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Variability in disease phenotypes within a single *PRNP* genotype suggests the existence of multiple natural sheep scrapie strains within Europe

Lorenzo González,[1] Sílvia Sisó,[1] Eva Monleón,[2] Cristina Casalone,[3] Lucien J. M. van Keulen,[4] Anne Balkema-Buschmann,[5] Angel Ortiz-Peláez,[6] Barbara Iulini,[3] Jan P. M. Langeveld,[4] Christine Hoffmann,[5] Juan J. Badiola,[2] Martin Jeffrey[1] and Cristina Acín[2]

Correspondence
Lorenzo González
l.gonzalez@vla.defra.gsi.gov.uk

[1]Veterinary Laboratories Agency (VLA), Pentlands Science Park, Penicuik EH26 0PZ, UK

[2]Centro de Investigación de Encefalopatías y Enfermedades Transmisibles Emergentes, Facultad de Veterinaria, Universidad de Zaragoza, (UNIZAR) C/ Miguel Servet, 50013-Zaragoza, Spain

[3]Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (IZS), Via Bologna 148, 10154-Torino, Italy

[4]Central Veterinary Institute, Wageningen University and Research Centre (CVI), PO Box 65, 8200 AB Lelystad, The Netherlands

[5]Friedrich-Loeffler-Institut (FLI), Südufer 10, 17493, Greifswald – Insel Riems, Germany

[6]VLA-Weybridge, Addlestone, Surrey KT15 3NB, UK

Variability of pathological phenotypes within classical sheep scrapie cases has been reported for some time, but in many instances it has been attributed to differences in the *PRNP* genotype of the host. To address this issue we have examined by immunohistochemistry (IHC) and Western blotting (WB) for the disease-associated form of the prion protein (PrP^d), the brains of 23 sheep from five European countries, all of which were of the same ARQ/ARQ genotype. As a result of IHC examinations, sheep were distributed into five groups with different phenotypes and the groups were the same regardless of the scoring method used, 'long' or 'short' PrP^d profiling. The groups made did not respond to the geographical origin of the cases and did not correlate with the vacuolar lesion profiles, which showed a high individual variability. Discriminatory IHC and WB methods coincided to detect a 'CH1641-like' case but otherwise correlated poorly in the classification of disease phenotypes. No other polymorphisms of the *PRNP* gene were found that could account for the pathological differences, except perhaps for a sheep from Spain with a mutation at codon 103 and a unique pathological phenotype. Preliminary evidence indicates that those different IHC phenotypes correlate with distinct biological properties on bioassay, suggesting that they are indicative of strain diversity. We therefore conclude that natural scrapie strains exist and that they can be revealed by detailed pathological examinations, which can be harmonized between laboratories to produce comparable results.

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INTRODUCTION

Scrapie is the prototype of the transmissible spongiform encephalopathies (TSEs), a group of neurodegenerative disorders affecting animals and man, generally believed to be caused by infectious proteinaceous agents termed prions (Prusiner, 1982). To explain strain diversity of prions the prion-only hypothesis postulates that, in the absence of a nucleic acid, the conformation of the abnormally folded, infectious form of the prion protein (PrP^{sc}) codes for strain-specific information (Wille *et al.*, 2002). Following inoculation and serial passage of different sheep scrapie isolates, around 20 scrapie strains (Bruce, 2003) have been isolated and characterized by their relative incubation periods and pattern of brain vacuolation in different inbred lines of mice (Fraser, 1976), and more recently by the immunohistochemical (IHC) pattern of disease-associated PrP (PrP^d) accumulation in the brain (Beck *et al.*, 2010). However, the significance of mouse-adapted scrapie strains remains obscure, since the disease phenotype arising from the

Supplementary material is available with the online version of this paper.

bioassay is most likely to result from the interaction between the agent and the host, rather than to represent exclusively agent properties. It is therefore uncertain whether the different murine scrapie strains are representative of strain diversity in the natural host or result from mutation or adaptation of the scrapie agent upon serial mouse passage.

In the sheep host, phenotypic differences are also observed between different TSEs, so that classical scrapie can be discriminated from Nor98 – also called 'atypical scrapie' – by their pathological, IHC and biochemical features in the brain (Benestad *et al.*, 2003, 2008). Experimental ovine BSE can also be differentiated from natural and experimental scrapie sources by their distinct IHC (Jeffrey *et al.*, 2001a, 2003, 2006; González *et al.*, 2005) and Western blotting (WB) characteristics (Stack *et al.*, 2002; Thuring *et al.*, 2004). Biochemical methods, however, do not provide a clear discrimination between scrapie isolates from different geographical origins or from sheep of different prion protein gene (*PRNP*) genotypes (Sweeney *et al.*, 2000; Nonno *et al.*, 2003). In contrast, differences in vacuolation profiles (Ligios *et al.*, 2002; Begara-McGorum *et al.*, 2002) and in patterns of PrP^d accumulation in the brain (van Keulen *et al.*, 1995; Ryder *et al.*, 2001; González *et al.*, 2002; Jeffrey *et al.*, 2006) have been reported between individual cases of both natural and experimental sheep scrapie. For some authors, those differences principally reflect strain diversity (González *et al.*, 2002, 2003, 2005; Jeffrey *et al.*, 2006), at least when dealing with experimentally induced disease, while others, mostly working on natural disease, suggest that they are mainly related to host *PRNP* gene polymorphisms (Ligios *et al.*, 2004; Spiropoulos *et al.*, 2007).

In 2006, a working group was established under the auspices of the Network of Excellence Neuroprion with the aim of standardizing criteria and methods for the definition and characterization of sheep scrapie strains in the natural host. This paper reports the outcome of one particular study carried out on classical scrapie within this collaborative network, the specific objectives of which were: (i) to determine the presence or absence of phenotypic variability in natural sheep scrapie cases from a variety of European geographical sources and a single *PRNP* genotype, and (ii) to standardize IHC approaches and evaluate IHC and WB methods for characterizing different naturally occurring sheep scrapie phenotypes.

RESULTS

Phenotypic variability was detected by the 'long' PrP^d profiling IHC method

The assessment of the 'long' PrP^d profile in blind-coded slides resulted in the identification of five different IHC phenotypes, according to which the sheep were grouped as follows (Tables 1 and 2):

Group 1: 15 sheep, five each from Scotland, Germany and Italy. These cases were characterized by prominent accumulations of extracellular PrP^d associated with glial cells in grey matter (stellate and subpial types) and white matter (perivascular, perivacuolar and subependymal types), mild intracellular (intraneuronal and intraglial) PrP^d deposits, mild extracellular PrP^d aggregates in the grey matter neuropil (diffuse particulate, linear, perineuronal and coalescing types), and absence of vascular plaques (Fig. 1a–c).

