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ICBAS | INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
SCHOOL OF MEDICINE AND BIOMEDICAL SCIENCES

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Mapping PRNP Polymorphisms in Portuguese Serra da Estrela Ovine Populations: Insights into Scrapie Susceptibility and Farm Animal Improvement

Soraia Filipa Serafim Rodrigues

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Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto

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Mapeamento de Polimorfismos do gene PRNP em Ovinos Portugueses Serra da Estrela: Perspetivas sobre a Suscetibilidade à Scrapie e o Melhoramento Animal

Scientific Areas: One Health
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Supervisor: Professor Doutor João Rodrigo Mesquita

Co-Supervisor: Dr. Guilherme Ramos Moreira

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ABSTRACT

Scrapie (classical and atypical) susceptibility in sheep is strongly influenced by PRNP gene polymorphisms. In Portugal, limited data exist for native breeds such as Serra da Estrela, despite their relevance to animal conservation and food production.

To assess scrapie susceptibility of Serra da Estrela sheep breed, the full coding region of PRNP gene of a 92 Serra da Estrela sheep cohort was sequenced, and subsequent SNP frequencies were analysed. A total of 27 SNPs were identified, including 20 nonsynonymous variants. Thirteen major haplotypes were observed. The ARR allele, which provides resistance to classical scrapie, was present in 58.7% of the population, with 18.5% of animals being homozygous (ARR/ARR). In contrast, the VRQ allele, associated with scrapie susceptibility, was absent. Several previously unreported SNPs were identified in this study, and their impact on prion protein aggregation propensity and structure was explored using PolyPhen-2 and AMYCO.

Earlier Portuguese surveys in this breed reported similar ARR/ARR (17%) and ARR/ARQ (42–44%) frequencies. Across other native and regional breeds, ARR is often rarer (e.g., 0.02–0.05 in Ethiopian sheep; low in many African and Indonesian populations), while ARQ predominates and VRQ may still be present. Thus, Serra da Estrela compares favourably for classical scrapie risk.

The high frequency of the ARR allele without full ARR fixation suggests that no selective breeding for scrapie resistance has been applied.

Serra da Estrela sheep already carry a high frequency of the protective ARR allele and lack VRQ but maintain rich PRNP diversity and novel variants. These results support the implementation of a gradual ARR-focused selection that strengthens resistance to classical (and better manages atypical) scrapie while consciously preserving genetic variability to ensure long-term breed sustainability.

KEYWORDS: PRNP gene; scrapie resistance; sheep; Serra da Estrela; single nucleotide polymorphisms (SNPs); animal improvement

RESUMO

A suscetibilidade à scrapie (clássica e atípica) em ovinos é fortemente influenciada por polimorfismos no gene PRNP. Em Portugal, os dados são limitados para raças autóctones como a Serra da Estrela, apesar da sua relevância na conservação e produção animal.

A região codificante completa do gene PRNP de 92 ovinos da raça Serra da Estrela foi sequenciada, e as frequências de SNPs foram analisadas. Foram identificados um total de 27 SNPs, incluindo 20 não sinónimos, e observados 13 haplótipos principais. O alelo ARR, que confere resistência à scrapie clássica, esteve presente em 58,7% da população, sendo 18,5% dos animais homozigóticos, enquanto o alelo VRQ (associado a suscetibilidade à scrapie) não foi detetado. Vários SNPs previamente não descritos foram identificados neste estudo, e o seu impacto na propensão para a agregação e na estrutura da proteína priónica foi explorado utilizando as ferramentas PolyPhen-2 e AMYCO.

Estudos portugueses anteriores nesta raça reportaram frequências semelhantes de ARR/ARR (17%) e ARR/ARQ (42–44%). Em outras raças autóctones e regionais, a frequência do alelo ARR é mas baixa (por exemplo, 0,02–0,05 em ovinos etíopes; baixo em muitas populações africanas e indonésias), enquanto o ARQ predomina e o VRQ pode ainda estar presente. Assim, a raça Serra da Estrela apresenta uma situação favorável no que respeita ao risco de scrapie clássica.

A elevada frequência do alelo ARR, sem fixação completa deste alelo, sugere que não foi aplicado melhoramento genético dirigido a aumentar a resistência à scrapie.

Os ovinos da raça Serra da Estrela apresentam uma elevada frequência do alelo protetor ARR e ausência do alelo VRQ, mantendo simultaneamente diversidade do PRNP e variantes novas. Estes resultados sustentam a implementação de uma seleção gradual focada no alelo ARR, que reforce a resistência à scrapie clássica (e permita uma melhor gestão da scrapie atípica), preservando a variabilidade genética e a sustentabilidade da raça.

PALAVRAS-CHAVE: gene PRNP; scrapie; ovinos; Serra da Estrela; SNPs; melhoramento animal

Case registry and activities developed during the internship

I had the opportunity to join the research team at ICBAS' Microbiology and Infectious Diseases laboratory (MIDlab) for 4 months, as an extracurricular internship, where I learned more about the molecular biology techniques used in research, including primer design, DNA extraction from various matrices using different methodologies, DNA purification, several PCR techniques (conventional, nested and semi nested, qPCR, RT-PCR and RTq-PCR, multiplexPCR, touchdownPCR, among others), electrophoresis gel preparation and interpretation, DNA sequencing (Sanger and Next Generation Sequencing), and I also had to chance to gain experience with several bioinformatic pipelines both in windows and linux environments. This time was spent working on several research projects in the genomics, parasitology, and virology areas, including laboratory analyses within the scope of infectious outbreaks surveillance. I soon gained proficiency in the lab's protocols and methodologies, and I was trusted with more autonomy. With the team's guidance and support, I seized the opportunity to propose and develop my own study, leveraging the skills and insights acquired during my internship. This project was developed over the following months and turned out to be the main theme of this dissertation, exploring the genetic susceptibility of scrapie in Serra da Estrela sheep. The findings originating from this study were presented in the format of a scientific poster at 7th International Conference of the European College of Veterinary Microbiology. These projects gave me insights into how scientific studies are planned, the logistics behind them, and allowed me to gain knowledge on systematic literature review, microbiology methodologies, interpretation and communication of results, data, scientific writing, data management, and statistical analysis. During this time, I was invited to participate in the Organizing Committee of the 13th Iberian Prio Congress that took place in ICBAS on the 22nd and 23rd of May, 2025. This experience played an important role in developing my knowledge in the subject of prion diseases and prion-like diseases and gave me the privilege to meet and discuss with some of the most knowledgeable researchers on the subject.

Now that I was a part of the research team of the MIDlab, it only made sense that I continued my road there, so for the 16 weeks of the curricular internship, I continued working on my research endeavours, working on multiple projects, and, as of the submission of this thesis I have several publications, both as first author and as co-author, namely articles in scientific journals (Table 1) and posters presented at scientific conferences (Table 2).

