Polymorphism Identification and Characterisation within Candidate Genes for Scrapie Susceptibility in Sheep

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χ^2	chi-squared
%	percent
∞	infinity
ø	average
Δ	difference
°C	degrees Celcius
µL	microliter
µm	micrometer
3'-UTR	3'-untranslated region
5'-UTR	5'-untranslated region
A	alanine
aa	amino acid
ACRS	amplification-created restriction site
AG	Aktiengesellschaft
APS	ammonium persulfate
AT	atypical scrapie
bp	base pair
bidest	double-distilled water
BLAST	basic local alignment search tool
BMELV	Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz
BS	German White Mountain sheep
BSE	bovine spongioform encephalopathy
Bt	*Bos taurus*
BTA	*Bos taurus* chromosome
C	cytosine
CFCA	cell free conversion assays
CHN1	chimerin 1 gene
CJD	Creuzfeldt-Jakob disease
CL	classical scrapie
cM	centimorgan
cm	centimeter
CNS	central nervous system
COF	Coburg Fox sheep
CPNE8	copine-8 gene
D	aspartic acid
Dde I	*Desulfovibrio desulfuricans*
DMNV	dorsal motor nucleus of the vagus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E.C.H.C.P.	European Commission on Health and Consumer Protection

EC	European Commission and Council Regulation
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin gallate
ELISA	enzyme linked immunosorbent assay
ENS	enteric nervous system
ER	endoplasmic reticulum
ERC	endosomal recycling compartment
et al.	*et alii*
EtBr	ethidium bromide
EU	European Union
Ex	exon
F	phenylalanine
fCJD	familial Creuzfeldt-Jakob disease
FDC	follicular dendritic cell
G	glycine
GALT	gut associated lymphoid tissue
GGH	German Grey Heath sheep
Gly	glycosylation
GmbH	Gesellschaft mit beschränkter Haftung
GPI	glycosylphoshatidylinositol anchor
Grb2	growth factor receptor-bound protein 2 gene
GWAS	genome wide association studies
H	histidine
Hinf I	*Haemophilus influenzae* Rf
HPLC	high-performance liquid chromatography
HS	Grey Horned Heath sheep
HSA	*Homo sapiens* chromosome
HSPG	heparan sulfate proteoglycan
HWD	Hardy-Weinberg disequilibrium
HWE	Hardy-Weinberg equilibrium
I	isoleucine
iCJD	iatrogenic Creuzfeldt-Jakob disease
IMLC	intermediolateral column
INC	inconclusive
Inc.	Incorporated
INNT	Institut für neue und neuartige Tierseuchenerreger
Intr	intron
ISGC	International Sheep Genome Consortium
K	lysine
k	1000
kDa	kilodalton
L	leucine
LAMR1	laminin receptor gene 1
LOD	logarithm of odds
LR	laminin receptor

LRP	laminin receptor precursor
LRS	lymphoreticular system
M	methionine
mAb	monoclonal antibody
Mb	Megabase
Mbi I	*Moraxella bovis*
MBM	meat and bone meal
min	minute
miRNA	microRNA
MIX	mixed breed sheep
ML	Merino Land sheep
mL	milliliter
mm	millimeter
MMU	*Mus musculus* chromosome
Mnt	meganucleotides
MOOR	German White Moorland sheep
mRNA	messenger RNA
N	asparagine
NaOH	sodium hydroxide
ng	nanogram
ns	no sample
NSP	National Scrapie Plan
nt	nucleotides
nvCJD	new variant Creuzfeldt-Jakob disease
Oa	*Ovis aries*
OAR	*Ovis aries* chromosome
OMS	East Friesian sheep
OPN	osteopontin gene
ORF	open reading frame
P	proline
p.a.	*per analysum*
PCR	polymerase chain reaction
Pint1	prion interactor 1 gene
PK	protein kinase
PNS	peripheral nervous system
pos	positive
PRND	prion protein doppel gene
PRNP	prion protein gene
Prom	Promoter
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{res}	protein kinase resistant core of PrP^{Sc}
PrP^{Sc}	disease-causing prion protein
Pwo	*Pyrococcus woesei*
Q	glutamine
QT	quantitative trait

QTL	quantitative trait locus
R	arginine
RABEPK	rab9 effector protein with Kelch motifs
RARB	retinoic acid receptor beta gene
RFLP	restriction fragment length polymorphism
RH	Rhoen sheep
RNA	ribonucleic acid
rpm	revolutions per minute
RPSA	ribosomal protein SA gene
RTN4	reticulon 4 gene
RV	Romanov sheep
S	serine
SAF	scrapie associated fibril
sCJD	sporadic Creuzfeldt-Jakob disease
SCRG1	scrapie responsive gene 1
SHROP	Shropshire sheep
siRNA	small interfering RNA
SKF	German Blackheaded Mutton sheep
SNP	single nucleotide polymorphism
SPRN	shadow of prion protein gene
SRM	specified risk material
Ssi I	*Staphylococcus sciuri*
STI1	stress-induced-phosphoprotein 1 gene
STMN2	stathmin-like 2 gene
SU	Suffolk sheep
T	thymine
Taq	*Thermus aquaticus*
TBE	Tris/borate/EDTA
TBM	tingible body macrophages
TEMED	Tetramethylethylenediamine
THRB	thyroid hormone receptor beta gene
TNT	tunnelling nanotube
TSE	transmissible spongiform encephalopathy
TX	Texel sheep
u	unknown
U	unit
UV	ultraviolet
V	valine
vCJD	variant Creuzfeldt-Jakob disease
VIM	vimentin gene
W	tryptophan
w/v	mass/volume
WB	Western blot

1 Introduction

1.1 Small Ruminant Scrapie

Scrapie is the oldest known transmissible spongiform encephalopathy (TSE) and affects sheep and goats (Table 1) (Chelle, 1942; Zlotnik and Stamp, 1961). TSEs may present as genetic, infectious or sporadic disorders in which spongiform degeneration and astrocytic gliosis can be found upon microscopic examination of the CNS (Zlotnik and Stamp, 1961), accompanied by deposition of a protease-resistant, aggregated and misfolded form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) (Basler et al., 1986; Brown et al., 1986; Diringer et al., 1983; Prusiner, 1982). Scrapie has never been shown to be a source of TSE infection in humans (Race et al., 2001; Raymond et al., 1997). However, it is widely accepted that meat and bone meal (MBM) from scrapie infected sheep was the source of the bovine spongiform encephalopathy (BSE) epidemic in the UK (Wilesmith et al., 1988). In turn, BSE was shown to cause a newly recognized TSE in humans, the new variant Creuzfeldt-Jakob disease (nvCJD) (Bruce et al., 1997). This prompted wide spread testing for BSE and scrapie in Europe's ruminant population through the establishment of a TSE monitoring and eradication program outlined in the European Parliament and Council Regulation No 999/2001 (EC 999/2001). The concern that scrapie could mask a BSE epidemic in the small ruminant population was substantiated by the identification of a naturally infected BSE positive goat in France in 2004 (Eloit et al., 2005). EC 999/2001 has been implemented to protect consumer and animal health.

1.2 Goal of this Study

It has been established for ovine classical scrapie that, to a high degree, susceptibility is dependent on certain nonsynonymous single nucleotide polymorphisms (SNPs) within the prion protein gene (*PRNP*) locus on ovine chromosome 13 (Belt et al., 1995; Diaz et al., 2005; Goldmann et al., 1994; Hunter et al., 1994). Particular *PRNP* genotypes confer a higher degree of classical scrapie susceptibility whereas others confer a higher degree of classical scrapie resistance in sheep (Baylis and Goldmann, 2004; Goldmann, 2008; Hunter, 1997; Mead, 2006; Tranulis, 2002). Active surveillance, *PRNP* genotyping and breeding programs were established within the EU as a result and implemented in an

effort to increase scrapie resistance in the sheep and goat breeding population (EC 999/2001). With the advent of atypical scrapie (Benestad et al., 2003) as well as accounts of putative resistant sheep succumbing to classical scrapie (Groschup et al., 2007; Ikeda et al., 1995), emphasis has turned to the search for other genetic factors that modulate classical and atypical scrapie susceptibility in sheep as well as in goats.

The goal of this study was to choose specific TSE candidate genes and survey them for mutations that possibly modulate scrapie susceptibility in sheep. In this study, five candidate genes for scrapie susceptibility were screened for SNPs. One SNP within each candidate gene was tested for its influence on susceptibility to classical as well as atypical scrapie in sheep.

2 Literature Review

The advent of TSE active surveillance revealed that scrapie in the EU small ruminant population was more widespread than expected. Also, it lead to an increased recognition of atypical scrapie, a separate scrapie strain affecting the sheep and goat population (Benestad et al., 2003; Götte et al., 2011), which in the field does not seem to be infectious (Lühken et al., 2007) but has proven so in a laboratory setting (Le Dur et al., 2005; Simmons et al., 2007). Classical and atypical scrapie represent separate scrapie strains capable of infecting individual animals dependent on their specific genotype at the prion protein gene (*PRNP*) locus (Arsac et al., 2009; Belt et al., 1995; Diaz et al., 2005; Goldmann et al., 1994; Gretzschel et al., 2005; Hunter et al., 1994; Lühken et al., 2007; Moum et al., 2005; Saunders et al., 2006).

Table 1: Overview of natural small ruminant TSEs.

Species	TSE	Year Identified	Reference
Sheep	classical scrapie	1732	(Schneider et al., 2007)
	atypical scrapie	1998	(Benestad et al., 2003)
Goats	classical scrapie	1942	(Chelle, 1942)
	BSE	2004	(Eloit et al., 2005)
	atypical scrapie	2007	(Seuberlich et al., 2007)

2.1 Historical Aspects of Sheep Scrapie

Recognition of scrapie as a small ruminant disease dates as far back as 1732 and initial investigations were conducted by veterinarians in England, France and Germany (Besnoit, 1899; Comber, 1772; Cuillé and Chelle, 1936; Schneider et al., 2007). Scrapie was shown to be a transmissible disease in 1936 when intraocular inoculation of brain and spinal tissue from an affected sheep transmitted scrapie to two healthy sheep (Cuillé and Chelle, 1936). However, epidemiological studies in the laboratory and in field cases showed that another factor must play a role in determining scrapie susceptibility (Hadlow, 1959; Kingsbury et al., 1983; Sigurdsson, 1954). Suspicion of a genetic component to scrapie susceptibility was expressed as early as 1826: “[Scrapie] is

caused by an external factor. However, one animal is affected more easily than another, i.e. has a certain disposition to [scrapie]“ (Waßmuth, 2001).

2.2 Prion Protein Encoding Gene

The genetic locus responsible for the largest effect on classicial scrapie susceptibility in sheep is the *PRNP* locus (Diaz et al., 2005). *PRNP*, the gene encoding the prion protein, is highly conserved in mammals (Schatzl et al., 1995; Wopfner et al., 1999). *PRNP* is located on mouse chromosome 2 (MMU2 F2|2), human chromosome 20 (HSA 20p13), bovine chromosome 13 (BTA 13q17) and sheep chromosome 13 (OAR 13q15) (Castiglioni et al., 1998; Iannuzzi et al., 1998; Liao et al., 1986; Robakis et al., 1986; Sparkes et al., 1986).

Ovine *PRNP* (Figure 1) is composed of three exons that are 52, 98 and 4028 base pairs (bp) in length (Lee et al., 1998). The 5’-untranslated region (5’-UTR) is 150 base pairs in length and includes exons 1 and 2. The open reading frame (ORF) is 768 bp and is found entirely within exon 3, followed by a 3220 bp 3’-UTR (Goldmann et al., 1990; Liao et al., 1986; Westaway et al., 1994).

In sheep and goats, *PRNP* has been shown to be polymorphic (Baylis and Goldmann, 2004; Bossers et al., 2000; Goldmann et al., 2011; Mead, 2006; Vaccari et al., 2009). Over 40 non-synonymous SNPs have been identified in the sheep *PRNP*, some of which are associated with susceptibility or resistance to scrapie (Belt et al., 1995; Goldmann, 2008; Hunter et al., 1994; Hunter et al., 1997b; Tranulis et al., 1999). In goats, 29 non-synonymous *PRNP* SNPs have been described worldwide, 25 of these in the European goat population (Acutis et al., 2008; Acutis et al., 2006b; Billinis et al., 2002; Fragkiadaki et al., 2011; Vaccari et al., 2009). Of these, 5 are suggested to modulate scrapie susceptibility (Acutis et al., 2012; Bouzalas et al., 2010; Goldmann et al., 1996; Goldmann et al., 2011; Vaccari et al., 2006; White et al., 2012).

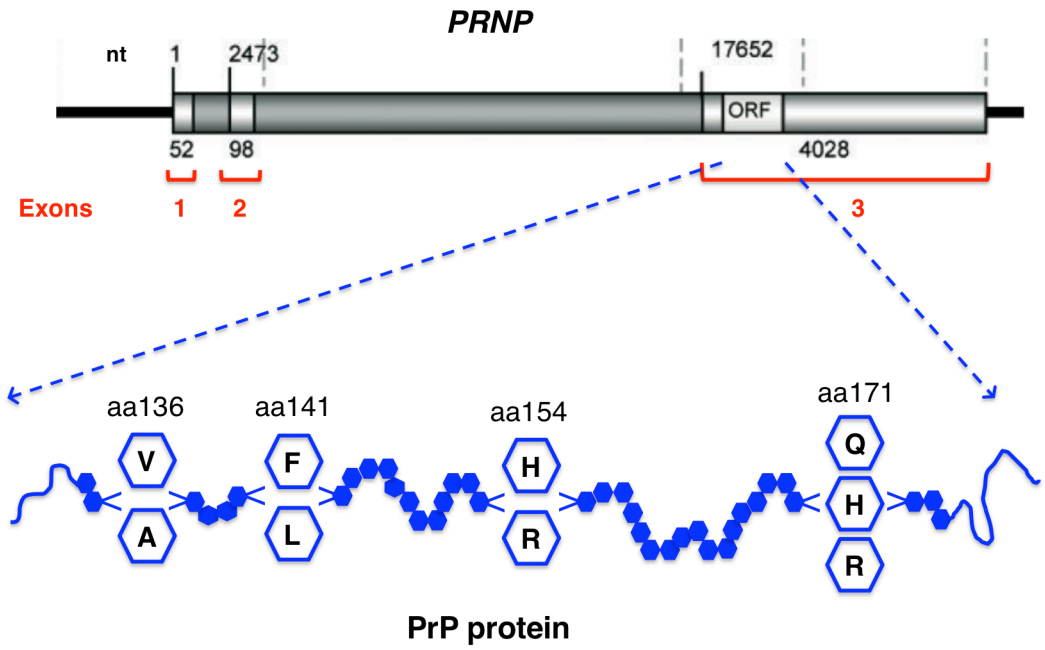

Figure 1: *PRNP* gene structure showing exon 1 (52 bp), exon 2 (98 bp) and exon 3 (4028 bp). The ORF codes for a 256 residue ovine prion protein (PrP) and is highly polymorphic. The polymorphic positions are labelled (136, 141, 154 and 171) that have, so far, been shown to modify scrapie susceptibility in sheep. V, A, F, L, H, R and Q represent the different allelic variants that can appear at each position (Saunders et al., 2009).

2.3 Cellular Prion Protein

Ovine *PRNP* encodes a 4.6 kb mRNA in the brain. The unprocessed ovine protein PrP^{C} comprises 256 amino acids (Figure 2). Many specific structural regions (Figure 2) can be identified in the protein: a carboxy-terminal secretion signal peptide (Hope et al., 1986), five repeat octapeptides (PHGGGWGQ) close to the amino-terminus, a propeptide removed in posttranslational processing, two glycosylation sites, one disulfide bond and one glycosylphoshatidylinositol (GPI) anchor (Stahl et al., 1987; Turk et al., 1988).

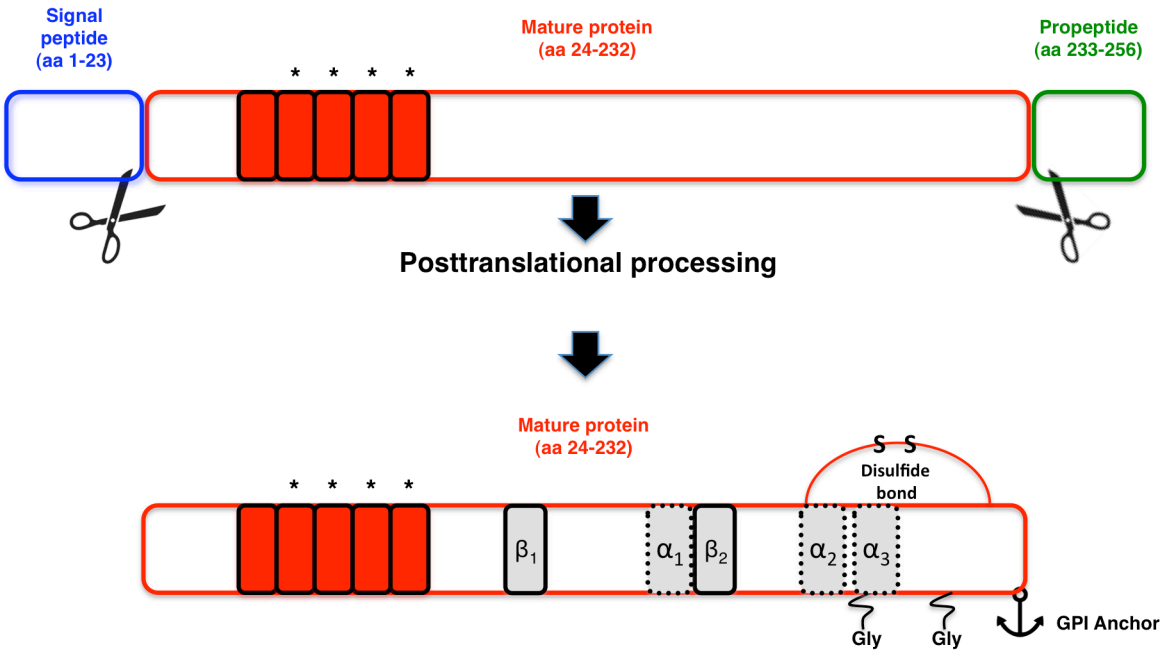

Figure 2: Posttranslational processing of the PrP protein into mature form. The mature ovine PrP contains 5 octapeptide repeats (red) where copper and zinc binding occur (*).Two β sheets (β_1 and β_2) and three α helices (α_1, α_2 and α_3) form the globular C-terminal end, where two glycosylation sites (Gly) are also found (Gasset et al., 1992; Haire et al., 2004).

The three dimensional structure of the prion protein is highly conserved among mammals, despite amino acid substitutions (Genoud et al., 2004; Gossert et al., 2005; Lopez Garcia et al., 2000; Lysek et al., 2005; Riek et al., 1996; Zahn et al., 2000). Ovine PrP^C consists of two regions (Haire et al., 2004; Lysek et al., 2005): a flexible N-terminal region extending to amino acid 145, and thereafter, a C-terminal globular region containing 3 α-helices and 2 β-sheets (Figure 3). Three α-helices are formed from amino acids 146 to 158, 174 to 196 and 203 to 228. Two short, anti-parallel β-sheets are built by amino acids 129 to 134 and 163 to 167 (Eghiaian et al., 2004). It is suggested that the percentage of α-helices in PrP^C is 43%. The mature PrP^C is fully digested after incubation with proteinase K and is soluble in detergent solutions (Meyer et al., 1986).

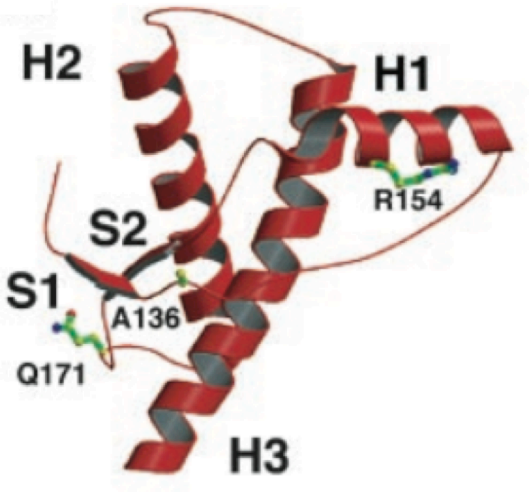

Figure 3: Structure of the globular C-terminal domain of the ovine PrP protein (Eghiaian et al., 2004). Shown are the positions of amino acids 136 (alanine), 154 (arginine) and 171 (glutamine). Beta sheets are labelled S1 and S2. Alpha helices are labelled H1, H2 and H3.

2.3.1 Cellular Biology of PrP^C

PrP^C is synthesized in the endoplasmic reticulum (ER) and transported through the Golgi apparatus to the cell membrane. During posttranslational processing (Figure 2), the immature PrP^C protein undergoes the following: the N-terminal signal peptide (aa 1-23) is removed during trafficking; N-linked glycosylation at aa 184 and 200 occurs; a disulfide bond is formed between aa 182 and aa 217; and following cleavage of the C-terminal propeptide (aa 234-aa 256), a glycosylphosphatidylinositol (GPI) anchor is attached at aa 233 (Campana et al., 2005). Mature PrP^C contains amino acids 24-233 of the original translation product.

The N-terminal signal peptide is responsible for transport of the PrP^C through the secretory pathway to the cell surface membrane (Nunziante et al., 2003). It appears that the GPI anchor associates most mature PrP^C with lipid rafts, which are cell membrane domains high in sphingolipids and cholesterol (Simons and Ikonen, 1997; Soto, 2006). These rafts are organized in the Golgi apparatus and are the preferential localisation of GPI-anchored proteins and proteins involved in signal transduction (Brown and London, 1998).