Group 2: three sheep from Spain and one from the Netherlands, which exhibited marked intraneuronal and intraglial PrP^d deposits, prominent coalescing aggregates and moderate perineuronal deposits, very little of any other extracellular or membrane-bound PrP^d types, and no vascular plaques (Fig. 1d–f).

Group 3: two sheep, one from Spain and one from the Netherlands, which showed conspicuous intraneuronal, intraglial, diffuse particulate and stellate PrP^d, absence of vascular plaques, and mild or moderate extracellular accumulations of PrP^d associated with astrocyte processes or in the neuropil (Fig. 1g–i).

The two animals in groups 4 (one sheep from Spain, O-758) and 5 (one sheep from the Netherlands, 559018) both showed vascular PrP^d plaques, mild glia-associated extracellular deposits and mild or moderate extracellular deposits in the grey matter. However, those two sheep differed markedly in the amount of intracellular PrP^d, which was much more prominent in the Spanish (Fig. 1j–l) than in the Dutch case (Fig. 1m–o).

The classification made by the UK laboratory, as described above, was completely coincident with that made by each participating laboratory. While the UK, Italian and German laboratories recorded a single phenotype amongst their sheep (which happened to be similar), the Spanish laboratory identified three different phenotypes (with the same sheep in each group as allocated in the blind trial), as did the Dutch laboratory amongst their three sheep.

Phenotypic variability was confirmed by the 'short' PrP^d profiling method

The five groups established by the UK laboratory on the basis of the correlation and cluster analyses of the individual 'short' PrP^d profiles (Table 3, Fig. 2 and Supplementary Fig. S1 and Supplementary Table S1, available in JGV Online) of 18 blind-coded sheep (all except the German sheep) coincided completely with those obtained by the 'long' PrP^d profiling method. Thus, the five from Italy and the five from UK were grouped together (group 1), as they all showed little intracellular PrP^d and abundant stellate and astrocyte-associated PrP^d (subpial, subependymal, perivascular and perivacuolar; Fig. 2a). It has to be noted, however, that the five Suffolk sheep from UK were more similar amongst themselves ($r=0.94\pm0.01$, mean correlation coefficient $\pm$ SEM) than to the sheep from Italy ($r=0.75\pm0.02$), which exhibited a higher intra-group variability ($r=0.80\pm0.04$). In any case, the PrP^d profiles of those

Table 1. Details of the sheep included in the study and some IHC, WB and *PRNP* genotyping results

Age, In years. F, Female; M, male. Flock, UK (Scotland); GE, Germany; IT, Italy; SP, Spain; NL, the Netherlands. Year, First detection of scrapie in the flock. Cases, Number of scrapie cases detected after culling the affected flocks or as a result of continued surveillance (see text). IHC, Allocation of the examined sheep to different groups (G1–G5) according to their PrP^d profiles. N/C, Signal ratio in WB analysis of the same sample with an N-terminal antibody (P4, German laboratory or 12B2, Dutch laboratory) and C-terminal antibody L42. MW, Molecular mass (kDa) of the unglycosylated band upon WB analysis. Di, Mo and Un, Relative percentages of di-, mono- and unglycosylated bands on WB analysis. WB, Allocation of the sheep to different groups (A–D) according to their glycoprofiles. *PRNP*, Genotype is ARQ/ARQ with any other specific polymorphisms indicated, when found.

Sheep no.	Breed	Age	Sex	Flock	Size	Year	Cases	IHC	N/C	MW	Di	Mo	Un	WB	*PRNP*
S68	Black-faced Merino	1.5	F	GE1	530	04	52	G1	4.9	19.3	44	30	26	A	
S73		2.5	F					G1	1.1	19.1	44	29	27	A	
S66		1.5	F					G1	6.3	18.8	47	28	25	B	
S82		4.5	F					G1	2.3	19.1	51	28	21	B	
S84		2.5	F					G1	3.2	18.9	44	29	27	A	
6529	Sarda	4.0	F	IT1	640	06	13	G1	1.2	18.7	51	31	18	B	L141F
33546		4.0	F	IT2	970	07	3	G1	0.9	18.7	53	31	16	C	
64757		5.0	F	IT3	560	07	11	G1	1.3	18.8	48	29	23	B	
79348	Biellese	5.0	F	IT4	1500	03	111	G1	1.2	18.8	45	30	25	A	
45231		5.0	F	IT4				G1	1.0	19.5	50	31	19	B	
X422	Suffolk	2.0	F	UK1	100	98	70	G1	1.0	19.2	39	27	34	A	
1366N		2.0	F					G1	1.0	19.1	49	30	21	B	
1354N		2.0	M					G1	1.0	18.8	48	29	23	B	
1360N		2.5	F					G1	0.9	19.0	48	30	22	B	
1358N		2.5	F					G1	1.1	18.9	48	29	23	B	
O-367	Rasa Aragonesa	4.0	M	SP1	260	03	65	G2	0.0	19.0	54	31	15	C	
O-788		4.0	F	SP2	920	06	18	G2	1.5	19.4	40	31	29	A	
O-940		3.0	F	SP3	1040	07	7	G2	1.7	19.5	40	36	24	A	
O-972		2.0	F	SP4	1000	07	45	G3	0.9	19.3	47	33	20	B	
O-758		6.0	F	SP5	650	06	3	G4	1.5	19.4	47	32	21	B	N103H
German scrapie WB control									1.1	19.0	49	31	20	B	
German BSE WB control									0.0	18.7	55	28	17	C	
603397	Texel	4.0	F	NL1	60	02	8	G2	1.0	19.8	48	34	18	C	
508192	Zwartbles	1.5	F	NL2	200	98	6	G3	1.2	19.9	49	35	16	C	
559018	Texel	4.0	F	NL3	150	00	6	G5	1.4	19.8	52	34	14	C	
Dutch scrapie WB control									1.3	20.8	53	31	16	C	
Dutch BSE WB control									0.2	18.2	65	27	8	D	

sheep were different from those of other groups (Table 3 and Supplementary Fig. S1 and Supplementary Table S1). Thus, the four sheep of group 2 showed prominent intracellular PrP^d deposits and little extracellular accumulations, mostly of the subpial and particulate types (Fig. 2b). The two sheep of group 3 displayed not only those two extracellular types, but also stellate and perivascular PrP^d, while intracellular – particularly intraneuronal – aggregates were scarce (Fig. 2c). The single sheep classified in group 4 according to the 'long' PrP^d profile again stood on its own when its 'short' profile was defined; although somewhat similar to sheep of group 2 (high intracellular and particulate PrP^d; see correlation value in Table 3), it showed conspicuous ependymal PrP^d deposits and vascular plaques (Fig. 2d). Finally, the other single sheep (group 5) showed high stellate and subpial PrP^d aggregates and moderate amounts of other extracellular PrP^d types. Its 'short' PrP^d profile was therefore similar to that of groups 1 and 3 (Table 3), although it showed higher intraneuronal PrP^d