Table 1. Published scientific papers during the internship at MIDlab

Title	Journal	DOI
Mapping PRNP Polymorphisms in Portuguese Serra da Estrela Ovine Populations: Insights into Scrapie Susceptibility and Farm Animal Improvement ¥	Animals - MDPI	10.3390/ani15182750
Molecular Screening of *Sarcocystis* spp. in Grazing Sheep (*Ovis aries*) and Shepherd Dogs (*Canis lupus familiaris*) from Central Portugal	Animals - MDPI	10.3390/ani15233479
Highly Virulent Newcastle Disease Virus in Eurasian Collared Doves in the North of Portugal	Animals - MDPI	10.3390/ani15243563

¥ First author

Table 2. Scientific posters presented at conferences during the internship at MIDlab

Title	Conference
Avian Orthoavulavirus 1 in Eurasian Collared Doves in the North of Portugal ¥	IV Encontro Anual AL4AnimalS 2025
Mapping PRNP Polymorphisms in Portuguese Ovine Populations: Insights into Scrapie Susceptibility and Disease Surveillance ¥	7th International Conference of the European College of Veterinary Microbiology
Molecular and Metagenomic Characterization of Avian Orthoavulavirus 1 in Columbiform Birds Presenting Neurological Signs	7th International Conference of the European College of Veterinary Microbiology
Molecular Surveillance of Viral Threats in Portuguese Rodents	Al4Animals II Thematic Meeting
First report on the occurrence of selected zoonotic arboviruses and associated risk factors in donkeys from eastern Algeria ¥	Al4Animals II Thematic Meeting
Coronavírus equino: um vírus entérico emergente?	Congresso Anual APMVE
Paslahepevirus balayani e *Rocahepevirus ratti* em Equinos em Portugal	VII Jornadas do Grupo de Trabalho de Investigação em Equídeos - FMV ULISBOA

¥ First author

At the time of submission, several manuscripts were under peer review or available as preprints (Table 3).

Table 3. Manuscripts submitted for peer review or available as preprints.

Title	Journal	Status
First report on the occurrence of selected zoonotic arboviruses and associated risk factors in donkeys from eastern Algeria	SSRN	Preprint (DOI: 10.2139/ssrn.5732925)
First Report on Occurrence of antibodies to Toxoplasma gondii and Associated Risk Factors in Donkeys (Equus asinus) from Northeastern Algeria	Veterinary Research Communications	Submitted for peer review
Absence of Anti-HEV Antibodies in Donkeys in Algeria: A First Serological Survey ¥	Veterinary Research Communications	Submitted for peer review

¥ First author

My *Curriculum Vitae* developed during these months can be found both at FCT's CienciaVitae (Ciência ID: 301A-0CBE-8F60) and at ORCID (ID: 0009-0000-2191-9831), as well as in my Research Gate profile.

As part of MIDlab's research team, I conducted fieldwork to collect biological samples for multiple projects, including the thesis research presented here. For a complementary study on cavernicolous bats, I participated in expeditions to caves and mines across Portugal under the guidance of a licensed specialist. Our protocols included collecting environmental (air, guano) and direct biological samples (anal and oral swabs) from bats. For the core work of this thesis, I performed independent field collection of blood samples from Serra da Estrela sheep.

In addition to academic investigation, I joined initiatives and programmes developed in the Department of Veterinary Sciences of ICBAS, such as Projeto SEI, Agro@TecVerde, and other programmes with external national and international researchers, welcoming and guiding them during their stay at ICBAS.

Projeto SEI is an initiative created by Câmara Municipal do Porto with the main objective to contribute to scientific literacy in the youth. Specifically, this project enables schools located in more socioeconomically disadvantaged contexts to offer their students direct contact with higher education, strengthening this connection and promoting future continuity of their studies. I had the opportunity to work with the students in this programme in a mentorship role, a commitment I will maintain during this school year.

Agro@TecVerde is a project in the scope of the Plano de Recuperação e Resiliência (PRR) that joins several higher education institutions to enhance the attractiveness of agricultural programs. In this framework, the MIDlab hosted open days for students in the last year of secondary education. I was responsible for hosting the students, with a guided visitation and showing them what "a day in the MIDlab" looks like.

Concurrently, I supported MIDlab's collaborative environment by assisting visiting national and international researchers. My responsibilities included welcoming them and facilitating their onboarding process. This role provided direct exposure to a wide range of ongoing research initiatives within the lab.

The experiences during these months contributed not only to my academic growth but also allowed me to develop soft skills such as scientific communication to diverse audiences (teenagers and adults), cross-cultural communication and adaptability, teamwork in multidisciplinary and multicultural environments, responsibility and professionalism, and general problem-solving skills.

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LIST OF ABREVIATIONS

bp	Base pairs
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FCT	Fundação para a Ciência e Tecnologia
HWE	Hardy-Weinberg equilibrium
ICBAS	School of Medicine and Biomedical Sciences
MIDlab	Microbiology and Infectious Diseases laboratory
min	Minute(s)
ORF	Open reading frame
PCR	Polymerase chain reaction
PRNP	Prion protein gene
PrP	Prion protein
PrP^{Sc}	Abnormal isoform of the prion protein
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RTq-PCR	Quantitative reverse transcription polymerase chain reaction
s	Seconds
SNP	Single-nucleotide polymorphisms
TSE	Transmissible spongiform encephalopathy
µL	Microliters

1. Introduction

1.1. Organization

The following chapters are structured to address and explore these themes systematically:

Chapter 1 – Introduction: This chapter provides a state-of-the-art literature review and historical context of the matter studied, detailing the background, the rationale for the investigation in this specific population, the research aims and objectives, and the overall scope of the study.

Chapter 2 – Materials and Methods: This section details the geographical area and study population, the sampling methodology, the laboratory techniques and resources used, and the tools applied for the analysis of the resulting data.

Chapter 3 – Results: This section summarizes the results of the genetic analyses, focusing on the characterization of genetic variation, allele frequencies, and population-level patterns observed in the studied samples. The data are presented using tables and figures to facilitate reading and interpretation.

Chapter 4 – Discussion: Here, the results obtained are interpreted in the context of existing literature, exploring their biological significance and implications. The findings are discussed in relation to previous studies, with particular emphasis on their relevance to the genetic characterization of the population and the limitations of the present study.

Chapter 5 – Conclusions: This section summarizes the main findings of the study and highlights their significance, outlining the key conclusions drawn from the results. It also addresses the broader implications of the work and suggests potential directions for future research.

1.2. Prions, TSEs, and Scrapie

Transmissible spongiform encephalopathies (TSEs), also called prion diseases, constitute a group of neurodegenerative afflictions. The defining neuropathological hallmarks include progressive brain degeneration with spongiform (vacuolar) change, neuronal loss, and gliosis as a result of the accumulation of an abnormally folded, protease-resistant isoform of prion protein (PrP^{Sc}) derived from the normal cellular prion protein. This infectious agent is thought to be a proteinaceous infectious particle ("prion") largely or entirely composed of misfolded PrP, lacking nucleic acid (Ganten & Ruckpaul, 2005).