PrP^C contains two glycosylation sites (Figure 2) and can therefore exist as di-, mono- or unglycosylated glycoforms. Differing ratios of the three glycoforms can be found in different species, strains or even tissues (Hill et al., 2003; Parchi et al., 1997), thus creating a specific glycoprofile of 3 bands upon Western blot analysis (Figure 12).

2.3.2 Prion Protein Expression

PrP^C is expressed in neurons (Kretzschmar et al., 1986), various cells in non-neuronal tissues (Simak et al., 2002), as well as in enterocytes (Morel et al., 2005). More recent studies have also located PrP^C in platelets, leukocytes and red blood cells (Barclay et al., 1999; Dodelet and Cashman, 1998; Panigaj et al., 2011). Cellular localisation depends largely on cell type. In neurons, PrP^C is mainly located on the cell surface (Sunyach et al., 2003; Galvan et al., 2005) whereas another study found PrP^C not only on the cell membrane of dendrites and axons but also in the protein synthesis and endocytic pathways (Mironov et al., 2003). In a neuroblastoma cell line, PrP^C is present predominantly in the late endosomes (Pimpinelli et al., 2005) and on early endocytic or recycling vesicles (Godsave et al., 2008).

2.3.3 Prion Protein Function

The functional role of PrP^C has yet to be elucidated. Because it is found in lipid rafts, it may have a function in signal transduction. Signaling proteins such as neuronal phosphoprotein synapsin Ib, growth factor receptor-bound protein 2 (Grb2), prion interactor 1 (Pint1) and stress-induced-phosphoprotein 1 (STI1) were shown to coimmunoprecipitate with PrP^C (Spielhaupter and Schatzl, 2001; Zanata et al., 2002). Interaction with these proteins may provide a neuroprotective effect (Jeong et al., 2012; Zanata et al., 2002). However, how a GPI-anchored protein such as PrP^C can transmit signals to the cytoplasm remains to be answered (Mouillet-Richard et al., 2000; Spielhaupter and Schatzl, 2001). Other studies (Brown et al., 1997; Kramer et al., 2001) have shown that the PrP^C octapeptide repeats bind copper for uptake into the cell (Figure 2). PrP^C binds less copper in the brains of mice infected with scrapie (Thackray et al., 2002). Patients affected by sporadic Creuzfeldt-Jakob disease (sCJD) show a 50% reduction of copper in the brain (Wong et al., 2001). Structural characteristics of PrP^C have been shown to bind copper and zinc, indicating a role in cell homeostasis of these metals (Pushie et al., 2011). PrP^C also plays a putative role in binding calcium (Whatley et al., 1995).

2.4 Disease-Causing Prion Protein

PrP^C and PrP^{Sc} are considered two isoforms of the prion protein, where PrP^{Sc} is the pathological misfolded isomer of PrP^C (Caughey and Raymond, 1991; Horiuchi et al., 2000; Oesch et al., 1985; Prusiner, 1982, 1998; Prusiner et al., 1982). To date, no genetic or posttranslational differences have been found between PrP^C and PrP^{Sc}. However, studies have shown differences in conformation within the C-terminal globular domain and biochemical properties (Table 2 and Table 1A) such as protease resistance and solubility (Cohen and Prusiner, 1998; Jackson et al., 1999). Detailed structural information for PrP^{Sc} has been difficult to attain due to its insolubility and propensity to form aggregates. The structure has been inferred through various protein-folding models based on the tendency of PrP^{Sc} to form fibrils. The fibrillar structure is similar for all amyloidogenic proteins and consists of a cross-ß structure of perpendicular ß-strands and parallel ß-sheets (Sunde et al., 1997). The α-helices comprise only about 20% of the C-terminal globular domain, whereas a much larger proportion of the C-terminus consists of β-sheets (Pan et al., 1993). Oligomerization with other PrP^{Sc} molecules stabilizes the β-sheets (Rigter et al., 2009).

Table 2: Characteristics of cellular PrP^C and disease-causing PrP^{Sc}.

PrP^C versus PrP^{Sc}		
	PrP^C	PrP^{Sc}
In detergents	soluble	insoluble
Proteolytic digestion	susceptible	resistant
α-helix content	43 %	20 %
Location	cell surface	fibrillar aggregates
Molecular weight	33-35 kDa	33-35 kDa
Molecular weight after PK digestion	degraded	27-30 kDa

2.4.1 $PrP^C \leftrightarrow PrP^{Sc}$ Interaction

The interaction of endogenous PrP^C and PrP^{Sc} is central to scrapie pathogenesis. The presence of host cell, membrane-anchored PrP^C is necessary for disease to occur (Sailer et al., 1994). PrP^C knockout mice do not develop disease after inoculation with PrP^{Sc} (Brandner et al., 1996). It is still unkown how $PrP^C \leftrightarrow PrP^{Sc}$ interaction spurs propagation of nascent infectious PrP^{Sc} molecules. Two models have been proposed: the template-assisted conversion model (Cohen et al., 1994) and the

nucleation/polymerization model (Caughey, 2003). In the first model, a transient PrP conformational intermediate interacts with an endogenous conversion co-factor, which is then capable of forming a heterodimer with PrP^{Sc}. This interaction causes a permanent conformational switch of the PrP intermediate to form a new PrP^{Sc} molecule. The PrP^{Sc} homodimer, consisting of the original and new PrP^{Sc} molecules, can dissociate and each PrP^{Sc} can drive formation of further PrP^{Sc} molecules. An exponential increase in PrP^{Sc} is the result (Cohen et al., 1994). The nucleation/polymerization model suggests that PrP^{C} and PrP^{Sc} coexist in solution, but that monomer PrP^{Sc} is unstable and transient. The presence of an ordered PrP^{Sc} aggregate stabilizes the monomer PrP^{Sc}, allowing it to retain its pathological conformation and join the aggregate .

Conformational change produces endogenous PrP^{Sc} from endogenous PrP^{C}. Endogenous PrP^{Sc} then propagates itself through further recruitment and autocatalytic conversion of additional PrP^{C} (Gambetti et al., 2011). Cell free conversion assays (CFCA) incubating only PrP^{C} and PrP^{Sc} are not very efficient (Kocisko et al., 1995) and PrP^{Sc} amounts must greatly exceed those of PrP^{C} in order to form *de novo* PrP^{Sc}. The low efficiency of this system is thought to explain the long incubation times in prion diesease (Graham et al., 2010). Conversion enhancement is achieved when certain subcellular co-factors are reintroduced into the CFCA, specifically those present in low-density subcellular fractions (Graham et al., 2010; Saborio et al., 1999). These results tend to support the template-assisted conversion model.

One study achieved amplification of infectious, PK-resistant protein when PrP^{C} from brain homogenate, its co-purifying lipid components and the addition of exogenous poly(A)RNA were combined and seeded with PrP^{Sc} in a cell-free system (Deleault et al., 2007). However, continual shaking alone of recombinant PrP^{C} eventually produces fibrils resembling PrP^{Sc} that can cause disease in transgenic mice overexpressing PrP^{C} (Bocharova et al., 2005; Legname et al., 2004).

Where PrP^{C} to PrP^{Sc} conversion occurs has not been completely elucidated. One study has ruled out the early and late endosomes as sites of conversion (Marijanovic et al., 2009), although this contradicts earlier studies (Godsave et al., 2008). Three cell lines infected with three different scrapie strains showed preferential accumulation of PrP^{Sc} in the endosomal recycling compartment (ERC). PrP^{Sc} amounts were reduced when trafficking of PrP to ERCs was inhibited. PrP^{Sc} amounts increased when release of PrP from the ERC was impaired, causing PrP to accumulate in the ERCs.

The GPI anchor has been implicated as necessary for PrP^{Sc} propagation (Chesebro et al., 2005). Monomeric anchorless PrP^{C} is excreted from cells. Transgenic mice with only this form of PrP never develop spongiform changes in the brain or clinical disease after being infected with PrP^{Sc}. However, large extracellular PrP^{Sc} plaques are still observed upon histological examination (Aguzzi, 2005). The GPI anchor may act in directing the PrP to the ERC and embedding it there in a cholesterol enriched membrane, such as lipid rafts, allowing sufficient time and an ideal environment for conversion. Cholesterol depletion has been shown to inhibit PrP^{Sc} formation (Taraboulos et al., 1995). Heterogeneous lipid rafts can be found in various subcellular locations such as ER, ERC as well as in the cellular membrane. GPI anchored proteins and their endocytic route is dependent on cell type and also correlates with length of time spent in lipid rafts (Fivaz et al., 2002). This may explain why PrP^{Sc} conversion occurs only in certain tissues such as neurons, or why certain cells are more susceptible to PrP^{Sc} accumulation (Marijanovic et al., 2009). These results could narrow the search for conversion co-factors to proteins found in the ERC.

2.5 PrP^{Sc} Invasion in Scrapie

2.5.1 Oral Uptake of the Scrapie Infectious Agent

Three hypotheses have been formulated for the uptake of the scrapie agent from the alimentary lumen through the mucosal barrier after ingestion. M-cells have been suspected to transport the PrP^{Sc} across the epithelium (Heppner et al., 2001; Neutra et al., 1996) where it can be taken up by macrophages or dendritic cells and transported to gut associated lymphoid tissue (GALT) (Andréoletti et al., 2000). Post digestion, smaller fragments of PrP^{Sc} could form complexes with ferritin, leading to endocytosis through a ferritin dependent mechanism (Mishra et al., 2004). Lastly, direct uptake could occur through dendritic cell processes extending between epithelial cells to the lumen, a process which has been shown for bacteria (Rescigno et al., 2001). *In vitro*, human enterocytes can internalize PrP^{Sc} derived from BSE brain homogenates through the laminin receptor (LRP/LR) (Morel et al., 2005). No PrP^{Sc} uptake occurred when human enterocytes were incubated with mouse prions or aggregates of purified BSE fibrils. LRP/LR is expressed in many ovine tissues including enterocytes, and colocalizes with scrapie- and BSE-derived PrP^{Sc} (Kolodziejczak et al., 2010; Qiao et al., 2009b).

2.5.2 GALT Invasion

PrP^{Sc} accumulation and propagation occur in the gut associated lymphoid tissue (GALT) of the tonsils and Peyer's patches as shown through infectivity and immunohistochemical studies (Andréoletti et al., 2000; van Keulen et al., 2002). PrP^{Sc} can initially be found in tingible body macrophages (TBM) and later in follicular dendritic cells (FDC) within the B-cell follicles. However, there is often minimal or no replication of PrP^{Sc} detected in the lymphoid tissues of cattle naturally infected with BSE (Iwata et al., 2006; Terry et al., 2003), sheep naturally infected with atypical scrapie (Andréoletti et al., 2011; Benestad et al., 2003) as well as ARR/VRQ sheep naturally infected with classical scrapie (van Keulen et al., 1996). In these animals, neuroinvasion does still occur albeit at a slower rate (Bossers et al., 1996). It is still unknown how PrP^{Sc} reaches the Peyer's patch follicles from the gut lumen. Uptake of PrP^{Sc} occurs in the absorptive epithelium within the first three hours of scrapie agent ingestion as seen by immunohistochemistry in a gut-loop model (Akesson et al., 2011; Jeffrey et al., 2006). Although the follicle-associated epithelium shows signs of activation and transcytosis, no PrP^{Sc} is detectable. However, thirty days after innoculation, activated macrophages in the Peyer's patch follicles contain *de novo* PrP^{Sc}.

Temporally, PrP^{Sc} detection initially occurs in the GALT, followed by further dissemination to non-GALT lymphoid tissues, presumably haematogenically (Hunter et al., 2002; Schmerr et al., 1999; Siso et al., 2009).

2.5.3 Neuroinvasive Routes of PrP^{Sc}

The primary neuronal tissue to harbor PrP^{Sc} is the enteric nervous system (ENS) at the level of the duodenum and ileum (Andréoletti et al., 2000; van Keulen et al., 2002). From here, the PrP^{Sc} is detected along efferent parasympathetic neurons to the dorsal motor nucleus of the vagus (DMNV) located in the brain stem as well as along efferent sympathetic neurons to the intermediolateral column (IMLC) in the spinal cord. Since some sheep can display PrP^{Sc} in the CNS without PrP^{Sc} being found in the lymphoid tissue, other neuroinvasive routes are considered. PrP^{Sc} has been identified within capillary endothelial cells in the hypothalamus as well as in various organs at an early stage after infection, which may indicate haematogenic neuroinvasion through the circulatory system (Siso et al., 2009; van Keulen et al., 2000).

Many cell types and neuroinvasive mechanisms have been proposed to be involved in cell-to-cell transportation of PrP^{Sc}. Tunnelling nanotubes (TNTs) are intercellular tube-like structures containing F-actin that can connect cells over large distances (Rustom et al., 2004). TNTs can form between dendritic cells and neurons in lymphoid tissue as well as between neurons. PrP^{Sc} can travel discretely along the TNT membrane or, encased in vesicles, transported within the TNT itself (Gousset et al., 2009). Since much of the PrP^{C} and PrP^{Sc} cycles through the cell in endosomes, these vesicles may be of endosomal origin. It has already been shown that the endosome is an important structure where conversion of PrP^{C} to PrP^{Sc} occurs (Sunyach et al., 2003). TNTs may represent the predominate or even exclusive mechanism of transferring infectious prions from immune cells to neurons (Gerdes, 2009).

2.5.4 Histopathological Changes in Infected Brains

Histopathological changes in scrapie-infected sheep are seen only in the CNS, despite PrP^{Sc} invasion via the peripheral nervous system (PNS) (Ligios et al., 2002; Wood et al., 1997). Histopathological changes can include diffuse or focal and eventually confluent grey matter vacuolation, salient neuronal loss and activation of astrocytes and microglia without lymphocytic infiltration. Synaptic dysfunction occurs long before neuronal death as well as varying degrees of PrP^{Sc} aggregation (Jeffrey and Gonzalez, 2007).

2.6 Ovine Scrapie

Two forms of ovine scrapie, classical and atypical scrapie, can presently affect sheep. Classical scrapie has been known as a disease affecting European sheep populations since the late 18^{th} century (Comber, 1772). Atypical scrapie has been recognized as a separate disease entity since 1998 (Benestad et al., 2003).

2.6.1 Classical Scrapie in Sheep

Classical scrapie was first described in 1772 as a disease in the British sheep population (Comber, 1772; Schneider et al., 2007). In the 18^{th} and early 19^{th} centuries, scrapie spread rapidly throughout Europe as a result of efforts to improve wool quality through both inbreeding and increased trade of purebred animals (Brown and Bradley, 1998).

2.6.1.1 Routes of Infection

Classical scrapie is mainly spread horizontally within and between flocks but also vertically from ewe to lamb (Dickinson et al., 1974; Pattison et al., 1972). Horizontal transmission occurs during lambing and through environmental contamination (Race et al., 1998; Ryder et al., 2004; Tuo et al., 2001). The main infectious route is oral (van Keulen et al., 2008). Preclinical sheep secrete PrP^{Sc} from the oral cavity and thus can infect flock mates or objects such as feeding troughs (Gough et al., 2012). PrP^{Sc} can persist in the environment and on farm objects that sheep come into frequent contact with (Maddison et al., 2010). Highly sensitive detection methods (Saborio et al., 2001) have also identified PrP^{Sc} in placenta, urine, feces and milk (Gough and Maddison, 2010).

2.6.1.2 Clinical Symptoms

Classical scrapie is endemic in many flocks and outbreaks affect multiple animals within one flock (Lühken et al., 2007). Most scrapie cases are diagnosed in 2 to 5 year old sheep, but have also occcurred in lambs as young as 6 months (Clark and Moar, 1992). The incubation times can vary between 18 months and 5 years and disease progression can be acute or chronic (Capucchio et al., 2001).

Clinical signs of scrapie can be very diverse and insiduous, which can hinder early detection of affected animals in the field (Jeffrey and Gonzalez, 2007). Early, subtle signs may include cardiac arrhythmia and abnormal rumination rates (Austin and Simmons, 1993). An increase in abomasal compaction incidence in sheep with naturally acquired scrapie infection has been observed (Sharp and Collings, 1987; van Keulen et al., 1995). Other nonspecific clinical signs involve weight loss in spite of maintained appetite and intermittent behavioral changes (Kimberlin, 1976). With disease progression, emaciation grows increasingly apparent and behavioral changes, such as hyperreaction to external stimuli or voluntary separation from the flock, become more pronounced and permanent. Conversely, somnolence and apathy could predominate. Affected animals spend more time grooming, rubbing against objects and scratching, resulting in alopecia and pruritis. Progressive ataxia is also a common symptom, characterised by hypermetria. In end stages, ataxia results in recumbency and finally death occurs (Sigurdsson, 1954; Ulvund, 2001; van Bogaert et al., 1978; Vargas et al., 2005). Sheep showing no clinical signs have been slaughtered and widespread vacuolation in the brain was nevertheless observed (Clark et al., 1994). Clinical

symptoms show great variation due to the interactions of age, breed, *PRNP* genotype, scrapie strain and disease progression (Masujin et al., 2009; Ulvund, 2001).

2.6.1.3 Genetic Susceptibility to Classical Scrapie

Studies of genetic susceptibility to classical scrapie identified certain nonsynonymous polymorphisms in *PRNP* codons 136, 154 and 171 that confer degrees of susceptibility or resistance to infection with classical scrapie (Belt et al., 1995; Clouscard et al., 1995; Goldmann et al., 1991; Hunter et al., 1989; Hunter et al., 1996; Hunter et al., 1992; Hunter et al., 1997a). The polymorphisms in these codons that modulate classical scrapie susceptibility are: at codon 136, a valine (V_{136}) or alanine (A_{136}); at codon 154, an arginine (R_{154}) or histidine (H_{154}); and at codon 171, a glutamine (Q_{171}), histidine (H_{171}) or arginine (R_{171}). A haplotype combination is $A_{136}R_{154}R_{171}$ and commonly designated ARR. Five haplotypes arising from these polymorphisms, namely VRQ, ARQ, AHQ, ARH and ARR, have been shown to modulate classical scrapie susceptibility in sheep. Many susceptibility studies in breeds such as Cheviot (Goldmann et al., 1991), Texel (Belt et al., 1995), Rygja (Tranulis et al., 1999), and Suffolk (Hunter et al., 1997b; Ikeda et al., 1995) show that the V_{136} allele strongly confers susceptibility. VRQ/VRQ animals are most susceptible to classical scrapie, the incubation time is short and death occurs quickly. Complete disease penetrance can occur in some flocks with high numbers of VV_{136} animals (Laplanche et al., 1993). However, in other flocks, survival times are increased in VA_{136} sheep bearing the H_{154} and, more importantly, R_{171} alleles. Homozygote ARR/ARR animals are thought to be completely resistant to classical scrapie or the incubation time is longer than life expectancy (Baylis et al., 2002; Goldmann et al., 1994; Hunter et al., 1996; Hunter et al., 1994). Genotype combinations including only the AHQ, ARH and ARQ alleles are associated with a slight genetic resistance to classical scrapie compared to genotypes including these same alleles combined with a VRQ allele. When the aforementioned alleles are combined with ARR (ARR/AHQ, ARR/ARH, ARR/ARQ), genetic resistance increases. In comparison, ARR/VRQ animals are more susceptible to classical scrapie. *PRNP* genotypes have been grouped according to their degree of genetic resistance to classical scrapie (Table 3) as observed for sheep flocks in the United Kingdom (http://www.defra.gov.uk/ahvla-en/disease-control/notifiable/scrapie/nsp/).

To date, four ARR/ARR sheep have been diagnosed with classical scrapie (E.C.H.C.P., 2012; Ikeda et al., 1995), although in two of these cases, the PrP^{Sc} showed slightly lower

PK resistance (Groschup et al., 2007). In addition, studies show flocks with low frequencies of the resistant/susceptible alleles ARR or VRQ, respectively, may not follow the NSP-scheme for classical scrapie resistance or susceptibility (Acin et al., 2004; Billinis et al., 2004; Lühken et al., 2007; Lühken et al., 2004).

Table 3: NSP scheme: *PRNP* genotypes and their risk levels for classical scrapie in sheep in the EU (E.C.H.C.P., 2011). *****Provisional classification for ARQ/ARQ pending scientific review.

Risk level groups lowest risk ➟ highest risk				
NSP1 Genetically most resistant	**NSP2** Genetically resistant	**NSP3** Genetically little resistance	**NSP4** Genetically susceptible	**NSP5** Genetically highly susceptible
ARR/ARR	ARR/ARQ	ARQ/ARQ*****	ARR/VRQ	ARQ/VRQ
	ARR/ARH	AHQ/AHQ		ARH/VRQ
	ARR/AHQ	ARH/ARH		AHQ/VRQ
		ARH/ARQ		VRQ/VRQ
		AHQ/ARH		
		AHQ/ARQ		

2.6.2 Ovine Atypical Scrapie

Five exceptional ovine scrapie cases were reported in Norway in 1998, differing from classical scrapie in their disease phenotype and biological properties of the scrapie strain. This new form of scrapie was denoted Nor98 or atypical scrapie (Benestad et al., 2003) and has since been recognized in many other European countries such as France and Germany (Arsac et al., 2007; Buschmann et al., 2004b), Belgium (De Bosschere et al., 2004), Ireland (Onnasch et al., 2004), Portugal (Orge et al., 2004) and others (Gavier-Widen et al., 2004; Konold et al., 2006). Age of the affected sheep were on average close to two years older than that observed for classical scrapie (Lühken et al., 2007). In most cases, only one sheep in the flock was affected and did not show some of the typical scrapie symptoms such as pruritus or behavioral changes (Gavier-Widen et al., 2004; Lühken et al., 2007). Multiple cases of atypical scrapie in the same flock tended to occur only in larger flocks. It has not yet been established if atypical scrapie is an infectious or sporadic disease.

