European small ruminant populations, and the recognition of the first BSE case of a goat in France [15] have emphasized the need to formulate suitable strategies against such diseases. The ineffectiveness of the tools used against "conventional" infectious disease has prompted the European Union (EU) to adopt innovative strategies against scrapie based on breeding programmes of sheep for the selection of genetically-resistant populations[1].

The susceptibility of sheep to TSE is greatly influenced by the host genotype at the PrP gene (*PRNP*) [17]. Polymorphisms at codon 136, 154 and 171 are combined in four main variants of the wild-type ARQ allele [6] (expressed in single-letter amino acid code at positions 136, 154 and 171): VRQ, AHQ, ARH and ARR. The ARR allele has been associated with the highest level of protection from classical scrapie, whereas VRQ, ARQ, AHQ and ARH are associated with different degrees of susceptibility [5]. Additional rare variations at the three standard codons, such as TRQ, ALQ ARK, VHQ, AHR and VRR, have been identified [4, 8, 13, 28] but their association with susceptibility is still unknown.

However, the variability of sheep *PRNP* is greater than that of these three codons. An additional 24 polymorphic codons have been described to date, giving rise to 43 allelic variants mainly derived from variations of the ARQ allele [21]. Moreover, studies have shown that reliance on only three PrP codon positions may not be sufficient to fully predict the susceptibility of sheep to TSE [20, 31, 40].

In particular, the $AF_{141}RQ$ allele (the ARQ allele with phenylalanine at codon 141 instead of leucine) was found to be associated with an increased susceptibility to the atypical form of scrapie named Nor98 [31]. Observations deriving from experimental challenges of sheep carrying mutations of the ARQ allele, suggest that alleles other than the ARR may have a protective effect against TSE [20, 40]. In particular, the $ARL_{168}Q$ allele was found to be associated with an increased resistance to experimental BSE [20]. Additionally, we recently observed in the Sarda breed that sheep carrying the $AT_{137}RQ$ or $ARQK_{176}$ alleles were protected following experimental challenge with classical scrapie or BSE [40]. Because of the small sample size used in these experimental challenges, larger studies under natural conditions are needed to confirm these observations.

Herein, we present the results of a multi-flock study, which shows the occurrence of PrP polymorphisms at codons other than 136, 154 and 171, inducing different degrees of protection against natural scrapie in sheep.

2. MATERIALS AND METHODS

2.1. Scrapie outbreaks overview

Five outbreaks of sheep scrapie (A, B, C, D and E), identified between 2004 and 2006 in the Tuscany Region (Siena province) in the framework of the official TSE surveillance, were included in the study. Outbreaks were included based on the following criteria: (i) the animals involved were from flocks of the Sarda breed; (ii) the outbreaks were associated with classical scrapie; (iii) there was a high scrapie prevalence rate; (iv) there was a large flock size; (v) the descriptive information was available, accurate and complete. Three outbreaks (A, C, D) were identified by passive surveillance in symptomatic animals, while the others were detected by active surveillance in fallen stocks.

All TSE eradication activities were carried out according to EU legislative guidelines. In outbreaks A, B, C and E, selective culling was applied. In these flocks, PrP genotyping at codons 136, 154 and 171 was performed on all animals. Sheep carrying susceptible genotypes were culled while those with resistant or semi-resistant PrP genotypes[2] were kept alive.

[1] Commission Decision 2003/100/EC of 13 February 2003 laying down minimum requirements for the establishment of breeding programmes for resistance to transmissible spongiform encephalopathies in sheep [on line] http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_041/l_04120030214en00410045.pdf [consulted 20 November 2008].

[2] Commission Regulation (EC) No. 260/2003 amending Regulation (EC) No. 999/2001 of the European Parliament and of the Council as regards the eradication of transmissible spongiform encephalopathies in ovine and caprine animals and rules for the trade in live ovine and caprine animals and bovine embryos [on line] http://eur-lex.europa.eu/pri/en/oj/dat/2003/l_037/l_03720030213en00070011.pdf [consulted 20 November 2008].

Table I. Information about the outbreaks included in the study.

Sheep	Flock					
	A	B	C	D	E	Total
Overall number	923	1774	570	3618	829	7714
No. tested for PrP genotype*	903	1707	566	1482	728	5386
No. tested for TSE	163	150	140	710	146	1309

* PrP genotype at codons 136, 154 and 171.