Prion formation results from a conformational conversion of the host-encoded cellular prion protein (PrP^{c}) into a misfolded, pathogenic isoform (PrP^{sc}). This process is uniquely driven by protein-protein interactions rather than nucleic acids. At the molecular level, PrP^{c} is a glycosylphosphatidylinositol (GPI)-anchored membrane protein predominantly composed of α-helical structures. PrP^{sc}, in contrast, exhibits a profound structural rearrangement characterized by an

increased β-sheet content, conferring partial resistance to proteolysis, decreased solubility, and a strong tendency to aggregate. Mechanistically, prion propagation is explained by the seeded polymerization (nucleation-dependent) model, in which PrP^{sc} acts as a conformational template or "seed." Upon direct interaction, PrP^{sc} induces the refolding of PrP^{c} into the pathogenic β-sheet-rich conformation. The newly converted PrP^{sc} molecules then oligomerize and elongate into fibrillar aggregates. Fragmentation of these aggregates generates additional seeds, thereby amplifying the conversion process in an autocatalytic manner. This continuous cycle of misfolding, aggregation, and fragmentation leads to the exponential accumulation of PrP^{sc} within neural tissue. The resulting aggregates interfere with membrane integrity, impair synaptic function, disrupt proteostasis, and ultimately trigger neuronal dysfunction and cell death, producing the progressive neurodegeneration characteristic of transmissible spongiform encephalopathies (Ganten & Ruckpaul, 2005; Iqbal et al., 2020; Prusiner, 1982). Classical scrapie is the oldest known TSE, with historical records dating back to the 18th century in Europe. It affects sheep and goats and is characterized by progressive neurodegeneration, often leading to behavioural changes, pruritus, ataxia, and ultimately death. The name "scrapie" derives from the intense itching behaviour observed in affected animals, who frequently scrape their bodies against surfaces (Cosier & Daraban, 2016; Iqbal et al., 2020). Other examples of TSEs are Creutzfeldt-Jakob disease, Kuru, Gerstmann-Sträussler-Scheinker (GSS) and fatal familial insomnia (FFI) in humans; bovine spongiform encephalopathy (BSE) in cattle; and chronic wasting disease (CWD) in Cervids (Ganten & Ruckpaul, 2005; Iqbal et al., 2020). In 1998, a novel, non-contagious form of scrapie was first identified in Norway. This condition, which predominantly affects older animals, was designated atypical scrapie (or Nor98), distinguishing it epidemiologically and pathologically from the classical form (Moum et al., 2005).

1.3. Genetics and susceptibility to scrapie

The prion protein is encoded by the PRNP gene, and genetic susceptibility to scrapie in sheep has been strongly linked to polymorphisms within this gene, making its study essential for understanding disease dynamics and improving breeding strategies for resistance (Cosier & Daraban, 2016; Iqbal et al., 2020).

Current evidence shows that key single- nucleotide changes in the PRNP gene occur mainly at codons 136 (A > V), 154 (R > H), and 171 (R > Q/H) (Adeola et al., 2023; Cassmann & Greenlee, 2020). Alterations at codons 141 and 154 have also been linked to different presentations of classical and atypical scrapie, most likely because of their effect on prion protein conformation (Yang et al., 2014). The substitution A > V at codon 136 has been associated with a higher risk of developing classical scrapie. Conversely, the Q > R substitution at codon 171 is recognized as a key protective polymorphism in sheep (Adeola et al., 2023; Cassmann & Greenlee, 2020). It is believed that the ancestral PRNP gene was $A_{136}R_{154}Q_{171}/A_{136}R_{154}Q_{171}$ (hereafter ARQ/ARQ) in sheep (Cosier & Daraban,

2016). The five most common haplotypes are ARR, ARQ, AHQ, ARH, and VRQ, defining genotypes that are conventionally categorized into five risk groups for classical scrapie. More recently, additional amino acid substitutions in certain nucleotide positions have also been reported to confer resistance to classical scrapie, even in animals carrying the ARQ/ARQ background (Cosier & Daraban, 2016; Iqbal et al., 2020).

1.4. PRNP gene investigation around the world and in Portugal

Numerous investigations have examined PRNP gene variation in sheep from different breeds and geographical regions, frequently assessing its connection with productive or phenotypic characteristics. In China, research has examined the specific PRNP gene in Chinese and Mongolian sheep (Li, Zhang, et al., 2018), as well as phenotypic traits in different Chinese breeds (Li, Erdenee, et al., 2018), Xinjiang local breeds (Lan et al., 2006), and Tan sheep from Ningxia (Xu et al., 2011). Other studies have focused on specific traits, such as milk production in Latxa dairy sheep (Vitezica et al., 2013) and Spanish Churra (Álvarez et al., 2006), reproductive traits in German breeds (Gerlinger et al., 2021), pathogenesis and neuropathological phenotypes (González et al., 2014), the Rasa Aragonesa breed from Spain (Acín et al., 2004), and general polymorphisms in Baltic breeds (Koynarski, 2020). Additional country-specific studies have been conducted in Greece (Argyriadou et al., 2019), Ireland (O'Doherty et al., 2001), Brazil (de Andrade et al., 2013), the Iberian Peninsula (Martin Palomino et al., 2019), Italy (Curcio et al., 2015), Türkiye (Meydan et al., 2017), and the native sheep breed of Palestine (Alsayed et al., 2019). Within China, further studies have examined sheep from Inner Mongolia (Han et al., 2011) and Northwest China (Zhao et al., 2012). In Africa, only a few studies have reported PRNP gene polymorphisms, including Algeria (Djaout et al., 2018), Ethiopia (Teferedegn et al., 2020), Morocco (Serrano et al., 2007), and four West African sheep populations: Burkina-Sahel, Djallonke, Mossi, and Touareg (Traoré et al., 2012).

Previous studies into the genetic basis for scrapie susceptibility in Portugal, in 2006 and 2010, investigated the distribution of genetic polymorphisms in the PrP locus in Portuguese sheep. However, the methods focused solely on the target codons 136, 154, and 171. These studies sampled sheep of various Portuguese breeds, with Serra da Estrela being the one with the highest frequency of ARR alleles, while having very low frequencies of the undesirable VRQ (Gama et al., 2006; Mesquita et al., 2010). Between 2002 and 2008, a total of 275,259 small ruminants were screened for atypical scrapie, and 326 sheep tested positive. The cases were associated with a variety of genotypes, most commonly ARR/AFRQ, followed by ARR/ARR, ARQ/AFRQ, and AFRQ/AFRQ (Orge et al., 2010). From 2002 to 2021, atypical scrapie cases were reported in 24 countries, with Portugal recording the highest number (696), followed by France (571) and the United Kingdom (368) (EFSA et al., 2021).

1.5. Serra da Estrela Sheep Breed

The Serra da Estrela is an autochthonous Portuguese sheep breed originating from the Serra da Estrela sub-region, where it has undergone long-term adaptation to local environmental and production conditions. The breed is officially recognized in the national Herd Book and comprises two coat-colour varieties, white and black (Figure 1). Both sexes are horned, typically presenting spiral-shaped horns. These sheep are classified as a medium-sized dairy breed, with adult live weights ranging from approximately 50-55 kg in females and 80-100 kg in males. The breed shows a well-defined dairy conformation, including a well-developed, globular udder with large, correctly positioned teats, supporting its high milk-producing capacity. The breed exhibits high fertility and prolificacy, with an extended reproductive season that allows sexual activity throughout the year, although mating under traditional systems occurs predominantly in spring. Milk yields frequently exceed 500 L per lactation (average duration of approximately 220 days), making this breed the principal genetic resource for the production of the traditional Serra da Estrela Protected Designation of Origin (DOP) cheese (ANCOSE, 2025).

