Intraepithelial and Interstitial Deposition of Pathological Prion Protein in Kidneys of Scrapie-Affected Sheep

Ciriaco Ligios1,*, Giovanna Maria Cancedda1, Ilan Margalith2, Cinzia Santucciu1, Laura Madau1, Caterina Maestrale1, Massimo Basagni3, Mariangela Saba1, Mathias Heikenwalder2*

1 Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy, 2 Department of Pathology, Institute of Neuropathology, UniversitätsSpital Zürich, Zürich, Switzerland, 3 Prion Diagnostica Srl, Rho, Italy

Prions have been documented in extra-neuronal and extra-lymphatic tissues of humans and various ruminants affected by Transmissible Spongiform Encephalopathy (TSE). The presence of prion infectivity detected in cervid and ovine blood tempted us to reason that kidney, the organ filtrating blood derived proteins, may accumulate disease associated PrPSc. We collected and screened kidneys of experimentally, naturally scrapie-affected and control sheep for renal deposition of PrPSc from distinct, geographically separated flocks. By performing Western blot, PET blot analysis and immunohistochemistry we found intraepithelial (cortex, medulla and papilla) and occasional interstitial (papilla) deposition of PrPSc in kidneys of scrapie-distinct, geographically separated flocks. By performing Western blot, PET blot analysis and immunohistochemistry we found that kidney, the organ filtrating blood derived proteins, may accumulate disease associated PrPSc. We collected and screened kidneys of experimentally, naturally scrapie-affected and control sheep for renal deposition of PrPSc from distinct, geographically separated flocks. By performing Western blot, PET blot analysis and immunohistochemistry we found that kidney, the organ filtrating blood derived proteins, may accumulate disease associated PrPSc.

INTRODUCTION

Scrapie, a fatal, transmissible spongiform encephalopathy (TSE) of sheep and goat caused by prions, has been intensively studied for more than a century [1]. It is broadly accepted that scrapie is caused by prions, as are bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD) of humans, including those of sporadic, familial, iatrogenic and variant etiology, chronic wasting disease (CWD) of deer, elk, and moose, and a variety of rarer diseases of several other animal species. Prions are thought to mainly consist of PrPSc, a misfolded and aggregated form of the cellular prion protein (PrPC) [2] and are used as a surrogate marker for prion affected animals. Many fundamental aspects of peripheral and central scrapie pathogenesis however are yet not understood. A particular important question relates to those factors permitting horizontal transmission of scrapie within sheep flocks. Although the main target of prion-induced pathology is the central nervous system (CNS), PrPSc can be readily demonstrated in the lymphoreticular system (LRS) in many TSEs [3,4–5,6]. Moreover, PrPSc and/or prion infectivity were demonstrated to accumulate at extraneural and extralymphatic sites, including skeletal muscle, mammary gland, placenta and salivary gland in sheep [7,8–9,10] as well as skeletal muscle [11,12–13,14] and blood in man [15,16] and mice [17] or in saliva [10] and skeletal muscles of deer [18]. Yet 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 thought to harbor very little-if any-prion infectivity, yet this assumption was shaken by the efficient blood-borne sheep-to-sheep transmission of scrapie [19], cervid [20] and BSE prions as well as blood-borne transmission incidents of variant CJD (vCJD) between humans [13,16,22]. In mice, sheep and deer, chronic lymphocytic inflammation can shift the distribution pattern of PrPSc or prion infectivity to non-lymphoid organs, being they in the kidney of mouse and deer [23,24], liver and pancreas of mouse [23], and mammary gland of sheep [8]. Extraneural deposition of PrPSc appears to become prominent in Creutzfeldt-Jakob disease when PrPSc is abundantly available as a substrate, as demonstrated in muscle with inclusion body myositis containing macrophages and lymphocytes [13]. In many instances ectopic prion infectivity and/or PrPSc was detected in pre-clinically prion-infected individuals [23,25]. Besides enabling high renal prion loads, chronic lymphocytic inflammation in kidney combined with pathological alterations of the renal filtration apparatus was demonstrated to induce prionuria in both preclinical and terminally scrapie-sick mice [25]. The latter studies yielded the first evidence that extrerey organs, when affected by chronic inflammation, can release prion infectivity into the environment. Alternatively, considering that in physiological conditions prions or PrPSc have been detected in blood of sheep and hamsters affected by scrapie [21,17], prionuria could occur in the absence of renal immunopathological changes [26]. This last data together with the recent finding of deposition of PrPSc in the salivary glands of sheep with scrapie [10] accentuate that prionuria may occur, hence

* To whom correspondence should be addressed. E-mail: ciriaco.ligios@izs-sardegna.it (CL); mathias.heikenwalder@usz.ch (MH).
RESULTS

Conventional and Sodium phosphotungstate (NaPTA)-Western blot analysis

Spongiosis, astrogliosis and PrP\textsuperscript{Sc} deposition were found by means of histopathological and immunohistochemical analysis in the brain of all analyzed 72 clinically scrapie-affected Sarda sheep. In addition, presence of PrP\textsuperscript{Sc} was confirmed by conventional and NaPTA-Western blot analysis in the brain, and by immunohistochemistry in the lymphoid tissues (e.g. spleen and palatine tonsils). Identical analysis was performed in sheep lacking clinical signs (sheep from Sardinian, scrapie-free flocks n = 30; sheep from scrapie-affected flocks from Abruzzo and Sardinia n = 67) and did not lead to the detection of PrP\textsuperscript{Sc} in brains and tonsils.