2.6.2.1 Routes of Infection

In atypical scrapie cases, lymphatic tissue consistently remains free of detectable PrP^{Sc} (De Bosschere et al., 2005), and may explain why atypical scrapie does not seem to be passed horizontally in a flock. Screening of peripheral tissues in neonatal lambs infected with atypical scrapie 12 and 24 months after oral challenge were negative. Atypical scrapie may be a sporadic disease, however, transmissibility has been shown in mice and sheep (Le Dur et al., 2005; Simmons et al., 2007). Mouse bioassays using peripheral, PrP^{Sc} negative tissues from atypical scrapie-infected lambs caused scrapie-like disease (Simmons et al., 2011), showing that atypical scrapie can be orally transmitted. Atypical scrapie may have the potential for natural or iatrogenic transmission.

2.6.2.2 Clinical Symptoms

Most atypical scrapie cases have been diagnosed through active surveillance of apparently healthy sheep at the time of slaughter (Benestad et al., 2008). Some reports of clinical signs have included ataxia, anxiety and loss of body condition (Benestad et al., 2003; Onnasch et al., 2004).

2.6.2.3 Genetic Susceptibility to Atypical Scrapie in Sheep

Three of the five initial atypical scrapie cases described in Norway carried the PrP genotype AHQ/AHQ and two were AHQ/ARQ. Both of these genotypes are rarely associated with scrapie in Norway (Benestad et al., 2003). Furthermore, atypical scrapie is strongly associated with an F variant at codon 141. F_{141} is only found with the ARQ haplotype, constituting the $AF_{141}RQ$ allele (Bossers et al., 1996; Lühken et al., 2007; Moum et al., 2005). Initial genotyping results showed the sheep most susceptible to atypical scrapie carried the AHQ/AHQ genotype, sheep with the VRQ haplotype seemed most resistant to atypical scrapie (Buschmann et al., 2004a; Moum et al., 2005; Orge et al., 2004). Genotypes seeming to confer susceptibility to atypical scrapie contrast with those conferring susceptibility to classical scrapie (Table A1).

2.6.3 *PRNP* Genotype Distributions in German Sheep

Genotyping results of randomly chosen, healthy, slaughtered sheep over 18 months of age in Germany for the past three years are shown below (Figure 4).

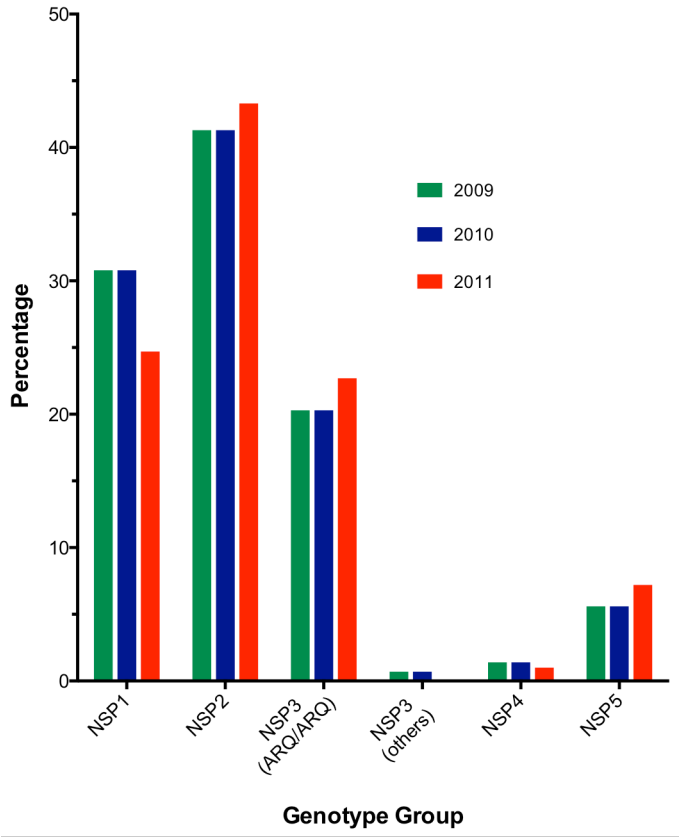

Figure 4: *PRNP* genotype frequencies in German slaughtered sheep according to random sampling results (E.C.H.C.P., 2010, 2011, 2012).

The genotype distribution of classical scrapie cases in Germany from 2002 to 2009 is shown in Figure 5 as reported by Germany to the European Commission Health and Consumers Directorate-General (E.C.H.C.P., 2010, 2011, 2012). Most classical scrapie cases in Germany have been found in sheep carrying the ARQ haplotype and the ARQ/ARQ genotype. The VRQ haplotype is found in a low percentage of classical scrapie cases, but is slighty more common in healthy flock mates. From 2002 to 2004, no classical scrapie case in Germany carried the VRQ haplotype (Lühken et al., 2007).

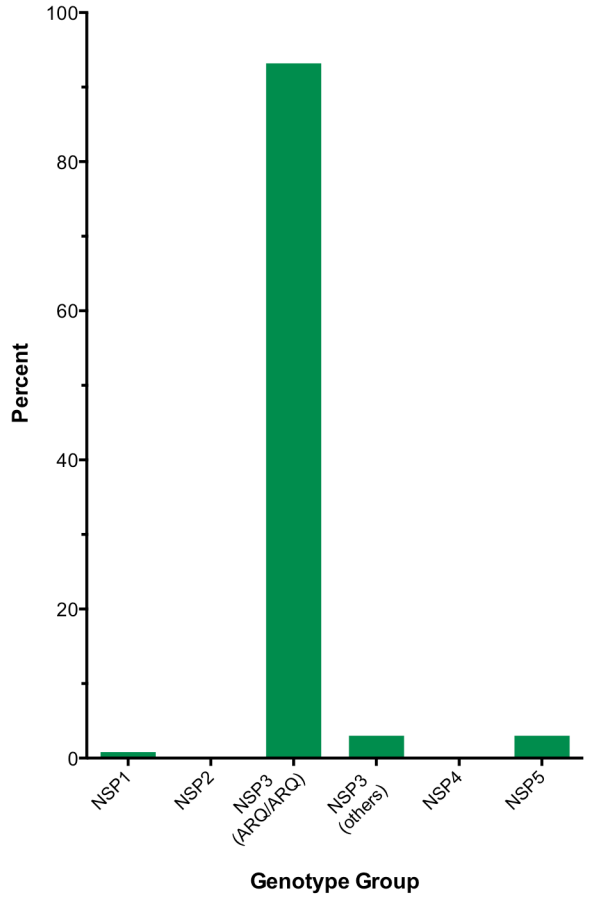

Figure 5: *PRNP* genotype distribution of classical scrapie cases in Germany 2002 to 2009 (E.C.H.C.P., 2010, 2011, 2012).

The genotype frequencies for atypical scrapie cases in Germany from 2002 to 2009 are shown in Table 4. The AHQ haplotype occurs more often in atypical scrapie cases compared to their healthy flock mates whereas the VRQ haplotype occurred infrequently in atypical scrapie cases. The ARR haplotype combined with either ARQ or AHQ seemed to confer a higher risk for atypical scrapie (Lühken et al., 2007).

Similar to atypical scrapie in Norway, the SNP at codon 141 coding for either leucine (L) or phenylalanine (F) is highly significant for atypical scrapie in Germany (Lühken et al., 2007; Moum et al., 2005): haplotypes $AF_{141}RQ$ and AHQ as well as the $AF_{141}RQ/AF_{141}RQ$ or $AF_{141}RQ/AHQ$ genotypes greatly increased risk for atypical scrapie. The genotype $AL_{141}RQ/AL_{141}RQ$ tends to confer higher but not absolute resistance to atypical scrapie in German sheep (Lühken et al., 2007).

Table 4: Genotype distribution (%) of atypical scrapie cases in Germany from 2002 to 2009 (G. Lühken, personal communication) according to NSP group and including F_{141} (here F).

Year	NSP1	AFRQ/ NSP1	NSP2	NSP3 (ARQ/ARQ)	AFRQ/ AFRQ	NSP3 (others)	AFRQ/ NSP3	NSP5
2002	11.1	0.0	33.3	0.0	11.1	33.3	11.1	0.0
2003	6.7	6.7	13.4	13.3	6.7	13.4	33.3	6.7
2004	8.3	5.6	11.1	5.6	0.0	50.0	19.4	0.0
2005	8.0	4.0	16.0	8.0	12.0	24.0	28.0	0.0
2006	21.7	4.3	39.1	0.0	4.3	26.1	4.3	0.0
2007	5.0	15.0	30.0	5.0	0.0	35.0	10.0	0.0
2008	0.0	0.0	0.0	0.0	0.0	42.9	57.2	0.0
2009	0.0	20.0	60.0	0.0	0.0	20.0	0.0	0.0

Figure 6 shows a comparison of genotype distributions in classical and atypical scrapie cases in Germany from 2002 to 2009. Sheep carrying genotypes considered protective against classical scrapie infection seem more susceptible to atypical scrapie (Benestad et al., 2003; Buschmann et al., 2004b; De Bosschere et al., 2005; Madec et al., 2004; Orge et al., 2004). However, German sheep carrying the VRQ allele do not seem as susceptible to classical scrapie as has been shown for other sheep populations (Baylis et al., 2004; Diaz et al., 2005). The ARQ/ARQ genotype shows the highest susceptibility to classical scrapie. This genotype confers a strong degree of resistance to atypical scrapie, along with the L_{141} allele, since the number of positive atypical scrapie cases of this genotype is low compared to the frequency it is found in the affected flocks (Lühken et al., 2007).

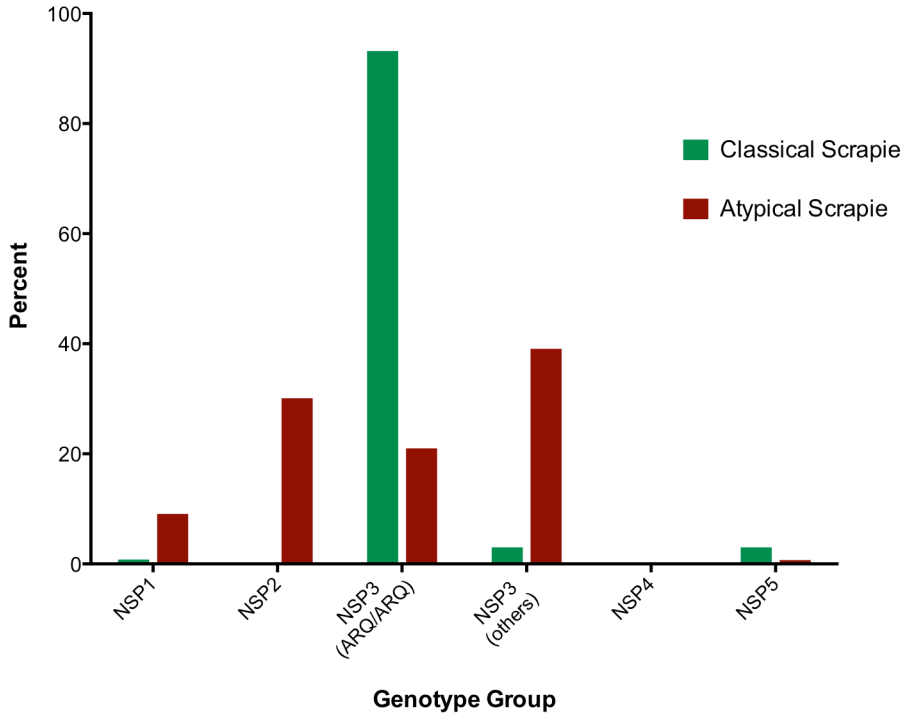

Figure 6: Genotype distribution in sheep positive for classical and atypical scrapie in Germany 2002 to 2009.
(G. Lühken, personal communication).

The frequency of affected genotypes may be influenced by the genotype mosaic present within a flock. Where VRQ is absent, ARQ/ARQ seems to be the most susceptible genotype for classical scrapie (Baylis et al., 2004; Hautaniemi et al., 2012; Hunter et al., 1997b; Westaway et al., 1994). It seems that in German sheep flocks, the absence of a high number of VRQ/VRQ animals may have shifted classical scrapie susceptibility to other genotypes (Lühken et al., 2004).

2.6.4 *PRNP* Genotype Distribution of Scrapie Positive Sheep in the EU

The genotype distributions of classical and atypical scrapie cases in the EU member states from 2002 to 2011 are shown in Figure 7. Breeding goals set out by the European Union (EC 999/2001) have had an effect on the frequency of certain genotypes. From 2005 to 2007, when the use of ARR/ARR rams for breeding was obligatory (2003/100/EC), the frequency of the ARR allele in certain populations increased by more than 10%, causing a concurrent decrease in the frequency of the ARQ allele, as exemplified in the Netherlands. The percentages of the genotypes ARR/ARR, ARR/AHQ and ARR/ARQ increased but ARH/ARH, ARQ/ARQ and VRQ/ARQ decreased. Prevalence of classical scrapie also decreased in the Netherlands during this time period (Hagenaars et al., 2010; Melchior et al., 2010).

In Cyprus, thorough genotyping of the entire sheep population has been ongoing since 2005. The frequency of the ARR/ARR genotype has increased from 15% in 2005 to 71.4% in 2011. In 2011, only 0.7% carried the ARQ/ARQ genotype as compared to 34% in 2005. Genotypes of the NSP2 group have decreased from 44% in 2005 to 26.7% in 2011. This trend has been accompanied by a significant decrease in the prevalence of classical scrapie in Cypriot sheep (E.C.H.C.P., 2006, 2012).

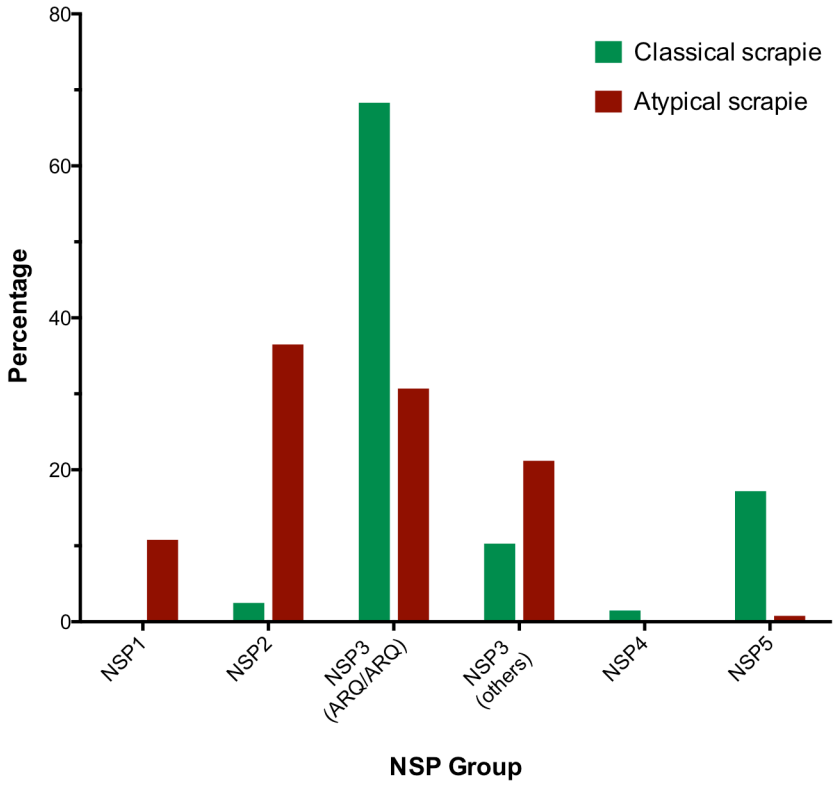

Figure 7: *PRNP* genotype distribution in sheep positive for classical and atypical scrapie in the EU 2002 to 2011 (E.C.H.C.P., 2012).

2.7 Caprine Scrapie

Scrapie has been shown to be transmissible to goats (Cuillé and Chelle, 1936) and most natural cases occur in goats cohoused with sheep affected by scrapie (Billinis et al., 2002; Chelle, 1942; Hadlow et al., 1980; Sofianidis et al., 2006; Toumazos and Alley, 1989). However, scrapie can also occur in goats that have not had contact with sheep (Fankhauser et al., 1982; Wood et al., 1992). Studies have shown that goats innoculated with scrapie can display a wide range of incubation times, with often up to 16 months difference between the shortest and longest times observed (Goldmann et al., 1998; Goldmann et al., 1996; Pattison and Millson, 1962).

Atypical scrapie has also been identified in goats in Switzerland (Seuberlich et al., 2007), Italy (Colussi et al., 2008) and other European countries (E.C.H.C.P., 2011; Fediaevsky et al., 2008).

2.7.1 Routes of Infection

Like classical scrapie in sheep, scrapie in goats can be spread horizontally or vertically (Pattison et al., 1972). Epidemiological studies show that in dairy goat herds, practices

such as cross-fostering or sharing of colostrum rapidly increase scrapie prevalence rates within the herd (Gonzalez et al., 2009).

2.7.2 Clinical Symptoms

Most common clinical symptoms in goats affected by scrapie are pruritis and emaciation, which can be observed in over 80% of cases (Capucchio et al., 2001). However, ataxia, hyperaesthesia, anxiety, aggression, blindness or tremor may be seen in more than 40% of cases (Andrews et al., 1992; Capucchio et al., 2001; Wood et al., 1992). Unlike in sheep, a scratch reflex can not usually be elicited in goats (Acutis et al., 2012).

2.7.3 Genetic Susceptibility to Classical and Atypical Scrapie in Goats

Preliminary results of *PRNP* genetic association with scrapie susceptibility in goats is emerging. However, due to low numbers of affected animals and/or few case control studies, statistical signinficance is limited (Bouzalas et al., 2011; Fragkiadaki et al., 2011).

A nonsynonymous polymorphism at codon 142 (M142I) has been shown to modulate incubation time in goats experimentally infected with two sheep scrapie strains, where M_{142} carriers displayed longer incubation times (Goldmann et al., 1996). In a study of a classical scrapie outbreak in a goat herd in the UK, *PRNP* codon 142 was shown to be associated with scrapie susceptibility but only in goats older than 60 months. M_{142} carriers showed a significantly lower prevalence of infection when compared to those homozygous for isoleucine (II_{142}). Age may have played a role in that increasing infection pressure as the disease spreads over time in a herd may overcome the protective effect of M_{142} (Gonzalez et al., 2009). In another field study, the M_{142} protective effect was only seen in French Alpine and Saanen goats concurrently homozygous for proline (P) at codon 240 (Barillet et al., 2009).

Codon 146 (N146S or D) may also modulate classical scrapie genetic susceptibility in Cypriot goats. None of the goats affected by scrapie carried S or D at codon 146, but these polymorphisms were observed in control and scrapie negative goats (Papasavva-Stylianou et al., 2007). In Greece, S_{146} was only recorded in scrapie negative goats (Fragkiadaki et al., 2011). Oral scrapie challenge of goats revealed that S_{146} heterozygotes remained clinically healthy and rectal biopsies remained negative for significantly longer compared to N_{146} homozygotes (White et al., 2012).

The R154H polymorphism is present in both sheep and goat *PRNP* (Billinis et al., 2002; Laplanche et al., 1993). In a case controlled study of 264 goats from a scrapie infected goat herd, risk of developing scrapie was lower in animals carrying one H_{154} allele, although this allele was present in the flock at a very low frequency (Barillet et al., 2009).

Studies of genetic susceptibility in goats to classical scrapie in both field observations and experimental challenge show an effect of the *PRNP* Q222K polymorphism (Acutis et al., 2006a; Acutis et al., 2012; Vaccari et al., 2006), where K_{222} confers a degree of resistance to infection. In a scrapie outbreak in Italian goat herds, no goats carrying the K_{222} haplotype were affected with scrapie (Acutis et al., 2006a; Vaccari et al., 2006). However, in France, goats heterozygous for K_{222} and affected by classical scrapie have been identified (Barillet et al., 2009). Another study in a Greek goat herd affected by classical scrapie, an aberrant PrP^{Sc} fragment that was not a classical or atypical scrapie isolate was found in 10 clinically healthy goats. Three from ten of the aberrant PrP^{Sc} goats carried the K_{222} polymorphism (Bouzalas et al., 2011). Also in Greece, scrapie positive goats from scrapie endemic flocks carried the K_{222} polymorphism, however no case control data from the flocks exists (Fragkiadaki et al., 2011). In goats orally challenged with scrapie, those carrying one K_{222} allele remained clinically healthy and rectal biopsy results remained negative significantly longer when compared to Q_{222} homozygotes (White et al., 2012).

Reports of atypical scrapie in goats are rare, inhibiting studies on genetic associations with scrapie susceptibility. However, the R154H polymorphism increases susceptibility to atypical scrapie in sheep (Moum et al., 2005). Three goats diagnosed with atypical scrapie all carried the H_{154} mutation (Le Dur et al., 2005; Seuberlich et al., 2007). In a case control study, all goats positive for atypical scrapie had the H_{154} mutation, showing this mutation has a highly significant effect on susceptibility of goats to atypical scrapie (Colussi et al., 2008).