Animals are traditionally reared under extensive or semi-extensive systems, grazing on diverse natural pastures characteristic of the Serra da Estrela region. This long-standing management system, combined with geographical isolation and controlled breeding practices, has contributed to the genetic distinctiveness of the Serra da Estrela breed and its importance as a reservoir of local ovine genetic diversity (ANCOSE, 2025).

Figure 1. Serra da Estrela, white (INIAV, 2025)

1.6. Aims of this study

This study aimed to comprehensively characterise the PRNP gene in the Serra da Estrela breed in Portugal. The objectives were to assess the distribution of known genotypes and to investigate the full spectrum of polymorphisms, including the atypical scrapie-linked L141F variant (Moum et al., 2005), and other potential susceptibility or protective alleles. To this end, we sequenced the entire PRNP coding region in a cohort of 92 animals to determine genotype and allele frequencies relative to

the wild-type sequence. Furthermore, we employed in silico tools to evaluate the potential biological impact of identified nonsynonymous single-nucleotide polymorphisms (nsSNPs) on prion protein structure and function.

2. Materials and Methods

2.1. Geographical Location

This study was conducted in the Serra da Estrela region, comprised of the municipalities of Fornos de Algodres, Gouveia and Seia of the Guarda district (Figure 2). It is the highest mountain range in mainland Portugal, reaching elevations of up to 2000 m above sea level. It is also the coldest region in the country, with average minimum temperatures ranging from −0.1 °C to 12 °C, and maximum temperatures between 6 °C and 22 °C. From March to October, daytime temperatures typically exceed 10 °C (CISE, 2025).

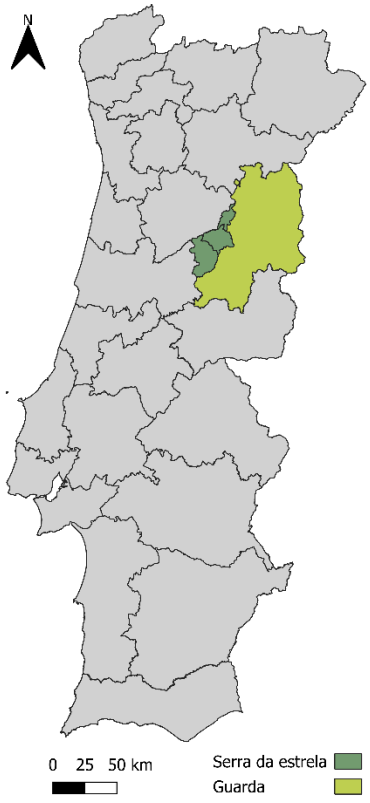

Figure 2. Serra da Estrela sub-region – created with qGIS v3.36.3 (Dawson et al., 2025)

2.2. The Serra da Estrela sheep

The study group was intentionally selected to ensure a genetically homogeneous sample of the Serra da Estrela breed. To this end, inclusion was restricted to purebred Serra da Estrela sheep that were officially registered in the ANCOSE (National Association of Serra da Estrela Sheep) genealogical book. This stringent selection criterion was applied to ensure breed purity, minimize potential genetic

confounding from recent or historical cross-breeding, and thereby enhance the reliability of subsequent genetic analyses. This approach allows for a clear interpretation of PRNP allele frequencies and their association with scrapie susceptibility specific to this defined population.

The sampled animals are typically managed under a traditional, low-mobility farming system. They remain confined to farm premises during the day and are sheltered in pens at night. This system provides a stable geographical context, making the population well-suited for structured, long-term disease monitoring and surveillance efforts. According to the most recent national livestock census (2020), the total registered population of the Serra da Estrela breed comprised 18,600 individuals (INIAV, 2025).

2.3. Sample Collection

A total of 92 animals were included in this study.

One of the advantages of working with an interdisciplinary team and department is the sample bank that can be shared for several studies, depending on the interest of each researcher.

In this study, brain tissue samples from 23 female sheep previously collected for another project were analysed. These samples were obtained post-mortem during routine slaughter for meat production in March 2024. The samples were transported that same day to the laboratory under refrigeration (4 °C) and subsequently stored at -20 °C until processed.

Additionally, 72 blood samples, obtained as remnants from the Brucellosis control program, were also used. Remnants from the 5 mL blood collected in EDTA tubes from the jugular vein of 72 sheep (all females) were randomly chosen in each of 12 different farms in the studied region to reach six samples per farm. Since three blood samples were fully clotted before processing, these samples were excluded from the study, resulting in a total of 69 blood samples. The whole blood samples were stored at -20 °C prior to DNA extraction.

All procedures for animal handling and sample collection followed the authorizations granted by the Órgão Responsável pelo Bem-estar Animal da ESAV (ORBEA-ESAV), in accordance with the applicable ethical and legal guidelines (license number: 03.01/ORBEA/2024; approval date: 22 March 2024).

2.4. Nucleic Acid Extraction

Table 4Total nucleic acid extraction relied on two automated systems at MIDlab: QIAcube (Qiagen® GmbH, Hilden, Germany) and the PurePrep96 (MolGen®, Veenendaal, The Netherlands). Their key operational differences are summarized in Table 4. Following an initial manual lysis step (tissue sample digestion with proteinase K in a chaotropic lysis buffer to disrupt cells and allow for the of release nucleic acids), subsequent purification was automated. Both systems executed sequential wash steps to remove contaminants and inhibitors, culminating in the elution of high-purity nucleic acids, ensuring a highly efficient and standardized extraction process.

Table 4. Different features in the nucleic acids extraction systems used in MIDlab.

Feature	QIAcube	PurePrep96
Binding phase	Silica spin columns	Magnetic beads
Typical format	12 samples/run	96-well plates
Driving force	Centrifugation/vacuum + pipetting robot	Magnet + pipetting robot

Brain sample DNA extraction was performed using a modified QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). A total of 420 µL of lysis buffer, along with 25 µL of proteinase K solution was added to the corresponding Eppendorf tube. The samples were vortexed for 30 s to ensure proper mixing, centrifuged at 6000× g for 2 min, and incubated at 57 °C for 15 min to facilitate lysis. A 350 µL aliquot of the resulting supernatant was transferred to a fresh microcentrifuge tube and mixed with an equivalent volume of RTL buffer. The tubes were vortexed again for 30 s and pulse-centrifuged, with subsequent steps being carried out in accordance with the QIAamp® DNA Mini Kit protocol using the automated QIAcube system (Qiagen® GmbH, Hilden, Germany).