Histopathological examination demonstrated the presence of focal interstitial inflammatory changes, moderate tubular degeneration as well as interstitial fibrosis in some kidneys from the 72 clinically scrapie-affected sheep. However, the damage was moderate in all cases and no overall differences were found when animals belonging to the different groups were compared to the controls (Table 1).

The analysis of PrP\textsuperscript{Sc} deposition in kidneys from the 72 clinically scrapie-affected Sarda sheep indicated 53 (73.6%) or 52 (71.2%) positive cases by using Western blot analysis after NaPTA precipitation or conventional Western blot analysis, respectively. The scrapie sheep positive for PrP\textsuperscript{Sc} in kidney were evenly distributed through all flocks investigated in Sardinia. In contrast, all 79 kidney homogenates derived from control Sarda sheep (49 distributed through all flocks investigated in Sardinia. In contrast, the immuno-histochemical profile and the histopathological features of the obex from these sheep were indicative of clinical stage.

The analysis of PrP\textsuperscript{Sc} deposition in kidneys from the 72 clinically scrapie-affected Sarda sheep indicated 53 (73.6%) or 52 (71.2%) positive cases by using Western blot analysis after NaPTA precipitation or conventional Western blot analysis, respectively. The scrapie sheep positive for PrP\textsuperscript{Sc} in kidney were evenly distributed through all flocks investigated in Sardinia. In contrast, all 79 kidney homogenates derived from control Sarda sheep (49 distributed through all flocks investigated in Sardinia. In contrast, the immuno-histochemical profile and the histopathological features of the obex from these sheep were indicative of clinical stage.

Table 1. Number (N), age, PrP genotype and clinical status of the sheep included in the study.

| Region | N flocks | N sheep | Genotypes (N) | Infection | Age (years) | Clinical signs |
|--------|----------|---------|---------------|-----------|-------------|----------------|
| Sardinia | 14 | 72 | ARQ/ARQ (63) ARQ/AHQ (9) | Natural | 2 to 5 | Yes |
| Sardinia | 1 | 9 | ARQ/ARQ | Experimental | 2 | Yes |
| Abruzzo | 1 | 3 | ARQ/ARQ | Natural | 1.5 to 4 | Yes |
| Sardinia | 1 | 11 | ARQ/ARQ | Natural | 2 to 5 | No |
| Sardinia | 2 | 30 | ARQ/ARQ (10) | No | 2 to 5 | No |
| Sardinia | 1 | 49* | ARQ/ARQ | No | 2 to 5 | No |
| Abruzzo | 1 | 18* | ARQ/ARQ (3) | No | 1.5 to 7 | No |
| | 1 | 8 | ARQ/ARQ (6) | | | |
| | 1 | 8 | ARQ/VRQ (1) | | | |

Sheep are grouped as scrapie-affected sheep or negative control. The number (N) of the flocks from which the sheep were selected is also indicated. Sheep from scrapie-affected flock.

* doi:10.1371/journal.pone.0000859.t001
Paraffin-embedded tissue (PET) blot analysis and Immunohistochemical analysis

In order to better define the localization and to verify the presence of PrPSc in kidneys with different methods, we performed PET blot analysis on paraffin sections of natural scrapie-sick and control sheep. As indicated PrPSc is clearly detectable in the tubular structures of the renal cortex and medulla (Figure 2B and C), including the papillae (Figure 2D). In the medulla the rate of immunostained tubular structures (collecting ducts) was higher than in the cortex, where immuno-positive signals were restricted to a few convoluted tubules. All negative animals were devoid of PK resistant PrPSc in the kidney.

Immunohistochemical and immunofluorescence analysis of consecutive paraffin sections from randomly selected kidneys of Sardinian naturally scrapie affected sheep (n = 33) demonstrated that 23 out of the 33 kidneys showed deposits of PrPSc positive for F99 antibody or for 2G11 (Figure 3A and B). Immunohistochemistry results were not always congruent with results gained from NaPTA and conventional Western blot analysis, though the rate of PrPSc positivity obtained by immunohistochemistry was comparable to Western blot analysis (69.6% versus 73.6% and 71.2%).

Moreover, all 23 kidneys accumulated PrPSc in both the cortex and the medulla, including the collecting ducts and the papillae, as demonstrated by PET blot and immunohistochemistry analysis (Figures 2 and 3). Kidney paraffin sections from control sheep remained negative for staining by 2G11 and F99 (Figures 2 and 3). The intensity of PrPSc staining varied among the different kidneys and regions (cortex, medulla, papillae). Notably, the immunohistochemical patterns were different depending on the antibody used. Our analysis demonstrated granular PrPSc deposits with the 2G11 antibody exclusively within the epithelial tubular cells of the convoluted tubules and the collecting ducts (Figure 3A). On the contrary, immunostaining with F99 showed presence of PrPSc staining on the surface of tubular cells of convoluted tubules (cortex) and in the collecting ducts (medulla and papillae) (Figure 3A). Moreover, with the same antibody in two cases PrPSc staining was demonstrated in the intertubular spaces of the renal papillae displaying a granular deposition, mostly around the basal membrane of the tubular structures (Figure 3A). Surprisingly, in these two cases, serial renal sections of the same area identified PrPSc granular deposits only within the tubular cells of the collecting ducts when probed with the 2G11 antibody. With both antibodies in the medulla the rate of immunostained tubular structures was higher than in the cortex, where immuno-positive signals were restricted to a few convoluted tubules.