2.8 TSE Prevalence in the EU and Germany

2.8.1 Sheep and Goat Populations

EU-wide active TSE surveillance (EC Regulation 999/2001) came into effect in April 2002 and has since revealed that scrapie is more widespread than previously thought (Baylis and McIntyre, 2004; Buschmann and Groschup, 2005). In 2011, the total EU sheep population as reported by 20 of the 27 member states (no data available for Czech Republic, Denmark, Estonia, Latvia, Luxembourg, Slovenia and Finland as of August 2, 2012) was 85 million (Eurostat, 2011b). Populations of ewes as well as goats used for breeding in the individual EU member states is shown in Table 5.

Table 5: Sheep and goat populations in each EU country for 2010 (E.C.H.C.P., 2011).

COUNTRY	SHEEP POPULATION	GOAT POPULATION
Austria	213 170	37 990
Belgium	121 470	27 190
Bulgaria	1 283 560	344 520
Cyprus	178 260	218 370
Czech Republic	105 190	8 680
Denmark	72 580	7 660
Estonia	49 960	2 570
Finland	62 170	4 620
France	6 814 190	1 159 800
Germany	1 468 150	149 900‡
Greece	8 116 400	4 109 150
Hungary	976 710	37 320
Ireland	2 966 830	6 270
Italy	5 891 940	797 480
Latvia	40 450	12 440
Lithuania	22 990	14 460
Luxembourg	4 330	1 220
Malta	7 520	5 000
Netherlands	644 800	223 250
Poland	208 000	93 640
Portugal	1 953 060	323 360
Romania	7 339 120	728 480
Slovakia	281 980	12 330
Slovenia	89 460	19 820
Spain	17 072 220	2 295 760
Sweden	230 900	+
United Kingdom	15 257 700	49 160

‡ estimated (Eurostat, 2011a); + no data available

2.8.2 Ovine TSE Prevalence and Incidence in the EU and Germany

The overall prevalence rate of ovine TSE cases per 10,000 sheep tested in 2010 in Europe, excluding Greece and Cyprus, was 14.2 (E.C.H.C.P., 2011). In 2010, Greece and Cyprus reported the most scrapie cases in sheep in the European Union. The prevalence rate of positive TSE cases per 10 000 tested sheep slaughtered for human consumption in Germany reported from 2002 to 2010 was 3.2 (E.C.H.C.P., 2011).

Relative to its sheep population, scrapie incidence in Cyprus remains the highest in Europe with 24.7 per 100,000 sheep, followed by Slovenia, Finland and Greece (Figure 8) (E.C.H.C.P., 2011).

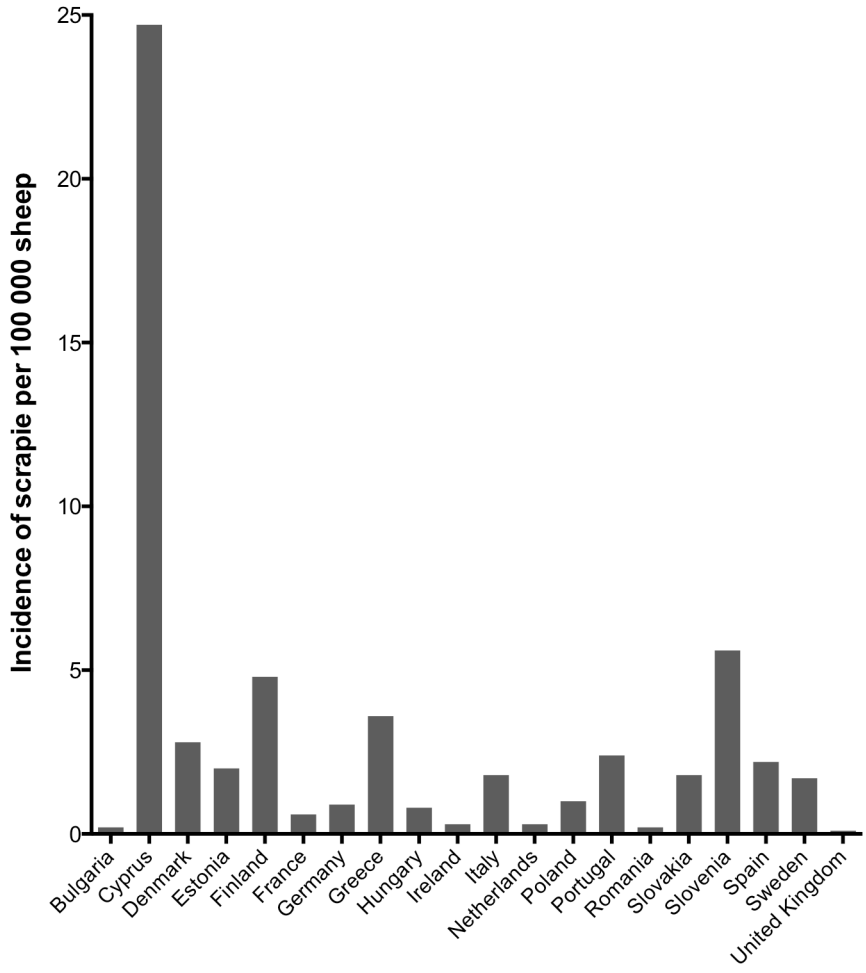

Figure 8: TSE incidence in sheep in 2010. Shown are only EU member states reporting cases in sheep in 2010 (E.C.H.C.P., 2011).

Scrapie incidence as reported for Germany from 2002 to 2011 is shown in Figure 9. Since 2008, overall scrapie incidence in Germany is again slowly increasing. As of November 2012, six scrapie cases have been reported in Germany (BMELV, October 2012).

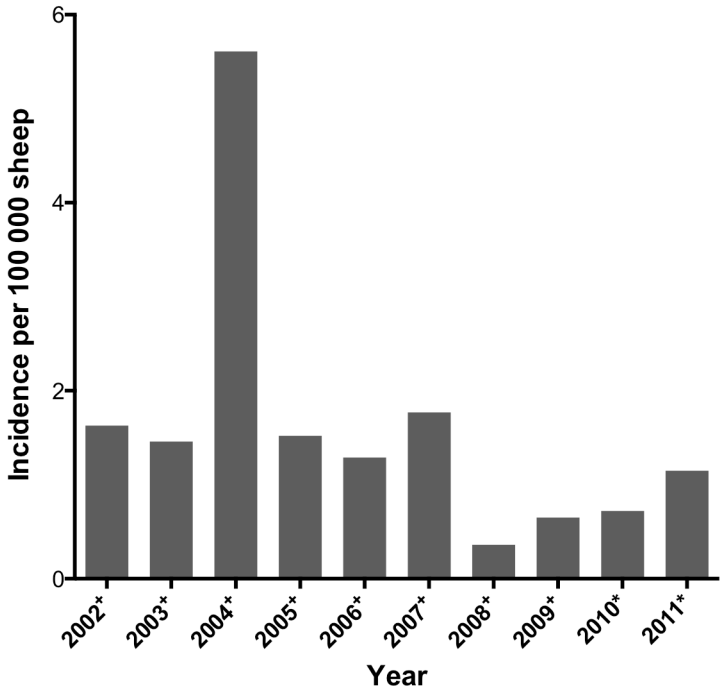

Figure 9: Overall ovine scrapie incidence in Germany 2002-2012. **+** (G. Lühken, personal communication) ***** (BMELV, October 2012).

Trends in the prevalence rates (cases per 10,000 sheep tested) of classical scrapie in sheep differ between member states. Some member states such as Cyprus, France, Netherlands, Germany, Belgium or Ireland have shown steadily decreasing classical scrapie prevalence rates between 2002 and 2011. In 2011, prevalence of classical scrapie in the UK increased for the first time since 2004, from 0.51 in 2010 to 72.5 in 2011. Testing in other states such as Spain and Greece have shown high numbers of classical scrapie cases and prevalence rates have since oscillated year for year around a relatively high average (27.8 and 306.4, respectively) (E.C.H.C.P., 2012).

Atypical scrapie consistently comprises a large portion of all scrapie cases in sheep in some member states such as France (28 of 44 TSE cases in 2010) or Portugal (46 of 47 cases in 2010). All reported scrapie cases in Italy, Denmark and Finland in 2010 were atypical (E.C.H.C.P., 2011; Hautaniemi et al., 2012). Monitoring for atypical scrapie is dependent on testing and sampling methods (E.C.H.C.P., 2011). Early active surveillance in the EU may have underestimated atypical scrapie prevalence, since its detection is highly dependent on method (Benestad et al., 2003; Buschmann et al., 2004a).

2.8.2.1 Distribution of Classical and Atypical Scrapie in Germany

Atypical scrapie has continuously made up an increasingly larger portion of the scrapie cases recorded in Germany since 2002 (Figure 10). This may also reflect the development of more sensitive tests for distinguishing atypical scrapie from classical scrapie (Buschmann et al., 2004a).

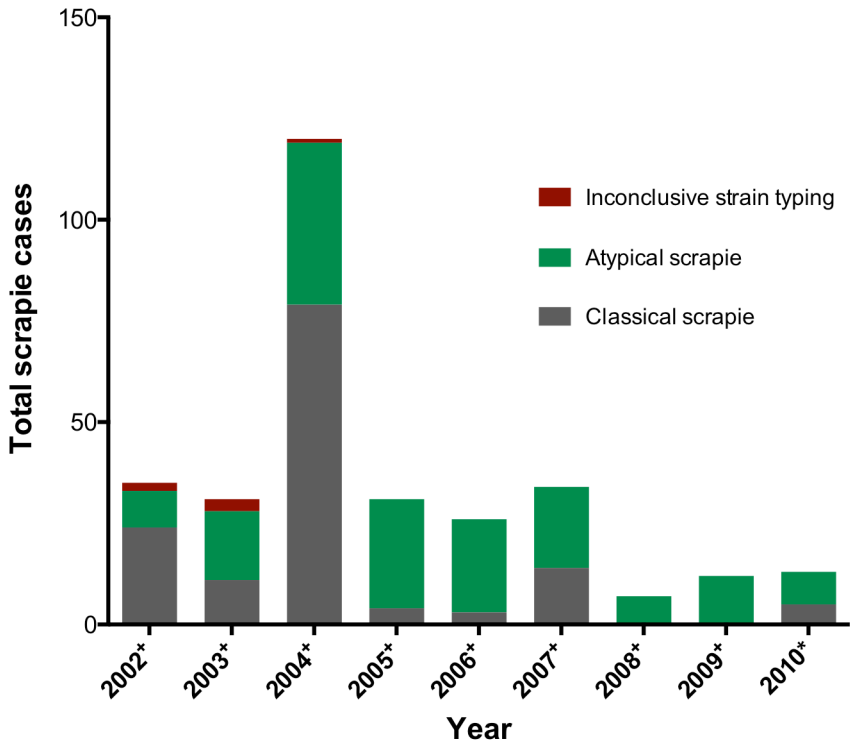

Figure 10: Distribution of scrapie cases by type in Germany.
+ (G. Lühken, personal communication)
* (E.C.H.C.P., 2011)

2.8.3 Scrapie Prevalence in Goats in the EU

The goat population in the EU in 2011 (no data for Belgium, Czech Republic, Denmark, Estonia, Ireland, Latvia, Luxembourg, Slovenia and Finland) was 13 million (Eurostat, 2011a). The total number of goats used for breeding purposes in each EU member state

is shown in Table 5. Prevalence rates of goat scrapie are shown in Figure 11 and are lower than in sheep. Cyprus shows by far the highest scrapie prevalence in goats compared to the remaining EU member states.

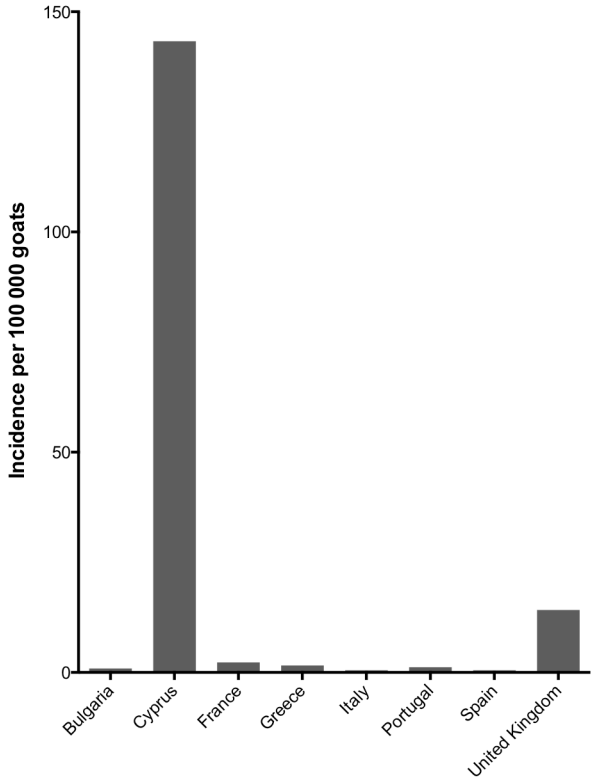

Figure 11: Shown are the scrapie prevalence rates in goats in the EU member states reporting scrapie in goats in 2010 (E.C.H.C.P., 2011).

2.9 BSE in Sheep and Goats

Small ruminants have proven susceptible to experimental BSE challenge (Foster et al., 1993) and BSE has been shown to spread naturally in a sheep flock experimentally infected with BSE (Bellworthy et al., 2005). Before the ban on MBM, sheep and goats were also exposed to contaminated feed supplements, which could have introduced BSE into the sheep and goat population (Kao et al., 2003; Wilesmith et al., 1988). One natural case of BSE was detected in a goat in France (Eloit et al., 2005). In 2010, discriminatory testing of 435 sheep TSE cases as well as 52 goat TSE cases revealed no positive BSE result, although 3 test results were inconclusive (E.C.H.C.P., 2011). Since clinical signs of scrapie and BSE in small ruminants are similar (Foster et al., 2001; Gonzalez et al., 2005), scrapie could mask a coexistent BSE epidemic (Houston and Gravenor, 2003), posing just as much a threat to consumers as BSE in cattle. Transgenic mice expressing human cellular prion protein exhibit a higher susceptibility to infection with sheep and goat passaged BSE than to bovine BSE (Padilla et al., 2011; Plinston et al., 2011).

2.9.1 Genetic Susceptibility to BSE in Sheep and Goats

Studies of sheep and goats experimentally innoculated with BSE have shown that PrP polymorphisms of the host affect incubation period (Foster et al., 2001; Goldmann et al., 2006; Gonzalez et al., 2005; Jeffrey et al., 2001). The mutation R_{171} provides a protective effect against experimental infection of sheep with BSE (Foster et al., 2001; Goldmann et al., 2006). ARR/ARR sheep can develop BSE after intracranial innoculation (Houston et al., 2003). However, ARR/ARR sheep innoculated orally or intraperitoneally with BSE proved resistant (Jeffrey et al., 2001). In goats, genetic susceptibility to BSE is similar to that in sheep, although in equivalent *PRNP* genotypes, goats displayed slightly longer incubation times (Foster et al., 1993).

L_{168}, an allele with low frequency in the study population of 33 ARQ/ARQ Cheviot sheep from New Zealand, has been shown to increase resistance to BSE infection but no association of codon 141 with BSE susceptibility could be found (Goldmann et al., 2006).

2.10 Evidence for Multiple Scrapie Strains

Western blot typing of different TSEs, as well as their stable characteristics such as incubation periods and brain lesion distribution upon innoculation into in-bred mouse models, reveals the presence of multiple scrapie strains in sheep populations (Masujin et al., 2009; Thackray et al., 2011). Different TSE strains most likely circulate together in a population as a mosaic of infectious agents (Groschup et al., 2007; Masujin et al., 2009; Thackray et al., 2011). TSE strains are thought to be encrypted in PrP^{Sc} conformations (Thackray et al., 2011). The successful strain causing disease in the host may be selected depending on the hosts' own PrP genotype and PrP^{C} conformation, as well as the specific cell or tissue environment where initial $PrP^{C} \rightarrow PrP^{Sc}$ conversion occurs. In this scenario, PrP genotype may be only one of many factors affecting susceptibility to a certain TSE strain (Collinge and Clarke, 2007; Gambetti et al., 2011). Classical and atypical scrapie most likely represent two different scrapie strains (Arsac et al., 2009; Götte et al., 2011; Le Dur et al., 2005; Wemheuer et al., 2011). Classical and atypical scrapie in sheep show different profiles on Western blotting (Figure 12). Differences in ovine classical and atypical scrapie are summarized in Table 1A.

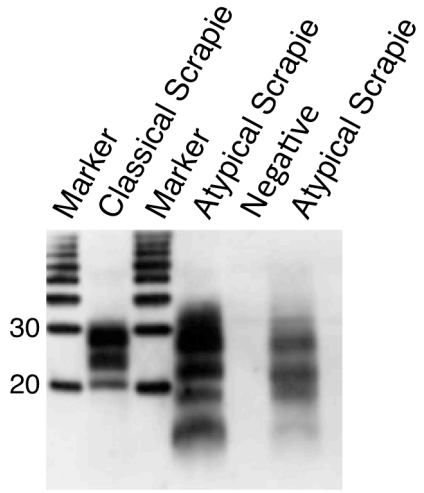

Figure 12: Western blot profiling of the protein kinase resistant core of PrP^{Sc} (PrP^{res}) from different ovine prion strains (De Bosschere et al., 2005).

2.11 Regulations for the Control and Eradication of Small Ruminant Scrapie in the European Union

Legislation regarding the prevention, control and eradication of TSEs binding for all EU member states are established in European Parliament and Council Regulation (EC) No 999/2001 (EC 999/2001). This legislation was introduced to protect human and animal health from the risk of TSEs and stipulates periodic updating in keeping with scientific information.

Live, slaughtered or fallen stock showing or having shown neurological or behavioral disorders, progressive deterioration and/or poor response to treatment where no alternative diagnosis may be made define an animal suspected of TSE infection in this regulation.

2.11.1 Specified Risk Material

Specified risk material (SRM) as defined by Regulation (EC) 999/2001 includes the skull with brain, eyes and tonsils, as well as the spinal cord and spleen of all sheep and goats over the age of 12 months. Where age is not known, this applies to all animals displaying at least one erupted incisor.

2.11.2 Scrapie Monitoring Framework

Scrapie monitoring and eradication measures are based on the Regulation (EC) 999/2001 as amended by Regulation (EC) No 2245/2003. Member states with large sheep populations are required to monitor scrapie utilizing approved rapid tests. Scrapie random sampling in monitored flocks must occur in a certain minimum number of slaughtered, fallen or otherwise perished sheep and goats over 18 months of age or where two permanent incisors are erupted through the gum. In Germany, a minimum of 10,000 sheep slaughtered for human consumption and all or up to 500 goats are to be randomly tested each year (EC No 727/2007).

Member states must name national reference laboratories for monitoring TSEs where samples giving positive rapid test results are sent for confirmation and discriminatory tests. Confirmation and discriminatory tests (EC No 36/2005) of a positive result from a rapid test include Western blot, immunohistochemistry and/or detection of typical scrapie fibrils. If the confirmatory test is positive, TSE strain typing must be carried out through Western blot or mouse bioassay (Beck et al., 2012). The national TSE reference

laboratory for Germany is the Institute of Novel and Emerging Infectious Diseases (INNT) of the Friedrich-Löffler-Institute in Greifswald on the Riems island.

After the occurrence of natural BSE identified in a slaughtered French goat (Eloit et al., 2005), (EC) No 214/2005 set sample size minimum limits on monitoring for TSEs in goats. Member states with large goat populations such as Spain and France were given minimum sampling sizes and in countries with small goat populations, such as Germany, all goats intended for slaughter were to be tested for TSE. This was replaced with (EC) No 727/2007, stating in countries with large goat populations (>750,000 animals) a minimum of 10,000 animals slaughtered for human consumption must be screened for TSEs.

2.11.3 Scrapie Eradication Measures

Commission Decision (EC) 2002/1003 requires all member states to complete a survey of the PrP genotype of each of its sheep breeds. Also, dependent on animal population (EC No 727/2007), either 600 or 100 animals, representative for the entire population, must be genotyped for *PRNP* at codons 136, 141, 154 and 171 each year.

2.11.3.1 Measures Following Confirmation of TSE in a Flock

Movement restrictions are imposed on a flock where TSE is suspected until results of testing become available (EC No 727/2007). If TSE is confirmed in a flock and BSE cannot be ruled out, all animals must be culled. If BSE is ruled out, options are either culling of the entire flock or culling of all animals with the following exceptions: ARR/ARR breeding rams, ARR/XXX breeding ewes not carrying a VRQ allele and ARR/XXX sheep intended for slaughter (EC 999/2001 as ammended by EC 260/2003 and EC No 727/2007). However, animals to be culled may also be slaughtered for human consumption if they are tested for TSE. For up to 50 of these culled or slaughtered animals, PrP genotype is to be determined. Exceptions to culling are possible for flocks with low ARR allele frequency and culling can be delayed up to 5 breeding years (EC No 1428/2007).

If the confirmed TSE in question is atypical scrapie, the infected animal may be culled as well as the parents and the last progeny if female. Or, the flock is subjected to increased TSE monitoring for a 2 year time period where all animals over 18 months, regardless of cause of death, are to be tested. Live animals and embryos originating from the flock are not to be traded to other member states (EC No 727/2007).

All positive TSE cases must be genotyped for PrP at codons 136, 154 and 171. In the case of atypical scrapie, PrP genotype at codon 141 must be determined (EC No 727/2007).

A certain percentage of animals over 18 months culled after TSE confirmation in a flock must be tested for TSE infection (EC No 727/2007).