Outbreak D underwent stamping-out and genotyping was performed in a random sample of these sheep. TSE diagnosis was carried out on the obex, by rapid tests in a random sample of culled sheep older than 18 months according to the manufacturer's recommendations. In flocks A, B, C and E, the TeSeE ELISA test (Bio-Rad, Marnes-la-Coquette, France) was used. In flock D, 144 animals were tested with the Prionics-Check Western blot (Prionics, Zurich, Switzerland), and the rest with the Bio-Rad TeSeE. No difference was found in the proportion of positive animals revealed by the two diagnostic tests (Chi-square test $P = 0.46$).

Positive animals were defined as scrapie cases with a molecular pattern of PrP^{Sc} compatible with classical scrapie, as assessed by discriminatory Western blot[3]. Negative animals were sheep that tested negative on the diagnostic test. Information about the size of the outbreaks and the number of animals that underwent genotyping and TSE diagnoses are summarised in Table I.

To estimate the occurrence of any variation of the PrP amino acid sequence, sequencing of the entire *PRNP* coding sequence (CDS) was performed on all positive cases and on a random sample of ARQ/ARQ negative sheep. The sample size was calculated assuming a type I error of 5%, 50% expected prevalence of PrP variation, and 7% accepted error. Using these parameters, the number of ARQ/ARQ negative sheep to be sequenced in each flock was not less than 64 (flock A), 67 (B), 43 (C), 105 (D) and 59 (E).

2.2. *PRNP* analysis by Real-Time PCR

DNA was extracted from 25 µL whole blood using the semi-automated ABI Prism 6100 Nucleic Acid Prep Station and the dedicated Blood Prep chemistry, following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

For the Allelic Discrimination Assay, 5 µL of genomic DNA were transferred into four different PCR mixtures (codon 136, codon 154, codon 171-1 and codon 171-2) (Tab. II), containing 1× TaqMan Universal PCR Master Mix, primers forward and reverse 900 nM each, variable concentrations of TaqMan® Minor Groove Binder (MGB)-probes (Tab. II) to a final volume of 25 µL. PCR mixtures were submitted to the same amplification protocol (2′ at 50 °C, 10′ at 95 °C, 15″ at 95 °C and 1′ at 62 °C for 40 cycles) with an ABI PRISM 7900HT thermal cycler (Applied Biosystems). The results were analysed by the SDS 2.1 software.

2.3. *PRNP* sequencing

PRNP CDS was amplified using 10 µL of extracted DNA, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of F1 (5′-CATTTATGACCTAGAATGTT TATAGCTGATGCCA-3′) and R1 (5′-TTGAATGA ATATTATGTGGCCTCCTTCCAGAC-3′) primers, 1X Gold Buffer and 5 units of AmpliTaq Gold® (Applied Biosystems) following standard amplification protocol (5′ at 95 °C, 30″ at 94 °C, 1′ at 66 °C and 1′ at 72 °C for 35 cycles). Sequencing reactions were carried out with primers T1 (5′-GGT CCTCATAGTCATTGCC-3′), T2 (5′-TGGTGGCT ACATGCTGGG-3′), T3 (5′-TTTACGTGGGCAT TTGATGC-3′) and T4 (5′-GGCTGCAGGTAGAC ACTCC-3′) using Big Dye Terminator Cycle sequencing Kit v1.1 and detected with ABI PRISM 3130 apparatus (Applied Biosystems).

2.4. *PRNP* nomenclature

PrP genotypes were reconstructed on the assumption that all polymorphisms are mutually exclusive.

In this study, PrP alleles are indicated with the three-letter code (e.g. ARQ or ARR) when only amino acids at positions 136, 154 and 171 are known or when the allele is intended to include all its possible variations. After sequence analysis, the alleles identical to

[3] Discriminatory WB developed at ISS (2007) Discriminatory testing handbook, version 2 March 2007 – TSEs strain characterisation in small ruminants [on line] http://www.defra.gov.uk/vla/science/docs/sci_tse_rl_handbookv2mar07.pdf [consulted 20 November 2008].

Table II. Primers and probe sequences used in the four different allelic discrimination assays were designed using GenBank M31313.