Quantification of renal PrPSc in scrapie-sick and control sheep

Furthermore we determined the relative amount of renal PrPSc in scrapie-sick sheep compared to brain derived PrPSc. Serial dilution experiments of PK digested brain homogenates spiked into kidneys from healthy sheep were performed, thereby generating a standard curve for the amount of PrPSc/mg of brain tissue from various brain samples (Figure 4). These results were compared to a selected

Table 2. Number (N) of examined Sarda breed sheep, with or without clinical signs, displaying PrPSc in the kidney, indicated as percentage (%).

| Number of examined sheep | Number of positive sheep for conventional Western blotting | Infection |
|-------------------------|----------------------------------------------------------|-----------|
| 72                     | 52 (71.2%)                                                | Natural   |
| 11                     | 3 (27%)                                                   | Natural   |

The results are only referred to conventional Western blot examinations.

doi:10.1371/journal.pone.0000859.t002

Figure 1. Western Blotting PrPSc deposition in scrapie-affected Sarda sheep. (A) Detection of PrPSc by conventional Western blot in the cortical and medullar part of kidneys. Brain and kidney homogenates were treated with (+) or without (−) proteinase K (PK), amounts of kidney and brain represented in each lane are 1mg and 1g respectively. S.b. = brain derived from a naturally scrapie-sick sheep; h.b. = brain derived from a scrapie-free sheep control; h.k. = kidney derived from a scrapie-free sheep control, s.k. = kidney derived from a naturally scrapie-sick sheep. kD = kilo Dalton, M = medulla, C = cortex. (B) Detection of PrPSc by NaPTA Western blot in kidney homogenate derived from different Sarda sheep. Substantial individual variation of the Western blotting signal was observed among the sheep examined. Spike control = brain homogenate derived from a scrapie-sick sheep spiked into negative control kidney homogenate. h.b. = brain derived from a scrapie-free sheep control. 2000–4000 mcg of renal homogenate were used as a starting material to perform NaPTA precipitation. doi:10.1371/journal.pone.0000859.g001
pool from independent animals of kidney homogenates loaded on the identical blot. After densiometric analysis, the relative amount of PrPSc found in μgs of kidney homogenate was quantified and blotted as percentage of PrPSc positive signal intensity found in μgs of brain homogenates (Figure 4). The relative amount of PrPSc found in kidney ranged from 5–10 fold to 100–500 fold lower than in brain. Even though 100–500 fold less PrPSc was detected in kidney when compared to brain, PrPSc was still detectable by NaPTA and conventional Western blot analysis.

Figure 2. PET blot analysis identifying PrPSc in the tubular structures of the cortex and medulla in naturally scrapie-sick Sarda sheep. (A) Brains of scrapie-sick (left) and healthy sheep (right) as a control for PET blot analysis procedure. Scale bar = 100 μm. (B) Low magnification of transverse paraffin sections from kidneys of a naturally scrapie-sick and a healthy sheep demonstrating PrPSc in the medullary and cortical regions of the kidney. The small transparent box indicates the region shown at higher magnification and resolution in figure C. Scale bar = 1 cm. (C) PrPSc deposits and tubular structure fit together in the cortical part of the kidney in scrapie-sick sheep. Scale bar = 100 μm. (D) PrPSc is also found to a high degree in the collecting ducts of the medulla (arrowhead) as well as in the papillae (arrow) of kidneys derived from naturally scrapie infected sheep. Scale bar = 30 μm.

doi:10.1371/journal.pone.0000859.g002

 Genetic analysis

Sequencing of the complete PrP gene (*PRNP*) of clinically scrapie-affected 16 sheep (8 of which harbouring PrPSc in the kidney) confirmed the ARQ/ARQ genotype for all of them and did not demonstrate the existence of additional polymorphisms. All remaining genotypes of sheep were determined at codon 136, 154 and 171 (Table 1).

DISCUSSION

Our study involved a high number of animals, providing good evidence that PrPSc can be found to high degree in the medullary and the cortical regions of kidneys derived from ARQ/ARQ scrapie-sick sheep. Moreover, we can demonstrate that the incidence of renal PrPSc deposition increases with the stage of disease.

Presence of PrPSc in kidney was detected in renal glomerula of cats [27] and cheetah [28] affected by feline spongiform encephalopathy by the means of immunohistochemical analysis. However, both findings are observations without Western blot or Histoblot and PET blot analysis, which are essential to prove the effective presence of PrPSc, a surrogate marker of prion infectivity. In addition, Herzog and colleagues [29] described PrPSc accumulation in kidney of primates experimentally inoculated with BSE by using an ELISA-based assay. However, also this technique did not allow to localize PrPSc nor to characterize renal PrPSc biochemically. In contrast to the described PrPSc deposition pattern in cats or cheetah, we do not find PrPSc positive signals in glomerula. This stresses the differences between various species in renal PrPSc deposition and may underline possible consequences for enabling horizontal prion transmission in particular species.