2.11.3.2 Breeding Program

Requirements for breeding programs for TSE resistance are outlined in both Commission Regulation 1492/2004 and Commission Regulation EC No 727/2007, which amended Regulation (EC) 999/2001. The aim is to increase the classical scrapie and BSE resistant allele ARR in the European sheep population (Melchior et al., 2010).

EC No 727/2007 dictates general breeding requirements for purebred sheep flocks with high genetic merit or those working towards such a status, meaning that all sheep are considered breeding animals belonging to certain purebred breeds under the auspices of a breeding association. A database must be established containing identification of all animals involved, their breed and genotyping results. A breeding program should be devised for each breed taking allele frequencies, breed rarity and inbreeding avoidance into account. All breeding rams must be genotyped and tagged with an individual identification. All genotyped rams carrying a VRQ allele must be slaughtered or castrated within 6 months of test results and are not allowed to leave holding except for slaughter. Female animals carrying a VRQ allele cannot leave the holding except for slaughter. Certain breeds displaying a low incidence of ARR alleles or where certain production traits may be lost are exempted from these requirements.

2.11.4 TSE Road Map 2

The EU Commission Decision 2008/341/EC of 25 April 2008 dictates common criteria, based on EC 999/2001, for national programs for the eradication, control and monitoring of TSEs. The EU Commission Decision 2008/425/EG regulates the submission of these national plans and harmonizes the reporting system to the EU Commission in order to provide epidemiological data on TSEs in each member country. The reporting system also facilitates the financing of the national eradication programs.

In July of 2010, the European Commission published a communication entitled TSE Road Map 2 [COM (2010) 384] (2010) outlining a strategy for the next 5 years, which

gradually repeals TSE control and surveillance measures in accordance with scientific advice. The current measures for livestock are being criticised as too excessive in comparison to the risks currently posed. For example, scrapie prevalence in Germany is low (3.2 per 10000 animals tested) and BSE has not yet been found in the German sheep or goat population after 7 years of active surveillance (E.C.H.C.P., 2011). The cost of conducting a TSE rapid test on a sheep is generally more than the slaughter value of the sheep itself (StMUG, 2011).

2.12 Other Genetic Factors Influencing Susceptibility to Scrapie

The discovery of the prion protein and the genetics of *PRNP* have been able to explain to a certain degree why some flock animals succumb to scrapie infection and others do not. However, it has been estimated that *PRNP* is responsible for approximately 79% of overall genetic susceptibility to scrapie in sheep (Diaz et al., 2005). In one Romanov sheep flock, not all animals with an expected equal degree of scrapie susceptibility and identical PrP genotypes developed clinical signs of scrapie (Elsen et al., 1999). When inbred mouse lines all carrying the same *PRNP* genotype are infected with the same prion strain, the incubation times vary from 100 to 500 days (Carlson et al., 1988; Dickinson, 1975; Westaway et al., 1987). Other genetic loci have been shown to modulate incubation times and/or disease pathogenesis (Booth et al., 2004; Cosseddu et al., 2007; Lloyd et al., 2001; Moreno et al., 2003; Moreno et al., 2010; Stephenson et al., 2000).

2.12.1 Quantitative Trait Locus Analysis

A quantitative trait (QT) is a phenotype that is influenced by more than one gene. Continuous variation in a quantitative trait is believed to be partly due to DNA sequence variations at multiple genes, called loci. QTs can act as precursors to risk of certain diseases, show a strong (>0.8) heritability (Bloom et al., 2013) and the genes underlying these traits can be mapped (Majumder and Ghosh, 2005). Quantitative trait loci (QTL) mapping is a particurlarly strong tool when inbred lines can be developed, such as the established mouse models for scrapie. QTL studies are highly useful in determining genomic regions putatively harboring genes involved in scrapie susceptibility. However, due to the scarcity of mapped markers on certain chromosomes, QTL regions identified can be up to 25 cM, which may contain a great number of genes (Lloyd et al., 2010).

Insufficient numbers of mapped markers is a problem in many species including sheep and cattle.

Many quantitative trait loci (QTL) for classical scrapie susceptibility have been identified in mice (Lloyd et al., 2001; Moreno et al., 2003; Moreno et al., 2010; Stephenson et al., 2000). Table 6 summarizes scrapie QTL studies. Regions on both mouse chromosomes 9 and 11 showed high LOD scores (Stephenson et al., 2000). Other mouse chromosome regions identified included chromosomes 2, 11 and 12 (Lloyd et al., 2001) as well as 5, 6, 7 and 8 (Moreno et al., 2003). Differing results for QTL studies can be attributed to use of different mouse lines and scrapie strains as well as innoculation routes (Iyegbe et al., 2010; Moreno et al., 2003). Candidate genes located in some of these QTL and further investigated in this study are: laminin receptor gene 1 (*LAMR1)* on mouse chromosome 6, scrapie responsive gene 1 (*SCRG1)* on mouse chromosome 8 and prion protein doppel (*PRND)* on mouse chromosome 2 (Table 6).

A study in sheep identified a QTL for classical scrapie incubation time on OAR 18 (Cosseddu et al., 2002). One study showed two QTLs (mouse chromosomes 4 and 8) being involved in increased incubation time in both classical scrapie and BSE (Moreno et al., 2003). Lloyd et al. (2002) identified QTLs on mouse chromosomes 2 and 11 significant for BSE incubation time in mice.

Table 6: Results of scrapie incubation time QTL studies in mice (continued on next page).

Mouse chromosome containing QTL (References)	QTL marker interval	Candidate genes in or near QTL (location)	Chromosome location (human, bovine, ovine)		
			HSA	BTA	OAR
MMU 2 (Cosseddu et al., 2007; Lloyd et al., 2001; Lloyd et al., 2002; Manolakou et al., 2001)	17 cM - 37 cM	*PRND* (131.7 Mb)	20p12	13q17	13q17
	61.2 cM - 98.4 cM	*VIM* (13.5 Mb)	10p13	13q16-17	13*
	6.4 - 29 cM	*CHN1* (73.44 Mb)	2q31.1	2	2
		RABEPK (34.6 Mb)	9q33.3	11	?
MMU 5 (Moreno et al., 2003)	51 cM - 72 cM	*OPN* (58 cM; 104.8 Mb)	4q22.1	6	6*
MMU 7 (Moreno et al., 2003)	2 cM - 18 cM	*SPRN* (147.33 Mb)	10	26	22q24
MMU 8 (Manolakou et al., 2001; Moreno et al., 2003)	24 cM - 64 cM	*SCRG1* (31 cM)	4q34.1	8	2*

Table 6: Results of scrapie incubation time QTL studies in mice (continued).

Mouse chromosome containing QTL (References)	QTL marker interval	Candidate genes in or near QTL (location)	Chromosome location (human, bovine, ovine)		
			HSA	BTA	OAR
MMU 9 (Stephenson et al., 2000)	17 cM - 34.4 cM	*LAMR1* (71 cM)	3p21	22	19q13
MMU 11 (Iyegbe et al., 2010; Lloyd et al., 2001; Lloyd et al., 2002; Stephenson et al., 2000)	1 cM - 65 cM 37 cM - 57 cM	*RTN4* (29.59 Mb)	2p16.3	11	3*
MMU 14 (Lloyd et al., 2002)	23.49 Mb (D14Mit138‡)	*RARB* (17.2 Mb)	3p24	27	?
		THRB (18.49 Mb)	3p24.2	27	?
		LAMR1 pseudogene (17.786 Mb; Gm18078+)	?	?	?
MMU 15 (Manolakou et al., 2001)	17.2 cM - 53.4 cM	*CPNE8*	12q12	5	?

Basic local alignment search tool (BLAST) results with OAR map v 2.0 (March 2011); ‡ LOD score reached level for suggestive linkage; + Gene number

2.12.2 Differential Display Studies

Differential gene expression studies can identify genes involved in scrapie pathogenesis. In classical scrapie, mRNA levels were compared in healthy and scrapie-infected animals, identifying genes that may modulate scrapie pathogenesis (Booth et al., 2004; Cosseddu et al., 2007; Stobart et al., 2007). Vimentin (*VIM*) was found to be differentially expressed between scrapie-infected and non-infected mice (Booth et al., 2004).

2.12.3 Functional Gene Studies

Gene function can be determined after establishment of a knock-out model for the gene in question. Also, protein-protein interactions can be useful in identifying functional gene relationships (Fields and Song, 1989; Limviphuvadh et al., 2007; Uetz et al., 2000). *LAMR1* was discovered to code for a protein acting as the PrP receptor after both proteins consistently coprecipitated (Rieger et al., 1997). In addition, localising a protein in the cell may give hints to functionality (Lechauve et al., 2009; Lin et al., 2008). For example, proteins located within lipid rafts usually play a role in signal transduction (Korade and Kenworthy, 2008).

2.13 Candidate Genes for Scrapie Susceptibility

2.13.1 Laminin Receptor Gene 1

The laminin receptor 1 gene (*LAMR1*; otherwise known as *RPSA*) is found distal on mouse chromosome 9 (Douville and Carbonetto, 1992) and at 3p22.1 on human chromosome 3 (Jackers et al., 1996b). It has also been mapped to bovine chromosome 22 (12.88 Mnt) and ovine chromosome 19q13 (Marcos-Carcavilla et al., 2008; Zimin et al., 2009). It lies just distal to a mice QTL for scrapie susceptibility but is located in a putative second QTL nearby (Stephenson et al., 2000).

The ovine *LAMR1* gene consists of 7 exons and results in the transcription of an mRNA 885 base pairs in length and encodes a 295 amino acid protein (Marcos-Carcavilla et al., 2008; Qiao et al., 2009a). A high level of conservation of the *LAMR1* nucleotide sequence is observed among mammalian species (Rao et al., 1989).

LAMR1 encodes the 37 kDa laminin receptor precursor (LRP), which constitutes the binding ligand of the mature 67 kDa laminin receptor (LR). Unmodified LRP is also a

ribosomal component, indicating a role in mRNA translation (Shmakov et al., 2000). Two LRP molecules are acylated to form the mature 67 kDa LR homodimer (Gauczynski et al., 2001; Hundt et al., 2001). Located on the cell surface, LR binds laminin, elastin, various viruses, epigallocatechin gallate (EGCG) as well as PrP and PrP^{Sc} (Gauczynski et al., 2006; Nelson et al., 2008; Rieger et al., 1999).

LRP and LR exhibit multiple functions depending on their ribosomal, nuclear or extracellular localisation (Nelson et al., 2008) and it seems threshhold levels of *LAMR1* are necessary for different functions. Yeast have two copies of *LAMR1*. When one is disrupted, cell growth is inhibited but knockout of both is lethal (Demianova et al., 1996). It is believed that the C-terminal domain, which is responsible for binding laminin, prions and various viruses, plays a role in cell viability in that apoptosis is prevented. Cleavage of this domain, resulting in the separation of LRP/LR from the cell surface, may promote apoptosis (Mathew et al., 2009). This domain is only present in vertebrates. Of those species showing a high susceptibility to prion disease, many have identical amino acids at positions 241, 272 and 290/291 of LRP (Marcos-Carcavilla et al., 2008; Zhou et al., 2010). Positions 241 and 272 fall within the heparan sulfate proteoglycan (HSPG) - dependent PrP binding domain (Hundt et al., 2001).

In approximately 40% of human small intestinal mucosa samples tested, apical expression of mature laminin receptor is observed. The samples also showed staining for the LRP in the region of the Golgi apparatus. (Shmakov et al., 2000). Human enterocytes take up bovine PrP^{Sc} via LRP/LR (Morel et al., 2005). In scrapie infected mice, tissues accumulating PrP^{Sc} such as spleen and brain display increased levels of LRP/LR (Rieger et al., 1997). On the surface of neuronal and non-neuronal cells, the laminin receptor LRP/LR is responsible for mediating PrP^{C} and PrP^{Sc} binding and internalization through endocytosis (Gauczynski et al., 2001). The inactivation of LRP/LR delays either PrP^{Sc} propagation or accumulation, causing prolonged incubation times in scrapie-infected mice (Pflanz et al., 2009). EGCG intervenes in the formation of PrP^{Sc} in scrapie-infected cells, possibly by occupying the laminin receptor (Rambold et al., 2008).

Ovine LRP amino acids 161-179 have been shown to bind prion proteins, which is also the binding site for laminin. In yeast, LRP binds to the PrP amino acids 144-179 directly. Amino acids 180-285 on LRP bind to PrP at amino acids 53-93, dependent on the presence of HSPGs. Yeast LRP also displays a transmembrane region encompassing amino acids 86 to 101. Heparan sulfate proteoglycans (HSPGs) act as coreceptors,

helping to form a more stable ligand-receptor complex between PrP^{C} or PrP^{Sc} and LRP/LR through the HSPG dependent binding sites (Gauczynski et al., 2001; Hundt et al., 2001; Rieger et al., 1997).

In spleen and brain from BSE-infected mice, LRP concentration is stable. In brains from scrapie infected mice, a 2-fold increase of *LAMR1* mRNA expression is observed (Rieger et al., 1997). In contrast, in the cerebellum of scrapie-infected sheep, levels of *LAMR1* mRNA were significantly reduced (Marcos-Carcavilla et al., 2008).

The genomes of many species contain *LAMR1* pseudogenes. To date, 63 *LAMR1* processed pseudogenes have been found in humans, at least one of those showing evidence of being transcribed (Asano et al., 2004; Balasubramanian et al., 2009; Jackers et al., 1996a). At least 25 *LAMR1* pseudogenes have been indentified in cattle through *in silico* analyses of the bovine genome (Van den Broeke et al., 2010). One of these has been shown to be transcribed (Germerodt et al., 2004). Eleven *LAMR1* pseudogenes are thought to be present in the ovine genome and five of them are transcribed (Marcos-Carcavilla et al., 2008; Van den Broeke et al., 2010).

2.13.2 Scrapie Responsive Gene 1

Scrapie responsive gene 1 (*SCRG1*) is located on mouse and bovine chromosome 8 (Dron et al., 2000; Zimin et al., 2009). Bovine chromosome 8 has been shown to be homologous to the short arm of ovine chromosome 2 (*OAR2p*) (Ansari et al., 1999). Two studies of mouse susceptibility to BSE (Manolakou et al., 2001) and scrapie (Moreno et al., 2003) each identified a QTL on mouse chromosome 8 containing the *SCRG1* gene.

Mouse and human *SCRG1* consist of three exons, where only exon 2 and 3 are protein coding (Dron et al., 2000). It encodes a protein of unknown function consisting of 98 amino acids (Dandoy-Dron et al., 1998; Dron et al., 1998). The hydrophobic N-terminal end could be anchored in the cell membrane or secreted as assumed from the presence of a cleavable signal peptide consisting of the first 20 amino acids (Dron et al., 1998). This gene is conserved in human, chimpanzee, cow, rat, and chicken (Dron et al., 2000).

In mice, *SCRG1* seems to be expressed only in brain tissue and has been identified in cell lines of glial origin (Dron et al., 2000). In humans, *SCRG1* is amply expressed in the brain and spinal cord, but also to a much lesser degree in the aorta and the testes (Dron et al., 1998). *SCRG1* was first identified in scrapie-infected mice using mRNA differential display (Dandoy-Dron et al., 1998). Expression of *SCRG1* in mice increased 2-3 fold in

the brain at 120 days post inoculation, when PrP^{Sc} can be found in the brain but approximately 30 days before clinical signs appear. In BSE infected mice, an increase of *SCRG1* expression was also observed (Dandoy-Dron et al., 2000). In one sample of brain from a CJD patient, *SCRG1* mRNA was also increased 3-fold compared to normal brain (Dandoy-Dron et al., 1998; Dron et al., 1998). This increase also coincides with astrocytosis and microglial activation, both of which may be involved in neuronal death. This gene may play a role in susceptibility to scrapie (Moreno et al., 2003) as well as in neurodegenrative processes (Dron et al., 2005).

More recently, it has been shown that the protein encoded by *SCRG1* plays a role in the neuronal autophagy cascade in TSE disease (Dron et al., 2006). In a *PRNP* knockout mouse model, where ectopic *PRND* expression causes similar pathological changes as in prion disease, *SCRG1* mRNA levels have been measured and were found to be stable, suggesting that the gene itself is not upregulated (Heitz et al., 2010). Rather, the autophagic pathway is interrupted, causing an accumulation of *SCRG1* protein. This interruption of the autophagic pathway may lead to cell death directly or initiate the Bax dependent apoptosis cascade, both of which are observed in TSE infected brains. Scrapie responsive gene 1 and its protein seem to be involved in general neurodegenerative processes but not directly involved in the regulation of PrP^{Sc}.

2.13.3 Prion-like Protein Gene (Doppel)

PRND is located on sheep chromosome 13 (OAR13q17), most likely between 20 (Comincini et al., 2001) and 52 kb (Essalmani et al., 2002) downstream from the *PRNP* gene. This discrepancy could be the result of an insertion or deletion event in the DNA material used in each study (Essalmani et al., 2002). The ovine gene (GenBank accession number AY017311) consists of 2 exons separated by an 1818 bp intron (Comincini et al., 2001). The coding sequence for doppel is exclusively contained in the second exon along with the 3'-UTR (Essalmani et al., 2002). Where *PRNP* is highly polymorphic, *PRND* is not, with only three synonymous ovine SNPs reported to date (Comincini et al., 2001; Mesquita et al., 2010).

PRND encodes the 178 aa doppel protein (Dpl), which shares approximately 25% amino acid homology to the PrP C-terminal region (Tranulis et al., 2001). Like prion protein, doppel protein is expressed during embryogenesis, but in contrast to PrP, at minimal levels in the adult brain. However, doppel protein is highly expressed in the testes of

post-pubertal sheep although its exact function is still unknown (Espenes et al., 2006). Male *PRND*$^{0/0}$ mice are infertile (Behrens et al., 2002).

PRNP promoter-induced *PRND* overexpression in *PRNP*$^{0/0}$ mice leads to Purkinje cell death and cerebral degeneration (Genoud et al., 2004; Moore et al., 1999; Rossi et al., 2001). The reintroduction of PrP reverses the ataxia phenotype, although Dpl expression levels remain the same (Moore et al., 1999). When *PRND* is also deleted from *PRNP*$^{0/0}$ mice, cerebellar degeneration does not occur (Genoud et al., 2004). Reintroduction of an N-terminal truncated PrP does not reverse cerebellar degeneration and ataxia (Shmerling et al., 1998).

Two synonymous mutations in the *PRND* coding sequence were identified in Italian Sarda sheep but were not associated with scrapie susceptibility (Comincini et al., 2001; Essalmani et al., 2002). In a study of 110 sporadic CJD (sCJD) patients compared to 102 healthy controls, a non-coding SNP in the *PRND* 3'-UTR was associated with sCJD occurrence (Jeong et al., 2005a).

2.13.4 Reticulon 4

Reticulon 4 gene (*RTN4* or Nogo) has been mapped in humans (HSA2p16.3), mice (MMU11) and cattle (BTA11) (Church et al., 2011; Oertle et al., 2003; Zimin et al., 2009). BTA 11 is homologous to the short arm of OAR 3 (OAR3p) (Ansari et al., 1999). *RTN4* is located within a mouse QTL for scrapie incubation time (Table 6). The human *RTN4* gene consists of 14 exons and encodes the Nogo-A protein (Diekmann et al., 2005). Typical for this gene is its transcription into at least 10 mRNA isoforms due to alternate splicing and the presence of multiple promotors. However, all vertebrate Nogo isoforms contain a highly conserved domain consisting of the carboxy terminal 201 amino acids (reticulon homology domain, RHD), whereby the amino terminal protein sequences showed no homology (Oertle et al., 2003).

RTN4 is highly expressed in the CNS (Fergani et al., 2005) and is mainly associated with membrane components of the cell such as the endoplasmic reticulum (Roebroek et al., 1998), Golgi apparatus and plasma membrane in oligodendrocytes (Fergani et al., 2005). Nogo-A acts as a potent inhibitor of neurite outgrowth through interaction with a GPI-anchored receptor in the CNS and is involved in apoptosis by sequestering and thus inhibiting anti-apoptotic proteins (Tagami et al., 2000).

Upregulation of *RTN4* occurs in the brains of scrapie-infected mice (Sorensen et al., 2008). Overexpression of two *RTN4* isoforms has been shown to reduce the amount of amyloid-ß-protein, which accumulates as senile plaques in the brains of Alzheimer patients (Selkoe, 2002).

Recently it has been shown that genetic knockdown of *RTN4* in a mouse model for Alzheimer's disease improves learning and memory deficits at an early stage of the disease (Masliah et al., 2010). Nogo is a potent inhibitor of axonal sprouting (Cafferty and Strittmatter, 2006) and may modulate disease progression in Alzheimer's. The compensatory reaction to neuronal loss inhibited by *RTN4* may also play a role following neurodegeneration in scrapie caused by PrP^{Sc} accumulation, thus modulating TSE disease progression (Masliah et al., 2010).

2.13.5 Vimentin

Vimentin (*VIM*) has been mapped in mice (MMU2, 7.0 cM, 87.8 Mb), humans (HSA10p13) and sheep (OAR 13q15) (Church et al., 2011; Deloukas et al., 2004; Iannuzzi et al., 2001), placing it just proximal to a mouse QTL for scrapie incubation time (Table 6) (Lloyd et al., 2001; Manolakou et al., 2001). The human and bovine *VIM* genes are comprised of 9 exons (Deloukas et al., 2004; Zimin et al., 2009) and encode a 466 amino acid protein (Geisler and Weber, 1981; Ikeda et al., 2010).