PCR mixture	Primer	MGB-probe	AA
Codon 136	136F: 5′-CTGCAGCTGGAGCAGTGGTA-3′ 136R: 5′-GATAGTAACGGTCCTCATAGTCATTGC-3′	136Ala: 5′FAM-TCRTGgCACTTCC-3′ (300 nM) 136Val: 5′VIC-CTCATGaCACTTCC-3′(200 nM)	Ala Val
Codon 154	154F: 5′TGGCAATGACTATGAGGACCG-3′ 154R: 5′-TGGTCTGTAGTACACTTGGTTGGG-3′	154Arg: 5′FAM-ACTATCgTGAAAACAT-3′(120 nM) 154His: 5′VIC-TACTATCaTGAAAACATG-3′(200 nM)	Arg His
Codon 171-1	171F: 5′-GTTACCCCAACCAAGTGTACTACAGA-3′ 171R: 5′-TGTTGACACAGTCATGCACAAAG-3′	171Arg: 5′FAM-CCAGTGGATCgGTATA-3′(150 nM) 171His: 5′-ACCAGTGGATCa TTAT-3′(120 nM)	Arg His
Codon 171-2	171F: 5′-GTTACCCCAACCAAGTGTACTACAGA-3′ 171R: 5′-TGTTGACACAGTCATGCACAAAG-3′	171Arg: 5′FAM-CCAGTGGATCgGTATA-VIC (150 nM) 171Gln: 5′VIC-ACCAGTGGATCaGTATA-3′ (200 nM)	Arg Gln

the wild-type allele (GenBank AJ000739) are indicated as ARQ_{wt}, while mutated alleles are indicated with the three-letter code plus the additional polymorphic amino acid and its position (e.g. $AF_{141}RQ$).

2.5. Descriptive epidemiology and statistical analysis

Categorical variables were described using the number of observations and percentages with a relative 95% confidence interval (CI 95%).

For each flock, the frequency distribution of PrP genotypes at codons 136, 154 and 171 was obtained. Prevalence of scrapie was estimated as the number of positive animals out of the total number of sheep tested for TSE. The frequency distribution of scrapie cases with respect to the PrP genotype, along with the proportion of positive or negative ARQ/ARQ sheep carrying any additional variation of the PrP gene were calculated.

Within each flock, the U-Mann Whitney test was performed to compare the age of PrP-sequenced ARQ/ARQ positive cases and negative controls. Bonferroni correction [9] for multiple comparisons was applied. Furthermore, the distributions of positive cases and controls by cohort of birth within each flock were obtained and differences were tested using the Cochrane-Armitage test.

To allow a suitable evaluation of the effect of PrP polymorphisms in influencing the susceptibility to scrapie, all polymorphic loci were preliminarily confirmed to follow the Hardy-Weinberg equilibrium, with a value of $P < 0.01$ considered as statistically significant.

For each flock, odds ratio (OR) and relative CI 95% were calculated to compare the risk of scrapie between ARQ/ARQ animals carrying additional variations and ARQ_{wt}/ARQ_{wt} flock mates. Scrapie status was considered as the outcome variable while the presence of additional variations as the explanatory variable.

Statistical analyses were carried out using STATA software version 8.2 (Stata Corporation, College Station, Texas, USA).

3. RESULTS

3.1. Outbreak overview and genotypes at codons 136, 154 and 171

PrP genotyping at the three codons, carried out on 5386 sheep from all flocks under study (Tab. I), revealed 11 of the 15 PrP genotypes commonly found in sheep (ARH/VRQ, AHQ/VRQ, ARH/ARH, VRQ/VRQ were absent). The allele frequencies in the flocks ranged between 50.8 and 60.7% for ARQ, 33.4 and 40.4% for ARR, 1.9 and 8.8% for AHQ, and 0 and 0.5% for both the VRQ and ARH alleles. The most frequent genotypes were ARQ/ARR and ARQ/ARQ, representing 44.7 and 33.1% of the overall population respectively. This was followed by ARR/ARR, ARQ/AHQ and AHQ/ARR. The VRQ allele, which is very rare in the Sarda breed, was absent in flock E.

Besides index cases, diagnostic tests revealed several additional scrapie cases among culled sheep. Overall, 175 sheep tested positive for classical scrapie. The prevalence rates of scrapie in sheep older than 18 months were the following: flock A 17.5% (CI 95% 11.6; 24.7); flock B 22.1% (CI 95% 15.8; 29.7); flock C 40.0% (CI 95% 31.8; 48.6); flock D 5.9% (CI 95% 4.3; 7.9); flock E 13.0% (CI 95% 8.0; 19.6). All positive cases carried at least one ARQ allele. ARQ/ARQ was the most frequently affected genotype ($n = 154$), followed by ARQ/AHQ ($n = 20$) and ARQ/ARR ($n = 1$).

