion of the PNS can take place in the intestinal wall. In sheep, the alimentary tract is considered the major route of entry of scrapie infection (Andreoletti et al., 2000). The early presence of infectivity and PrP^{Sc} in aggregated intestinal lymphoid tissue after oral exposure to TSE infectious material has

identified gut-associated lymphoid tissue (GALT) as the most probable site of entry of the scrapie agent (Beekes et al., 2000; Kimberlin et al., 1983).

It is still obscure how TSE agents cross the mucosal barrier after they are ingested but there are three possible ways. The agent can cross the intestinal wall by the 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. However, transport of TSE agents 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. Finally a another 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 (Van Keulen et al., 2008). However even if it has been demonstrated for bacteria but has yet to be shown for TSE agents.

After crossing the mucosal barrier, infectivity and PrP^{Sc} first accumulates in Peyer's patches. 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 Peyer's patches. The first immunohistochemical evidence of PrP^{Sc} in the in Peyer's patches consists of the intracellular accumulation within the tangible body macrophages (TBM) in the B-cell follicles. At a later stage, PrP^{Sc} is also found on the plasmalemma of the follicular dendritic cells (FDC) in the germinal centres of the B-cell follicles (Andréoletti et al., 2000; Jeffrey et al., 2007; Van Keulen et al., 1996; Van Keulen et al., 2000). The replication of TSE agents in the Peyer's patches and of all the GALT is thought to be crucial to the further neuro-invasion.

FDCs are specialized to trap and retain unprocessed antigens, in the form of immune complexes, and to present these to B cells in the course of the selection of clones producing high-avidity antibodies and the generation and maintenance of B-cell memory. Some conventional viruses, including HIV-1, have also been shown to be trapped and retained by the FDC (Racz et al., 1995). The association of high levels of PrP^C with FDC networks in uninfected mice suggests that this protein may play a part in the trapping function (Racz et al., 1995).

However, the absence of agent replication in the GALT does not fully prevent neuro-invasion. For instance in sheep of the VRQ/ARR PrP genotype (carrying both the VRQ allele associated with high scrapie susceptibility and the ARR allele associated with high scrapie resistance) there is minimal or no involvement of the lymphoid tissues in agent replication (Van Keulen et al., 1996). However, these sheep do get natural scrapie, albeit at an older age (Bossers et al., 1996).

Although the ENS is capable of functioning independently, it is modulated by the central nervous system (CNS) by means of the parasympathetic and sympathetic efferent nerves of the autonomic nervous system that are connected to the enteric plexi (Van Keulen et al., 2008).

After infection of the ENS, TSE agents ascend through the parasympathetic and sympathetic efferent neuronal pathways to the brain and (via the ganglion mesentericum craniale/coeliacum) to the spinal cord (Van Keulen et al., 2008). The portal of entry of TSE agents in the brain is thus the dorsal motor nucleus of the vagus (DMNV) in the medulla oblongata at the level of the obex and the intermediolateral column (IMLC) in the thoracic segments of the spinal cord. From these sites in the CNS, infection spreads in both an ascending and descending direction to eventually involve the entire neuraxis.

The observations of initial ENS infection and spread through autonomic efferent nerve fibers do not preclude other possible routes of neuro-invasion. Theoretically it is also

possible that the scrapie agent travels to the CNS through other peripheral nerve endings originating from infected non-GALT lymphoid tissues. Another possible route to the CNS would be through haematogenic spread during the phase of scrapie infectivity in the blood (Sisò et al., 2009)

Diagnostic methods: principles and application

Historically, the initial diagnosis of TSE was based on histopathologic changes, but, given the pivotal role played by prion protein in pathogenesis, the characterization of PrP^{C} and PrP^{Sc} has been a prime research element. Over time, many specific antibodies against PrP, the only certain marker for TSE, have been produced. Immuno-biological techniques, such as IHC and WB analyses, ELISA and the conformation-dependent immunoassay (CDI) (Grassi et al., 2006) are being increasingly used to aid diagnosis for screening purposes, to study pathogenesis, and to provide comparative information at molecular level. There are now many tests that have been validated and officially approved, and several more are in the development stage. Most diagnostic methods for PrP^{Sc} detection rely on its protease resistance and identification by use of PrP-antibodies. However, a fundamental difficulty with antibodies to TSE is that those currently used cannot specifically discriminate PrP^{C} from PrP^{Sc}.

There are currently no reliable non-invasive tests for the diagnosis of TSE in live animals or humans. Clinical signs of disease, although characteristic, are insufficient for a definitive diagnosis. It is assumed that the immune system fails to generate a specific response to PrP^{Sc} because the protein is not recognized as ‘‘foreign.’’ Presently, immunologic tests that are based on detection of an immune response cannot be used for diagnosis of TSEs. It has been shown that PrP^{Sc} is present in blood and that TSE can be transmitted by blood transfusion (Houston et al., 2008). However, the PrP^{Sc} concentration in blood is 100 to 1,000 folds lower than that in the brain (Grassi, 2003).

The PrP^{Sc} is also detected at low concentration in urine of cervids and rodens affected by TSE, (Haley NJ et al., 2009; Gregori et al., 2008). Even though the development of *in vivo* tests for urine or blood, i.e. protein misfolding cyclic amplification (PMCA), has made progress (Castilla et al., 2006) sufficient sensitivity and consistency have not yet been achieved. Some tests are already commercially available, but their performance is not yet clearly established. Detection of PrP^{Sc} in tissue biopsy specimens currently remains the only reliable method for *in vivo* diagnosing TSE.

Tonsillar biopsy specimens are used in cervids, small ruminants, and humans. In sheep, biopsy of the lymphoid deposits on the third eyelid also can be used (O'Rourke et al., 2000). but in many cases, insufficient lymphoid tissue is obtained. The early distribution of PrP^{Sc} in the lymphoid tissue is not homogeneous; therefore, it is considered that when applying IHC analysis, a minimal number (usually 4 to 6) of follicles with germinal centers needs to be examined to provide a reliable negative diagnosis. Brain biopsy is the last resort for confirmation of TSE in humans, and only a positive diagnosis is reliable.

Positive diagnosis of scrapie can be based on histopathologic changes alone when the characteristic vacuolar changes in the brain, with the typical neuroanatomic distribution, are recognized. Other histologic features of TSE, such as presence of florid plaques, astrogliosis, and neuronal loss, when present, support a positive diagnosis, but are not diagnostic in the absence of TSE vacuolation.

Recognizing the presence of disease-specific immunostaining in the target areas and the characteristic patterns is the most specific test for TSE available so far. Immunohistochemical analysis usually is conducted on formalin-fixed samples. It involves pre-treating of the sections with formic acid and hydrated autoclaving for epitope demasking and to remove the normal PrP (Haritani et al., 1994).

In the early days, histologic examination and detection of *Scrapie-associated fibrils* (SAF) by use of negative-contrast electron microscopy were the only available tests for diagnosis of TSE. Today, SAF is rarely used because of its comparatively low sensitivity, but it may be a method of choice when only severely autolysed or only formalin-fixed material is available.

Genetic basis of scrapie

The PrP gene in sheep, goats and cattle has been mapped to chromosome 13 (Iannuzzi et al., 1998). Disease susceptibility and resistance follow genetic rules based on the PrP genotype. It is a sign of the close association between PrP alleles and sheep scrapie susceptibility that the crucial amino acid variations in ovine PrP were discovered soon after the gene sequence became available (Goldmann et al., 1990).