Blood DNA extraction was performed using the PurePrep96 magnetic-bead-based extraction system (MolGen®, Veenendaal, The Netherlands). Whole blood in EDTA tubes was resuspended and 200 µL of the sample was used for extraction, relying on the kit Bio-Extract® SuperBall® (MolGen®, Veenendaal, The Netherlands).

2.5. Polymerase Chain Reaction (PCR) and DNA Sequencing

To identify polymorphic sites in the PRNP gene, PCR was carried out using a single pair of primers (Table 5) that amplify the 771 bp open reading frame (ORF), corresponding to the same region of the *Ovis aries* PRNP sequence available in the NCBI database (Gene ID: EF153678)(Adeola et al., 2023).

Table 5. Primers used to amplify the PNRP gene region studied.

Primer name	Sequence
PRNP-Forward	5'-CATTTATGACCTAGAATGTTTATAGCTGATGCCA-3'
PRNP-Reverse	5'-TTGAATGAATATTATGTGGCCTCCTTCCAGAC-3'

The 25 µL PCR mixture was obtained with the Takara Bio Inc. kit "TaKaRa LA Taq® DNA Polymerase" kit (Takara Bio Inc., Otsu, Japan), and 2 µL of genomic DNA was used in each reaction, 0.6 µM of each primer, 2.5 mM dNTPs, and 1.25 units of TaKaRa La Taq® DNA polymerase in the 10X LA PCR Buffer II with 2.5 mM added MgCl2. The PCR conditions were: 96 °C for 5 min, 35 cycles of denaturation at 96 °C for 30 s, 57 °C for 15 s, 72 °C for 1 min 30 s, and final extension of 72 °C for 4 min. The amplified DNA fragments were identified by electrophoresis on 1.5% agarose gels, stained with Xpert Green Safe DNA gel dye (GRiSP®, Porto, Portugal), at 100 V for 30 min. UV light irradiation was used to visualize the results, confirming the gene sequences were correctly amplified.

Amplicons of the expected size (771 bp) were purified using the Exo/SAP Go PCR purification kit (Grisp®, Porto, Portugal). After purification, bidirectional sequencing was performed using the Sanger sequencing method. Alignment and deconvolution of Sanger chromatogram trace files were performed using Tracy 0.7.6 (GEAR-Genomics) (Rausch et al., 2020). Sequence editing was conducted with the BioEdit Sequence Alignment Editor v7.1.9 (Ibis Biosciences), and alignment and comparison of SNPs and their translated proteins were conducted using the MEGA-X v10.2.6 software package.

2.6. Statistical Analysis

Haplotype distributions of the PRNP gene were obtained using DNA SNP Version 6.12. Hardy--Weinberg exact test was conducted using R v4.2.2. Genotype differences, allele, and haplotype frequencies of the PRNP gene were analysed using Microsoft Office 365 Excel v2506.

2.7. Evaluation of Nonsynonymous SNPs in the PRNP Gene

Evaluation of 20 nonsynonymous SNPs in the PRNP gene was carried out using the predictive online software PolyPhen-2 v2.0 (http://genetics.bwh.harvard.edu/pph2/), a tool for annotating nonsynonymous SNPs and predicting damaging effects of missense mutations in humans, which has been used before to assess SNPs in sheep (Adeola et al., 2023; Adzhubei et al., 2010). PolyPhen-2 is a computational tool used to predict whether a single amino acid substitution is likely to be benign or damaging to protein structure and function. The method integrates information from sequence conservation, protein structural features, and annotated functional domains to generate a probabilistic assessment of variant impact. At the sequence level, PolyPhen-2 evaluates the evolutionary conservation of the affected residue using multiple sequence alignments of homologous proteins, under the assumption that highly conserved positions are more likely to be functionally important. It also considers the physicochemical differences between the wild-type and mutant amino acids, such as charge, size, and hydrophobicity, as well as the local sequence context. When three-dimensional structural data or reliable models are available, the variant is mapped onto the protein structure to assess its potential impact on structural integrity, including its location within the protein core or surface, proximity to ligand-binding or active sites, presence in transmembrane regions, and involvement in secondary structural elements. In addition, PolyPhen-2 incorporates functional annotations from curated databases, assigning higher scores to variants that disrupt known functional domains or motifs. All features are combined using a Naïve Bayes-based machine-learning classifier, trained on curated datasets of known disease-associated and neutral variants, to produce a quantitative score and a qualitative classification (benign, possibly damaging, or probably damaging). The model is calibrated and validated against independent datasets to optimize predictive performance and guide threshold selection in genetic analyses (Adzhubei et al., 2010).

The evaluation of the effect of mutations on the aggregation properties of ovine prion protein was explored using AMYCO. This is a web-based tool designed to predict how amino acid substitutions

affect the aggregation propensity of prion-like domains (PrLDs) in proteins, with a particular focus on disease-related changes. The prediction algorithm integrates two main components: (1) PrLD composition change, which evaluates how a mutation alters the overall amino acid composition of the PrLD, considering that certain residues (e.g., Q/N-rich) promote aggregation while others are disruptive, and (2) localized amyloid propensity, which assesses how mutations affect short sequence segments prone to form β-sheet-rich aggregates. These features are combined into a refined AMYCO score that correlates with intracellular aggregation behaviour. In a typical workflow, users submit a wild-type sequence along with one or more mutations; AMYCO identifies the PrLD region, computes aggregation propensities for the wild-type and mutant sequences, and reports whether each mutation is predicted to increase or decrease aggregation propensity. Overall, AMYCO integrates global PrLD composition and local amyloid-forming segment analysis to provide both qualitative and quantitative predictions of mutation effects on prion-like aggregation (Iglesias et al., 2019).

3. Results

3.1. Identification of Polymorphic Sites of the PRNP Gene in 92 Serra da Estrela Sheep

The allele sequences derived from 92 sheep were deposited in GenBank under accession numbers PV854868 to PV855051.

A total of 27 SNPs, including 20 non-synonymous SNPs, were identified (Table 6). Due to low genotype and allele frequencies, the Hardy-Weinberg exact test was used to determine if the observed genotype frequencies in the population deviate significantly from the frequencies expected under Hardy-Weinberg equilibrium (HWE), and eight SNPs, including four non-synonymous (Q171H, N176K, Q226H, and G256R), did not conform to HWE.

Table 6. Genotype and allele frequencies of 27 PRNP polymorphisms in Serra da Estrela sheep. Each SNP shows nucleotide position and codon position, i.e., G220A is the SNP (G > A) in nucleotide 220 corresponding to the G > S amino acid mutation coded from codon.