Table 2. Summary of IHC results after completion of the 'long' PrP^d profiling examinations

Groups 1–5: groups to which the 23 examined sheep were allocated according to their features of PrP^d accumulation in the whole of the brain ('long' PrP^d profile; for details and descriptions, see text). ITNR, Intraneuronal; ITGL, intraglial (intramicroglial and intraastrocytic combined); STEL, stellate; ASTR, astrocyte-associated PrP^d (subpial, subependymal, perivascular and perivacuolar). PART, particulate; PNER, perineuronal; COAL, coalescing; VSPL, vascular plaques (for detailed description of these PrP^d types see González *et al.*, 2005 and Sisó *et al.*, 2010). −, Absent; −/+, trace; +, mild; ++, moderate; +++, conspicuous; ++++, prominent; +++++, severe.

	ITNR	ITGL	STEL	ASTR	PART	PNER	COAL	VSPL
Group 1 (n=15)	+	+	+++	++++	++	+	−/+	−
Group 2 (n=4)	++++	++++	+	+	++	+++	++++	−
Group 3 (n=2)	+++++	++++	+++++	++	+++++	++	−/+	−
Group 4 (n=1)	+++++	+++++	+	+	++	++	++	+++
Group 5 (n=1)	++	+	++	+	+++	++	+++	++

deposits (Fig. 2e); it has also to be noted that the vascular plaques observed in other brain areas were not present in the forebrain sections used for the 'short' profile at the UK laboratory.

When performing the inter-laboratory comparison of the 'short' profiles, some minor differences were observed (Fig. 3). In the five sheep from Italy (group 1), the Italian laboratory recognized less subpial and more perivacuolar PrP^d than the UK laboratory (Fig. 3a). In the four sheep of group 2, the Dutch and Spanish participants recognized less subpial and more particulate PrP^d types than did the UK laboratory (Fig. 3b), and also less subpial PrP^d in the two sheep of group 3 (Fig. 3c). The Spanish laboratory described more intramicroglial and perivacuolar and less particulate and ependymal PrP^d than the UK laboratory in the only sheep of group 4 (Fig. 3d) and, finally, the Dutch laboratory found less stellate and subpial but more particulate PrP^d than the UK laboratory in the only sheep of group 5. In addition, the sections from this last sheep examined by the Dutch laboratory showed vascular PrP^d plaques, which had not been observed in the forebrain sections examined by the VLA (Fig. 3e).

Therefore, the 'short' profiles, while giving less information overall, nevertheless provided a reproducible means of assessing IHC phenotypic characteristics of natural sheep scrapie cases.

Variability in PrP^d profiles in the brain did not correlate with vacuolation and WB results

For the 18 sheep considered (excluding German sheep), overall neuropil vacuolation was most prominent in the dorsal motor nucleus of the vagus (2.9 ± 0.2, mean ± SEM) followed by the thalamic nuclei (2.7 ± 0.3) and the nucleus of the spinal tract of the trigeminal nerve (2.6 ± 0.3), while the cerebral cortex (1.3 ± 0.3) and the posterior olivary nuclei (1.3 ± 0.2) were the least affected (see details in Supplementary Tables S2 and S3, available in JGV Online). There was, however, substantial individual variability in the vacuolar lesion profiles, so that out of a possible total of 153 analyses between pairs of sheep only one correlation exceeded 0.9 (two Italian sheep), seven ranked between 0.8 and 0.9 (four of them between Scottish Suffolk sheep), and five between 0.7 and 0.8 (all of them between sheep of the same countries). The best multiple correlations were obtained when sheep were grouped by the country of origin (Supplementary Tables S2 and S3), although the only correlation above 0.75 was between the five Suffolk sheep from the UK, all of which came from the same flock. When sheep were grouped according to their IHC profiles, as described above, the intra-group correlations for vaculoar profiles were very low (Supplementary Tables S2 and S3).

With the exception of one animal, the discriminatory WB analyses provided a scrapie signature for all sheep examined (Table 1), with P4/L42 or 12B2/L42 signal ratios between 0.9 and 6.3, more in line with those of the scrapie controls (1.1 and 1.3) than those of the BSE controls (0 and 0.2) from the German and Dutch laboratories, respectively. Similarly, the molecular masses of the unglycosylated band were all in the range of 18.7 to 19.5 kDa for the 19 samples examined by the German laboratory, and between 19.8 and 19.9 kDa for the three samples examined by the Dutch laboratory. Those values were closer to those of the respective scrapie controls than to the BSE controls, although in the case of the German controls the scrapie and BSE values were very similar (Table 1). As for the proportion of di-, mono- and unglycosylated bands, those 22 sheep could be classified into three groups according to the cluster analysis performed (Table 1 and Fig. 4; details and statistical analysis in Supplementary Methods and