Figure 3. PrPSc detected by immunohistochemistry in scrapie-affected Sarda sheep. (A) Immunohistochemical analysis of the cortex, medulla and papillae of naturally scrapie-sick sheep and control sheep derived from scrapie-free flocks. Antibodies 2G11 and F99 were used. Scale bar = 30 μm. (B) Immunofluorescence using 2G11 identifies tubular deposits in the medullar-cortical junction of the kidney of a scrapie-sick sheep. Scrapie-free control is devoid of detectable positive signal.

doi:10.1371/journal.pone.0000859.g003

Presence of PrPSc in kidney was detected in renal glomerula of cats [27] and cheetah [28] affected by feline spongiform encephalopathy by the means of immunohistochemical analysis. However, both findings are observations without Western blot or Histoblot and PET blot analysis, which are essential to prove the effective presence of PrPSc, a surrogate marker of prion infectivity. In addition, Herzog and colleagues [29] described PrPSc accumulation in kidney of primates experimentally inoculated with BSE by using an ELISA-based assay. However, also this technique did not allow to localize PrPSc nor to characterize renal PrPSc biochemically. In contrast to the described PrPSc deposition pattern in cats or cheetah, we do not find PrPSc positive signals in glomerula. This stresses the differences between various species in renal PrPSc deposition and may underline possible consequences for enabling horizontal prion transmission in particular species. By
The quantification of PrPSc signal intensity found in kidney homogenates in naturally affected by scrapie. Sisoé et al. [30], found a higher presence of PrPSc in sheep experimentally inoculated with an ARQ/ARQ Suffolk isolate, the deposition of PrPSc might be mostly influenced by the source of the etiological agent. Further studies are needed to establish whether renal PrPSc deposition occurs in a similar manner in VRQ/VRQ sheep naturally affected by scrapie.

Most importantly, our survey demonstrated that renal PrPSc deposition is mostly relegate to the clinical stage of the disease, indeed only 3 out of the 11 asymptomatic sheep harbored PrPSc in the kidney. In addition the immunohistochemical patterns that were observed in brain of these 3 sheep were indicative of clinical scrapie, suggesting a later stage of the disease.

In contrast to the previously published studies, we conclusively demonstrate for the first time, by using immunohistochemical, immunofluorescent and PET blot analysis, intraepithelial, but not glomerular, deposition of PrPSc in kidney. However, the deposition patterns detected by immunohistochemistry vary with the epitope specificity of the detecting antibody, indicative of different PrPSc isoforms or differential processing in the kidney of naturally scrapie-sick sheep. This finding is again in contrast to the previously published study that indicated differences in staining intensity but not in staining pattern [30]. In this case it is reasonable to suppose that differences in immunohistochemical procedures (e.g. formalin fixation, antibodies, etc.) can play an important role.

Indeed, using the same antibodies (F99 and G11) Vascellari et al. [10] found analogous PrPSc deposition patterns in the salivary glands of ARQ/ARQ sheep with scrapie. This might suggest, on one side, a similar mechanisms of PrPSc accumulation in the kidney and in the salivary glands, on the other, a similar role of the filtrating epithelial cells and of the secreting epithelial cells in spreading prions.

Since prion infectivity was demonstrated in blood of sheep [21], in the light of these results it could be hypothesized that blood contains prions which could be filtered and accumulated by the kidney, resulting in renal PrPSc accumulation. A multitude of experimental paradigms shows that prion diseases can be transmitted from one individual to another. Many of the parameters controlling transmissibility are relatively well understood. Firstly the size of the infectious inoculum, secondly the route, with intracerebral inoculation being more efficient than oral, intraperitoneal and intravenous injection, thirdly the polymorphisms in the Prnp gene of both donor and recipient.

For a living organism to initiate horizontal transmission under field conditions of any infectious agent, including prions, the agent must be released from the organism into the environment, through respiratory aerosols, bodily secretions, or excrements. The unsuccessful attempts at eradicating scrapie from Icelandic flocks in the 20th century bear witness to the likelihood of environmental prion contamination. Additional pathways of horizontal transmission of sheep scrapie may include parasitic vectors [31,32] and placental cannibalism within flocks [9]. The importance of the two latter routes in naturally occurring transmission of field cases, however, is supported by limited evidence.

Previous studies in sheep, humans, and genetically modified mice have pointed to inflammatory conditions as a cofactor enabling prion replication in disparate tissues, including liver, pancreas, skeletal muscle, mammary gland, and kidney [11,25,6]. The function of the latter organs is excretion, and indeed chronic lymphofollicular nephritis leads to prionemia in prion-infected mice [24]. As a first step towards investigating the relevance of prionemia in horizontal transmission of scrapie, we undertook to investigate the presence of PrPSc in sheep kidney.