3.2. Beyond the three codon nomenclature of ARQ/ARQ sheep

To investigate the frequency and effect of PrP polymorphisms at codons other than 136, 154 and 171, all scrapie cases ($n = 175$) and 378 ARQ/ARQ negative sheep were analysed by sequencing the *PRNP* CDS. Among positive cases, additional polymorphisms, beyond the three codons, were detected only in sheep of the ARQ/ARQ genotype. Therefore, statistical analyses refer only to ARQ/ARQ sheep. The PrP genotype after sequencing of negative and scrapie-affected ARQ/ARQ sheep is reported in Table III. Remarkably, additional PrP polymorphisms were detected in only 6 out of 154 scrapie-affected sheep, with in-flock frequencies ranging from 0 to 30%. In contrast, these polymorphisms occurred in 204 out of 378 scrapie-negative sheep, with frequencies ranging from 28.9 to 85.1%. Overall, eight different alleles, combined into 17 genotypes, were observed: ARQ_{wt}, $T_{112}ARQ$, $V_{127}ARQ$,

Table III. PrP genotype distribution in ARQ/ARQ scrapie cases and negative sheep.

Genotype	ARQ/ARQ positive cases per flock					ARQ/ARQ negative sheep per flock				
	A *n* (%)	B *n* (%)	C *n* (%)	D *n* (%)	E *n* (%)	A *n* (%)	B *n* (%)	C *n* (%)	D *n* (%)	E *n* (%)
ARQ_{wt}/ ARQ_{wt}	23 (100)	31 (97)	48 (96)	39 (100)	7 (70)	54 (71)	36 (48)	7 (15)	59 (52)	18 (27)
ARQ_{wt}/ $T_{112}ARQ$									1 (1)	
ARQ_{wt} /$V_{127}ARQ$					1 (10)	1 (1)	1 (1)		1 (1)	
ARQ_{wt} /$AT_{137}RQ$						7 (9)	10 (13)	14 (30)	10 (9)	13 (19)
ARQ_{wt} /$AF_{141}RQ$		1 (3)	2 (4)		1 (10)	3 (4)	8 (11)	4 (9)	13 (12)	16 (24)
ARQ_{wt} /$AK_{142}RQ$									1 (1)	
ARQ_{wt} /$AR_{143}RQ$						6 (8)			1 (1)	
ARQ_{wt} /$ARQK_{176}$						2 (3)	12 (16)	20 (43)	26 (23)	15 (22)
$T_{112}ARQ/AT_{137}RQ$							2 (3)			
$AT_{137}RQ/AT_{137}RQ$										1 (1)
$AT_{137}RQ/AF_{141}RQ$						1 (1)		1 (2)		2 (3)
$AT_{137}RQ/AR_{143}RQ$						1 (1)				
$AT_{137}RQ/ARQK_{176}$						1 (1)	2 (3)	1 (2)		
$AF_{141}RQ/V_{127}ARQ$							1 (1)			
$AF_{141}RQ/AF_{141}RQ$					1 (10)		1 (1)			1 (1)
$AF_{141}RQ/ARQK_{176}$							1 (1)			1 (1)
$ARQK_{176}/ARQK_{176}$							1 (1)		1 (1)	
TOTAL	23	32	50	39	10	76	75	47	113	67

$AT_{137}RQ$, $AF_{141}RQ$, $AK_{142}RQ$, $AR_{143}RQ$ and $ARQK_{176}$. Among positive sheep, only the ARQ_{wt}, $V_{127}ARQ$ and $AF_{141}RQ$ alleles were observed; conversely, all allelic variants were found among negative sheep.

Genotypes carrying variations at codons 137, 141 or 176 were the most frequent and were observed in all flocks. In particular, the overall occurrence of $ARQK_{176}$/ARQ (indicating the $ARQK_{176}$ allele associated with ARQ or any variation of it) was higher (A: 3.9%; B: 22.7%; C: 44.7%; D: 23.9%; E: 23.9%) than that of $AT_{137}RQ$/ARQ (A: 13.2%; B: 18.7%; C: 34.0%; D: 8.8%: E: 23.9%) and $AF_{141}RQ$/ARQ (A: 5.3%; B: 13.3%; C: 10.6%; D: 11.5%; E: 29.9%).