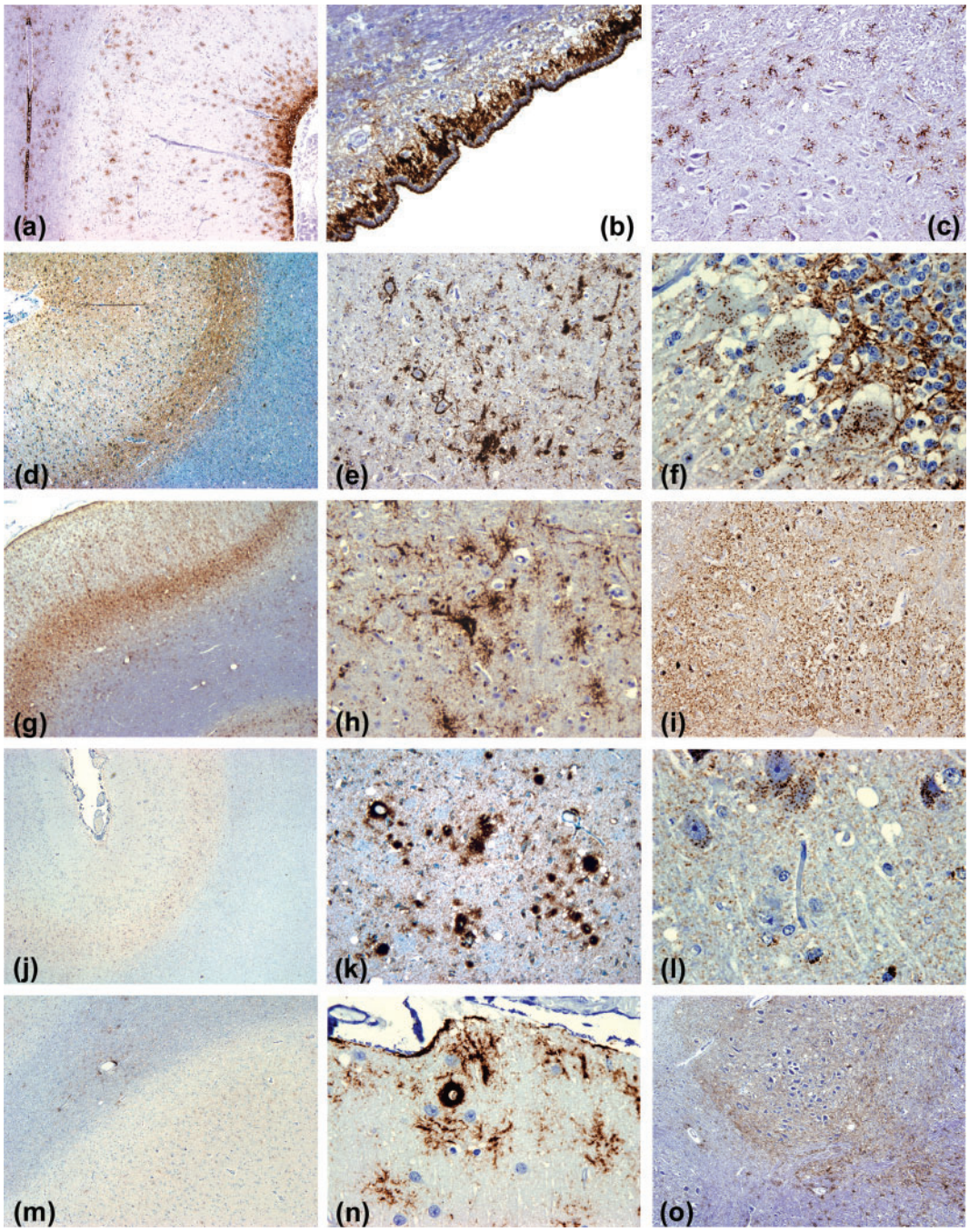

Fig. 1. Diversity of IHC phenotypes amongst the sheep examined by the 'long' PrP^d profiling method. Group 1: abundant subpial, stellate, perivascular and perivacuolar PrP^d types in cerebral cortex (a), conspicuous subependymal accumulations in corpus callosum (b), and stellate with little intraneuronal PrP^d in the obex (c). Group 2: diffuse particulate and intraneuronal PrP^d in cerebral cortex (d), perineuronal and coalescing aggregates in hypothalamus (e), prominent intraneuronal deposits in Purkinje cells (f). Group 3: abundant stellate, particulate and intracellular PrP^d in cerebral cortex (g and h), and diffuse particulate and intraneuronal deposits in the obex (i). Group 4: moderate particulate and intraneuronal and intraglial PrP^d in cerebral cortex (j and l), and prominent vascular plaques in thalamus (k). Group 5: mild perivascular and perivacuolar PrP^d in cerebral cortex (m), stellate, subpial and vascular plaque in cerebellum (n), and diffuse particulate with little intraneuronal in the obex (o). IHC with R145 PrP antibody and haematoxylin counterstaining; magnifications: a, d, g, j, m (×10); c, e, i, k, o (×25); b, h (×50); f, l, n (×100).

Table 3. Summary of the correlation analysis of the 'short' PrP^d profile results

Groups 1–5: are as described in the text and in Table 2 (German sheep are not included in Group 1, see text). Correlation values (*r* coefficient) are expressed as mean ± SEM. Values in bold indicate the best correlations obtained. NA, Not applicable as those two groups were made up of a single sheep.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1	**0.81 ± 0.02**				
Group 2	−0.03 ± 0.02	**0.96 ± 0.01**			
Group 3	0.58 ± 0.03	0.32 ± 0.02	**0.88**		
Group 4	−0.35 ± 0.02	0.70 ± 0.01	−0.14 ± 0.04	NA	
Group 5	0.51 ± 0.04	0.34 ± 0.03	0.67 ± 0.02	−0.04	NA

Supplementary Table S4, available in JGV Online). Group A (low di- and high unglycosylated bands) contained three German, one Scottish and one Italian cases (IHC group 1), and two Spanish cases of IHC group 2. PrP^{res} from sheep of group B (a mixture of IHC groups 1, 3 and 4) was significantly less di- and monoglycosylated than PrP^{res} from group C, which showed significantly lower proportions of unglycosylated PrP^{res}. Group C included the three Dutch cases, which had been classified in different IHC groups, one sheep from Italy and the Dutch scrapie and German BSE controls. The Dutch BSE control showed the highest diglycosylated and lowest unglycosylated PrP^{res} of all samples analysed.

One of the 23 sheep examined (O-367 from Spain) showed a molecular mass of the unglycosylated band (19 kDa) and a glycoprofile (Fig. 4) that were similar to those of sheep in group C of the cluster analysis. This animal, however, accumulated PrP^{res} in the brain that did not react with antibody P4 in the WB analysis (Table 1). On extended IHC examinations (data not shown), the lack of P4 reactivity in the WB corresponded with an absence of intracellular PrP^d labelling in the brain not only with 12B2 PrP antibody, but also with 9A2 and IDPrP4, which bind to amino acid sequences of ovine PrP separated only about seven amino acids C-terminally from the epitopes of P4 and 12B2. However, those intracellular deposits were prominent with PrP core specific antibody Bar224. In addition, intracellular PrP^d aggregates were detected with all antibodies in all lymphoid tissues available for examination. This sheep had a similar 'short' and 'long' PrP^d profiles as the other three sheep of group 2 (two from Spain and one from the Netherlands), which in turn did not show any differential antibody reactivity on WB or IHC.

PRNP polymorphisms did not account for PrP^d profile variation

Twenty-one of the 23 sheep examined did not show any polymorphisms within the whole ORF of PrP. One Italian sheep (6529) was an LF heterozygote at codon 141 (L, leucine; F; phenylalanine), but it had the same IHC characteristics as all other Italian, German and Scottish cases, and fell within WB group 2 according to the cluster analysis of glycoprofiles. Within the same WB group was also classified one sheep from Spain (O-758), which had a mutation at codon 103, resulting in an NH genotype (N, asparagine; H, histidine); interestingly, the IHC phenotype of this sheep was unique (group 4, see above).