SNP	Genotype frequency *n* (%)			Allele frequency		HWE*
G220A	GG	GA	AA	G	A	Yes
G74S	91(98.9%)	1(1.1%)	0(0.0%)	183(99.5%)	1(0.5%)	
A302G	AA	AG	GG	A	G	Yes
Q101R	89(96.7%)	3(3.3%)	0(0.0%)	181(98.4%)	3(1.6%)	
T335C	TT	TC	CC	T	C	Yes
M112T	91(98.9%)	1(1.1%)	0(0.0%)	183(99.5%)	1(0.5%)	
A341G	AA	AG	GG	A	G	Yes
H114R	91(98.9%)	1(1.1%)	0(0.0%)	183(99.5%)	1(0.5%)	
G361T	GG	GT	TT	G	T	Yes
A121S	91(98.9%)	1(1.1%)	0(0.0%)	0(0.0%)	1(0.5%)	
C407T	CC	CT	TT	C	T	Yes
A136V	90(97.8%)	2(2.2%)	0(0.0%)	182(98.9%)	2(1.1%)	
C421T	CC	CT	TT	C	T	Yes
L141F	84(91.3%)	8(8.7%)	0(0.0%)	176(95.7%)	8(4.3%)	

A428G H143R	AA 81(88.0%)	AG 10(10.9%)	GG 1(1.1%)	A 172(93.5%)	G 12(6.5%)	Yes
G461A R154H	GG 86(93.5%)	GA 5(5.4%)	AA 1(1.1%)	G 177(96.2%)	A 7(3.8%)	Yes
C511A Q171K	CC 91(98.9%)	CA 1(1.1%)	AA 0(0.0%)	C 183(99.5%)	A 1(0.5%)	Yes
A512G Q171R	AA 34(37.0%)	AG 41(44.6%)	GG 17(18.5%)	A 109(59.2%)	G 75(40.8%)	Yes
G513T Q171H	GG 88(95.7%)	GT 2(2.2%)	TT 2(2.2%)	G 178(96.7%)	T 6(3.3%)	No
A515T Y172F	AA 91(98.9%)	AT 1(1.1%)	TT 0(0.0%)	A 183(99.5%)	T 1(0.5%)	Yes
G525A 175 Q	GG 91(98.9%)	GA 1(1.1%)	AA 0(0.0%)	G 183(99.5%)	A 1(0.5%)	Yes
C528A N176K	CC 84(91.3%)	CA 5(5.4%)	AA 3(3.3%)	C 173(94.0%)	A 11(6.0%)	No
A566T Q189L	AA 91(98.9%)	AT 1(1.1%)	TT 0(0.0%)	A 183(99.5%)	T 1(0.5%)	Yes
G594C 198 G	GG 91(98.9%)	GC 0(0.0%)	CC 1(1.1%)	G 182(98.9%)	C 2(1.1%)	No
G630A 210 E	GG 91(98.9%)	GA 1(1.1%)	AA 0(0.0%)	G 183(99.5%)	A 1(0.5%)	Yes
G637C 212 V	GG 91(98.9%)	GC 0(0.0%)	CC 1(1.1%)	G 182(98.9%)	C 2(1.1%)	No
G668A R223K	GG 91(98.9%)	GA 1(1.1%)	AA 0(0.0%)	G 183(99.5%)	A 1(0.5%)	Yes
G678C Q226H	GG 91(98.9%)	GC 0(0.0%)	CC 1(1.1%)	G 182(98.9%)	C 2(1.1%)	No
A691C 231 R	AA 58(63.0%)	AC 27(29.3%)	CC 7(7.6%)	A 143(77.7%)	C 41(22.3%)	Yes
C711G 237 L	CC 56(60.9%)	CG 13(14.1%)	GG 23(25.0%)	C 125(67.9%)	G 59(32.1%)	No
T715C S239P	TT 91(98.9%)	TC 1(1.1%)	CC 0(0.0%)	T 183(99.5%)	C 1(0.5%)	Yes
T723C 241 P	TT 91(98.9%)	TC 0(0.0%)	CC 1(1.1%)	T 182(98.9%)	C 2(1.1%)	No
T734T L245P	TT 90(97.8%)	TC 2(2.2%)	CC 0(0.0%)	T 182(98.9%)	C 2(1.1%)	Yes
G766A G256R	GG 83(90.2%)	GA 3(3.3%)	AA 6(6.5%)	G 169(91.8%)	A 15(8.2%)	No

* HWE: Hardy-Weinberg Equilibrium

The haplotype distribution of the 20 nonsynonymous PRNP variants was investigated (Table 7). Variants occurring as singletons were excluded, and analysis of the other nine sites identified 13 major haplotypes. The most frequent was QALHRQNG (40.2%), whereas QALHRRNG (27.7%) and QALHRQNR (3.8%) appeared at lower proportions.

Table 7. Haplotype frequencies of non-synonymous SNPs of PRNP gene in Serra da Estrela sheep. SNPs with allele frequency of one (1) are aggregated in the "Others" category

Haplotypes	A302G Q101R	C407T A136V	C421T L141F	A428G H143R	G461A R154H	A512G Q171R	G513T Q171H	C528A N176K	G766A G256R	*n* = 184
Hap 1	Q	A	L	H	R		Q	N	G	74 (0.4022)
Hap 2	Q	A	L	H	R	R		N	G	51 (0.2772)
Hap 3	Q	A	L	H	R		Q	N	R	7 (0.0380)
Hap 4	Q	A	L	R	R	R		N	G	6 (0.0326)
Hap 5	Q	A	L	H	R		Q	K	G	8 (0.0435)
Hap 6	Q	A	L	H	R	R		N	R	5 (0.0272)
Hap 7	Q	A	L	R	R		Q	N	G	5 (0.0272)
Hap 8	Q	A	F	H	R		Q	N	G	3 (0.0163)
Hap 9	Q	A	L	H	H		Q	N	G	2 (0.0109)
Hap 10	R	A	L	H	R	R		N	G	2 (0.0109)
Hap 11	Q	A	L	H	R		H	N	G	2 (0.0109)
Hap 12	Q	V	L	H	R	R		N	G	2 (0.0109)
Hap 13	Q	A	L	H	H	R		N	G	2 (0.0109)
Others										15

3.2. Estimation of Potential Scrapie Vulnerability

In line with the British National Scrapie Plan (NSP), the European Commission has classified ovine PrP genotypes into five categories reflecting increasing levels of susceptibility to scrapie according to the three main codons (136, 154, and 171). NSP1 comprises the most resistant genotype (ARR/ARR). NSP2 includes genotypes with high resistance, namely ARR/ARQ, ARR/ARH, and ARR/AHQ. NSP3 encompasses genotypes with reduced resistance and is subdivided into NSP3 ARQ/ARQ and NSP3 others, which include AHQ/AHQ, ARH/ARH, ARH/ARQ, AHQ/ARH, and AHQ/ARQ. NSP4 corresponds to a genetically susceptible genotype (ARR/VRQ), while NSP5 includes the most susceptible genotypes, namely ARQ/VRQ, ARH/VRQ, AHQ/VRQ, and VRQ/VRQ (Cosseddu et al., 2007).

In this study 17 sheep (18.5%) had the highly resistant genotype ARR/ARR, 37 sheep (40.2%) had genotypes in the second risk class (ARR/ARH and ARR/ARQ), and 33 sheep (35.9%) in the third risk class, which are considered to have a low genetic resistance to classical scrapie and, therefore, their use in breeding programs should be avoided (Table 8). There were no sheep with genotypes in the fourth and fifth risk classes; however, five sheep showed genotypes not classified in this index (all heterozygous ARQ).