The degree of amino acid sequence similarity of PrP between species will often have consequences for the transmissibility of TSE between them. It is also often the case that the same or a similar polymorphism in two species will have the same or a similar effect on their TSE susceptibility. Observations regarding the genetic variation of PrP in related species are therefore important in the attempt to understand and control susceptibility. The PrP gene may be of crucial importance to disease but it is most likely not the only gene exerting influence on it. Several chromosomal regions have been associated with susceptibility (Manolakou et al., 2001; Stephenson et al., 2000), but no other gene has yet been unambiguously associated with disease.

The ancestral allele at these codons is thought to be ARQ ($ARQ_{wildtype}$) where A indicates the amino-acid Alanine, R Arginine and Q Glutamine codified at codons 136, 154 and 171, respectively (Belt et al. 1995). To make a slightly generalized assessment of genetic susceptibility to scrapie, it is believed that substitution of A with Valine (V) increases susceptibility to scrapie, substitution of R with Histidine (H) at codon 154 is

associated with resistance, although this latter data has not been universally accepted (Dawson et al., 1998). Substitution of Q with H at codon 171 does not modify susceptibility to scrapie, whereas substitution of Q with R gives resistance to scrapie (Baylis and Goldmann, 2004). However, up to now many other polymorphic codons have been described in the *PRNP* gene, even if they are much less frequent in sheep population (Maestrale et al., 2009).

These polymorphisms combined to form five alleles (ARQ, VRQ, AHQ, ARR, ARH) and those can be arranged in 15 genotypes, ARR/ARR, VRQ/ARQ, or ARH/AHQ. Even after more than 15 years of PrP genotype analysis these are still the only alleles (based on the three codons) with significant frequencies in the world. Novel combinations such as VRR and AHR (with two mutations at the same allele) have been described for breeds in Germany and USA (DeSilva et al., 2003; Lan et al., 2006), but they appear to be very rare indeed. The same is true for new mutations such as TRQ, ARK, and ALQ (Acin et al., 2004; Billinis et al., 2004; DeSilva et al., 2003).

A five-group risk classification system has been developed based on these 15 genotypes which was modified to be applied in breeding and eradication programmes like the National Scrapie Plan (NSP) of Great Britain and equivalent plans in other member states of the EU (Dawson et al., 1998; Detweiler et al., 2003). The highest risk group (R5) is reserved for animals that are at the greatest risk of developing scrapie (Baylis et al., 2004). In the UK and in many outbreaks outside of the UK, such as in France, Ireland and Norway (Elsen et al., 1999; O'Doherty et al., 2002; Tranulis et al., 1999) the highest risk group included the VRQ/VRQ animals. The risk in this group is so high that scrapie was for a long time regarded as a genetic disease. It has however been shown conclusively that VRQ/VRQ sheep can survive into old age when their environment is free of scrapie infection (Foster et al., 2006; Hunter et al., 1997). The other three genotypes in R5 are VRQ/ARQ, VRQ/ARH and VRQ/AHQ.

Whereas the risk estimates are similar to the VRQ/VRQ homozygote in the first two genotypes, the risk for the VRQ/AHQ is surprisingly low (Baylis et al., 2004). It suggests that the AHQ allele has a clear resistance effect compared to the ARQ allele when it is combined with VRQ. R4 animals are classified as being at a lower risk of being affected with scrapie than R5 but the risk to their progeny is still significant, especially as R5 offspring can be produced from their breeding. The VRQ/ARR genotype represents this group. Risk estimates indicate a very significant susceptibility reduction through the presence of the ARR allele. R3 animals have average resistance to scrapie and the same is true for their offspring, which will always belong to the R3 group. The six genotypes in this group include the ARQ/ARQ as well as the less common ARH/ARH genotype. It is of particular interest to find that an ARQ/ARQ homozygote in the UK has only average susceptibility whereas outbreaks in other countries have convincingly shown that ARQ/ARQ sheep can have similar risk to VRQ carriers. Scrapie-affected ARQ/ARQ sheep can be found in populations where VRQ carriers and ARQ/ARQ animals are similarly exposed to the agent or in populations where only ARQ/ARQ are exposed because of the absence of the VRQ allele altogether (mostly due to breed). For example, in the French "Langlade" flock, ARQ/ARQ animals were affected in parallel to VRQ carriers (Elsen et al., 1999). In a selection of Spanish flocks of the Rasa breed 95% of scrapie affected animals were ARQ/ARQ, but the VRQ/ARQ flock-mates were not affected (Acin et al., 2004). In an Irish study, the combined risk for ARQ/ARQ and ARQ/ARH animals was similar to the risk of VRQ/ARQ animals (O'Doherty et al., 2002). Equally, scrapie in Germany, Spain and Greece appears to involve ARQ/ARQ animals in a high percentage of cases (Billinis et al., 2004; Lühken et al., 2007). As in the case of scrapie attacking the R5 and R4 groups, in the R3 group a risk reduction (partial resistance) of about three times can be found in ARQ/AHQ animals (Billinis et al., 2004). R2 animals (genotypes ARR/ARQ,

ARR/AHQ and ARR/ARH) are quite resistant to scrapie but their offspring can be of a higher risk classification (R3). Occasionally R2 animals are found with scrapie (Baylis et al., 2004) which up to recently distinguished them from R1 animals (ARR/ARR) which were regarded as fully resistant to natural scrapie, although not to experimental exposure to BSE (Houston et al., 2003). It has recently been shown for two ARR/ARR animals from two different populations that they carried classical scrapie, even if both were subclinical cases (Groschup et al., 2007). Although there has not been a reason to fundamentally change the risk classification system for classical scrapie as developed by Dawson et al. 1998, it is now clear that breeds from different countries carry different PrP variants and that there is as yet no proper adjustment of the risk group assignment to novel PrP genotypes. The additional alleles in codons 136, 154 and 171, TRQ, ALQ, ARK, VRR, AHR (Alvarez et al., 2006; DeSilva et al., 2003; Kutzer et al., 2002) alone could lead to 55 additional genotype combinations. Although some of the alleles have low frequency and as consequence the frequency of certain genotype combinations will be also very low, it should not be forgotten that at the level of individual flocks their frequency could become high through simple founder effects, such as the introduction of a specific ram. With the genetic association to classical scrapie as described above it becomes very relevant to know the allele frequencies of the major variants in different populations and huge effort has gone into genotyping as many breeds (common and rare) as possible around the world. A survey conducted of more than 20 studies (Acutis et al., 2004; Bossers et al., 1999; DeLima et al., 2007; Thorgeirsdottir et al., 1999; Tranulis et al., 1999; Townsend et al., 2005) reporting on sheep populations from 15 different countries in Europe, America and Asia revealed that so far all but one (Iceland) presented significant frequencies of all five major alleles (ARQ; ARR; AHQ; VRQ; ARH) in their sheep. Iceland appears not to have any ARR animals (Thorgeirsdottir et al., 1999). The allele frequency averaged over all animals in

these studies were 56% ARQ, 30% ARR, 6% AHQ, 5% VRQ and 3% ARH. The ARK and ARH allele had average frequencies of 0.4% and 0.02%, respectively. Regional differences are apparent, such as a high frequency (~ 9%) of the ARH allele combined with a low frequency (~ 1.5%) of the AHQ allele in Asian breeds. There is a lower than average frequency (~ 22%) of ARR in the Mediterranean countries and higher than average frequency (~ 11%) of VRQ in Norway. The variation is however huge and individual flocks or breeds – especially rare breeds – may deviate significantly from these averages. Of course these figures are due to change over the next decades when genotyping and breeding programmes take effect.