DISCUSSION

Conjecture about the extent of scrapie strain diversity within sheep scrapie was raised when numerous murine strains were recognized following serial passage at limiting dilution in inbred mice of different *PRNP* genetic backgrounds (Fraser, 1976; Bruce, 2003). Historically, however, no effort has been made to characterize the nature of the disease in first mouse passages or in the sheep donors. In other words, the definition of prion strains has been largely based on a product resulting from the interaction between the agent and a host of a different species. Without knowing the precise effect of the species barrier and the ability of mice to respond distinctly to different sheep scrapie isolates, the validity of mouse bioassay in terms of strain interpretation is, at least, questionable.

More recently, pathological and biochemical characterization of the disease produced by different sheep scrapie isolates has been attempted. Differences in WB glycoprofiles, antibody reactivity and degree of resistance to proteinase K digestion have been reported to provide discrimination between classical scrapie, experimental ovine BSE, CH1641 and CH1641-like isolates (Stack *et al.*, 2002; Baron & Biacabe, 2007; Langeveld & Jeffrey, unpublished observations), and Nor98/atypical scrapie (Buschmann *et al.*, 2004; Benestad *et al.*, 2008). In addition, differences in pathological phenotypes, both in terms of vacuolar and, particularly, PrP^d profiles in the brain, have been observed between cases of TSEs, either natural or experimentally induced, in the natural sheep host. These pathological differences apply not only to clearly distinct and perhaps unrelated conditions, that is, classical scrapie, ovine BSE, Nor98 and CH1641 infection, but also between cases of classical scrapie itself. Amongst these, some studies conclude that the main force driving the phenotypic variability is the *PRNP* genotype of the sheep (Spiropoulos *et al.*, 2007). If that were the case, two possible interpretations could be made: (i) the classical scrapie strain is

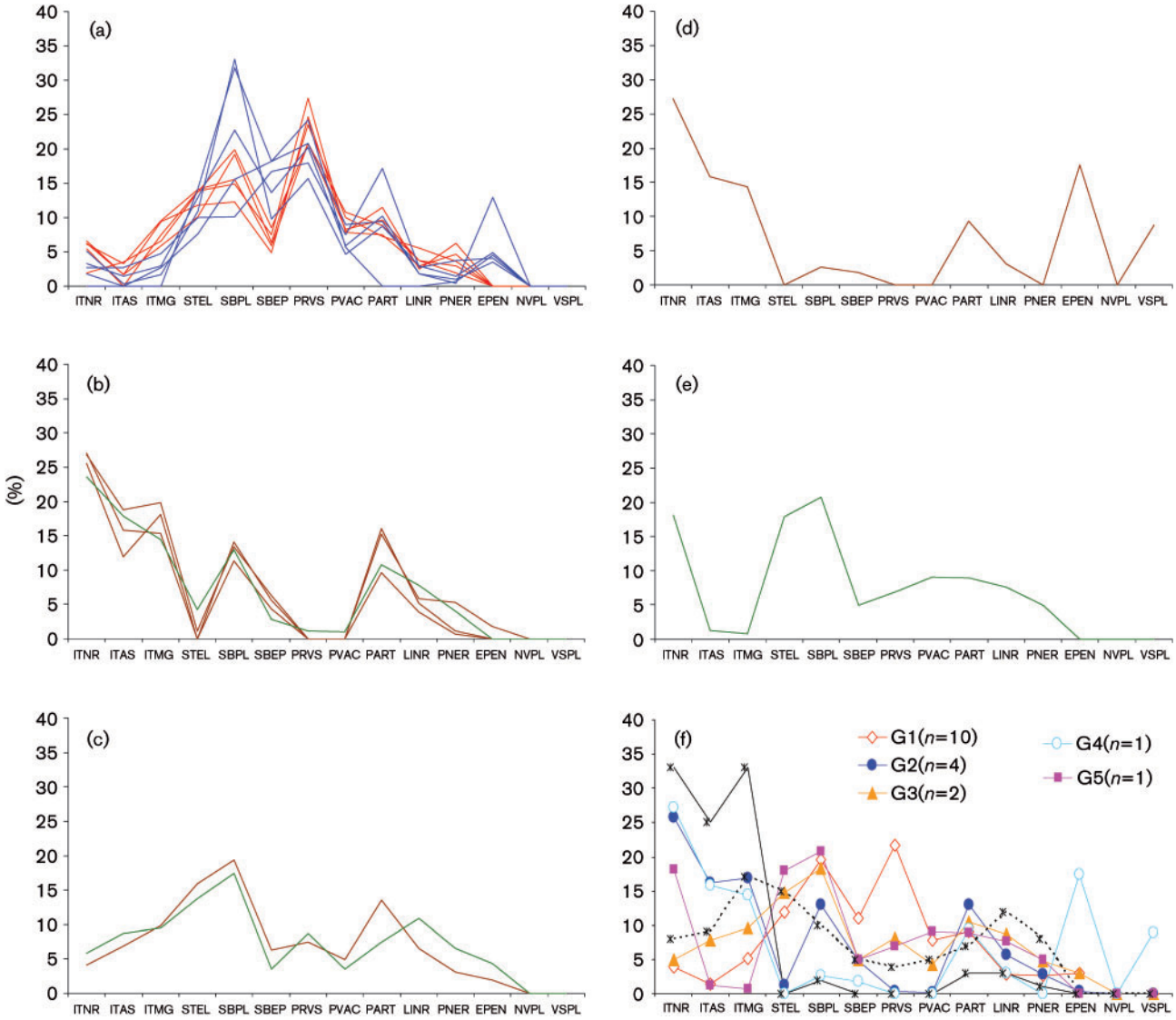

Fig. 2. 'Short' PrP^d profiles of 18 sheep from four different countries (sheep from Germany are excluded, see text). (a) Group 1: five sheep from Italy (blue lines) and five from UK (red lines). (b) Group 2: three sheep from Spain (brown lines) and one from the Netherlands (green line). (c) Group 3: one sheep from Spain and one from the Netherlands. (d) One sheep from Spain (O-758; group 4). (e) One sheep from the Netherlands (559018; group 5). (f) Average 'short' PrP^d profiles of the five groups. Groups 1–5 as defined in the text and in Table 2; for comparison, the 'short' profiles of BSE (dotted black line) and CH1641 (continuous black line) in ARQ/ARQ sheep are given [extracted from Sisó *et al.* (2010)]. *x*-Axis shows the different PrP^d types considered: ITNR, intraneuronal; ITAS, intraastrocytic; ITMG, intramicroglial; STEL, stellate; SBPL, subpial; SBEP, subependymal; PRVS, perivascular; PVAC, perivacuolar; PART, particulate; LINR, linear; PNER, perineuronal; EPEN, ependymal; NVPL, non-vascular plaques; VSPL, vascular plaques. *y*-Axis indicates the percentage value for each PrP^d type.

unique or almost unique, and phenotypic variability is genotype-related, or (ii) different strains of classical scrapie target sheep of different genotypes. Resolving this dilemma, by focusing on scrapie infected sheep of a single *PRNP* genotype but broad geographical and breeding origin, was the main purpose of the study reported here.