Work in progress will define the spatiotemporal mechanisms of renal PrPSc deposition in subclinical and naturally scrapie-sick sheep as well as the epidemiological importance of this finding. Due to prion infectivity detected in sheep blood, it is not surprising that renal prion deposition can occur independent of lymphocytic inflammatory disorders. Potentially, renal prion deposition could be a result of blood borne prions. Prions were recently demonstrated in blood of 50–60% of subclinical and in >80% of terminal hamsters [17]. This could explain why also subclinical sheep could accumulate prions in kidney but to less degree and

---

Figure 4. Quantification of PrPSc in renal homogenates of naturally scrapie-sick sheep. (A) Conventional Western blot analysis of various brain dilutions after proteinase K (PK) digestion. Undigested brain is loaded to control for PK digest (lane 1). Kidney homogenates from independent scrapie sick animals were loaded (lanes 7–10). (B) Relative quantification of PrPSc signal intensity found in kidney homogenates in relation to brain derived PrPSc. Our results indicate that the highest amounts of renal PrPSc detected were appr. up to 20% of the PrPSc signal detected in brain.

doi:10.1371/journal.pone.0000859.g004

---

PLoS ONE | www.plosone.org 5 September 2007 | Issue 9 | e859

---

© 2007 A. S. et al.; licensee BioMed Central Ltd. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
incidence. Alternatively, ovine kidneys could potentially contain renal cells that are capable of prion replication itself.

It will be extremely important to evaluate whether inflammation can further increase renal deposition of PrPSc and whether prion infectivity can be detected in kidney and urine of naturally scrapie-sick sheep, during the clinical and preclinical stage. Our data suggest that prionuria might indeed be possible in an unexpected high percentage of affected animals, presenting an important cofactor for horizontal prion transmission.

**MATERIALS AND METHODS**

**Sampling**

Our study was carried on experimentally and naturally infected sheep as well as appropriate control sheep located in Sardinia and Abruzzo (Italy). In Sardinia, we set out to investigate naturally scrapie-sick Sarda sheep (n = 72) from 14 distinct flocks as well as experimentally scrapie-sick Sarda sheep (n = 9), inoculated with 25 ml of a 10% brain homogenate (pooled from natural ARQ/ARQ sheep scrapie cases) at 40 days of age. Additionally, to verify whether our hypothesis was valid in different and geographically separated breed we investigated an additional scrapie outbreak in Abruzzo, where we analyzed Comisana-crossed breed sheep with natural clinical scrapie (n = 3), (Table 1).

All the sheep (n = 75) coming from these different sources were sacrificed at clinical stage of the disease. Lastly, to define whether PrPSc deposition already occurs at asymptomatic stage of the disease, we analyzed 11 asymptomatic scrapie-affected sheep from a single flock located in Sardinia. In addition, 30 control Sarda breed sheep from flocks without scrapie cases and 67 healthy sheep from 2 scrapie affected flocks, located respectively in Abruzzo (n = 18 sheep) and in Sardinia (n = 49 sheep) were analyzed, other details are reported in Table 1.

All the 9 ARQ/ARQ orally infected sheep, after an average incubation period of 650 days (+/- 25 days) showed clinical signs of scrapie and were sacrificed at terminal stage (mean clinical course = 27 days). The 75 naturally scrapie-affected sheep with clinical signs were monitored in the open field on a constant basis and sacrificed at terminal stage, approximately 40 days after the onset of the first clinical signs.

From all sheep we collected brain, palatine tonsil, kidney and spleen, which were partly frozen at -20°C or -80°C and partly fixed in 10% neutral buffered formalin. For investigating the regional distribution of PrPSc in kidneys we separated cortex and medulla by macro-dissecting transversal sections of whole kidneys and used pooled (cortex and medulla) or separated regions for Western blot analysis.

**Conventional WB analysis**

Frozen kidney, nervous and LRS tissue samples were thawed and then submitted to an appropriate Western-blotting (WB) protocol for PrPSc detection. For the examination of kidney, this was carried out by means of a modified PriOnics check protocol (Prionics AG, Switzerland), with 1 g of kidney being first incubated in TBS buffer (10 mM Tris HCl, 133 mM NaCl, pH 7.4) containing 1.5 mM CaCl2 and 2.5 mg/ml (final concentration) of type XI collagenase (Invitrogen, USA) for electrophoresis under constant application of 120 V for 45 min. Electroblotting was performed onto polyvinylidene fluoride membranes (150 V for 1 h). For PrPSc detection, membranes were incubated over night at 4°C with P4 monoclonal antibody (MoAb) (1:15,000), which is specific for the aa89-104 of the ovine PrP [33]. After washing with a solution of 10 mM Tris HCl, 133 mM NaCl, and 0.2% Tween 20 (TBST), the secondary Ab diluted 1:5000 was added to the reaction, with membranes being subsequently washed 4 times for 5 min, equilibrated in luminescence buffer, placed in chemiluminescent substrate (Roche, Switzerland), and finally exposed to an X-Ray film. Examination of the obex was carried out using the PriOnics check kit, in accordance with the manufacturer’s instructions.