VIM expression is characteristic for activated astrocytes (Myerowitz et al., 2002; Ridet et al., 1997). Expression is increased in the brains of mice infected with mouse-adapted BSE (Booth et al., 2004).

VIM encodes intermediate filaments required for astrocytic activation (Kraft et al., 2012), which occurs in the brain in response to PrP^{Sc} accumulation (Field and Peat, 1969). Vimentin is a member of the intermediate filament protein family and has been shown to be an important regulator of cell mobility (Tapscott et al., 1981). Cells can respond to a high amount of damaged or abnormal protein, such as PrP^{Sc}, in the cytoplasm by forming a proteinaceous body called an aggresome, where the abnormal protein is held within these aggressomes by a filamentous cage containing vimentin (Johnston et al., 1998). Accumulation of such aggresomes can impair the ubiquitin-proteasome pathway (Bence et al., 2001). Vimentin may functionally interact with PrP^{Sc} in forming aggresomes. Recently, in an Alzheimer's disease mouse model, *VIM* could be knocked out, resulting in reduced astrocytic activation and increased accumulation of amyloid

precursor protein (Kraft et al., 2012). If a similar mechanism exists in scrapie infected brains, TSE disease progression could be modulated by *VIM*.

3 Material and Methods

3.1 DNA Samples

Table 7 shows an overview of ovine DNA samples used in this study. One reference sample of DNA from a Merino Land (ML) sheep was used to test designed PCR primers and establish the PCR reaction for each candidate gene. Ten ovine DNA samples were then sequenced and compared in order to identify single nucleotide polymorphisms (SNPs). SNP validity was tested using sheep family DNA. Lastly, DNA sample sets from 217 scrapie positive sheep as well as from 456 (or 196 for the *PRND* gene) healthy cohort sheep were available for SNP genotyping. In the case of rare SNP alleles, 192 representative DNA samples from 7 Hessian breeds were also genotyped.

Table 7: Overview of ovine DNA sample groups used in this study

DNA samples used in this study	Number of individuals	Description
1) Reference DNA	1	female Merino Land sheep (3.1.1)
2) Sequencing DNA	11	nine different sheep breeds (3.1.2)
3) Sheep Family DNA	32	4 rams, 12 ewes, 16 lambs (3.1.3)
4) Scrapie Positive Sheep	214	from scrapie DNA bank (3.1.4)
5) Scrapie Flock Mate Sheep	654	from scrapie DNA bank (3.1.4)
6) Hessian Breeds	192	seven different breeds from Hesse (3.1.5)

3.1.1 Reference DNA

The reference DNA originated from one female Merino Land (ML) sheep (ear tag number 035), which was kept at the Oberer Hardthof Teaching and Research Facility at Justus-Liebig University, Giessen, Germany. DNA samples from this sheep were consistently used to establish PCR and sequencing reactions for each candidate gene.

3.1.2 DNA Samples for Sequencing

DNA samples routinely sequenced in this study included DNA from 11 individual sheep representing nine breeds (Table 8). Nine sheep were breeding stock on farms in Hesse, Germany and were listed in the Hessian Association of Sheep Breeders and Owners breeding stock register (3.1.5). The remaining two Merino Land (ML) sheep were held at the Oberer Hardthof Teaching and Research Facility at Justus-Liebig University, Giessen, Germany. DNA samples from these animals were routinely sequenced for each candidate gene to provide data for SNP identification and were then further used as positive controls in genotyping of the scrapie DNA bank samples.

Table 8**:** DNA samples from 9 different sheep breeds plus reference sample routinely sequenced and used as controls in genotyping.

Individuals (#)	Sheep Breed	Schafrasse	Abbreviation
1	Coburg Fox	Coburger Fuchsschaf	COF
1	German Blackheaded Mutton	Schwarzköpfiges Fleischschaf	SKF
1	German White Mountain	Weißes Bergschaf	BS
1	Grey Horned Heath	Graue gehörnte Heidschnucke	HS
3	Merino Land	Merinolandschaf	ML
1	Rhoen	Rhönschaf	RH
1	Romanov	Romanov	RV
1	Suffolk	Suffolk	SU
1	Texel	Texel	TX

3.1.3 Sheep Family DNA

Blood for DNA extraction was collected from Merino Land (ML) and Rhoen (RH) sheep families totalling 32 individuals. Pedigree relationships are illustrated in Figure 13. These families were genotyped for each candidate gene SNP to prove Mendelian inheritance segregation of the SNP in question. All sheep were kept at the Oberer Hardthof Teaching and Research Facility at Justus-Liebig University, Giessen, Germany.

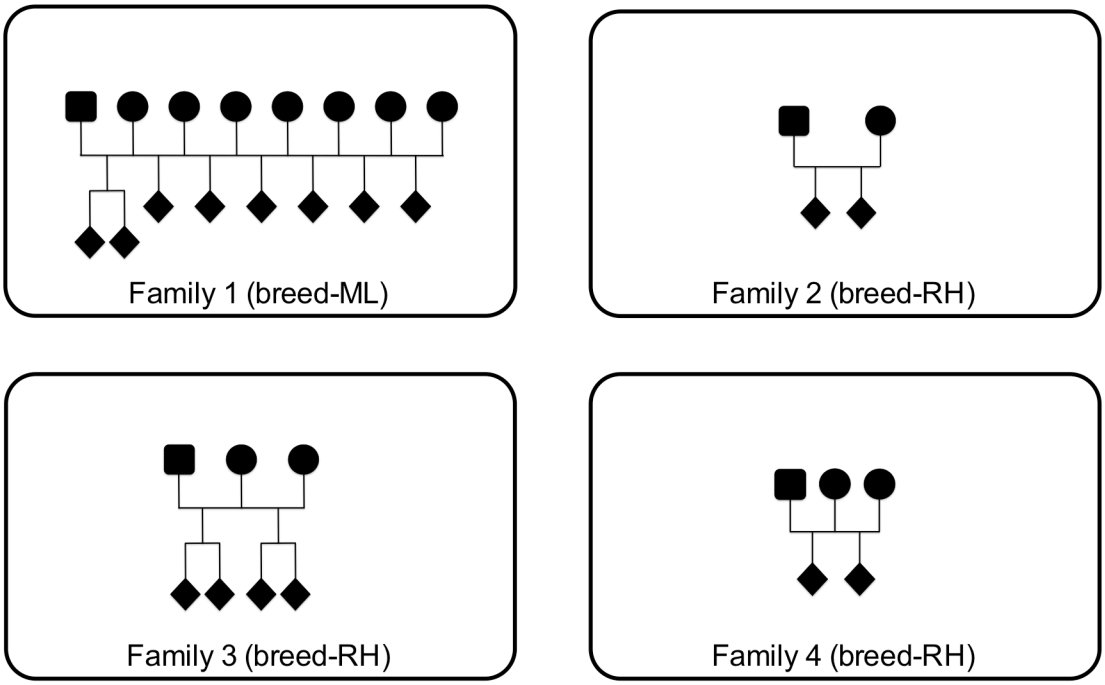

Figure 13: Pedigree relationships within four sheep families (square = ram; round = ewe; diamond = offspring).

3.1.4 Scrapie Sample DNA Bank

All scrapie DNA samples used in this study originated from an existing ovine scrapie DNA bank kept at the Department of Animal Breeding and Genetics, Justus-Liebig University of Giessen, Germany. Samples are made up of DNA from scrapie positive sheep and their apparently healthy, cohort flock mates. Each animal is genotyped with respect to PrP at codons 136, 141, 154 and 171 by sequencing and/or restriction fragment length polymorphism (RFLP) analysis as previously described (Lühken et al., 2007). All available information for each animal is included in the database: sex, breed (exact from breeder or phenotype estimated), age (exact from breeder or dentition estimated), scrapie status (positive or negative) and scrapie type (classical or atypical) were recorded for each animal (Lühken et al., 2007). Most scrapie affected flocks included in the DNA bank were of mixed (MIX) breed. In addition to sheep breeds already mentioned (Table 8), flocks affected by scrapie included in the DNA bank were of the following breeds: Shropshire (SHROP), German Grey Heath (GGH), East Friesian (OMS), and German White Moorland (MOOR). Table 9 summarizes the samples used in this study taken from the scrapie sample DNA bank.

3.1.4.1 Scrapie Positive Sheep DNA

Samples with a positive or questionable result from a previous TSE rapid test were sent to the INNT for verification and determination of scrapie type as described elsewhere (Buschmann et al., 2004a; Gretzschel et al., 2005). DNA samples from scrapie positive sheep identified in German sheep flocks were kindly provided by the research group of Prof. Dr. Martin Groschup, Institute for Novel and Emerging Infectious Diseases (INNT), Greifswald, Germany.

3.1.4.2 Scrapie Sheep DNA

For each scrapie positive DNA sample described in 3.1.4.1, an average of five DNA samples from flock mates were used for association analyses of candidate genes in this study. DNA control samples from apparently healthy flock mates were collected and extracted in a previous project (Lühken et al., 2007). Flock mates were age- and breed-matched to the positive animal whenever possible.

For all candidate genes with the exception of *PRND*, flock mate samples were chosen independent of PrP genotype at *PRNP* codons 136, 141, 154 and 171. Due to the location of both *PRNP* and *PRND* on sheep chromosome 13 (OAR13), for each scrapie positive DNA sample, one *PRNP*-matched DNA sample from a flock mate was chosen for the *PRND* gene (Table 9).

3.1.5 Hessian Breeds

Blood samples of sheep listed in the Hessian Association of Sheep Breeders and Owners (Hessischen Verbandes für Schafzucht und Schafhaltung e.V.) breeding register and their lambs were collected by Hessian Sheep Health Service veterinarians between 2002 and 2005 at the request of the breeders. These samples form part of a DNA bank for sheep breeds in Hesse (Lipsky et al., 2008). These include 32 samples of DNA from each of the following sheep breeds: Coburg Fox (COF), German Grey Heath (GGH), Merino Land (ML), Rhoen (RH), and German Blackheaded Mutton (SKF) as well as a total of 36 samples from Suffolk (SU) and Texel (TX) sheep. Samples were genotyped for an SNP identified in the *PRND* gene to determine its frequency.

Table 9: Information on samples from scrapie DNA bank used for association analyses in this study (continued over next two pages).

Flock Number	TSE Type (pos animal)	Breed	Total TSE Positive Animals	Total Negative Animals	
				All other genes	*PRND*
2	AT	Mix	1	5	1
3	AT	Mix	1	5	1
4	AT	COF	1	5	1
5	AT & INC	Mix	2	5	2
6	AT	ML	1	5	1
7	AT	COF	1	5	1
8	AT	Mix	1	5	1
9	AT	Mix	2	5	2
10	AT	Mix	1	0	1
12	AT	ML	1	5	1
14	AT	Mix	2	5	2
15	INC	Mix	1	5	1
16	CL	Mix	4	5	4
17	AT	Mix	1	5	1
18	AT	Mix	2	5	2
19	CL	Mix	1	5	1
21	CL	SHROP	1	5	1
22	AT	Mix	1	5	1
23	AT	MOOR	1	5	1
24	CL	Mix	4	5	4
25	AT	ML	4	5	4
26	AT	Mix	1	5	1
27	AT	MLW	1	5	1
28	INC	Mix	1	5	1
29	AT	Mix	1	5	1
30	AT	Mix	1	5	1
31	AT	ML	3	5	3
32	AT	COF	1	5	1
33	AT	u	1	0	0
34	AT	Mix	1	5	1
35	CL	Mix	51	50	51
36	CL	TX	2	5	2
37	CL	Mix	1	5	1
38	AT	Mix	2	5	2
41	AT	GGH	1	5	1
42	AT	Mix	1	5	1
43	AT	Mix	1	5	1
44	AT	ML	1	5	1
45	AT	Mix	1	5	1
46	AT	Mix	1	5	1

Flock Number	TSE Type (pos animal)	Breed	Total TSE Positive Animals	Total Negative Animals	
				All other genes	*PRND*
47	AT	ML	1	5	1
50	AT	ML	1	5	1
51	AT	Mix	1	5	1
52	ns	Mix	0	5	1
54	CL	Mix	1	5	1
55	AT	Mix	1	5	1
57	AT	OMS	1	5	1
58	AT	Mix	1	5	1
59	CL	Mix	3	5	3
60	AT	Mix	1	5	1
61	AT	Mix	1	1	1
62	CL	Mix	17	17	15
63	AT	GGH	1	5	1
64	AT	ML	1	5	1
65	AT	Mix	1	5	1
66	AT	Mix	1	5	1
67	CL	Mix	1	5	1
68	AT	Mix	1	5	1
69	AT	Mix	1	5	1
70	AT	Mix	1	5	1
71	AT	Mix	1	5	1
72	CL (11) INC (3)	Mix	14	14	13
73	AT	TX	1	5	1
74	AT	ML	1	5	1
75	CL	Mix	1	5	1
77	AT	BS	1	4	1
78	AT	ML	1	5	1
79	AT	ML	1	5	1
80	AT	Mix	1	5	1
81	AT	Mix	1	2	1
83	AT	Mix	1	5	1
84	AT	u	1	5	1
85	AT	Mix	1	5	1
86	CL	Mix	2	5	1
87	AT	SHROP	1	4	1
88	CL	Mix	1	3	1
89	AT	Mix	1	5	1
90	AT	Mix	1	5	1
91	AT	Mix	1	8	1
92	AT	Mix	1	5	1
93	AT	Mix	1	2	1
94	AT	Mix	1	5	1

Flock Number	TSE Type (pos animal)	Breed	Total TSE Positive Animals	Total Negative Animals	
				All other genes	*PRND*
96	AT	Mix	1	0	1
97	AT	Mix	1	0	1
98	AT	Mix	1	0	1
99	AT	Mix	1	0	1
100	AT	Mix	2	0	2
101	AT	Mix	1	0	1
102	AT	Mix	1	0	1
103	AT	Mix	1	0	1
104	AT	Mix	1	0	1
105	CL	Mix	1	0	1
106	AT	Mix	1	0	1
107	AT	Mix	1	0	1
108	AT	ML	1	0	1
109	AT	Mix	1	0	1
110	AT	Mix	1	0	1
111	AT	Mix	1	0	1
112	AT	Mix	1	0	1
113	AT	Mix	1	0	1
114	AT	ML/Mix	1	0	1

AT=atypical scrapie; CL=classical scrapie; INC=scrapie type inconclusive; ns=no sample; u=unknown

3.2 Materials

3.2.1 Equipment

Item	Company
ABI Prism® 377 DNA Sequencer	Applied Biosystems, Darmstadt
Analysis Scale AC211S	Satorius AG, Göttingen
Double Water Still 2302 Distillation Unit	Gesellschaft für Labortechnik GmbH, Burgwedel
Borosilicat Glass Plates (36 cm)	Applied Biosystems, Darmstadt
CAS-1200™ Pipetting Robot	Corbett Life Science, Sydney, Australia
Centrifuge Biofuge 13R	Heraeus Instruments GmbH, Hanau
Centrifuge 5804 with Rotor A-2-DWP	Eppendorf AG, Hamburg
Centrifuge 5810R with Rotor A-4-62	Eppendorf AG, Hamburg
Centrifuge/Vortex Combi-Spin FVL 2400	Peqlab, Erlangen
Combitips® Plus	Eppendorf AG, Hamburg
Digital Graphic Printer UP-D895 (Sony)	Biometra, Göttingen
Distriman Multipipette	Gilson International B.V., Bad Camberg
Distritips Micro, Mini, and Maxi	Gilson International B.V., Bad Camberg

Item	Company
Electrophoresis Chamber (horizontal), mini & midi	Von Kreutz Labortechnik GmbH, Reiskirchen
Electrophoresis Chamber IBI Model HRH	Intl. Biotechnologies, Inc., New Haven, CT, USA
Filter 595 (∅ 240 mm)	Schleicher und Schuell MicroScience GmbH, Dassel
Filter Tips Biosphere® (100 µL)	Sarstedt, Nümbrecht
Filter Tips (10 µL, 1000 µL)	nerbe plus GmbH, Winsen/Luhe
Filter Tips (20 µL, 200 µL)	Peqlab, Erlangen
Gel Combs (various sizes)	Von Keutz Labortechnik GmbH, Reiskirchen
Gel Trays (various sizes)	Von Keutz Labortechnik GmbH, Reiskirchen
Ice Machine Scotsman AF10	Kälte Mack, Maintal
iCycler 96 well Thermocycler	Bio-Rad, München
Kimwipes®	Kimberly-Clark, Roswell, USA
Latex Gloves	G. Kisker GbR, Steinfurt
Magnetic Mixer MR2002	Heidolph, Kelkheim
Membrane Filter (0.2 µm)	Schleicher und Schuell MicroScience GmbH, Dassel
Microwave	Clatronic, Kempten
Monovette K-EDTA 9 mL	Sarstedt, Nümbrecht
Multipipette	Eppendorf AG, Hamburg
Mylar Shark Tooth Gel Comb (36-well, 0.2 mm)	Applied Biosystems, Darmstadt
Mylar Spacer (0.2 mm)	Applied Biosystems, Darmstadt
Nitril Gloves (Latex-free)	MAGV, Rabenau-Londorf
PCR Cap Strips	VWR International GmbH, Darmstadt
PCR-Cups Soft Tube® with Soft Strip Cap	Biozym, Hess. Oldendorf
PCR Plate PP (96-well)	nerbe plus GmbH, Winsen/Luhe
PCR Strips (0.2 mL)	VWR International GmbH, Darmstadt
Photopaper Type V UPP-110HG (Sony)	MS Laborgeräte Schröder, Wiesloch
Pipetman P10, P20, P100, P200, P1000	Gilson International B.V., Bad Camberg
Pipette Research Pro (0,5-10 µL and 50-1200 µL)	Eppendorf AG, Hamburg
Power Supply for Electrophoresis Chamber Consort E425	MAGV, Rabenau-Londorf
Power Supply for Electrophoresis Chamber Power Pac 1000 and 3000	Bio-Rad, München
Quali PCR Plates (96-well)	G. Kisker GbR, Steinfurt
Reagent Flasks	Sarstedt, Nümbrecht
Thermocycler PE 9600	Perkin Elmer Applied Biosystems GmbH, Weiterstadt
Thermowell Plates (96-well)	Corning Inc., Corning, New York, USA
Transilluminator Ti5	Biometra, Göttingen

Item	Company
Transferpipette®-8 (2.5 mL-25 mL)	Brand GmbH & Co KG, Wertheim
Vortex Reax 2000	Heidolph, Kelkheim
Water bath GFL	MAGV, Rabenau-Londorf
Water bath Julabo 22A	Julabo Labortechnik, Saalbach
Water bath	Köttermann Labortechnik, Uetze-Hänigsen

3.2.2 Chemicals and Reaction Kits

All chemicals are grade p.a. (*per analysum*).

Item	Company
Acrylamide/Bisacrylamide 29:1 (30% w/v)	Bio-Rad, München
Agarose NEEO Ultra Quality	Carl Roth GmbH, Karlsruhe
Ammonium persulfate (APS)	Amresco, Ohio, USA
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Darmstadt
Boric Acid	AppliChem, Darmstadt
Bromophenol blue	E. Merck AG, Darmstadt
Bromophenol blue	United States Biomedical Corp., Ohio, USA
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe
EDTA dipotassium salt dihydrate	AppliChem, Darmstadt
Ethanol	Carl Roth GmbH, Karlsruhe
Ethanol	E. Merck AG, Darmstadt
Ethidium bromide	Serva Feinbiochemica GmbH, Heidelberg
Fast Ruler™ DNA Ladder Low Range	Fermentas, St. Leon-Rot
Formamide	AppliChem, Darmstadt or Carl Roth GmbH, Karlsruhe
Gene Ruler™ 100nt DNA Ladder plus	Fermentas, St. Leon-Rot
Glycerol	Carl Roth GmbH, Karlsruhe
HPLC Water	KMF Laborchemie Handels GmbH, Sankt Augustin
Invisorb® Blood Mini HTS 96 Kit / C-Sheep	Invitek, Berlin
Magnesium Solution [25 mM $Mg(OAc)_2$]	Eppendorf AG, Hamburg
MSB® HTS PCRapace/ C-Kit	Invitek, Berlin
Multiplex PCR Kit	Qiagen, Hilden
N,N,N′,N′-Tetramethylendiamine (TEMED)	Amresco, Ohio, USA or AppliChem, Darmstadt
NuSieve® GTG® Agarose	Cambrex Bio Science, Inc., Rockland, Maine, USA
Rotiphorese® 10X TBE Buffer [1 M Tris-Borate (pH 8.3), 20 mM EDTA, distilled, deionized water]	Carl Roth GmbH, Karlsruhe
Sodium Acetate	Merck KgaA, Darmstadt

Item	Company
Tris(hydroxymethyl)aminomethane (Tris)	MP Biomedicals, LLC, Eschwege
Urea	AppliChem, Darmstadt or Bio-Rad, München
Water - Molecular biology grade	Carl Roth GmbH, Karlsruhe
Wizard® SV Gel and PCR Clean-Up System	Promega GmbH, Mannheim
Xylencyanol FF	E. Merck AG, Darmstadt or United States Biomedical Corp., Ohio, USA

3.2.3 DNA Size Markers

Item	Company
FastRuler™ DNA Ladder, Low range	Fermentas, St. Leon-Rot
GeneRuler™ 100nt DNA Ladder Plus	Fermentas, St. Leon-Rot
pUC Mix Marker 8	Fermentas, St. Leon-Rot
pUC19 DNA/MspI (HpaII) Marker	Fermentas, St. Leon-Rot

3.2.4 Enzymes

Item	Company
BseR I Restriction enzyme	New England Biolabs, Frankfurt a.M.
Dde I Restriction enzyme	New England Biolabs, Frankfurt a.M.
Hinf I Restriction enzyme	Fermentas, St. Leon-Rot
Mbi I Restriction enzyme	Fermentas, St. Leon-Rot
Proteinase K	Invitek, Berlin
Pwo (*Pyrococcus woesei*) Polymerase	Roche Diagnostics GmbH, Mannheim
Ssi I Restriction enzyme	Fermentas, St. Leon-Rot
Taq (*Thermus aquaticus*) Poymerase	Eppendorf, Hamburg

3.2.5 Oligonucleotides

Item	Company
unlabelled Oligonucleotides	biomers.net GmbH

3.2.6 Computer and Internet-Based Programs

Program	Company
BLAST 2.2.25	(Zhang et al., 2000)
ChromasPro Version 1.32	Technelysium Pty Ltd.