Five different IHC phenotypes were observed amongst the 23 sheep studied by the 'long' PrP^d profiling protocol and there was a complete agreement on the groupings made by the five participating laboratories, each on their own sheep. Furthermore, the allocation of groups was the same when the 'short' PrP^d profiling method was applied, and did not correlate with the geographical origin of the animals. When 'short' PrP^d profiles performed by each laboratory were formally compared with those done by the UK laboratory, only minor deviations were found, which did not affect the classification of the sheep. The inter-laboratory differences

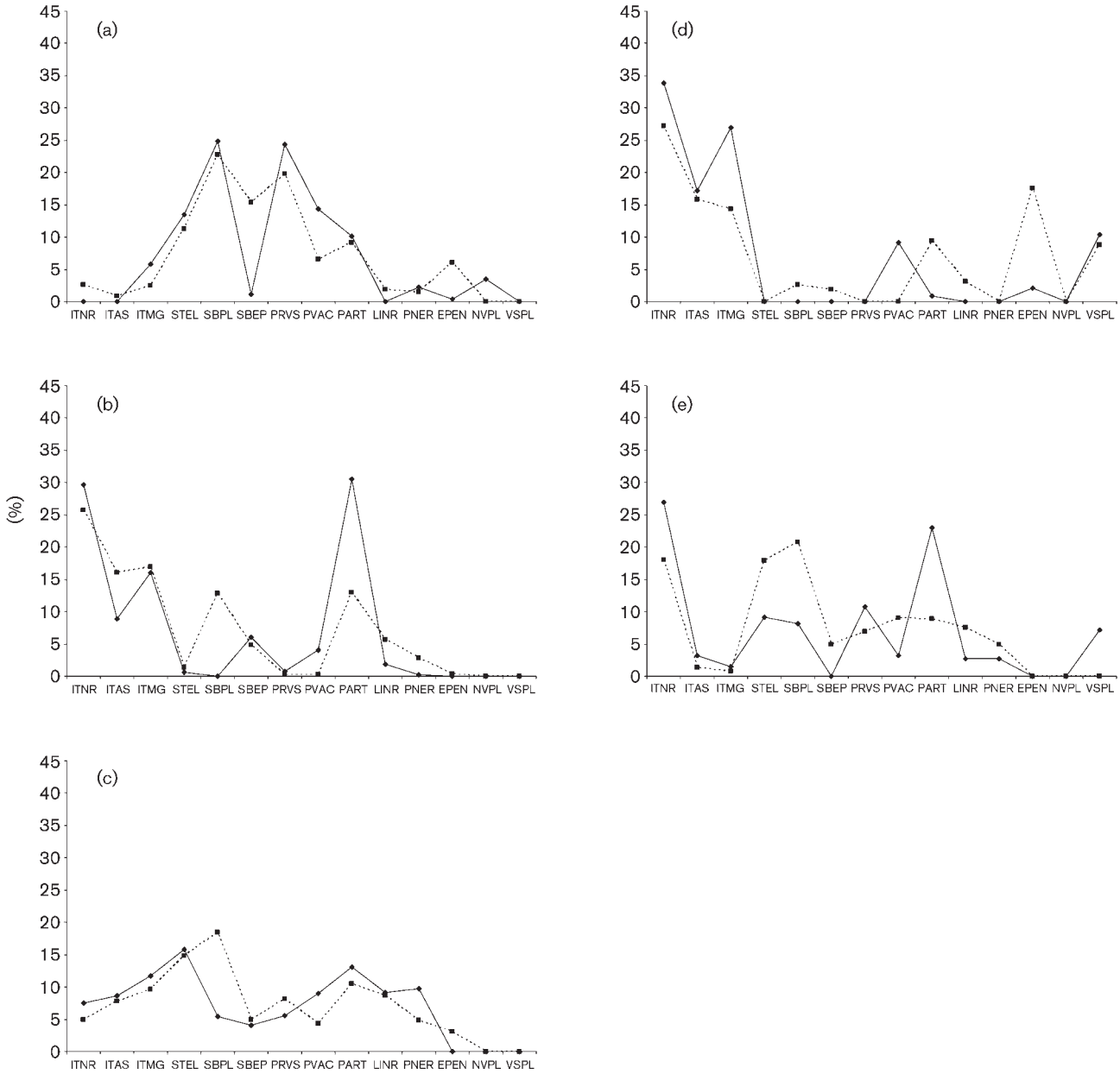

Fig. 3. Comparison of 'short' PrP^d profiles carried out on the same sheep at each participating laboratory (diamonds and solid lines) and at the UK participating laboratory (squares and dotted lines). (a) Five sheep from Italy (group 1). (b) Three sheep from Spain and one from the Netherlands (group 2). (c) One sheep from Spain and one from the Netherlands (group 3). (d) One sheep from Spain (group 4). (e) One sheep from the Netherlands (group 5). Groups 1–5 as defined in the text and in Table 2. For those groups with more than one sheep, profiles reflect average values for each PrP^d type. Axes as in Fig. 2 legend.

may have been partially due to the use of different IHC protocols and antibodies, and to the fact that the tissue slides examined by the UK laboratory were semi-serial rather than identical from those examined by the respective laboratories. This is particularly relevant in the case of focal lesions, such as vascular plaques, which in the case of the single sheep of group 5 were present in the cerebral cortex slides examined by the Dutch laboratory but not in those looked at by the UK laboratory.