Table 8. Genotypes for the 136, 154, and 171 codons in Serra da Estrela sheep, sorted by classical scrapie risk class. Adapted (Cosseddu et al., 2007).

Risk Class	Genotype	*n* (%)
1	ARR/ARR	17 (18.48)
2	ARR/ARH	2 (2.17)
	ARR/ARQ	35 (38.04
3	ARQ/ARH	1 (1.09)
	ARQ/AHQ	1 (1.09)
	AHQ/AHQ	1 (1.09)
	ARH/ARH	2 (2.17)

	ARQ/ARQ	28 (30.43)
other	ARQ/VRR	2 (2.17)
	ARQ/ARK	1 (1.09)
	ARQ/AHR	2 (2.17)

3.3. Impact of Nonsynonymous SNPs of the PRNP Gene in Serra da Estrela Sheep

Four different results predictions were observed, as shown in Table 9: 12 benign (M112T, H114R, A136V, L141F, H143R, Q171R, Q171K, N176K, Q189L, V213L, R223K, and Q226H), one possibly damaging (Q171H), six probably damaging (Q74S, Q101R, A121S, R154H, Y172F, and S239P), and two unknown (L245P, and G256R).

AMYCO results are shown in Figure 3.

Table 9. PolyPhen-2 prediction of the effect of amino acid substitutions of PRNP nonsynonymous SNPs (AA1: ancestral amino acid; AA2: amino acid derived from nonsynonymous SNP).

Position	AA_1	AA_2	Score	Prediction
74	G	S	1.000	Probably Damaging
101	Q	R	0.621	Probably Damaging
112	M	T	0.007	Benign
114	H	R	0.331	Benign
121	A	S	1.000	Probably Damaging
136	A	V	0.156	Benign
141	L	F	0.411	Benign
143	H	R	0.241	Benign
154	R	H	0.997	Probably Damaging
171	Q	R	0.005	Benign
171	Q	K	0.040	Benign
171	Q	H	0.586	Possibly Damaging
172	Y	F	0.999	Probably Damaging
176	N	K	0.002	Benign
189	Q	L	0.277	Benign
213	V	L	0.002	Benign
223	R	K	0.000	Benign
226	Q	H	0.199	Benign
239	S	P	0.988	Probably Damaging
245	L	P	not available	Unknown
256	G	R	not available	Unknown

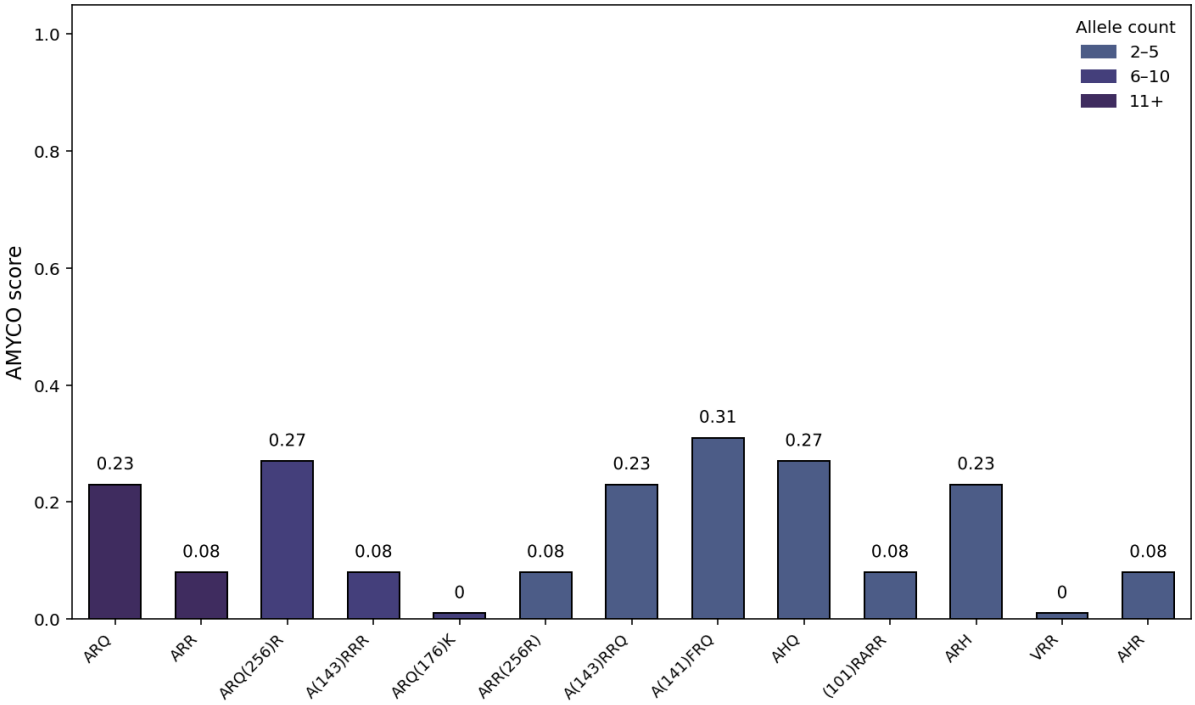

Figure 3. AMYCO scores (as ordered in Table 2) of the haplotypes observed in Serra da Estrela sheep.

4. Discussion

Classical scrapie is a fatal disease, and the main eradication strategies include genotyping and the subsequent selection of animals carrying the ARR/ARR genotype (Iqbal et al., 2020; Regulation (EC) No 999/2001 of the European Parliament and of the Council, 2025). To date, very limited data are available on the presence and characterization of the PRNP gene in sheep of Portugal, specifically the Serra da Estrela breed. To shed light on this matter, the present study analysed the nucleotide sequence of 92 Serra da Estrela sheep and revealed 27 SNPs, 20 of which are nonsynonymous. Results show that the Serra da Estrela sheep breed presents a high frequency of the resistant ARR allele, observed in 58.7% of the sampled population, with 18.5% of individuals being homozygous (ARR/ARR). Previous studies in Portugal had similar values in the same breed as in this study: in 2010, 17.4% ARR/ARR and 42.0% ARR/ARQ were found (Mesquita et al., 2010), and, in 2006, 17.2% ARR/ARR and 43.8% ARR/ARQ (Gama et al., 2006). Since the frequencies of these codons have apparently remained stable throughout these last two decades, it is tempting to speculate that no animal improvement strategies regarding scrapie prevention have been implemented in the Serra da Estrela sheep breed.

Other than the 136, 154, and 171 codons associated with classical scrapie susceptibility, this study also assessed other codons associated with resistance. Two potential resistant variants were observed, namely M112T (0.5% allele frequency) and N176K (6% allele frequency) (Teferedegn et al., 2020). Curiously, previous studies in Portugal or Spain did not take into consideration these two

codons. However, in Italy, these two SNPs have already been reported (M112T with a frequency of up to 31.8% depending on the breed, and N176K of 4.6% in the Sarda breed) (Curcio et al., 2015), and, in Morocco, 2.27% frequency was observed for N176K (Serrano et al., 2007). Since current knowledge suggests these two mutations have a protective effect (Teferedegn et al., 2020), it would be valuable to consider them in future breeding strategies and to use them as surveillance markers in upcoming studies. Although their current frequencies are very low, they can be monitored longitudinally to safeguard genetic diversity and guide future decisions. However, at present, the available data do not support diverting selection pressure away from the ARR allele toward these rare polymorphisms, especially in a breed that already shows a high frequency of ARR.