Program	Company
ExPASy	http://www.expasy.org/ (Gasteiger et al., 2003)
GenBank	http://www.ncbi.nlm.nih.gov/genbank/
Geneious™ 5.3	Biomatters Ltd., Auckland, New Zealand
Genotyper® V 3.7 NT	Applied Biosystems, Darmstadt
Livestock Genomics	http://www.livestockgenomics.csiro.au/sheep/
Mutation Surveyor®	SoftGenetics, LLC., PA, USA
NEBCutter v2.0	New England Biolabs, Ipswich, MA, USA
Online Encyclopedia for Genetic Epidemiology Studies	http://www.oege.org/software/hwe-mr-calc.shtml (Rodriguez et al., 2009)
Primer3	(Rozen and Skaletsky, 2000)
Prosite	http://prosite.expasy.org/ (Sigrist et al., 2013)
SAS® 8.01	SAS Institute Inc., Cary, NC, USA
SPSS® 18	International Business Machines Corp., Armonk, NY, USA
UniProt	http://www.uniprot.org/ (UniProt, 2012)

3.3 Methods

3.3.1 DNA Extraction

3.3.1.1 From Peripheral Leukocytes

Whole blood sample monovettes were centrifuged at 3000-3500 rpm for 15 minutes at 4°C. Using a capped 1000 µl pipette tip, the buffy coat was transferred to a 1.5 mL eppendorf tube and stored at -20°C. Genomic DNA was isolated from peripheral leukocytes using a modified salt extraction protocol (Montgomery and Sise, 1990) in that no EDTA was added before the samples were incubated with proteinase K.

3.3.1.2 Brain Stem Tissue

DNA from scrapie positive sheep was kindly provided by the research group of Martin Groschup (INNT) at the Friedrich-Loeffler-Institut, Greifswald, Germany. Samples of brain stem tissue from sheep showing positive results after testing with the Bio-Rad Platelia rapid test were sent to the Institute for Novel and Emerging Infectious Diseases (INNT) in Greifswald, Germany. Immunohistochemistry (Hardt et al., 2000) and/or SAF immunoblot confirmed scrapie. DNA was extracted from brain stem tissue samples as described (Buschmann et al., 2004b).

3.3.2 DNA Dilution

A total of fourteen 96-well plates were created using the CAS-1200 Pipetting Robot (Corbett Life Science, Sydney, Australia). Each plate well contained 20 μL of DNA diluted to 10 ng/μL (Table 10).

Table 10: Overview of scrapie DNA sample plates with total numbers of sheep DNA samples.

	Number of Plates	Total Sheep	Total Herds	Scrapie Negative	Scrapie Positive		Number Control DNA Wells	Number Negative Control Wells
					AT	CL		
Scrapie Positive Samples	3	214	81	–	97	117*	–	3
Flock Mate Samples	6	458	81	458	–	–	77	6
PRND Flock Mate Samples	3	196	81	196	–	–	9	4
Hessian Samples	2	192	–	–	–	–	–	–

* 9 positive samples inconclusive (INC) as to scrapie type; AT = atypical scrapie; CL = classical scrapie

3.3.3 Screening for DNA Polymorphisms in Candidate Genes

3.3.3.1 Primer Design

Primers were designed with the help of the Primer3 program (Rozen and Skaletsky, 2000). Optimal primer conditions were chosen to be approximately 21 bp in length with an approximate annealing temperature of 60°C and a GC clamp of 1 or 2. Candidate gene sequences were taken from GenBank reference sequences (Table 11).

Where ovine gene sequences were not available, sequences from cattle, human and/or mouse were aligned using ChromasPro. Primers were then chosen using the bovine sequences from homologous domains between the different species. PCR products were designed to be approximately 800 bp in size and to cover as many exons as possible.

To avoid targeting pseudogenes, a primer pair was designed with the forward primer in an exon and the reverse primer in an intron. The intron sequences of known genes of other species were aligned and primers were chosen from intron segments showing a high amount of similarity. Table 11 lists candidate genes analyzed in this study, the primer pairs designed to amplify candidate gene domains as well as the GenBank sequences from which the primers originated. Primers were diluted to a concentration of 10 µM with HPLC water upon arrival and stored at -20°C or at 4°C when in use.

Table 11**:** Primer information for the candidate genes analyzed in this study.

GENE	GENOMIC REGION	FORWARD (5'→3') (Origin, *Species*)	REVERSE (5'→3') (Origin, *Species*)	FRAG–MENT SIZE (bp)
LAMR1	Exon 5 → Exon 7	LAMR1 Ex5f **TCAGTGGGTCTGATGTGGTG** (NM174379, *Bt*)	LAMR1 Ex7r **GTAGGAACCACCACCGAGTG** (NM174379, *Bt)*	374
LAMR1	Exon 5 → Intron 6	LAMR1 Ex5f **TCAGTGGGTCTGATGTGGTG** (NM174379, *Bt*)	LAMR1 In6r **GATGTGGCTGATGACCTCCT** (NW930073, *Bt*)	542
SCRG1	Exon 1 → Intron 1	SCRG1 Ex1f **GTTGGACTAACTTTGCTGCTAGG** (XM593394, *Bt*)	SCRG1 In1r **GGAGTTTGCTGAAACTTGTCAC** (XM593394, *Bt*)	310
PRND	Prom → Exon 1	Prom f **GAATTCACATTCAGGAAGAGTGATG** (AF394223, *Oa*)	Ex 1r **AGGTGCGTCAGAGAGATCCTAA** (AF394223, *Oa*)	465
PRND	Exon 2	Ex 2f **TCCGACACAATGAGGAAACA** (AF394223, *Oa*)	Ex 2r **TAGCATCTGGCCCACCATAT** (AF394223, *Oa*)	830
PRND	Exon 2 → 3'UTR	PRND Ex2f B **TGTGACTTTTGGTTGGAAAGG** (AF394223, *Oa*)	PRND 3UTRr **TAAGCCAGGGTTGAATTATGG** (AF394223, *Oa*)	576
RTN4	Exon 6 → Exon 8	RTN4 Ex6f **TGTGGGTATTTACCTATGTTGGTG** (BC116111, *Bt*)	RTN4 Ex8r **GGACTTGCAAATAAGAATGTTAAAGA** (BC116111, *Bt*)	1201
RTN4	Intron 7 → Intron 7	RTN4 Intr7f **GGCTAACAAATCTTAGAACTGTGA** (BC116111, *Bt*)	RTN4 Intr7r **TGAACGGCATCAGGTAATTT** (BC116111, *Bt*)	166
VIM	Exon 1 → Exon 2	VIM Ex1f **GAGCTACGTGACCACATCCA** (AF251147, *Oa*)	VIM Ex 2r **TGGAGCAGCAGAACAAGATC** (AF251147, *Oa*)	307

Origin denotes GenBank accession numbers from which the primer pairs were designed. *Bt* = *Bos taurus*; *Oa* = *Ovis aries*; A denotes a mismatch base created for amplification-created restriction site analysis (Haliassos et al., 1989).

3.3.3.2 Polymerase Chain Reaction

To establish an optimal polymerase chain reaction (PCR) protocol for each primer pair (Mullis and Faloona, 1987), a test PCR reaction was mixed containing 0.5 µM each forward and reverse primer, 10 ng DNA, 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 1x Taq puffer advanced (2 mM Mg^{2+}), 1 unit (U) *Taq* polymerase and bidest water to a reaction volume of 25 µL.

An initial temperature gradient PCR (Table 12) was conducted to identify the ideal annealing temperature using the iCycler thermocycler.

According to PCR results as seen on an ethidium bromide (EtBr)-stained agarose gel, different strategies were undertaken to optimize the PCR product quality. DMSO was added to the PCR reaction mix of certain primer pairs (Table 13) to enhance primer binding (Filichkin and Gelvin, 1992).

Following establishment of an efficacious PCR protocol for each candidate gene, PCR was repeated with a reaction volume of 50 µL in preparation for sequencing.

Table 12**:** Temperature gradient PCR program.

Cycles		Temp (°C)	Time
1	Initial Denaturation	94	02:00
30	Denaturation	94	00:30
	Annealing	50-65	00:30
	Elongation	72	01:30
1	Final Elongation	72	10:00
1		6	∞

3.3.3.3 PCR Fragment Assessment and Estimation

PCR fragments were visualized for quality assessment and quantity estimation on agarose gels stained with ethidium bromide (EtBr). DNA size markers containing premeasured DNA amounts migrated alongside PCR products for size and concentration determination of the product. PCR products were run on 1% to 3.5% agarose gels (Table 13) in horizontal electrophoresis chambers with 1X TBE buffer (Sambrook et al., 1989). The gels were stained in an ethidium bromide (EtBr) bath and PCR products were visualized under UV light.

3.3.3.4 PCR Purification

PCR products to be sequenced were purified using the Wizard SV Gel and PCR Clean-Up System (Boom et al., 1990) and eluted with 40 µl of nuclease-free water.

Table 13: Established PCR reactions and conditions for the different candidate gene primer pairs with a final volume of 25 µl.

Gene	Primer Pair	Annealing Temperature (°C)	Number Cycles	Primer Concentration (µM)	Units (U) *Taq* Polymerase	DNA (ng)	Modifications	Product Size (nt)	Agarose Gel (%)
LAMR1	Ex 5f → Ex 7r	58.9	40	0.5	1.0	12.5	10% DMSO	374	1.2
LAMR1	Ex 5f → In 6r	63.1	40	0.75	1.5	12.0	5% DMSO	542	1.5
SCRG1	Ex 1f → In 1r	59.5	35	1.2	1.0	12.0	-	310	2.0
PRND	Prom f→ Ex 1r	59.5	35	1.25	1.0	15.0	5% DMSO	465	1.5
PRND	Ex 2f → Ex 2r	60.0	35	1.25	1.0	13.0	8% DMSO	830	1.0
PRND	Ex 2f B →3'UTRr	59.5	35	0.5	1.25	12.5	-	576	1.5
RTN4	Ex 6f → Ex 8r	62.3	30	0.5	1.0	12.5	-	1201	1.5
RTN4	In 7f →In 7r	55.0	40	1.0	1.0	25.0	5% DMSO	166	3.5
VIM	Ex 1f → Ex 2r	60.1	35	1.0	1.0	12.5	4% DMSO	307	1.5

3.3.3.5 Sequencing of PCR Products

Sequencing reactions and program (Table 14) were based on the Sanger method (Sanger and Coulson, 1975).

All purified PCR products were sequenced with the ABI PRISM 377 DNA Sequencer both in the 5'→3' and the 3'→5' direction. Sequencing was conducted using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Kit which included the Premix and sequencing buffer. Reaction mix included 4 µl Premix, 2 µl sequencing buffer, 0.5 µl each PCR primer of 10 µM, between 8 and 40 ng purified PCR product, and HPLC water to an end volume of 20 µl. Purified PCR product concentration required for sequencing was determined by dividing the size of the PCR product by 20 (Anonymous, 2000). Premix contained dye terminated dNTP's where end hydroxyl groups were replaced by one of four dye molecules for each of the nucleotide bases, as well as normal dNTPs.

Table 14: Sequencing reaction cycle program.

Sequencing reaction		
Initial denaturation	96°C	1 min
25 cycles		
Denaturation	96°C	10 sec
Annealing	50-60°C*	5 sec
Elongation	60°C	4 min
Final Elongation	4°C	∞

* if primer ≤ 20 nt, then annealing temperature 50°C; if primer = 20-23 nt, then annealing temperature 55°C; if primer ≥ 23 nt, then annealing temperature 60°C

Reactions were precipitated immediately in 2 µl sodium acetate (3M) and 50 µl nondenatured 100% ethanol (Sambrook et al., 1989). After centrifugation for 15 min at 11900 rpm, the pellet was washed twice in 150 µl ethanol, centrifuged at 11900 rpm for 5 min and dried in a heat block at 50°C for 15 min. The pellet was stored at -20°C until loading onto 5% polyacrylamide gel in the sequencer occurred.

3.3.3.6 Sequencing Gel Casting and Loading

Preparation of the 0.2 mm, 5% polyacrylamide sequencing gel followed the manufacturer's protocol (Anonymous, 2000). Glass plates were cleaned with an anionic

detergent, rinsed with deionized water and the inner surfaces treated with 6N sodium hydroxide (NaOH). A 5% polyacrylamide gel with 7 M urea was prepared by mixing 8.4 mL polyacrylamide (acrylamide:bisacrylamide 29:1; 30% w/v), 21 g urea, 6 mL 10x TBE (Tris-base, boric acid, EDTA) and 20 mL HPLC water, with subsequent degassing and filtering (0.2 μm) prior to gel casting. Immediately before gel casting, the polymerisation initiators TEMED (20 μl) and APS (300 μL) were added to the degassed polyacrylamide gel solution. Gel polymerized following dispersion between glass plates, which were then washed with isopropanol and set into the ABI PRISM 377 DNA sequencer. Buffer chambers were filled with 1x TBE buffer.

Sequencing reaction pellets were dissolved in 4 µl loading dye included in the sequencing kit, loaded onto the gel and run for approximately six to eight hours.

3.3.3.7 Sequence Analysis

Candidate gene segment sequences were extracted with the Genotyper V 3.7 NT program and visualized with the program ChromasPro Version 1.32. The use of basic local alignment search tool (BLAST) verified sequences attained (BLAST 2.2.25).

3.3.3.8 SNP Identification

Sequences from a minimum of 11 DNA samples from 9 different sheep breeds were aligned together with GenBank reference sequences using ChromasPro Version 1.32. Scanning for SNPs followed with the help of the program ChromasPro Version 1.32, Geneious 5.3 and/or Mutational Surveyor. One SNP was selected for genotyping based on the occurrence of all three genetic variants as well as position in the candidate gene. SNPs located within exons were prioritized over those in introns.

3.3.4 SNP Genotyping

Candidate gene SNPs were genotyped in scrapie positive sheep and their flock mates in order to investigate whether the candidate gene in question influenced susceptibility to classical or atypical scrapie.

3.3.4.1 Restriction Fragment Length Polymorphism Analysis

Restriction fragment length polymorphism (RFLP) analysis (Saiki et al., 1985) was used to genotype candidate gene SNPs in the scrapie DNA bank sample population. Restriction enzymes for each identified SNP were chosen with NEBcutter V2.0 (Vincze et al., 2003).

Eight microliters of PCR product from each sample were incubated overnight with 2 μl of enzyme mix (5U of enzyme, 1 μl puffer and 1 μl HPLC water) in a water bath at the appropriate temperature for the enzyme. This reaction was run on a 2.5 to 3.5% agarose gel and visualization under UV light occurred through previous staining in an EtBr bath. RFLP conditions for all candidate gene SNPs analyzed are shown below (Table 15).

Table 15: Overview of RFLP conditions for each candidate gene.

Gene	Primers	Enzyme	Units Per Reaction	Incubation Temperature	Agarose Gel (%)
LAMR1	LAMR1 Ex 5f	*Mbi I*	3	37°C	3.5%
	LAMR1 In 6r	*BseR I*	5	37°C	3.5%
SCRG1	SCRG1 Ex1f SCRG1 In1r	*Ssi I*	5	37°C	3.5%
PRND	PRND Exon2f B PRND 3UTRr	*Dde I*	5	37°C	2.5%
RTN4	RTN4 In7f RTN4 In7r	*Hinf I*	2	37°C	3.5%

3.3.4.2 Amplification-created restriction site (ACRS)-RFLP Analysis

Amplification-created restriction site (ACRS)-RFLP analysis was implemented for *RTN4* as no restriction enzyme recognition site existed for the SNP investigated. By designing new primers containing a mismatch base, a cutting site for one of the two possible alleles was created (Haliassos et al., 1989). For genotyping of the *RTN4* C/T SNP, new primers were designed where the last position of the forward primer contained a mismatch base, replacing a T with an A (Figure 14). The T in the original sequence is replaced by A in the amplified PCR product and is situated 2 bp upstream of the C/T SNP. Together with the SNP T-allele, a cutting site for *Hinf I* was created. PCR amplification leads to the creation of an allele specific cutting site for *Hinf I*, when the T-allele is present.

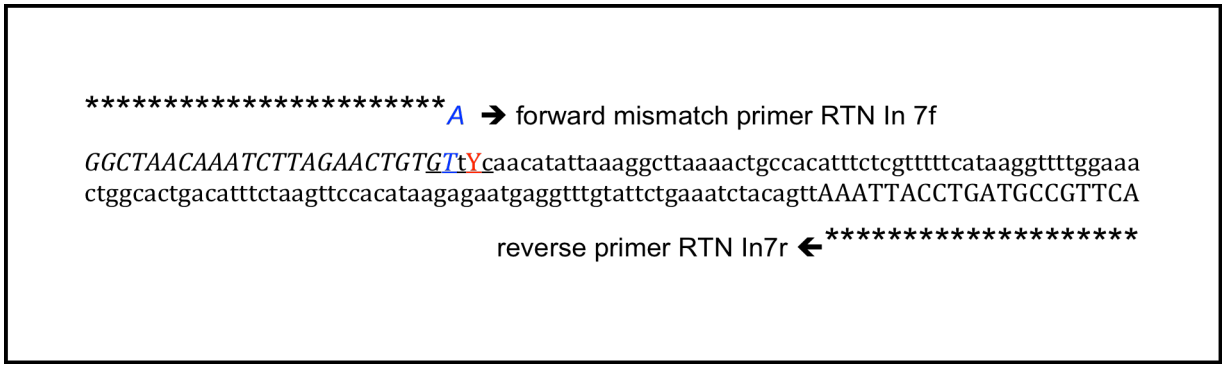

Figure 14: ACRS-RFLP design for candidate gene *RTN4*. Capital letters represent primer sequence with * denoting the same base as the gene sequence. Lower case letters represent *RTN4* intron 7 sequence amplified by RTN In7f and In7r. Blue represents the mismatched base. The **Y** is the C/T SNP in intron 7. Cutting site for *Hinf I*, created when **Y** = T, is underlined.

3.3.4.3 SNP Verification

The validity of SNPs analyzed in this study was tested using RFLP or ACRS-RFLP on PCR products from sheep family DNA (Figure 13). SNP validation occurred through demonstration of a Mendelian inheritance pattern.

3.3.5 Functional Analysis of SNPs

The DNA sequences attained for the candidate genes in this study were scanned using the internet based program ExPASy to investigate if the identified SNP was located within a functional or regulatory motif (Gasteiger et al., 2003). Translated products of exons containing identified SNPs were also scanned for functional and conserved domains using the internet based programs Prosite (Sigrist et al., 2013) and Uniprot (UniProt, 2012).

3.3.6 Statistical and Association Analyses

All SNP genotype and allele frequencies were calculated using the FREQ command with SAS version 8.01 or SPSS version 18.0. Frequencies were calculated according to scrapie type (classical or atypical) and status (negative or positive).

Pearson's chi-squared (Chi^2 or χ^2) test was used to calculate possible non-random association between identified SNPs in the candidate genes and scrapie status (Pearson, 1900). Genotype and allele frequencies in scrapie positive sheep were compared to those in the scrapie negative cohort flock mates. Association analyses were calculated with SAS version 8.01 or SPSS version 18.0.

Fisher's exact test (Fisher, 1922) was useful to analyze small sample sizes or where there were large differences in the number of observed genotypes. In these cases, Fisher's exact test was more appropriate to test associations.

Deviations from the Hardy-Weinberg equilibrium (HWE) model (Hardy, 1908; Weinberg, 1908) were estimated for the control groups (Gomes et al., 1999; Lunetta, 2008) consisting of healthy flock mates using SAS version 8.01, SPSS version 18.0 and/or the Online Encyclopedia for Genetic Epidemiology studies (Rodriguez et al., 2009).

4 Results

Table 16 summarizes all the SNPs identified in this study as well as their locations within the amplified and sequenced genomic segment for each candidate gene. Homologous sequence (BLAST) results for sequences obtained for each candidate gene are shown in the Appendix (Table A2). All sequences attained in this study, with the exception of the *RTN4* sequence, were submitted to GenBank (Table 16). The length of the analysed *RTN4* sequence (166 bp) precluded GenBank submission.

Table 16: Overview of sequenced candidate gene segments and the corresponding GenBank accession number where appropriate. SNP alleles, positions and SNP location within gene structures are listed.

Candidate Gene	Segment Sequenced	SNP Alleles	GenBank Accession Number	SNP Position (bp)	Location
LAMR1	Exon 5 to Intron 6	A/G	KC904790	119	Exon 5
LAMR1	Exon 5 to Intron 6	C/T	KC904790	311	Exon 6
SCRG1	Exon 1 to Intron 1	G/A	KC904792	228	Intron 1
PRND	Exon 2 to 3'-UTR	C/T	KC904791	262	3'UTR
RTN4	Exon 6 to Exon 8	G/A	NT022184.15 HSA 2 genomic contig*	34022706	Intron 7
RTN4	Exon 6 to Exon 8	C/T	NT022184.15 HSA 2 genomic contig*	34022716	Intron 7
VIM	Exon 1 to Exon 2	C/T	KC904793	43	Exon 1

*Sequence attained in this study was too short (<200 bp) to submit to GenBank.