As reported elsewhere (Begara-McGorum *et al.*, 2002), a high individual variability was observed in this European collaboration in terms of vacuolar lesion profiles. Any attempt to group the sheep examined according to this parameter resulted in low correlation values, and the best figures were obtained when grouping the animals by country of origin, which in itself may suggest some kind of 'observer bias'. Also, the lack of correlation between histopathological and IHC results (see Supplementary

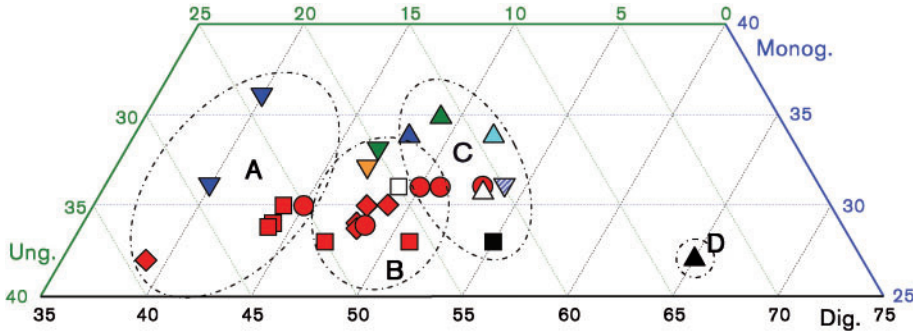

Fig. 4. WB glycoprofiles (%) of the 23 sheep and four controls examined. Triplot illustration restricted to the area of interest. Ung., Unglycosylated PrP^{res} (green axis and down cross gridlines); Dig., diglycosylated PrP^{res} (black axis and up cross gridlines); Monog., monoglycosylated PrP^{res} (blue axis and horizontal gridlines). Individual results from sheep from UK (diamonds), Germany (squares), Italy (circles), Spain (downward triangles), Netherlands (upward triangles). Colours indicate IHC groups as defined in the text and in Table 2: group 1, red; group 2, dark blue, including sheep O-367 that showed a P4/L42 ratio=0 (barred down triangle); group 3, green; group 4, gold; group 5, light blue; scrapie controls, white; BSE controls, black. Dotted ellipses enclose groups A–D determined by the cluster analysis of glycoprofiles, according to which the two Italian sheep at the intersection of groups B and C actually belong to group B.

Tables S2 and S3) indicates a dissociation between vacuolation and PrP^{d} accumulation, as already suggested (Jeffrey *et al.*, 2010).

Observations on natural scrapie made by others have led to the conclusion that PrP polymorphisms at codons 136, 154 and 171 are the main driver of IHC phenotypical variability (Ligios *et al.*, 2004; Spiropoulos *et al.*, 2007). Our results clearly contradict that notion, at least for sheep of the wild-type genotype, as animals examined in this study were all ARQ homozygotes at the same three codons, respectively (A, alanine; R, arginine and Q, glutamine). Furthermore, no other polymorphisms were found in the ORF of the *PRNP* gene with the exception of two sheep: one was an Italian sheep with IHC features undistinguishable from those of all other 14 sheep of group 1 and no biochemical peculiarities, and the other was a Spanish sheep, the only individual of group 4. This sheep showed a previously undescribed mutation at codon 103, which is homologous to human codon 100, and it is known that peptide 100–111 of human PrP^{c} has specific properties on the ability of recruiting PrP^{d} in buffer and plasma (Lau *et al.*, 2007). Therefore, a polymorphism at sheep codon 103 that leads to a change from a polar amino acid with no charge (N) to another with positive charge (H) could play an important role in PrP^{d} propagation. Whether this could account for the unique IHC phenotype of that sheep is at present unknown.

Pathological phenotype diversity, in the terms described above, was not mirrored by WB analyses. The molecular masses of the unglycosylated band were uninformative, and the three groups established on the basis of their glycoprofiles did not match the IHC classification, so that sheep with very similar PrP^{d} profiles fell within different WB groups, and some sheep with very different PrP^{d} profiles (e.g. the three Dutch cases) were classified in the same WB group. Only one (O-367) of the 23 sheep studied showed discordant properties, as it did not react to N-terminal antibodies, neither in WB (P4 antibody) nor for intracellular PrP^{d} in IHC (12B2, 9A2 and IDPrP4 antibodies). Because of its glycoprofile pattern and the IHC characteristics both in brain and lymphoid tissues, this case resembles the so-called CH1641-like scrapie rather than ovine BSE, and should be further investigated by rodent bioassay. Whatever the strain affecting this sheep, its PrP^{d} profile was grouped alongside with those of three other sheep (group 2), and provides an example of how IHC phenotypes of classical sheep scrapie need to be defined not just on PrP^{d} profile criteria, but also on epitope mapping (Jeffrey *et al.*, 2001a, 2006) results. The emergence of such CH1641-like scrapie cases in sheep of the ARQ/ARQ PrP genotype has been previously documented (Stack *et al.*, 2006; Baron *et al.*, 2008).

To summarize, phenotypic variability amongst classical scrapie cases exists irrespective of the *PRNP* genotype, at least amongst ARQ/ARQ sheep. Whether that heterogeneity reflects actual field strain diversity or is due to other unknown or uncontrolled factors is uncertain. However, though far from complete, some additional bioassay data are available to this respect. Thus, Italian and Scottish Suffolk sheep scrapie isolates with identical PrP^{d} phenotypes as those reported here (group 1), showed the same biological properties as they did not transmit to wild-type mice, and showed poor and good transmission to Tg338 mice and bank voles, respectively (Di Bari *et al.*, 2008). Moreover, preliminary data on transmission to RIII, VM and Tg338 mice from the three Dutch sheep, which had different epidemiological origins and were classed in different IHC groups, strongly suggest that they have different biological properties (van Keulen and others, unpublished observations).

In conclusion, evidence from this study and on-going bioassays suggests that, for natural classical scrapie, immunohistochemical (PrP^d profiles) similarities and differences (i) are unrelated to *PRNP* genotype and geographical origin, (ii) cannot be picked up by or do not correlate with the results of standard biochemical tests (WB) or histopathological examinations (vacuolar profile), and (iii) coincide with biological properties, suggesting that they are related to strain diversity in the natural host. Moreover, interpretation of the different PrP^d types can be harmonized between laboratories to provide comparable profiles, and these should be used to characterize natural scrapie isolates to evaluate the results of rodent bioassays in the definition of sheep scrapie strains.

METHODS

Selection of natural scrapie cases. The study was carried out on 23 sheep whose details are summarized in Table 1. All of them, except for the sheep from Germany, showed clinical signs consistent with scrapie and all showed moderate to high levels of PrP^d in the brain. All sheep were of the ARQ/ARQ *PRNP* genotype and originated from 14 different flocks in five European countries, as follows:

Germany. Five female sheep from a black-faced Merino flock located in the North-east of the country. After confirmation of scrapie in 2004, all 530 animals in the flock were culled and examined for PrP^d detection, with 46 positive results amongst the ARQ/ARQ sheep. The five sheep with the highest magnitudes of PrP^d labelling were selected for this study, although they were not showing clinical signs; their ages ranged between 1 and 4 years.

Italy. Five female sheep from four different flocks widely spread throughout the Italian peninsula and Sardinia. The two sheep originating from the same flock (Biellese breed) were 5 years old, and the other three were of the Sarda breed and aged 4–5 years. The size of the flocks ranged from 560 to 1500 sheep, and the scrapie outbreaks were diagnosed between 2003 and 2007. The flocks were culled and examined, leading to detection of three to 111 further infected animals, most of them of the ARQ/ARQ genotype.