Atypical scrapie

Currently, sheep prion disorders of sheep may be divided into those classically recognized and the recent descriptions of Nor 98 or atypical scrapie (Benestad et al., 2003). Classical scrapie is transmissible and contagious, characterized by variable neurological signs, classical spongiform changes in the brain and accumulation of PrPd in many tissues, and PrPres is detected following robust digestion conditions (Buschmann et al., 2006). Nor 98 scrapie, or the unfortunately adopted term atypical scrapie, has mostly been recognized by screening methods applied to cull animals (Everest et al., 2006), and only limited information is therefore available about the neurological signs that may occur. Classical vacuolation is absent, although a different form of microvacuolation is inconsistently present (Benestad et al., 2003). PrPres can be detected but only under mild denaturing conditions, and PrPd is not detected outside the central nervous system (CNS). Epidemiology shows that atypical scrapie is present throughout Europe at about 1/10 000 of the sheep population, irrespective of the concurrent presence of classical scrapie. Although infectious, this prion disorder does

not appear to be contagious and mainly involves sheep PrP genotypes considered resistant to classical scrapie (Jeffrey et al., 2007).

The most frequently found atypical phenotype is designated Nor98 since it was initially discovered in Norway and subsequently found in other EU member states (De Bosschere et al., 2004; Madec et al., 2004; Orge et al., 2004). In atypical scrapie cases, sheep carrying PrP haplotypes known to be less susceptible (AHQ) or resistant (ARR) to classical scrapie have been affected, while the VRQ haplotype seemed to confer at least partial resistance to atypical scrapie strains (Benestad et al., 2003); Buschmann A., et al. 2004; De Bosschere H., et al. 2005). In addition to the variations at codons 136, 154 and 171, further ovine PrP polymorphisms were described (Goldmann et al., 2005) . Among these, an amino acid substitution (L→F) at codon 141 showed an association with the susceptibility to Nor98 (Moum et al., 2005). The F141 allele has been shown to occur in combination with the allelesA136, R154 and Q171, forming the PrP haplotype AFRQ (Bossers et al., 1996; Hunter et al., 1996). It has been shown that Nor98 and other atypical cases can be efficiently transmitted to transgenic mice expressing sheep PrP (Le Dur et al., 2005). No data are yet available on whether this disease is transmitted naturally within a sheep flock.

Aim of the study

- Estimate the frequencies of the genotypes at codons 136, 154 and 171 of the *PRNP* gene in Sarda breed sheep

- By using experimental and natural scrapie cases, estimate the association of these different genotypes with scrapie occurrence in Sarda breed sheep

- Estimate the frequencies of the polymorphisms beyond those at codon 136, 154 and 171 of the *PRNP* gene in Sarda breed sheep

- By using experimental and natural scrapie cases, estimate the association of these additional polymorphisms with scrapie occurrence in Sarda breed sheep.

Materials and Methods

Sampling

Breeding rams

To study the frequencies of the genotypes of the *PRNP* gene, we analysed the PrP gene at codons 136, 154, 171 of 884 Sardinian breeding rams (333 used for artificial insemination and 551 for natural mating), which were selected at random from the 1459 rams which were used in the flock-book during the reproduction period (May-July) of the year 2002-2004.

Naturally occurring scrapie and sampling (Group n. 1)

To study the association of the genotypes at codons 136, 141 and 171 with presence/absence of scrapie in sheep, firstly, our investigation was carried out on 1,050 Sarda breed sheep of different ages (from 7 months up to 6 years) and belonging to 8 scrapie-affected Sarda sheep flocks located in Sardinia (Italy) and showing different prevalences of clinical scrapie.

Criteria for the collection of samples were established for each flock in relation to epidemiological and clinico-pathological features of the single outbreaks. In addition, sheep with different genotypes were sampled from the same flocks.

These flocks were randomly selected in Sardinia from 1998 to 2003.

Inside the framework of appropriate actions for eradicating scrapie, as was stipulated until 2003 in Italian regulations, all sheep belonging to an infected flock must be sacrificed.

All the animals of these 8 flocks were euthanized and a number of them (n. 1,050) were randomly selected to collect the obex region of the brain, the palatine tonsils and the retropharyngeal lymph nodes, which were partly frozen at –20 °C and partly fixed in 10% neutral buffered formalin. These organs were examined by Western Blotting (WB) and Immunohistochemistry (IHC) for the detection of PrP^{Sc} in order to perform the diagnosis of scrapie.

In addition, a group of 49 clinically ill sheep affected with natural scrapie, originating from the aforementioned flocks and aged between 3 and 5 years, were also included in the study. All of the animals were euthanized and nervous and lymphoid tissues were collected to confirm scrapie by WB and IHC. Details of the sampling are summarized in the table 1.

Naturally occurring scrapie and sampling (Group n. 2)

To study the association of certain polymorphisms beyond those at codons 136, 154 and 171, another part of the study was carried out on 24 scrapie-affected Sarda sheep flocks located in Sardinia. These flocks, which were randomly selected in Sardinia from 1998 to 2006, had a different history of clinical cases of scrapie before the diagnosis of the *index* case. The flocks were confirmed to be scrapie-affected after the notification of clinically suspected cases or throughout the TSE active surveillance, examining the fallen stock and the healthy slaughtered animals.

Inside the framework of appropriate actions for eradicating scrapie, as was stipulated in EC regulations in 2003 in, all sheep carrying susceptible $AX_{154}Q/AX_{154}Q$ genotypes must be sacrificed. To identify these sheep, EDTA-treated blood was collected from each sheep to isolate the genomic DNA for determining the *PRNP* genotype.

All the above susceptible animals were euthanized and a number of them (n. 1,762) were randomly selected to collect the obex region of the brain, the palatine tonsils and

the retropharyngeal lymph nodes, which were partly frozen at –20 °C and partly fixed in 10% neutral buffered formalin. These organs were examined by Western Blotting (WB) and Immunohistochemistry (IHC) for the detection of PrP^{Sc} in order to perform the diagnosis of scrapie.

Additionally, we selected 96 sheep from the same flocks with clinical signs indicative of scrapie. All these animals were sacrificed and nervous and lymphoid tissues were collected to confirm scrapie by WB and IHC.

Experimentally occurring scrapie and sampling (Group 3)

Twenty ARQ/ARQ Sarda sheep, 20 ARQ/ARR and 20 ARR/ARR which were orally inoculated with 25 ml of a 10% scrapie brain-pooled homogenate in the framework of a separate, larger study. Of 20 ARQ/ARQ sheep, 17 had the $ARQ/ARQ_{wildtype}$ genotype, while 2 carried both the N176K and the L141F dimorphisms and 1 carried only the N176K dimorphism.

Of these animals, 17 were euthanized when advanced clinical signs of scrapie developed, while one carrying the genotype $AF_{141}RQN_{176}/AL_{141}RQK_{176}$ and the other $ARQN_{176}/ARQK_{176}$ were sacrificed at 700 days post-inoculum without clinical signs.

40 sheep carrying ARQ/ARR and ARR/ARR genotype were sacrificed 700 days post inoculum without clinical signs.

From all these 60 sheep a representative set of nervous and lymphoid tissue samples were collected and then submitted to WB and IHC analysis.

In addition 9 sheep, 3 Sarda breed sheep VRQ/AHQ and 6 Cheviot breed sheep (4 VRQ/VRQ, 1 $AL_{141}RQ/AF_{141}RQ$ and 1 $AF_{141}RQ/AF_{141}RQ$) were orally inoculated with 25 ml of a 10% scrapie brain-pooled homogenate from scrapie-affected sheep. Of these animals, 3 Sarda sheep are still alive and 3 of the 6 Cheviot sheep were euthanized

between 109 to 714 days post-inoculum without clinical signs. Nervous and lymphoid tissue samples were collected and then submitted to WB and IHC analysis.

From 3 of the 6 Cheviot sheep, placentas were collected in the peripartal period and submitted to W.B. analysis and to DNA analysis to determine fetal genotype.

Cheviot sheep were included in the study in order to demonstrate whether the breed of the host can influence the association between scrapie and certain polymorphisms.