**Western blot analysis with Sodium phosphotungstate (NaPTA) precipitation**

10% homogenates of brain and kidney were prepared in 2% Sarcosyl or PBS. Gross cellular debris were removed by centrifugation at 500 g for 1 min. The resulting supernatant was adjusted to 300 µl with PBS, and mixed 1:1 with 4% Sarkosyl in PBS. Samples were incubated for 15 min at 37°C under constant agitation. Benzonase and MgCl2 were added to a final concentration of 50 U/ml and 12.75 mM respectively, and incubated for 30 min at 37°C under continuous agitation. Pre-warmed PTA stock solution (pH 7.4) was added to a final concentration of 0.3% and the sample was incubated at 37°C for 30 min with constant agitation, followed by centrifugation at 37°C for 30 min at maximum speed (14,000 g) in an Eppendorf microcentrifuge. The pellet was resuspended in 30 µl 0.1% Sarkosyl in PBS and digested with 30 or 50 µg/ml proteinase K (PK) for 30 min at 37°C with agitation. The sample was heated at 95°C for 5 min in SDS-containing loading buffer before loading onto 10%, 12% or 16% Novex SDS polyacrylamide gels (Invitrogen, USA), then wet-blotting were performed as described in the previous paragraph using as primary antibody POM1/POM19 or POM19 alone, which bind on distinct epitopes on the globular domain of PrP [54].

**Quantification of total brain or kidney protein**

From 10% homogenates of kidney and brain was performed with a BCA™ Protein Assay Kit, according to the manufacturer's protocol (23-225 BCA™ Protein Assay Kit, Pierce).

**Quantification of PrPSc in brain and kidney**

To analyze and to quantify PrPSc in ovine kidney homogenates of natural scrapie sick sheep 250–400 µg total protein of selected kidney samples were digested with proteinase K (50 µg/ml) for 30 min at 37°C and loaded on a pre-casted 12%–SDS gel (NuPAGE). Proteins were transferred to nitrocellulose (Schleicher-Schuell, Germany) by wet-blotting. Membranes were blocked with TBST containing 5% Top Block or 5% bovine serum albumine (BSA) (Juro, Switzerland). As primary MoAb was applied P4 and also POM1 and/or POM19. On the same blot serial dilutions of brain homogenate (2–0.125 µg), spiked into 300 µg kidney protein derived from a sheep without clinical signs, were PK digested, loaded and developed. To control PK digest, undigested and digested brain homogenate as well as a dual color marker were loaded. Blots were developed until the three characteristic, shifted, PK resistant bands appeared in the kidney homogenates. Signals...
received from serial brain dilutions were quantified in the non-saturated, linear range after development using the VersaDoc imaging system (Biorad) and Quantity one® software (Biorad). Thereby a standard curve was generated, allowing relative quantification of PrPSc/μg of brain or kidney tissue.

**Immunohistochemical techniques**

Tissues were formalin-fixed, treated for 1 hr in 98% formic acid, and embedded in paraffin using conventional protocol. For histopathology, nervous and renal specimens were cut into 5 μm-thick sections and stained by hematoxylin and eosin (HE) and then examined by light microscope examination. For immunofluorescence, tissue sections, mounted onto positively charged glass slides, were autoclaved in a citrate buffer (pH 6.1) at 121°C for 10 min to expose epitopes. Slides were immersed in a solution of 0.3% H2O2 for 10 min at room temperature (RT) to quench endogenous peroxidase activity, rinsed with PBS and non-specific sites were blocked in 5% BSA in PBS. Slides were then incubated with anti-PrP monoclonal antibody 2G11 followed by an incubation with an Alexa Fluor conjugate 594 secondary antibody. Negative tissue controls included kidney sections from ARR/ARR scrapie-negative sheep. For immunohistochemistry, tissue sections were first dried overnight at 37°C, rehydrated and then autoclaved at 121°C for 10 min in a solution of 0.01 M citric acid (pH 6.1). Further steps included utilization of a biotin-streptavidin detection method (Vector Laboratories, Inc., U.S.A.), using the following primary monoclonal antibodies (MoAbs): 2G11 (1 μg/ml) (1: 500) and (1 μg/ml) F99 (1:300). 2G11 and F99 antibodies have epitopes on aa151-159 and aa220-225 of the ovine PrP, respectively. Immune reactions were visualized by 3,3′-diaminobenzidine (DAB) chromogen solution (Dako, Denmark). Tissue sections from ARR/ARR scrapie-negative sheep were included in each run as control. Another series of controls was set up by omitting the primary MoAbs.

**Paraffin-embedded tissue (PET) blot analysis**

2 μm thick paraffin sections were collected on a nitrocellulose membrane and dried overnight at 37°C and just before use for 1h at 56°C. Deparaffinization in Xylool, twice for 5 min, followed by 100% isopropanol, twice for 2 min, 95% isopropanol, once for 2 min and 70% isopropanol (once for 2 min). The membranes were then washed in 0.1% Tween-20 in distilled water for 10 min and air-dried over night. This procedure was followed by a washing step in 1× Tris-buffered Saline with 0.5% Tween-20 (TBST) for 1 hr and a PK digestion (Roche) (250 μg Proteinase K/ml) in digestion buffer (100 mM NaCl, 0.1% Brij 35 in Tris-HCl, pH 7.8) at 55°C for 8 hrs. PK digestion was followed by washing steps in TBST (3×10 min). Denaturation 3M Guanidinium thiocyanate (Sigma) in 10 mM Tris-HCl, pH 7.8 for 10 min was followed by further washing steps in 1× TBST (3×10 min) and blocking with 5% low fat milk in 1× TBST (blocking buffer), for 1 hr. Thereafter the primary MoAb POM1 or 2G11 (POM1: 200 ng/ml; 2G11: 500 ng/ml) in 1% low fat milk in 1× TBST was incubated overnight at RT. Washing was performed thereafter in 1× TBST (3×10 min) followed by blocking in 1% low fat milk 1× TBST for 5 min. Incubation of the secondary antibody AB (Vector or DAKO D0486, AP-conjugated goat ant mouse; 1:1000) was performed with the membrane for 2 hrs–3 hrs at RT, followed by washing with 5% low fat milk in 1× TBST (3×10 min). For development of signal Vecastain ABC-AMA P (Vector) was incubated for 10 min at RT followed by incubation with BCP/NBT for 30 min at RT. After a final washing step in 5% low fat milk in 1× TBST the blots were air-dried and flattened with a glass plate for visual inspection. Alternatively, for development of signal B3 DIG was incubated for 10 min followed by incubation with NBT and BCIP for 1 hr. After a final washing step in distilled water for at least 30 min the blots were air-dried and flattened with a glass plate for visual inspection. As positive and negative controls the brain of terminal scrapie-sick sheep and ARR/ARR sheep were used, respectively.