Additionally, codon 141, associated with atypical scrapie, was also evaluated. The ancestral haplotype codes for leucine (L), but an SNP in nucleotide 421 (C > T) will result in coding phenylalanine (F). This mutation has been associated with susceptibility to atypical scrapie, with AF141RQ and AL141HQ being the variants with the highest susceptibility (Moum et al., 2005; Yang et al., 2014). In the present study, we found an allele frequency of 4.3% for L141F, 2.7% for AF141RQ haplotype, and 2.2% for AL141HQ haplotype. Since there are limited data on this polymorphism in Portugal and research focuses on the main 136, 154, and 171 codons, it would be a good recommendation to have this codon included in future studies to identify susceptibility to atypical scrapie, parallel to classical scrapie. This should also have an impact when applying animal improvement strategies, to avoid accidentally increasing the frequency of haplotypes with increased susceptibility to atypical scrapie;, as previous studies have found cases of atypical scrapie in sheep with ARR/ARR genotypes, it is worth the discussion on whether increasing this genotype frequency to protect from classical scrapie will increase the cases of atypical scrapie in a country with a higher number of reports when compared with other countries.

Other nonsynonymous SNPs found in this study have been described before: Q101R (1.6% allele frequency), H143R (6.5%), and Q189L (0.5%) (Goldmann, 2008; Houston et al., 2015). Noteworthy, to the best of our knowledge, the remaining nine nonsynonymous SNPs (G74S, H114R, A121S, Y172F, R223K, Q226H, S239P, L245P, and G256R) observed in this study have not been described before; lastly, it is of note that all these, with the exception of the SNPs at codons 245 (1.1% allele frequency), 226 (1.1%), and 256 (8.2%), were found in only one allele (0.5%).

Some of the results were marked as unknown by PolyPhen-2, which typically indicates that the algorithm was unable to predict the impact of the substitution due to insufficient sequence conservation data. This often occurs when the affected region lacks reliable multiple sequence alignment, commonly seen in proteins with low-complexity regions, repetitive sequences, or non-globular domains (Adzhubei et al., 2010). A likely explanation in our case is the sequence divergence between the sheep PRNP protein and its human counterpart, which serves as the reference for the

tool's predictions. AMYCO results support the current knowledge of ARR, resulting in the coding of prion proteins less likely to form aggregates and, therefore, more resistant to scrapie. On the other hand, some nonsynonymous SNPs (G256R, for example) might increase aggregation propensity, and others (N176K, for example) might decrease it. A previous study made simulations of breeding strategies that assumed a selection nucleus would be formed, where all breeding males and females, as well as replacement lambs, would be genotyped, with a stable flock of 3000 males and 120 rams; ARR/ARR rams would be preferentially used in ewes carrying the ARR allele. For a breed with similar genotype frequencies (Merino Branco), it was estimated that eight years would be needed to obtain a homozygous classical scrapie-resistant line (Gama et al., 2006). However, the benefits of implementing a classical scrapie resistance breeding program in Portugal, where no clinical cases have been reported, must be balanced against potential drawbacks. Chief among these is the risk of increased inbreeding and reduced genetic diversity, particularly in native sheep breeds such as Serra da Estrela. Additionally, reduced selection pressure for production and adaptation traits, and possible genetic correlations with scrapie resistance, highlight the need for a gradual, balanced approach. Breeding programs should aim to integrate disease resistance with the preservation of genetic variability, productivity, and adaptability, rather than rushing toward full ARR homozygosity. There is also the issue of the farmers' compliance: farmers who might see these programs as overzealous, expensive, and time-consuming. An example of a more gradual and balanced approach, given that reproductive practices in this region, predominantly rely on a limited number of breeding males within each flock (INIAV, 2025), involving genotyping males prior to their use in reproduction, selecting exclusively those homozygous for the AL141RR allele. According to this study's results Serra da Estrela already has a 57.8% ARR allele frequency (18.5% ARR/ARR haplotype frequency), and the undesirable VRQ was not detected; this could mean that genotyping the males would ensure the increase in the AL_{141}RR allele in the flocks without the need to genotype females and lambs, resulting in a slower road towards classical and atypical scrapie resistance, but with better farmer compliance, safeguarding genetic variability, and ensuring preservation of the breed.

This study has some limitations that should be considered. First, both PolyPhen-2 and AMYCO were originally developed for predicting the functional effects of variants in the human PRNP gene, and their application to ovine sequences must be interpreted with caution. Although predictions such as the damaging effect of R154H or the reduced aggregation propensity of ARR highlight potential structural consequences, they do not fully reflect the biological complexity of scrapie. A well-known example of this complexity is the ARR genotype, which provides protection against classical scrapie but has been associated with increased susceptibility to the atypical form (Orge et al., 2010). In our work, these tools were therefore used in an exploratory way, to generate hypotheses rather than to produce definitive conclusions about susceptibility. In addition, eight SNPs identified in this study did

not conform to Hardy-Weinberg equilibrium. While this may be partly explained by low allele frequencies and the limited sample size, deviations could also reflect underlying biological processes such as natural selection, genetic drift, or non-random mating. For example, Q171H, predicted by PolyPhen-2 as possibly damaging, may represent a deleterious variant under negative selection, consistent with the observed departure from equilibrium. Together, these findings suggest that some PRNP polymorphisms could be subject to selective pressures, potentially influencing prion protein function and disease susceptibility. Furthermore, the limited sample size may result in insufficient statistical power for certain low-frequency SNPs, and rare alleles may have been underrepresented or missed entirely. A larger dataset covering additional flocks and regions would be recommended to validate these results, improve statistical power, and provide a more comprehensive picture of genetic diversity and scrapie susceptibility in this native Portuguese breed.

5. Conclusions

This dissertation focused on providing the most comprehensive characterisation to date of PRNP polymorphisms in Serra da Estrela sheep, identifying both known and novel variants with potential implications for scrapie susceptibility. The high frequency of the protective ARR allele, alongside the absence of the highly undesirable VRQ variant, highlights a favourable genetic profile for resistance to classical scrapie. Additionally, the identification of polymorphisms not previously reported, including those with potential protective or deleterious effects, broadens current knowledge of PRNP diversity and highlights the importance of monitoring beyond the traditionally targeted codons.

From a practical standpoint, these findings support the implementation of gradual, balanced breeding strategies that prioritise the use of ARR/ARR rams. Such an approach would enhance scrapie resistance while preserving genetic diversity and ensuring farmer compliance. By integrating genetic surveillance into breeding programs, it is possible to strengthen the long-term sustainability, health, and economic value of this culturally important Portuguese breed.

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Soraia Filipa Serafim Rodrigues

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