Detection of PrP^{Sc} and PrP gene analysis

Western-blotting

Nervous and lymphoid tissue samples were unthawed and then submitted to an appropriate Western-blotting (WB) protocol. The examination of lymphoid tissues was carried out by means of a modified Prionics check kit (Prionics AG), with 1 g of lymphoid tissue being first incubated in TBS buffer (10mM Tris HCl, 133 mM NaCl, pH 7.4) containing 2 mM $CaCl_2$ and 2.5 mg/ml (final concentration) of type XI collagenase 1.6U/mg (Sigma) for 2h at 37°C.

Samples were subsequently centrifuged at 30,000 g for 30 min at 4°C, with deriving pellets being resuspended in homogenation buffer (20% w/v) which is included in the Prionics check kit (Prionics AG, Switzerland), and homogenated using an automatic system (Fasth PCPM). A 500 µl volume of this homogenate was incubated at 50°C for 40 min with 100 µl of digestion buffer and 100 µl of proteinase K, both of which were also included in the Prionics check kit. The reaction was blocked with 100 µl of digestion stop. After digestion, further concentration steps were performed by centrifugation at 30,000 g for 30 min at 4°C. Pellets were resuspended in 20 µl of NuPage and 10 µl of them were loaded on NuPAGE Novex Bis-Tris Gels 17 wells (Invitrogen) for electrophoresis, under constant application of 120 V for 45 min.

Electroblotting was performed onto polyvinylidene fluoride membranes (150 V for 1 hour). For PrP^{Sc} detection, membranes were incubated over-night at 4° C with P4 MoAb (1:5,000). After washing with a solution of 10 mM Tris HCl, 133 mM NaCl and 0.2% Tween 20 (TBST), the secondary Ab diluted 1:5,000 was added to the reaction, with membranes being subsequently washed 4 times for 5 min, equilibrated in luminescence buffer, placed in CDP star and finally exposed to an X-Ray film. Examination of the obex was carried out using the aforementioned Prionics check kit in accordance with the manufacturer's instructions.

Immunohistochemistry

After fixation, all tissue specimens were embedded in paraffin, cut into 5 micron-thick sections and placed on poly-L-lysine-coated glass slides and then submitted to immunohistochemistry (IHC) protocol for PrP^{Sc} detection. To this aim, tissue sections were first dried overnight at 37 °C, re-hydrated and then autoclaved at 121 °C for 30 min in a solution of 0.01 M citric acid (pH 6.1). Further steps included utilization of a biotin-streptavidin method (Vector Laboratories, Inc.) and monoclonal antibody F99 (VMRD, Inc.), which was applied with a final dilution of 1:800. Immune reactions were visualized by 3-3'-diaminobenzidine (DAB, Dako, Denmark) chromogen solution. Tissue sections from scrapie-affected and scrapie-negative ARR/ARR sheep were included in each run as positive and negative controls respectively.

Genetic analysis

PRNP genotypes at codons 136, 154 and 171 were determined in all the sheep belonging to the 8 scrapie-affected flocks.

Additionally, the PrP gene sequence of all the 256 sick sheep and of all the euthanized sheep with preclinical scrapie as well as of 320 flock-mate negative controls were determined

DNA extraction. Total DNA was extracted from 100 µl of whole EDTA-treated blood by Micro DNeasy Kit (Qiagen), according to the manufacture's instructions. DNA concentration and quality were determined by spectrophotometrical scanning and by examination following 0.8% agarose gel electrophoresis.

Detection of single nucleotide polymorphisms (SNPs) at codons 136, 154 and 171 by Real Time PCR. DNA (30ng) was amplified by 4 different multiplex Real Time PCRs, using specific primers and MGB-probes (Applied Biosystems) for each codon.

For codon 136 the primers were F136 5'-GATAGTAACGGTCCTCATAGTCATTGC-3' and R136 5'-CTGCAGCTGGAGCAGTGGTA-3'; the MGB-probes were A_{136} (FAM) 5'-TCATGgCACTTCC-3' for codon codifying for A and V_{136} (VIC) 5'-CTCATGaCACTTCC-3' for codon codifying for V. For codon 154 the primers were F154 5'-TGGCAATGACTATGAGGACCG-3' and R154 5'-TGGTCTGTAGTACACTTGGTTGGG-3'; the MGB-probes were R_{154} (FAM) 5'-ACTATCgTGAAAACAT-3' for codon codifying for R and H_{154} (VIC) 5'-TACTATCaTGAAAACATG-3' for codon codifying for H. For codon 171 we performed 2 distinct multiplex Real Time PCRs. In the first amplification, the primers were F171 5'-GTTACCCCAACCAAGTGTACTACAGA-3' and R171 5'-TGTTGACACAGTCATGCACAAAG-3'; the MGB-probes were R_{171} (FAM) 5'-CCAGTGGATCgGTATA-3' for codon codifying for R and H_{171} (VIC) 5'-ACCAGTGGATCAtTAT-3' for codon codifying for H.

In the second PCR amplification we used the same primers (F171 and R171) while the MGB-probes were R_{171} (FAM) 5'-CCAGTGGATCgGTATA-3' for codon codifying for R and Q_{171} (VIC) 5'-ACCAGTGGATCaGTATA-3' for codon codifying for Q.

Each 25 µl PCR contained 30ng of genomic DNA, 900 nM primers, 200nM MGB probes and 12,5 µl of 2X TaqMan PCR MasterMix (Applied Biosystems). Amplification was done under the following conditions: 50°C, 2 min; 95°C, 10 min; by 35 cycles of 95°C, 15 sec and 60°C, 1 min in GeneAmp® PCRSystem 9700 (Applied Biosystems). The genotype for each codon was detected using ABI Prism 7900HT Sequence Detection System and SDS. 2.2.1 Software (Applied Biosystems).

Sequencing of PRNP gene For the sequencing of the PRNP gene, PCR amplification was performed by using the following primers: PrP1(+) 5'-CAGGTTAACACCCTCTTTATTTTGCAG -3' and PrP2(-) 5'-ACCTCTAGAAGATAATGAAAACAGGAAG-3'. The PrP1(+) and PrP2(-) anneal at the 5' and 3' flanking regions of the PrP-coding sequence amplifying a product of 800 bp. Reactions were set up in a 50 µl of reaction volume containing 10 ng genomic DNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1,5 mM MgCl, 250 µM each deoxynucleotide triphosphate (Applied Biosystems), 1U of Taq Gold DNA polymerase (Applied Biosystems) and 1 µM each sense and antisense primers Amplification was performed in GeneAmp® PCRSystem 9700 (Applied Biosystems) with a heat step of 10 min at 95°C, 30 cycles of 30 sec at 95° C, 30 sec at 58° C, 45 sec at 72° C, and an elongation step of 7 min at 72°C.

Products were visualized by staining with ethidium bromide after the electrophoresis of a 5 µl of reaction mixture on 2% agarose gels. Before sequencing, 5µl of the amplification products were purified with 2 µl of ExoSAP-IT® (USB Corporation) at 37 °C for 15 min and 80°C for 15 min in thermal cycle. Sequencing reactions were

carried out by using Dye Terminator version 3.1 Cycle Sequencing kit according to the manufacturer's guidelines (Applied Biosystems) and detected with an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

Each sequence was aligned with the wildtype sequence (GenBank n.AF195247) of the Sarda sheep, using Mutation Surveyor^{MT} Software (SoftGenetics, LLC).