**Genetic analysis**

The PrP genotypes of all investigated sheep were determined focusing on the PrP gene (PRNP) polymorphisms at codons 136, 154, and 171, keeping in mind that the polymorphic site which is known to influence susceptibility/resistance to scrapie in Sarda breed sheep is represented by codon 171, with 171 Q/Q homozygous sheep being the most susceptible to disease. DNA was isolated from EDTA-treated blood and submitted to appropriate TaqMan allelic discrimination using ABI Prism (Applied Biosystem, USA), as described elsewhere [35]. Additionally, from 16 scrapie affected sheep, of which only 8 with PrPSc in the kidney, genomic DNA was isolated from the brain tissue by the DNA isolation kit (Qiagen, USA). PCR amplification of the PrP gene was performed using the following primers: PrP1(+) 5′-ATGGTGAAAAGCCACATAGGCAGT-3′ and PrP2(−) 5′-CTATCCCTACTATGAGAAAAATGAG-3′. The PrP1(+) and PrP2(−) anneal at the extreme 5′ and 3′ regions of the PrP-coding sequence, respectively. Amplification reactions were performed for 30 cycles of 30 sec at 94°C, 30 sec at 59°C, and 45 sec at 72°C. PrP gene polymorphisms were detected by DNA sequencing on both strands of the PCR products (Applied Biosystems, USA).

**ACKNOWLEDGMENTS**

We would like to thank Pedra Schwarz, Rita Moos, Simona Macciocu and Giulia Demurtas for excellent technical assistance and Adriano Aguzzi for critically reading the manuscript.

**Author Contributions**

Conceived and designed the experiments: MH CL. Performed the experiments: GC IM CS LM CM MS IM. Contributed reagents/materials/analysis tools: MB. Wrote the paper: CL.

**REFERENCES**

1. Aguzzi A, Polymenidou M (2004) Mammalian prion biology. One century of evolving concepts. Cell 116: 313–27.
2. Prusiner SB (1996) Prions. Proc Natl Acad Sci U S A. 10: 13363–83.
3. Aguzzi A (2003) Prions and the immune system: a journey through gut, spleen, and nerves. Adv Immunol 80: 123-71.
4. Andreolotti O, Berthon P, Levavaeseur E, Marc D, Lanter F, et al. (2002) Phenotyping of protein-prion (PrPSc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep. J Histochem Cytochem 50: 1357–70.
5. Andreolotti O, Berthon P, Marc D, Sarradin P, Grosclaude J, et al. (2000) Early accumulation of PrPSc in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. J Gen Virol 81 Pt 12: 3115–26.
6. van Keulen LJ, Schreuder BEG, Meleno RH, Moosjhaers G, Vromans MEW, et al. (1996) Immunohistochemical Detection of Prion Protein in Lymphoid Tissues of Sheep With Natural Scrapie. Journal of Clinical Microbiology 34: 1228–1231.
7. Andreolotti O, Simon S, Lacroux C, Morel N, Tahuoret G, et al. (2004) PrPSc accumulation in myocytes from sheep incubating natural scrapie. Nat Med 10: 591–3.
8. Lajos C, Sigurdson CJ, Santucciu C, Carcassola G, Manci G, et al. (2005) PrPSc in mammary glands of sheep affected by scrapie and mastitis. Nat Med 11: 1137–8.
9. Tao W, O’Rourke KL, Zhuang D, Cheevers WP, Spraker TR, et al. (2002) Pregnancy status and fetal prion genetics determine PrPSc accumulation in placentomes of scrapie-infected sheep. Proc Natl Acad Sci U S A 99: 6310–5.

10. Vascellari M, Nonno R, Mutinelli F, Bigolaro M, Di Bari MA, et al. (2007) PrPSc in salivary glands of scrapie-affected sheep. J Virol. 81: 4672–6.

11. Bosque PJ, Kyoo C, Telling G, Peretz D, Legname G, et al. (2002) Prions in skeletal muscle. Proc Natl Acad Sci U S A 99: 3812–7.

12. Glatzel M, Abela E, Mannes M, Aguzzi A (2003) Extraneural pathologic prion protein in sporadic Creutzfeldt-Jakob disease. N Engl J Med 349: 1812–20.