In negative sheep, the ARQ_{wt}/ARQ_{wt} genotype was the most frequent in flocks A, B, D and E, while in flock C, sheep with the $ARQ_{wt}/AT_{137}RQ$ and $ARQ_{wt}/ARQK_{176}$ genotypes were the more numerous. All the other genotypes had a frequency lower than 3%, with the exception of $AR_{143}RQ$/ARQ which was reported almost exclusively in flock A, with a frequency of 9.2%.

Differences of PrP genotype distribution in negative sheep between the flocks were tested only for those genotypes found in all flocks (ARQ_{wt}/ARQ_{wt}, $ARQ_{wt}/AT_{137}RQ$, $ARQ_{wt}/ARQK_{176}$, $ARQ_{wt}/AF_{141}RQ$). Statistically significant differences resulted for all genotypes ($P \leq 0.001$) with the exception of the $ARQ_{wt}/AT_{137}RQ$.

No statistical difference in the age of sequenced ARQ/ARQ animals between scrapie cases (mean age ± standard deviation in years A: 2.9 ± 1.4; B: 2.6 ± 1.3; C: 2.5 ± 0.9; D: 3.0 ± 0.6; E: 4.0 ± 0.0) and negative sheep (A: 2.4 ± 1.0; B: 3.5 ± 1.9; C: 3.0 ± 1.3; D: 3.5 ± 1.6; E: 3.9 ± 0.3) was found within each flock. Differences in the distribution of positive cases and negative controls by cohort of birth were not significant in any flocks, except in flock B ($P = 0.015$).

3.3. Scrapie risk in ARQ/ARQ genotypes

To estimate the risk of scrapie associated with polymorphisms on the ARQ allele, the OR value for ARQ/ARQ genotypes carrying

Table IV. OR point estimates and relative CI 95% for sheep carrying the $AT_{137}RQ/ARQ$, $AF_{141}RQ/ARQ$ and $ARQK_{176}/ARQ$ genotypes.

Flock	$AT_{137}RQ/ARQ$	$AF_{141}RQ/ARQ$	$ARQK_{176}/ARQ$
A	0.00 (0.00; 0.94) *	0.00 (0.00; 2.38)	0.00 (0.00; 3.18)
B	0.00 (0.00; 0.33) *	0.11 (0.01; 0.82) *	0.00 (0.00; 0.29) *
C	0.00 (0.00; 0.04) *	0.06 (0.01; 0.47) *	0.00 (0.00; 0.03) *
D	0.00 (0.00; 0.56) *	0.00 (0.00; 0.46) *	0.00 (0.00; 0.22) *
E	0.00 (0.00; 0.68) *	0.26 (0.02; 1.63)	0.00 (0.00; 0.68) *

* Refers to statistical significant estimates ($P \leq 0.05$).

at least a polymorphic allele with respect to the ARQ_{wt}/ARQ_{wt} genotype was calculated. The OR estimates showed that the probability of testing positive for scrapie was significantly lower in sheep carrying the ARQ/ARQ genotype with any additional polymorphism, as compared to the ARQ_{wt}/ARQ_{wt} sheep. In particular, the protective effect was very high in flocks B (OR = 0.03; CI 95% 0.00; 0.20), C (OR = 0.01; CI 95% 0.00; 0.41) and E (OR = 0.16; CI 95% 0.02; 0.80) and appeared even complete in flocks A (OR = 0.00; CI 95% 0.00; 0.42) and D (OR = 0.00; CI 95% 0.00; 0.11). It should be noted that because it is difficult to interpret risk with an OR estimate value of 0, closer attention was paid to the value of the upper limit of the CI 95%.

We further calculated the OR associated with ARQ/ARQ genotypes containing each observed allelic variant, compared to ARQ_{wt}/ARQ_{wt}. For each of the seven PrP polymorphisms, the OR values estimated in the flocks were below 1, indicating a lower susceptibility compared to ARQ_{wt}/ARQ_{wt}. However significant OR were obtained only for the $AT_{137}RQ/ARQ$, $AF_{141}RQ/ARQ$ and $ARQK_{176}/ARQ$ genotypes (Tab. IV). In particular, a strong protective effect of $AT_{137}RQ/ARQ$ was evident in all flocks, with OR = 0 and the upper 95% confidence limit ranging between 0.04 and 0.94. Similarly, a null value of the OR was observed for $ARQK_{176}/ARQ$ in all outbreaks, with estimates statistically significant in flocks B, C and E, and the upper 95% confidence limits between 0.03 and 0.68. Finally, the protective effect of $AF_{141}RQ/ARQ$ was statistically significant only in flocks B, C and D. In flock B and C, OR estimates were higher than those reported for $AT_{137}RQ/ARQ$ and $ARQK_{176}/ARQ$.