Cloning of the PRNP gene. In order to identify the polymorphisms per allele, the amplicons with 2 different polymorphisms were directly inserted into the pCR®4-TOPO vector, followed by transformation of One Shot® TOP10 Competent Cells using the TOPO TA Cloning Kit (Invitrogen). After blue/white colony selection, microbial colonies were tested by PCR for the presence of 800bp insert. From each sample, five clones were analysed by sequencing. To do this, plasmid DNA was isolated using QIAprep Spin Miniprep Kit (Qiagen) and sequenced directly using PrP1 (+) and PrP2 (-) (0,17 μM each), according to the aforementioned method.

Statistical analysis

A logistic regression was used to model the risk of being scrapie-affected according to animal genotype. Firstly, the frequency of scrapie cases in the group of ARQ/$ARQ_{mutated}$ sheep (including those with the AX_{154}/AH_{154}Q) were compared with that of the group of sheep carrying the ARQ/$ARQ_{wildtype}$ genotype.

Then, to detect whether there are differences in risk among the different mutated genotypes, using the same statistical methodology, the frequency of scrapie cases recorded in the group of ARQ/$ARQ_{wildtype}$ sheep was compared with those observed in 4 different groups of sheep, each carrying only one following dimorphisms: M137T, L141F, R154H, N176K. Odds ratios (OR) were calculated using the ARQ/$ARQ_{wildtype}$ genotype as reference.

Results

Breeding rams

Three alleles were found in our survey ARR, ARQ, AHQ. The allelic frequencies in the flock-book rams are reported in graphic A. The allelic frequencies between the AI and NM rams did not show significant differences and this confirms that they belong to the same population. The VRQ allele, which is linked to the highest susceptibility to scrapie, was not found in this survey, suggesting that its frequency is very low.

Naturally occurring scrapie (Group n. 1)

Of the 1050 clinically healthy sheep under study, 981 turned out to be PrP^{Sc} negative, by means of both IHC and W.B., in all investigated LRS as well as at the level of the obex region of the brain. The distribution of PrP genotypes within this group of animals is shown in table 2.

Sixty-nine out of the 1050 aforementioned sheep were found to be scrapie-infected (table 2) since PrP^{Sc} was detected by means of IHC and /or WB in one or more LRS districts as well as at the level of the obex region of the brain. All 8 of the flocks investigated here included at least one of the above 69 animals that were of the ARQ/ARQ (n=60), ARQ/AHQ (n=7) and AHQ/AHQ (N=2) genotypes, with no sheep of remaining genotypes apparently harbouring PrP^{Sc} in any LRS or nervous tissue region (Table 2). Only 9 out of the 69 clinically healthy scrapie-infected sheep showed PrP^{Sc} deposition in their LRS tissues, but not in the obex region of their brain (Table 2). On the other hand, 18 of these 69 animals, all exhibiting ARQ/ARQ homozygosis, with the exception of only one ARQ/AHQ sheep, showed IHC and Immunobiochemical evidence of PrP^{Sc} deposition within the obex region of the brain without any apparent PrP^{Sc} accumulation in their lymphoid tissues.

Three of 49 clinically sick animals, all of ARQ/ARQ genotype, did not harbour any PrP^{Sc} aggregates within their LRS tissues such as palatine tonsils, retropharyngeal and mesenteric lymphnodes, and spleen as shown by both IHC and WB.

Naturally occurring scrapie (Group n. 2)

Among the 1,762 susceptible sheep, scrapie was confirmed in 160 clinically healthy sheep (Table 3). The 96 sheep showing clinical signs consistent with scrapie displayed PrP^{Sc} in the nervous and lymphoid tissues. All these scrapie-affected sheep (n. 256) showed immunohistochemical and immunobiochemical features of classical scrapie, as confirmed by the current Italian statutory diagnostic criteria in the Italian TSE Reference Laboratory.

The mean age of the 96 clinically scrapie-affected sheep was 3.26 years (range 2 to 6 years) while the 160 asymptomatic scrapie-affected sheep and the 320 flock-mate negative controls had a mean age of 3.31 years (range 2 to 8 years) and of 4 years (range 2 to 9 years) respectively.

Through the genetic analysis of all the scrapie-affected sheep (n. 256) and of the 320 flock-mate negative controls, we identified 229 sheep with at least one of the 7 different dimorphisms, namely M112T, M137T, L141F, I142K, H143R, R154H and N176K (Table 3). The cloning of each allele of all genotypes with 2 different polymorphic codons did not find 2 substitutions at the same allele.

Eighteen different genotypes were recorded, with a different distribution according to the health status of the animals (see Table 3). They differed from the $ARQ/ARQ_{wildtype}$ for the presence of 1 or 2 of the 7 aforementioned dimorphisms, which were of homozygous or heterozygous state.

Only 64 of the infected sheep carried the ARQ/$ARQ_{mutated}$ genotype or the $AX_{154}Q/AH_{154}Q$ genotype, compared to 165 of the flock-mate negative controls (Table 4).

Among the scrapie-affected sheep, based on the mutation carried, we identified 5 main groups of genotypes (Table 4).

It is noteworthy that all the scrapie-affected sheep with the substitutions at codons 112, 137 and 176 were clinically healthy and only 3 of the 29 carrying the substitution at codon 141 showed clinical signs of scrapie (Table 3).

In addition, the scrapie-affected sheep carrying the genotype $ARQK_{176}/ARQK_{176}$ displayed PrP^{Sc} only in the tonsils, while the 4 $AM_{137}RQ/AT_{137}RQ$ sheep had PrP^{Sc} deposition only in the brain.

Among the asymptomatics, the sheep carrying the ARQ/$ARQ_{mutated}$ genotype or the $AX_{154}Q/AH_{154}Q$ genotype had a higher mean age at diagnosis compared with those ARQ/$ARQ_{wildtype}$ (4.3 years *vs*. 3.3 years respectively).

The logistic regression analysis showed that the group of sheep with the ARQ/$ARQ_{mutated}$ or the $AX_{154}Q/AH_{154}Q$ genotype had a significant 3-fold lower risk (OR = 0.315; P<0.0001) than sheep with the wildtype genotype (Table 5). Moreover, a lower risk of scrapie was associated with the genotypes $AX_{137}RQ/AT_{137}RQ$, $AX_{141}RQ/AF_{141}RQ$ (although not significant), $AX_{154}Q/AH_{154}Q$ and $ARQX_{176}/ARQK_{176}$ (Table 5).

Experimentally occurring scrapie (Group 3)

Of the 20 experimentally scrapie-inoculated sheep, only the 17 with the ARQ/$ARQ_{wildtype}$ genotype developed clinical signs of scrapie after a mean incubation period of 600 (SD +/- 20) days. By contrast, 2 of the remaining 3 sheep, one carrying the genotype $AF_{141}RQN_{176}/AL_{141}RQK_{176}$ and the other $ARQN_{176}/ARQK_{176}$, did not

show neurological signs and were sacrificed at 700 days post-inoculum. There was no PrP^{Sc} deposition in the lymphoid and nervous tissues of both animals. The last sheep, which was $AF_{141}RQN_{176}/AL_{141}RQK_{176,}$ is still alive 1300 days after *inoculum*. Table 6.

None of the 4 Cheviot experimentally scrapie-inoculated sheep of VRQ/VRQ genotype showed clinical signs of scrapie. One of them, which died for causes unrelated with scrapie at 275 days after inoculum, showed PrP^{Sc} in the lymphoid tissue without the involvement of CNS, while the remaining 3 are still alive (Table 6).

The Cheviot sheep carrying the genotype $AL_{141}RQ/AF_{141}RQ$ and that $AF_{141}RQ/AF_{141}RQ$ were sacrificed at 714 at 109 days post inoculum, respectively. PrP^{Sc} was detected in the lymphoid and nervous tissues of both animals.

The 3 VRQ/AHQ Sarda breed sheep experimentally scrapie-inoculated are still alive (Table 6).