13. Kovacs GG, Lindeck-Pozza E, Chimelli L, Araujo AQ, Gahbaur AA, et al. (2004) Creutzfeldt-Jakob disease and inclusion body myositis: Abundant disease-associated prion protein in muscle. Ann Neurol 55: 121–5.

14. Peden AH, Ritchie DL, Head MW, Ironside JW (2006) Detection and localization of PrPSc in the skeletal muscle of patients with variant, iatrogenic, and sporadic forms of Creutzfeldt-Jakob disease. Am J Pathol 168: 927–35.

15. Llewelyn CA, Hewitt PE, Knight RS, Amar K, Cousens S, et al. (2004) Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. Lancet 364: 527–9.

16. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW (2004) Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. Lancet 364: 527–9.

17. Saa P, Castilla J, Soto C (2006) Presymptomatic detection of prions in blood. Science 313: 92–4.

18. Angers RC, Browning SR, Seward TS, Sigurdson CJ, Miller MW, et al. (2006) Prions in Skeletal Muscles of Deer with Chronic Wasting Disease. Science. 24: 1117.

19. Hunter N, Foster J, Chong A, McCutcheon S, Parham D, Eaton S, et al. (2002) Transmission of prion diseases by blood transfusion. J Gen Virol 83: 2987–905.

20. Mathiason CK, Powers JG, Dahmes SJ, Osborn DA, Miller KV, et al. (2006) Infectious prions in the saliva and blood of deer with chronic wasting disease. Science 314: 133–6.

21. Houston F, Foster JD, Chong A, Hunter N, Bostock CJ (2000) Transmission of BSE by blood transfusion in deer. Lancet 356: 999–1000.

22. Wroe SJ, Pal S, Siddique D, Hyare H, MacFarlane R, et al. (2006) Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. Lancet 368: 2061–7.

23. Hamir AN, Kunkle RA, Miller JM, Hall SM (2006) Abnormal prion protein in ectopic lymphoid tissue in a kidney of an asymptomatic white-tailed deer experimentally inoculated with the agent of chronic wasting disease. Vet Pathol 43: 367–9.

24. Seeger H, Heikenwalder M, Zeller N, Kranich J, Schwarz P, et al. (2005) Coincident scrapie infection and nephritis lead to urinary prion excretion. Science 310: 324–6.

25. Heikenwalder M, Zeller N, Seeger H, Prinz M, Klohn PC, et al. (2005) Chronic lymphocytic inflammation specifies the organ tropism of prions. Science 307: 1107–10.

26. Kayihimali Z, Ben-Hur T, Grigoriadis NC, Engelerstein R, Gahbaur B (2006) Urine from scrape-prion-infected hamsters comprises low levels of prion infectivity. Neurodegener Dis 3: 123–8.

27. Ryder SJ, Wells GAD, Bradshaw JM, Pearson GR (2001) Inconsistent detection of PrP in extraneural tissue of cats with feline spongiform encephalopathy. Vet Rec 148: 437–441.

28. Leznik S, Benseik A, Monks E, Petit T, Baron T (2003) First case of feline spongiform encephalopathy in a captive cheetah born in France: PrPSc analysis in various tissues revealed unexpected targeting of kidney and adrenal gland. Histochem Cell Biol 119: 415–22.

29. Herzog C, Rivière J, Lesouza-Entchegaray N, Charbonnier M, Leblanc V, et al. (2005) PrPSc distribution in a primate model of variant, sporadic and iatrogenic Creutzfeldt-Jakob Disease. J Virol 82: 14339–45.

30. Sio S, Gonzalez L, Jeffrey M, Martin S, Chianini F, et al. (2006) Prion protein in kidneys of scrapie-infected sheep. Vet Rec 159: 327–8.

31. Lapu O (2005) Risk analysis of ectoparasites acting as vectors for chronic wasting disease. Med Hypotheses 65: 47–54.

32. Winnieks HM, Sigurdarson S, Rubenstein R, Kascak RJ, Carp RI (1996) Mites as vectors for scrapie [letter]. Lancet 347: 1114.

33. Thuring CM, Bensch J, Monks E, Petit T, Baron T (1995) First case of feline spongiform encephalopathy in a captive cheetah born in France: PrPSc analysis in various tissues revealed unexpected targeting of kidney and adrenal gland. Histochem Cell Biol 119: 415–22.

34. Maestrale C, Vaccari G, Carcassola G, Mela P, Vargiu MP, et al. (2004) PrPSc distribution in a primate model of variant, sporadic, and iatrogenic Creutzfeldt-Jakob Disease. J Virol 22: 14339–45.

35. Polymenidou M, Stoeck K, Glatzel M, Vey M, Bellon A, Aguzzi A (2005) Coexistence of multiple PrPSc types in individuals with Creutzfeldt-Jakob disease. Lancet Neurol 4: 903–14.

36. Maestrale C, Vaccari G, Carcassola G, Mela P, Vargiu MP, et al. (2004) Standardizzazione di un metodo di discriminazione allelica TaqMan per la determinazione dei polimorfismi del gene della proteina prionica (PrP) in individui con Creutzfeldt-Jakob disease. PLoS ONE | www.plosone.org 8 September 2007 | Issue 9 | e859