Since scrapie typically affects adult sheep, age could represent a potential source of bias. OR values stratified by age on the overall population were estimated and showed that age did not have a confounding effect (data not shown).

4. DISCUSSION

It has been noted from the earliest reports of scrapie in sheep, that family lines have a strong influence on the occurrence of the disease [25]. Since that time, knowledge on the genetics of sheep scrapie has so improved, that innovative strategies based on breeding programmes for prion disease resistance in sheep are ongoing in Europe. Only three classical scrapie cases carrying the ARR/ARR genotype have been reported to date [23, 26]. Recently, it was proposed that polymorphisms besides those at the standard three codons may influence the susceptibility of sheep to prion diseases [20, 40]. We therefore investigated the influence of PrP polymorphisms at codons other than 136, 154 and 171 on the susceptibility of sheep to classical scrapie. Our results show a high frequency of variants of the ARQ allele and demonstrate a clear protective effect of some of them.

In our study, the $AT_{137}RQ$ and $ARQK_{176}$ PrP alleles were associated with the strongest protection. Indeed, despite their high frequency in the flocks under study, no sheep carrying these variants was found positive to scrapie. Moreover, no cases have been reported elsewhere with the $AT_{137}RQ$ and $ARQK_{176}$ PrP alleles. Interestingly, the protective effect of

these alleles has been previously observed in sheep challenged with classical scrapie or BSE [40].

A significant protective influence was also observed for the $AF_{141}RQ$ allele. However, OR values indicate that the degree of protection conferred by this allele is lower than that given by $AT_{137}RQ$ and $ARQK_{176}$. Indeed, classical scrapie cases in sheep carrying the $AF_{141}RQ$ allele were observed in the flocks under study, as well as in other outbreaks [10, 30, 34, 39]. These results were consistent with previous studies that reported a lower risk of $AF_{141}RQ$ compared to ARQ_{wt} [30].

A protective effect of $AK_{142}RQ$ could not be assessed because of the low frequencies of this allele. Nevertheless, we previously reported that sheep carrying the ARQ/$AK_{142}RQ$ genotype may have been protected after experimental challenge with scrapie [40].

Similarly, we could not assess the influence of the $T_{112}ARQ$, $V_{127}ARQ$ and $AR_{143}RQ$ alleles due to their low frequency. Although no positive sheep were detected carrying these alleles in the flocks under study, the $T_{112}ARQ$, $V_{127}ARQ$ [40] and $AR_{143}RQ$ [1] alleles have been previously described in classical scrapie affected sheep.

Studies in sheep have demonstrated that, when scrapie is present in a flock, alleles conferring resistance have higher fitness compared to those associated with susceptibility to the disease [16]. The flocks under study were from one of the areas most affected by scrapie in Italy [2, 39]. In these outbreaks, a high prevalence rate of scrapie was found, suggesting that the infection was present for a long time. It could therefore be speculated that the high frequency of some variants of the ARQ allele exerting a remarkable protective effect, might be the result of the genetic selection driven by scrapie.

It should be emphasized that the fitness of different *PRNP* genotypes may change significantly depending on the prion strain. It is well known that sheep carrying the VRQ allele are highly susceptible to experimental challenge with the scrapie source SSBP/1 [25] while they show a much lower disease incidence following injection with either BSE or the scrapie isolate CH1641, which have their main genetic target in the ARQ allele [18, 25]. Similarly, the ARR allele confers strong protection from classical scrapie, but not from the atypical scrapie agent Nor98 [30, 34]. It is noteworthy that in our study the $AF_{141}RQ$ allele exerted a protective effect with respect to classical scrapie, while it represents the main target of Nor98 [31], thus offering another example of the specificity of the association of PrP genotypes and prion strains.

Studies on the molecular evolution of the sheep PrP gene suggested that the high degree of polymorphisms in sheep *PRNP* has occurred by balancing selection [37], a process which operates to maintain polymorphisms within a population. Along this line, it could be hypothesized that the maintenance of *PRNP* variability might have been beneficial in sheep populations, which have to cope with a variety of scrapie strains having different *PRNP* targets. Here we show that more than one resistant allele does exist, one of which, $AF_{141}RQ$, confers differential susceptibility to classical scrapie and Nor98, thus suggesting a potential explanation for the maintenance of multiple alleles in sheep populations.