Of the 3 placentas analysed by means of WB only the one which had the ARQ/VRQ fetal genotype displayed PrP^{Sc}. The other 2 placentas, which had the VRQ/ARR fetal genotype, were PrP^{Sc} negative.

Discussion

The data from the genotyping of the breeding rams show that the frequency of susceptible alleles is relatively high if compared to other European milking breeds such as the Lacaune in France (Palhière et al., 2002).

However, it should be stressed that in Sarda sheep the most susceptible VRQ allele was not found in this survey. Recently, the selection scheme applied to the Sardinian breed has achieved important gains in milk yield (Salaris et al., 2007). Other traits of economic interest such as fat and protein content and udder morphology are being implemented as selection objectives.

On the light of these results the population consists of 35.7 % of 3 susceptible genotypes animals, of 48.1 % of 2 heterozygous resistance/susceptible animals and of 16.2 % of one resistant genotypes homozygous animals. The selection pressure that should be applied in the flock-book population to increase the number of resistant homozygous rams is clearly related to number of outbreaks detected in the commercial flocks. However considering that in every country the precise prevalence of scrapie is still not known, it is difficult to estimate with accuracy the number of rams to conform with the European legislation. Our results suggest that because at present there is only a small number of resistant ram, a large reduction may occur in the genetic gain from other traits of economic interest as well as a loss of genetic variability, if high selection pressure is applied to increase the number of resistant rams.

On the basis of the data reported here, all the scrapie-affected sheep (either preclinically or clinically) were of the ARQ/ARQ, ARQ/AHQ, and AHQ/AHQ genotypes, thus confirming that QQ171 homozygosity is a necessary prerequisite for acquiring infection and developing clinical disease in Sarda sheep, similar to what was reported previously

for clinically affected animals belonging to the same breed (Vaccari et al., 2001). It is worth mentioning that two VRQ/ARQ sheep, which belonged to one of the 8 flocks (group 1) were apparently PrP^{Sc} negative.

In flocks with mostly ARQ carriers, the prevalence have been analysed for naturally-affected flocks in Belgium (Roels et al., 1999), Germany (Reckzeh et al., 2007), Iceland (Thorgeirsdottir et al., 1999; Georgsson et al., 2008), Italy (Vascellari et al., 2005; Ligios et al., 2006), Norway (Ersdal et al., 2003), Shetland (UK) (Jeffrey et al., 2002), and the United States (Caplazi et al., 2004), and in Netherlands (Langeveld et al., 2006). For these flocks, the prevalence varied between 3% (Jeffrey et al. 2002) 58.3% (Georgsoon et al., 2008). As consequence, the prevalence found the Sardinian flocks under study in this survey should be considered within the same range, demonstrating that variability of scrapie prevalence is communally very high. On of the reason of this variability is depending on the several factor, i.e. genetic of the host, strains of the scrapie agent, management of the flock, that can influence the occurrence of the disease. Using data from naturally infected flocks and experimentally infected sheep, this study suggests that the presence of additional polymorphisms in the $ARQ/ARQ_{wildtype}$ genotype results in a comprehensively lower risk of scrapie and that the dimorphisms N176K and M137T are associated with the lowest risk of scrapie.

A significantly lower risk is also associated with the R154H dimorphism. The L141F mutation, which has a protective effect not statistically significant, may determine a longer incubation period since most of the affected sheep carrying that mutation were asymptomatic.

Because of the low frequencies, up to now there has been little data on the role of additional polymorphisms in modulating scrapie. Indeed, a study on the association between natural scrapie and *PRNP* polymorphisms in Icelandic sheep failed to find associations between some rare polymorphisms and scrapie, while a protective effect of

AHQ allele was demonstrated (Thorgeirsdottir S. et al., 1999). Similarly, in our work the low number (n. 3) of examined sheep exclusively carrying the M112T dimorphism, which was recently associated with prolonged survival of experimental scrapie-exposed ARQ/ARQ Suffolk sheep (Laegreid et al., 2008), made statistical analysis impracticable. However, we found one $T_{112}AL_{141}RQ/M_{112}AF_{141}RQ$ sheep with PrP^{Sc} in the tonsils.

A protective effect of P168L polymorphism in experimentally challenged sheep with BSE agent (Goldman et al., 2006), and of M137T, I142K and N176K in sheep experimentally inoculated with scrapie and BSE (Vaccari et al., 2007) were reported. These last findings demonstrate an effect on the clinical occurrence of scrapie and BSE, in addition our study in experimental inoculated sheep reasonably excludes, by exhaustive WB and IHC analysis, the possibility that sheep with the dimorphisms N176K replicate PrP^{Sc} without clinical signs. The lack of asymptomatic PrP^{Sc} replication in N176K sheep is also supported by analysis in the 24 naturally scrapie-affected flocks, in which 33 clinically healthy animals with this genotype were found without PrP^{Sc} in nervous and lymphoid tissues.

Although in the experimentally inoculated sheep the dimorphism N176K seems to be associated with absolute resistance to scrapie, we found 1 K176K sheep with natural scrapie suggesting that the protective effect of these substitutions may be sporadically less efficient in natural conditions.

The same experience has been recorded for the ARR/ARR genotype, which is universally considered to be the most scrapie resistant genotype. Indeed, no ARR/ARR sheep have been described as scrapie affected after experimental inoculation, though sick sheep with this genotype have recently been described in natural conditions (Groschup et al., 2007).

All our experimentally inoculated sheep carrying the 176 dimorphism were heterozygous as were those used by Vaccari et al. (2007). However, the scrapie-affected sheep with substitution at codon 176 described in this work were of homozygous state. Thus, we cannot rule out the possibility that being homozygous might result in less resistance. In fact, data obtained in intra-cerebrally BSE-challenged sheep with different genotypes demonstrated that ARR/ARR sheep succumbed, while the ARQ/ARR sheep did not appear to be affected by BSE (Houston et al., 2003).

All 4 sheep carrying the dimorphism at codon 137 were heterozygous and displayed PrP^{Sc} only in their brains without apparent involvement of the lymphoid tissues. The lack of PrP^{Sc} deposition in the lymphoid tissues is not surprising as it is a frequent condition which has often been described in other studies. Such a condition does not appear to be related to particular scrapie strains or host polymorphisms, since it has been frequently observed in ARQ/ARQ scrapie affected Sarda sheep (Ligios et al., 2006) and, occasionally, in VRQ breeds (Jeffrey et al., 2002; Ersdal et al., 2006).

In our study, all the WB and IHC examinations support the idea that one strain is involved, which is in agreement with a recent survey of Sarda breed sheep (Nonno et al, 2003). Therefore, the possible influence of the presence of different scrapie strains influencing the number of scrapie cases can be reasonably excluded.

Whether the association of the dimorphisms M137T and N176K with lower disease susceptibility is valid for all scrapie sources or whether this association is reserved only for certain scrapie isolates needs to be addressed in other experiments.

Until now all the breeding programs designed in Europe and USA for eradicating and controlling the occurrence of scrapie have aimed at indiscriminately eliminating sheep carrying the ARQ allele. In the light of our results, the ARQ/ARQ sheep should be reassessed as a genetic category, which may potentially include animals with different scrapie susceptibility because of the presence of additional polymorphisms. This may

allow the application of alternative strategies for breeding programmes, for instance in some rare breeds or in particular farms that show high frequency of the scrapie susceptible-associated ARQ allele.

However, a great deal more evidence is required to support these findings before they result in new strategies for the ovine breeding program for scrapie resistance.

Studies of SSBP/1 scrapie and PrP genotypes in Cheviot sheep was demonstrated to result in an association between Val 136 allele and increased susceptibility to disease (Goldmann et al., 1991).