UK. Four female and one male Suffolk sheep aged between 2 and 3 years, all originating from a well studied (Jeffrey *et al.*, 2001b), closed, experimental Scottish flock with an average size of 100 animals, in which scrapie was originally detected in 1990. From 2001 to 2006, ~70 scrapie cases were recorded, all in the ARQ/ARQ sheep aged between 2 and 3 years.

Spain. Four females aged 2–6 years and one 4-year-old male, all of the Rasa Aragonesa breed, from five different flocks located within a 100 km radius in North Eastern Spain. The size of the flocks ranged from 260 to 1040 sheep, and the scrapie outbreaks, almost exclusively affecting the ARQ/ARQ sheep, were diagnosed between 2003 and 2007. The flocks were subjected to monitoring with further identification of scrapie cases ranging from three to 65.

The Netherlands. Three female sheep between 1.5 and 4 years of age from three different flocks. One of them is a closed experimental flock with an average size of 60 animals; between 2004 and 2008, 38 scrapie cases were diagnosed in this flock, mostly in the VRQ/VRQ and the VRQ/ARQ animals (V, valine), and also in some of the ARQ/ARQ sheep. Another was a flock of 200 sheep, in which the index scrapie case was diagnosed in 1998 in an ARH/ARH (H, histidine) sheep; five further cases were detected the same year in sheep of the ARQ/ARH and ARQ/ARQ genotypes and the flock was depopulated. The third was a commercial flock of 150 sheep, in which, between 2000 and 2006, 31 cases of scrapie were diagnosed, all of them in the ARQ/ARQ sheep.

Histopathology and IHC. Sheep were euthanized by barbiturate overdose. Sagittally sliced hemi-brains obtained at post-mortem were fixed in formaldehyde and processed for paraffin-wax embedding by standard procedures. Sections of frontal cortex, corpus striatum, diencephalon, midbrain, cerebellum and medulla at the obex, were cut at 4 μm and stained with haematoxylin and eosin to perform examinations for spongiform change at 10 defined neuroanatomic areas (see Supplementary Table S2). These were performed by each participating laboratory following the scoring system described by Ligios *et al.* (2002). Vacuolar lesion profiles from individual sheep were compared by Spearman non-parametric correlation analyses (InStat GraphPad Software) between all possible pairs of sheep in order to establish the best-fitting groups. The five German scrapie cases were not considered in this comparative analysis as, probably due to their preclinical status, vacuolar lesions were minimal.

Two different approaches were followed for the characterization and comparison of IHC phenotypes between the different sheep examined:

(i) 'long' PrP^d profile: unlabelled sections of the above-indicated six brain areas from the 18 sheep from the other four countries were submitted to the UK participating laboratory, where they were mixed with the five sheep from the Scottish flock and blind coded. The sections were processed following a previously described IHC protocol (González *et al.*, 2002), using PrP rat mAb R145 (Jeffrey *et al.*, 2006). The magnitude of accumulation of eight different morphological and cell-associated types of PrP^d was subjectively scored by two pathologists independently; the PrP^d types considered were: intraneuronal, intraglial (intraastocytic and intramicroglial combined), stellate, astrocyte-associated (subpial, subependymal, perivascular and perivacuolar combined), particulate, perineuronal, coalescing and vascular plaques. These types have been described in detail previously (González *et al.*, 2002, 2005; Sisó *et al.*, 2010).

Tissue sections from the same brain areas were also examined by the Dutch, German, Italian and Spanish laboratories, each of them on their own sheep and with their own protocols. Although a formal description of the IHC phenotypes observed was not requested (hence there was no formal comparison with those described by the UK participant), each laboratory was asked to classify their scrapie cases according to the phenotypic similarities and differences observed.

(ii) 'short' PrP^d profile: the 'long' PrP^d profiles require a significant investment of time to perform and potentially may incur greater inter-observer error. To circumvent this problem, tissue sections of the forebrain were examined in order to construct the 'short' PrP^d profile of each sheep. This approach has been described in detail elsewhere (Sisó *et al.*, 2010), and had been previously shown at a training workshop attended by representatives of all participating institutes, held at the UK participating laboratory in November 2006. Briefly, it involves the scoring of 14 different morphological types of PrP^d in 11 defined areas of two forebrain sections (cerebral cortex and corpus striatum). The mean value for each PrP^d type is then calculated and converted into percentage values by reference to the total magnitude of PrP^d, in order to minimize the effect of variability in absolute magnitudes of PrP^d accumulation. The profile results from the graphical representation of those values.

Each participant was requested to produce their own 'short' PrP^d profile, except for the German laboratory, whose sheep were disregarded due to the low levels of PrP^d immunoreactivity in those forebrain areas, which is consistent with the fact that these were preclinical scrapie cases (Sisó *et al.*, 2009); they showed, however,

enough PrP^d in mid- and hind-brain areas as to enable their classification by the 'long' profile. To provide an inter-laboratory comparison of the 'short' PrP^d profiles, blind-coded sections of 13 sheep (those from Italy, Spain and the Netherlands) were also examined by the VLA. To classify these 13 sheep and the five from UK into groups, the 'short' PrP^d profiles from individual blind-coded sheep were compared by Spearman non-parametric correlation analyses between all possible pairs of sheep. In addition, the 'short' PrP^d profiles were subjected to cluster analysis (Stata 10; StataCorp).

Western blotting (WB) and *PRNP* genotyping. Frozen brain samples of cerebellum from 20 of 23 sheep were sent to the German laboratory, where they were analysed by discriminatory WB procedures as described elsewhere (Gretzschel *et al.*, 2005); brainstem samples from the three sheep from the Netherlands were analysed at their laboratory of origin by a similar WB procedure (Langeveld *et al.*, 2006). The parameters considered in the analysis of results were the molecular mass of the unglycosylated band of the protease-resistant core of PrP (PrP^{res}), the PrP^{res} glycoprofile and the N-terminal/C-terminal antibody reactivity ratios (details of the antibodies used and their PrP epitope recognition, as well as of the statistical methods used to analyse the results are given as Supplementary Methods and Supplementary Table S4).

In order to ascertain the existence of polymorphisms at codons other than 136, 154 and 171 of PrP, blood or brain tissue samples from all the 23 sheep were taken for PCR amplification and sequencing of the whole ORF of the *PRNP* gene on an Applied Biosystems 3130 Genetic Analyzer with the BigDye terminator v3.1 cycle sequencing kit as per the manufacturer's protocol. Except for the three sheep from the Netherlands and the five from the UK, which were genotyped at the Spanish laboratory, all analyses were done at each of the participating laboratories as described elsewhere (Acín *et al.*, 2004; Acutis *et al.*, 2004; Lühken *et al.*, 2004).

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