It is noteworthy that polymorphisms A136V, M137T, L141F and I142K, are located in the loop connecting the first β sheet and the first α helix (β1- α1) and that they all have a significant influence on scrapie susceptibility, thus suggesting the importance of this PrP region in the disease mechanisms. Similarly, polymorphism N176K lies in the β2 - α2 loop, a region in which other critical polymorphisms (P168L and Q171R/H/K) are located and which is key in conditioning the PrP^C three-dimensional structure [22], the formation of fibrils [35], the replication of prions [27] and the transmission barrier [3, 7, 12].

In the present study, the diagnosis was performed by rapid tests on the obex. It is well known that PrP^{Sc} accumulation in the lymphoreticular system (LRS) precedes neuroinvasion. Therefore, the unavailability of LRS tissues might have prevented the identification of scrapie incubating animals, leading to misclassification of some sheep within the control group. However, it is reported that the presence of PrP^{Sc} in the LRS varies according to genotype and that resistant and semi-resistant

genotypes show much lower – or even undetectable – levels of PrP^{Sc} compared to susceptible ones [23, 29]. Moreover, data from the oral experimental challenge of sheep with scrapie revealed no PrP^{Sc} deposition in the LRS of animals carrying the $AT_{137}RQ$ or $ARQK_{176}$ alleles, in contrast to ARQ_{wt}/ARQ_{wt} [40]. All the above indicates that misclassification of animals carrying the $AT_{137}RQ$ or $ARQK_{176}$ alleles as a consequence of the lack of diagnosis on LRS, is unlikely.

The relatively low availability of ARQ/ARQ negative controls to be sequenced and the wide range of birth cohorts have impaired our ability to perform a cohort matched analysis which would have led to a satisfactory control of age as a potential confounder. Nevertheless, bias associated with age appears to have scarcely affected our estimates. As a matter of fact, no difference in the age between positive cases and negative controls nor in its distribution by cohort was found in four flocks (A, C, D, E), including those showing the strongest protective effect of additional polymorphisms (A and D). Only in flock B could the existence of bias in the estimates not be excluded due to differences in the distribution of scrapie cases and controls by cohort.

This is the first study revealing ovine PrP alleles other than the ARR that exhibit significant protection against natural scrapie. This finding may suggest that more than one PrP allele can be selected in breeding programmes aimed at increasing resistance of sheep populations.

The $AT_{137}RQ$ allele has been observed in several breeds (Swifter, Textel, Benthein, short tailed and mixed breed) and countries (Germany, Iceland, Italy, Netherlands, United Kingdom and USA) [10, 13, 24, 36, 38, 41] with frequencies ranging from 1 to 5%. In particular, it was found in 19.4% of flocks examined in the United Kingdom [19]. $ARQK_{176}$ has been reported in European countries (Italy, Spain) and New Zealand and in several breeds (Sarda, Rasa Aragonesa, Ojinegras, Maellana) [1, 41]. In the five examined flocks, the frequencies of $AT_{137}RQ/ARQ$ and $ARQK_{176}/ARQ$ genotypes ranged from 8.8 to 34.0% and from 3.9 to 44.7% of all ARQ/ARQ sheep, respectively.

All flocks under study were located in a restricted area of the Tuscany region with a high scrapie incidence. In recent years, several scrapie isolates from that area have been characterised by both molecular assays and transmission to rodent models. Their characteristics have always appeared very homogeneous [3, 14, 32]. Given the influence of the prion strain in the genetic susceptibility of sheep, further investigations with other scrapie sources are required. Interestingly, ongoing studies aimed at comparing the influence of $AT_{137}RQ$ and $ARQK_{176}$ variations with the ARR allele in sheep experimentally infected with classical scrapie, BSE [40] and L-type BSE[4] suggest that these alleles are associated with increased resistance to more than one prion strain in sheep. If future investigations in other countries and with other scrapie sources confirm the protective effect of these alleles, breeding programmes could take advantage of the existence of additional resistant genotypes. The availability of more than one protective allele would offer the opportunity to maintain a higher variability of *PRNP* also against the possible emergence of TSE strains with different genetic targeting and would be useful in sheep breeds with a low frequency of the ARR allele.

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