After that, Goldmann et al. (1994) demonstrated that there was a more complex host genetic control when the agent strain is changed. Indeed, Cheviot sheep inoculated with the SSBP/1 scrapie strain have a short (2 to 3 years) incubation time mostly depending on the presence of VV at codon 136. Diversely, sheep challenged with the scrapie source CH1641 had disease primarily according to the codon 171 genotype, with the shortest incubation period in those that were QQ 171. These data showed that the modulation of incubation period by codon 136 dimorphism appears minor compared with the major effect of codon 171 dimorphism (Goldmann et al., 1994).

In our experimentally infected animals, we found 1 VRQ/VRQ sheep with deposition of PrP^{Sc} only in lymphoid tissues (Table 6) and another with the placenta accumulating PrP^{Sc}. These findings demonstrated that VRQ/VRQ sheep can be experimentally infected with the Sardinian scrapie strain from ARQ/ARQ sheep, although no Sarda sheep with V at codon 136 has yet been found to be affected by scrapie in the frame work of the TSE surveillance plan (data from Italian Reference Laboratory for TSE) . In our study there was a lack of clinical signs in the VRQ sheep after a post-inoculum period (907 and 1636 days in VRQ/VRQ Cheviot and VRQ/AHQ Sarda, respectively) longer than that observed in those ARQ/ARQ Sarda belonging to the same Group 1. This result demonstrated that in Cheviot, valine at codon 136 may prolong the

incubation time of the disease when they are orally challenged with an ARQ/ARQ isolate. Although we cannot exclude influences due to breed, a similar conclusion could be expanded to Sarda sheep. Indeed, ARQ/ARQ Cheviot and ARQ/ARQ Sarda sheep inoculated with scrapie-infected milk became sick after a similar incubation time (Ligios et al., 2009).

The successful transmission of the ARQ/ARQ Sardinian isolate to recipient with heterologous genotypes suggests that PrP genotype of the host plays a fundamental role in scrapie occurrence (Goldmann et al., 1994). This also confirm that a given scrapie strain adapts itself to a particular PrP genotype more than to a sheep breed.

With regards to the VRQ/AHQ Sarda sheep, we have not yet established whether they are infected. This data needs to assess how the heterozygote valine genotype modulate the susceptibility/resistance to scrapie in Sarda breed. However, it seems that AHQ allele combined with VRQ has a stronger protective effect than associated with ARQ allele (Gooldman, 2008).

Placenta collected in the peri-partal period of the VRQ/VRQ Cheviot sheep showed PrP^{Sc} deposition in the cotyledons corresponding to the foetus carrying the VRQ/ARQ genotype but not in those of VRQ/ARR genotype, as was previously reported by Andreoletti et al. (2002).

Finally, it should be stressed that ARQ/ARQ Cheviot sheep with L141F and F141F mutation did not develop clinical signs of the disease, throughout the course of the experiment even if they were found with scrapie (Table 6). This result confirm the hypothesis that L141F mutation may determine a longer incubation period, as established in the Sarda sheep with natural scrapie (Group 2 sheep).

Conclusions

- The study of the frequencies of the genotypes at codons 136, 154 and 171 of the *PRNP* gene in Sarda breed sheep established that allelic frequency of ARR is less than 40%. The presence of V at 136 codon is very rare.

- By using experimental and natural scrapie cases, we demonstrated that the ARQ allele is associated to scrapie in Sarda breed sheep. The presence of only one ARR allele in the genotype gives total resistance to natural and experimental scrapie.

- The frequencies of the polymorphisms beyond those at codon 136, 154 and 171 of the *PRNP* gene in Sarda breed sheep are low.

- By using experimental and natural scrapie cases we found an association of these additional polymorphisms with scrapie resistance in Sarda breed sheep.

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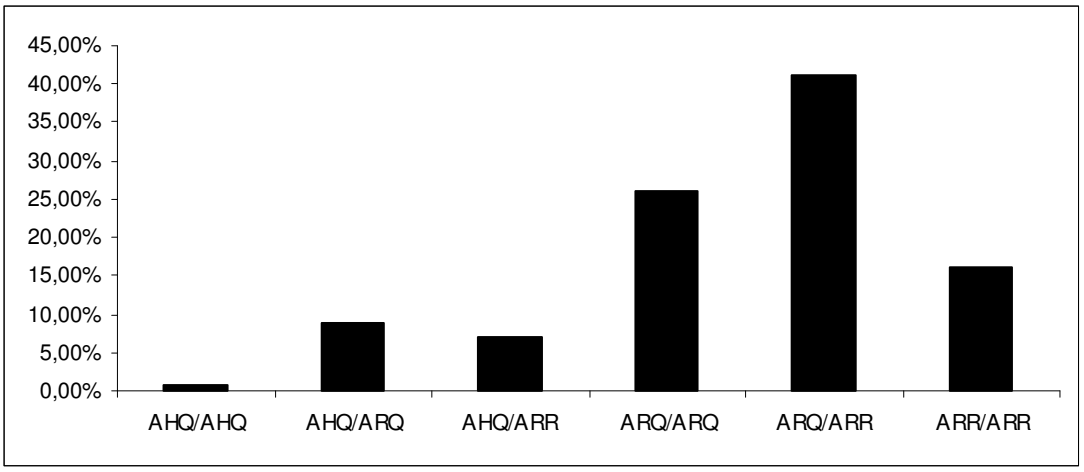
Table 1. Tissues investigated and analyses performed, along with age and PrP genotype of the sheep under study (Group 1).

Flock	No of examined sheep and age range (months)	Genotype of examined sheep at PrP codons 154 and 171 (No)	No of scrapie-affected sheep	Investigated tissues	
				IHC	W B
A	61 (22 to 42)	ARQ/ARQ (29) ARQ/ARR (25) ARR/ARR (7)	12 (41.3%)	Obex, tonsil, retropharyngeal and mesenteric lymph nodes, spleen, ileocoecal junction	Obex, tonsil, retropharyngeal and mesenteric lymph nodes, spleen
B	90 (12/72)	ARQ/ARQ (34) ARQ/ARR (44) ARR/ARR (12)	4 (11.7%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
C	198 (9 to 48)	ARQ/ARQ (44) ARQ/ARR (103) ARR/ARR (51)	3 (6.8%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
D	167 (24 to 48)	ARQ/ARQ (87) ARQ/ARR (67) ARR/ARR (13)	7 (8.0%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
E	100 (48 to 72)	ARQ/ARQ (100)	12 (12%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
F	50 (18 to 96)	ARQ/ARQ (50)	5 (10%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
G	84 (18 to 72)	ARQ/ARQ (56) ARQ/AHQ (25) AHQ/AHQ (3)	8 (9.5%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes
H	300 (36 to 60)	ARQ/ARQ (105) ARQAR/R (151) ARR/ARR (44)	18 (17.1%)	Obex, tonsil, retropharyngeal lymph nodes	Obex, tonsil, retropharyngeal lymph nodes



Graphic A. Frequencies of PrP genotypes in Sarda breeding rams

Table 2. PrP genotypes and results of PrP^{Sc} IHC and WB in the obex, LRS tissues**,** and ENS plexuses of the 69 preclinical scrapie-affected sheep from the 8 flocks under study (Group 1).

No of sheep	Genotype	Obex		LRS		ENS
		IHC	WB	IHC	WB	IHC
17	ARQ/ARQ	+	+	-	-	+
1	AHQ/ARQ	+	+	-	-	+
34	ARQ/ARQ	+	+	+	+	+
2	AHQ/AHQ	+	+	+	+	+
6	AHQ/ARQ	+	+	+	+	+
9	ARQ/ARQ	-	-	+	+	+

Table 3. Genotypes and alleles of the *PRNP* gene and their frequencies in scrapie affected sheep and in flock-mate negative controls (Group 2). In this survey, additional polymorphisms were found at codons 112, 137, 141, 142, 143, 154 and 176 that resulted in 18 different genotypes. Note that only the sheep with polymorphisms at codons 141 or 154 displayed clinical signs.

Genotypes	**Scrapie-affected sheep**						**Flock-mate negative controls**	
	Symptomatics		**Asymptomatics**		**Total**			
	N°	**%**	**N°**	**%**	**N°**	**%**	**N°**	**%**
$ARQ/ARQ_{wildetype}$	82	85.4	110	68.75	192	75	155	48.44
$M_{112}ARQ/T_{112}ARQ$	0	0	0	0	0	0	3	0.94
$AM_{137}RQ/AT_{137}RQ$	0	0	4	2.5	4	1.56	24	7.5
$AT_{137}RQ/AT_{137}RQ$	0	0	0	0	0	0	2	0.62
$AL_{141}RQ/AF_{141}RQ$	3	3.12	25	15.62	28	10.94	36	11.25
$AF_{141}RQ/AF_{141}RQ$	0	0	1	0.62	1	0.39	1	0.31
$AH_{143}RQ/AR_{143}RQ$	0	0	0	0	0	0	4	1.25
$AR_{154}Q/AH_{154}Q$	11	11.46	16	10	27	10.55	40	12.5
$AH_{154}Q/ AH_{154}Q$	0	0	2	1.25	2	0.78	3	0.94
$ARQN_{176}/ARQK_{176}$	0	0	0	0	0	0	33	10.31
$ARQK_{176}/ARQK_{176}$	0	0	1	0.62	1	0.39	0	0
$T_{112}M_{137}ARQ/M_{112}T_{137}ARQ$	0	0	0	0	0	0	1	0.31
$T_{112}AL_{141}RQ/M_{112}AF_{141}RQ$	0	0	1	0.62	1	0.39	2	0.62
$T_{112}ARQN_{176}/M_{112}ARQK_{176}$	0	0	0	0	0	0	4	1.25
$AT_{137}R_{154}Q/AM_{137}H_{154}Q$	0	0	0	0	0	0	4	1.25
$AF_{141}R_{154}Q/AL_{141}H_{154}Q$	0	0	0	0	0	0	3	0.94
$AK_{142}R_{154}Q/AI_{142}H_{154}Q$	0	0	0	0	0	0	1	0.31
$AF_{141}RQN_{176}/AL_{141}RQK_{176}$	0	0	0	0	0	0	2	0.62
$AR_{154}QN_{176}/AH_{154}QK_{176}$	0	0	0	0	0	0	2	0.62
Total	96	#	160	#	256	#	320	#
Alleles								
$ARQ_{wildetype}$	178	92.71	265	82.81	443	86.52	450	70.31
$T_{112}ARQ$	0	0	1	0.31	1	0.19	10	1.56
$AT_{137}RQ$	0	0	4	1.25	4	0.78	33	5.16
$AF_{141}RQ$	3	1.56	28	8.75	31	6.05	45	7.03
$AK_{142}RQ$	0	0	0	0	0	0	1	0.16
$AR_{143}RQ$	0	0	0	0	0	0	4	0.62
$AH_{154}Q$	11	5.73	20	6.25	31	6.05	56	8.75
$ARQK_{176}$	0	0	2	0.62	2	0.39	41	6.41

Table 4. Results of the *PRNP* sequencing of AXQ/AXQ scrapie affected sheep and flock-mates negative controls (Group 2). A: total number and frequency (%) of genotypes with at least one polymorphism as well as of the wildtype genotype in scrapie affected sheep and in flock-mate negative controls. B: number and frequency (%) of the genotypes with only 1 (homozygous or heterozygous) or 2 polymorphic codons within the scrapie affected and flock-mate negative controls (see the text). The symbol X indicates one of the two amino-acids that could be codified at that codon.

A	**Number and frequency of scrapie affected-sheep**	**Number and frequency of flock-mate negative controls**	**Total**
$ARQ/ARQ_{wildetype}$	192	155	347
$ARQ/ARQ_{mutated}$ and $AH_{154}Q/AX_{154}Q$	64	165	229
Total	256	320	576
B			
$AX_{137}RQ/AT_{137}RQ$	4	26	30
$AX_{141}RQ/AF_{141}RQ$	29	37	66
$AX_{154}Q/AH_{154}Q$	29	43	72
$ARQX_{176}/RQK_{176}$	1	33	34
2 polymorphic codons	1	26	27
Total	64	165	229

Table 5. Contrasts, odds ratios (OR), confidence limits and *p-values* of the logistic regression analysis in Group 2 sheep. A: OR for scrapie in affected flocks, comparing the group of sheep carrying the ARQ/$ARQ_{wildtype}$ genotype (reference category) to that of sheep with ARQ/$ARQ_{mutated}$ or $AX_{154}Q/AH_{154}Q$ genotype. B: OR for scrapie comparing ARQ/$ARQ_{wildtype}$ sheep to those having additional polymorphisms at codon 137, 141, 176 or the $AX_{154}Q/AH_{154}Q$ genotype. The symbol X indicates one of the two amino-acids that could be codified at that codon. C.I. = confidence interval.

A

Contrasts	Odds Ratio point estimate	99,9% C.I. for Odds Ratio		*p-value*
		Lower Limit	Upper Limit	
ARQ/$ARQ_{mutated}$ and $AX_{154}Q/AH_{154}Q$ *vs* ARQ/$ARQ_{wildtype}$	0.315	0.173	0.576	< 0.0001

B

Contrasts	Odds Ratio point estimate	95% C.I. for Odds Ratio		*p-value*
		Lower Limit	Upper Limit	
$AX_{137}RQ/AT_{137}RQ$ *vs* ARQ/$ARQ_{wildtype}$	0.125	0.043	0.365	0.0001
$AX_{141}RQ/AF_{141}RQ$ *vs* ARQ/$ARQ_{wildtype}$	0.636	0.375	1.079	0.0935
$AX_{154}Q/AH_{154}Q$ *vs* ARQ/$ARQ_{wildtype}$	0.568	0.335	0.961	0.0351
$ARQX_{176}/ARQK_{176}$ *vs* ARQ/$ARQ_{wildtype}$	0.025	0.003	0.182	0.0003

Table 6. Details of the experimentally scrapie-inoculated sheep, including WB results (Group 3). Each sheep received a dose of ARQ/ARQ scrapie- brain pooled homogenate (see the text). ND= not done. * Standard Deviation.

No sheep	Breed	Genotype	Examined	Days P.I. at examination	Clinical status	WB/IHC results	
						SNC	Lymphoid tissue
1	Cheviot	VRQ/VRQ	Yes	275	Asymptomatic	-	+
3	Cheviot	VRQ/VRQ	No	907	Alive	ND	ND
3	Sarda	VRQ/AHQ	No	1636	Alive	ND	ND
1	Cheviot	$AL_{141}RQ/AF_{141}RQ$	Yes	714	Asymptomatic	+	+
1	Cheviot	$AF_{141}RQ/AF_{141}RQ$	Yes	109	Asymptomatic	+	+
17	Sarda	ARQ/ARQ	Yes	600+/-20*	Symptomatic	+	+
1	Sarda	$AF_{141}RQN_{176}/AL_{141}RQK_{176}$	Yes	700	Asymptomatic	-	-
1	Sarda	$ARQN_{176}/ARQK_{176}$	Yes	700	Asymptomatic	-	-
1	Sarda	$AF_{141}RQN_{176}/AL_{141}RQK_{176}$	No	1557	Asymptomatic	-	-
20	Sarda	ARQ/ARR	Yes	700	Asymptomatic	-	-
20	Sarda	ARR/ARR	Yes	700	Asymptomatic	-	-