4.1 Laminin Receptor Gene 1

4.1.1 *LAMR1* Sequences and SNPs

4.1.1.1 Exon 5 to Exon 7

BLAST results of the gene segment sequenced with primer pair LAMR1 Ex5f and LAMR1 Ex7r (GenBank KC904790) showed a 99% homology with no gaps to *Ovis aries* cDNA sequence (GenBank GW996948.1) and an 87% similarity to *Homo sapiens* *LAMR1* pseudogene 9 (GenBank NR026890.1). Sequences were also compared to the

genomic human sequence (GenBank U43901). A pseudogene was amplified, as it was apparent that the obtained sequence was devoid of introns 5 and 6 (Figure 15).

```
Query  1    CCCGTGGGAGGTCATGCCGGACCTCTACTTCTACAGGGACCCCGAGGAG~ATTGAAAAGGA   60
            ||||||||||||||||||||||||||||||||||||||||||||||||||~||||||||||||
Sbjct  141  CCCGTGGGAGGTCATGCCGGACCTCTACTTCTACAGGGACCCCGAGGAG~ATTGAAAAGGA  200

Query  61   AGAGCAGGCAGCAGCCGAGAAGGCTGTGACCAAGGAGGAGTTTCAGGGCGAATGGACCGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  AGAGCAGGCAGCAGCCGAGAAGGCTGTGACCAAGGAGGAGTTTCAGGGCGAATGGACCGC  260

Query  121  TCCAGCTCCAGAGTTCACGGCTGCCCNGCCTGAGGTGGCAGACTGGTCCGAAGGTGTGCA  180
            |||||||||||||||||||||||| |||||||||||||||||||| ||||||||||||
Sbjct  261  TCCAGCTCCAGAGTTCACGGCTGCCCAGCCTGAGGTGGCAGACTGGTCTGAAGGTGTGCA  320

Query  181  GGTGCCTTCCGTGCCCATTCAGCAGTTCCCCACTG~AAGACTGGAGTGCTCAGCCTTCCAC  240
            ||||||||||||||||||||||||||||||||||~||||||||||||||||||||||||||
Sbjct  321  GGTGCCTTCCGTGCCCATTCAGCAGTTCCCCACTG~AAGACTGGAGTGCTCAGCCTTCCAC  380

Query  241  TGAAGACTGGTCTGCAGCCCCCACTGCCCAGGCCACGGAATGGGTAGGAACCACCNCCGA  300
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  381  TGAAGACTGGTCTGCAGCCCCCACTGCCCAGGCCACGGAATGGGTAGGAACCACCACCGA  440

Query  301  GTGG  304
            ||||
Sbjct  441  GTGG  444
```

Figure 15: Alignment of reference DNA *LAMR1* Ex 6f-Ex 7r sequence (Query) with ovine mRNA *LAMR1* sequence (Sbjct; Genbank accession no. GW996948.1). ~ indicates missing intron sequence.

4.1.1.2 Exon 5 to Intron 6

Pseudogene amplification was avoided using this primer pair. BLAST results for the sequence obtained (Figure 16) are shown in Table A2. This sequence was submitted to GenBank and assigned accession number KC904790.

A synonymous C/T polymorphism (Figure 16) was identified at position 311 of the sequence obtained (KC904790), corresponding to bp 692 of the *Ovis aries* mRNA sequence (NM001105263.1). This SNP is located at position 69 in exon 6 which is the third position of codon 232 (NP001098733.1). Both C or T in the third position codes for threonine (T).

A second SNP could be identified at position 119 of this sequence (KC904790), constituting a G/A polymorphism (results not shown). This G/A SNP is located at the second nucleotide position in the last codon of exon 5 and is nonsynonymous. An adenine at this position codes for glutamic acid (E) but a guanine at this position codes for glycine (G).

```
001 TCAGTGGGCC TGATGTGGTG GTGCTCGCCC GGGAAGTCCT GCGCATGCGC  Exon 5
051 GGCACCATCT CCCGAGAACA CCCGTGGGAG GTCATGCCGG ACCTCTACTT
101 CTACAGGGAC CCCGAGGAGg tgagctccgt ccacagaggc gtgttggcac
151 tcacataagt acatactggg tacttcttac tgccaagaac agaaattaat
201 ctgtcaatgc atataaacta aaaactattt tcctaaacta gATTGAAAAG  Exon 6
251 GAAGAGCAGG CAGCAGCCGA GAAGGCTGTG ACCAAGGAGG AGTTTCAGGG
301 CGAATGGACY GCTCCAGCTC CAGAGTTCAC GGCTGCCCAG CCTGAGGTGG
351 CAGACTGGTC TGAAGGTGTG CAGGTGCCTT CCGTGCCCAT TCAGCAGTTC
401 CCCACTGgta cgtatcagga tccaagggca tccagctggt ggtttagaac
451 tgctcttctc agtctgacat atctgtagga ataaaagcag attggtgcag
501 tggggttaca aaagtaactg gggatgtggc tgatgacctc ctt
```

Figure 16: Sequence (KC904790) for ovine *LAMR1* obtained with primer pair Ex 5f and In 6r. **Y** represents the C/T SNP. Exons 5 and 6 represented by capital letters, introns 5 and 6 by lower case letters. Primers are underlined.

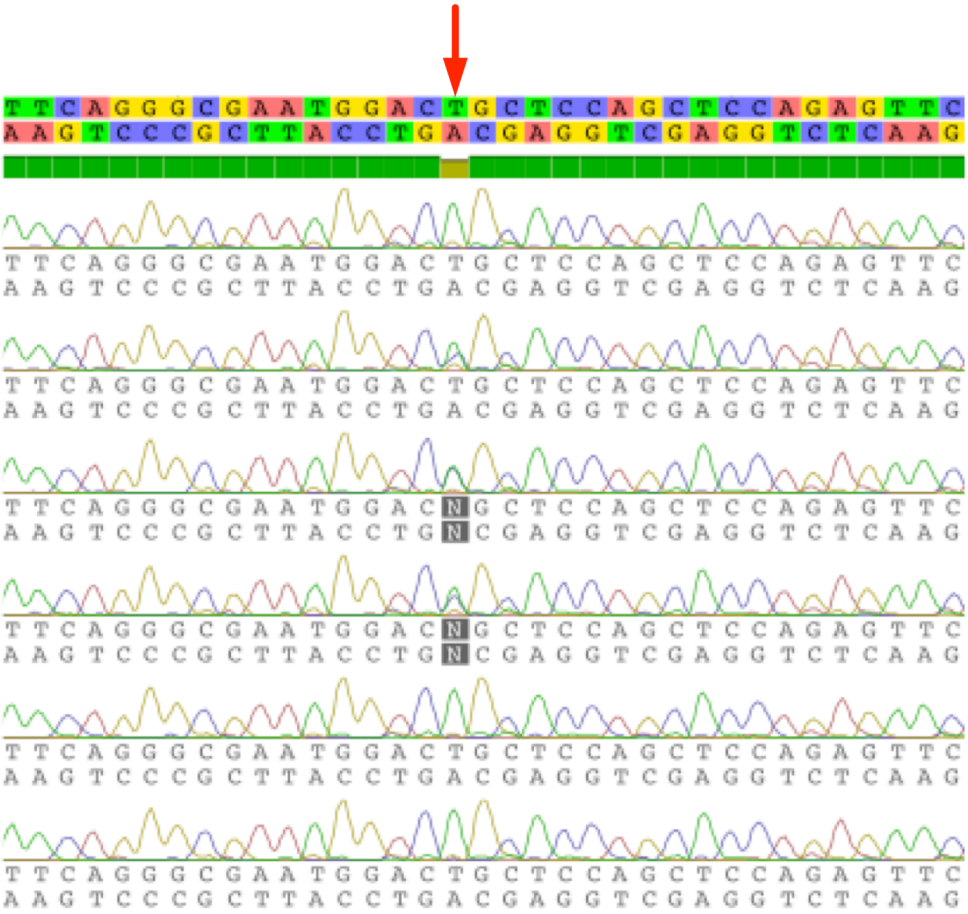

Figure 17: Alignment of six *LAMR1* Ex 5 f sequences. The arrow shows the C/T SNP.

4.1.2 Investigation of *LAMR1* SNP C/T by RFLP Analysis

An RFLP was developed using the enzyme *Mbi I*, which cuts at 5'-CCG↓CTC-3', with C indicating the SNP within the restriction enzyme site (Figure 18). A total of 640 animals could be genotyped.

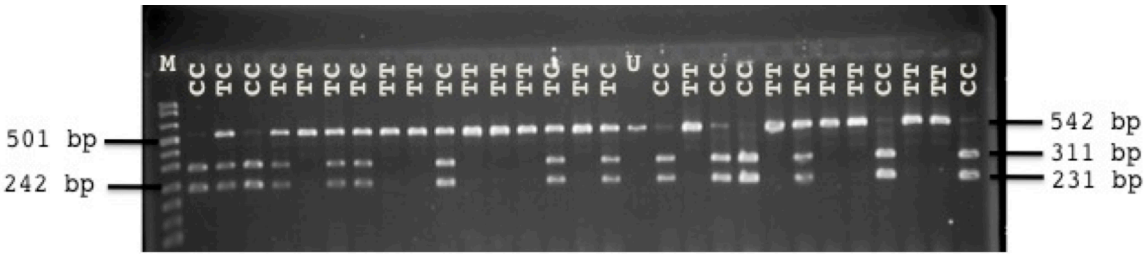

Figure 18: *LAMR1* RFLP analysis. 3.5% agarose gel showing *LAMR1* genotypes for C/T SNP after digestion with enzyme *Mbi I*. Fragment sizes are labelled on right. **M** = pUC Mix marker 8. **U** = undigested PCR product.

4.1.3 Genotype and Allele Frequencies of C/T SNP and their Influence on Scrapie Susceptibility

Genotype and allele frequencies of the C/T SNP identified for all scrapie groups are shown in Table 17 and Table 18, respectively. Cumulative genotype frequencies were as follows: 16.4% CC, 47.7% TC, 35.9% TT and the cumulative allele frequency for C was 40.2% and for T 59.8% in all animals. No significant differences were found in genotype (Table 17) or allele (Table 18) frequencies between classical or atypical positive scrapie groups when compared to the corresponding group of scrapie negative cohort flock mates.

Table 17: *LAMR1* genotype frequencies for C/T SNP for both classical scrapie as well as atypical scrapie groups. Total sheep shows total number of animals genotyped in each subgroup and p values are listed for both cases.

	LAMR1 Genotype Frequency	Total Sheep (n)	**CC (%)**	**CT (%)**	**TT (%)**	p-value
classical scrapie	negative	137	16.8	43.1	40.1	1.00
	positive	106	17.0	43.4	39.6	
atypical scrapie	negative	304	16.1	50.0	33.9	0.49
	positive	93	11.8	55.9	32.3	

Table 18**:** *LAMR1* allele frequencies for C/T SNP with P values are listed for classical and atypical scrapie groups.

	LAMR1 Allele Frequency	Total Sheep (n)	**C (%)**	**T (%)**	p-value
classical scrapie	negative	137	38.3	61.7	0.94
	positive	106	38.7	61.3	
atypical scrapie	negative	304	41.1	58.9	0.75
	positive	93	39.8	60.2	

4.1.4 HWE of Healthy Flock Mates

Observed and expected genotype frequencies of the *LAMR1* C/T SNP according to Hardy-Weinberg equilibrium in the healthy cohort flock mates are shown below (Table 19).

Table 19: Observed and expected *LAMR1* C/T genotype frequencies in the classical and atypical healthy cohort flock mates.

	LAMR1	**CC (%)**	**CT (%)**	**TT (%)**	p-value
classical scrapie	observed	16.8	43.1	40.1	0.38
	expected	14.7	47.3	38.0	
atypical scrapie	observed	16.1	50.0	33.9	0.74
	expected	16.9	48.4	34.7	

4.1.5 Investigation of *LAMR1* SNP G/A

A second, nonsynonymous G/A SNP was identified within the same amplified *LAMR1* gene segment and located at the second nucleotide position of the last codon in exon 5 (position 119 in GenBank KC904790). The current ovine *LAMR1* sequence (GenBank

GQ202529.1) found in the GenBank displays an adenine at this position (bp 12705). This codon codes for glutamic acid (E) when an adenine is found at this position but codes for glycine (G) when a guanine is present. Of the 11 sequencing DNA samples, 4 were heterozygote A/G and 7 were homozygote A/A but G/G homozygotes were not observed. An RFLP with the enzyme BseR I [5'-GAGGAG(N)^{10}↓-3'] was conducted on PCR products from the 11 sheep samples sequenced as well as on 5 additional samples amplified from the Hessian sheep sample DNA group to try to identify all three genotypes (results not shown). Five A/A homozygote animals could be genotyped but it was not possible to distinguish between the A/G heterozygote or the putative G/G homozygote genotypes from this RFLP even after increasing enzyme concentrations from 4 U to 5 U per digest reaction.

4.2 Scrapie Responsive Gene 1

4.2.1 *SCRG1* Sequencing and SNPs

4.2.1.1 Exon 1 to Intron 1

Primers SCRG1 Ex1f and SCRG1 In1rB amplified a 310 bp product (Figure 19) assigned GenBank accession number KC904792. BLAST results of the sequenced products are shown in Table A2. Only one SNP was found in this amplified segment of the ovine *SCRG1* gene. A G/A polymorphism was identified at position 228 of the sequence (Figure 20). Figure 20 shows the reverse sequence originating from the reverse primer In 1rB due to the higher quality sequence achieved. Position 228 in this sequence corresponds to bp 9501 of the GenBank cattle sequence NC007306.

```
001 GTTGGACTAA CTTTGCTGCT AGGAGTCCAA GCCATGCCTG CAAACCGCCT   Ex 1
051 TTCCTGCTAC AGAAAAATAC TAAAAGATCG CAACTGTCAC AGTCTTCCAG
101 AAGGAGTAGC TGACCTGACA AAGATTGATG TCAATGTCCA GGATCACTTC
151 TGGGATGGGA AGGGATGTGA GATGATCTGT TACTGCAACT TCAGCGAACT
201 GCTCTGCTGC CCAAAgtaag gaaatgcrgt cacaagacgt atggttgtaa   In 1
251 aatgtatgca taacgacttt cttaagacac cttcacaagg agtttgctga
301 aacttgtcac
```

Figure 19: Forward *SCRG1* sequence of PCR product obtained with primer pair Ex 1f and In 1rB. SNP G/A represented by **r** and located at bp 13 of intron 1. Exon bases are in capital letters and intron bases in lowercase letters.

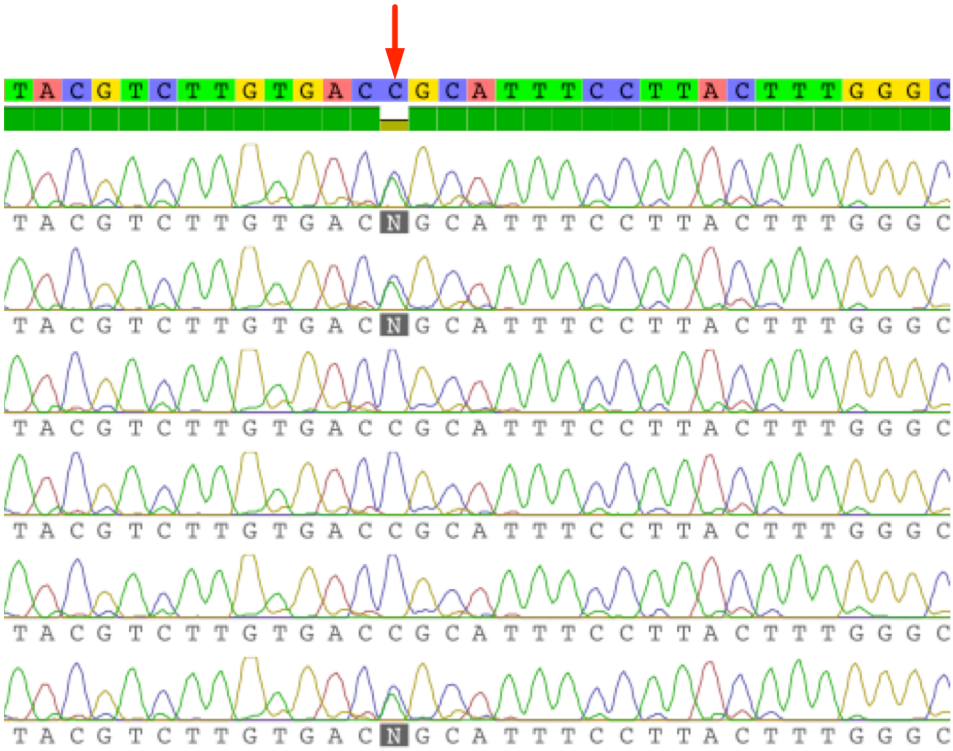

Figure 20**:** Alignment of six *SCRG1* reverse sequences showing the SNP (arrow).

4.2.2 Demonstration of *SCRG1* SNP by RFLP Analysis

An RFLP was designed using the enzyme *Ssi I*, which cuts at 3'–GGC↓G–5', where the underlined G represents the G/A SNP identified (Figure 21).

A total of 556 animals could be genotyped.

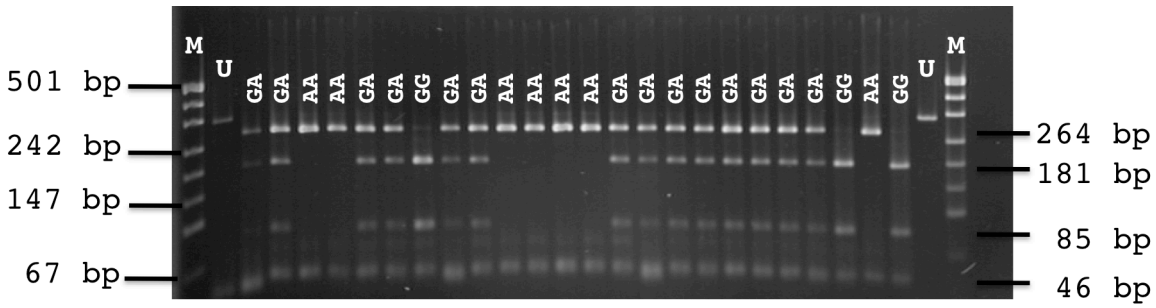

Figure 21: *SCRG1* RFLP analysis. 3.5% agarose gel showing *SCRG1* genotypes for G/A SNP in intron 1 after digestion with enzyme *Ssi I*. Fragment sizes (bp) are 264, 181, 85 and 46. **M** = marker pUC19, fragment sizes are labelled on left. **U** = undigested PCR product (310 bp).

4.2.3 Genotype and Allele Frequencies of *SCRG1* G/A and their Influence on Scrapie Susceptibility

Genotype and allele frequencies are shown in Table 20 and Table 21, respectively. The cumulative genotype frequencies in all animals were as follows: 27.9% GG, 46.9% GA, 25.2% AA and the cumulative allele frequencies were 51.3% for G and 48.7% for A. No statistically significant differences in genotype (Table 20) or allele (Table 21) frequencies were observed.

Table 20: *SCRG1* genotype frequencies for identified SNP for classical and atypical scrapie. Total sheep shows total number of animals genotyped in each subgroup and p values are shown.

SCRG1 Genotype Frequency		Total Sheep (n)	**GG (%)**	**GA (%)**	**AA (%)**	p-value
classical scrapie	negative	115	22.6	47.0	30.4	0.64
	positive	80	17.5	52.5	30.0	
atypical scrapie	negative	287	31.0	45.6	23.3	0.38
	positive	74	36.5	47.3	16.2	

Table 21: *SCRG1* allele frequencies for identified SNP. Total sheep shows total number of animals genotyped in each subgroup.

	SCRG1 Allele Frequency	Total Sheep (n)	**G (%)**	**A (%)**	p-value
classical scrapie	negative	115	46.1	53.9	0.65
	positive	80	43.8	56.3	
atypical scrapie	negative	287	53.8	46.2	0.17
	positive	74	60.1	39.9	

4.2.4 HWE of Healthy Flock Mates

Observed and expected genotype frequencies according to Hardy-Weinberg equilibrium in the healthy cohort flock mates are shown below (Table 22).

Table 22: Observed and expected *SCRG1* genotype frequencies in the classical and atypical healthy cohort flock mates.

	SCRG1	**GG (%)**	**GA (%)**	**AA (%)**	p-value
classical scrapie	observed	22.6	47.0	30.4	0.59
	expected	21.2	49.7	29.1	
atypical scrapie	observed	31.0	45.6	23.3	0.41
	expected	29.0	49.7	21.3	

4.2.5 Putative Location of Ovine *SCRG1*

The ovine *SCRG1* sequence determined in this study was used to search (BLAST) the *O. aries* genome assembly version 2.0 (www.livestockgenomics.csiro.au; March 2011) database, resulting in a putative location for this gene on OAR 2 (Table 6).

4.3 Prion protein 2 (*PRND* – Doppel)

4.3.1 *PRND* Sequencing and SNPs

4.3.1.1 Promoter to Exon 1

BLAST results of sequences obtained showed 99% homology to ovine *PRND* partial sequence AY184242.1 (Table A2). A total of 24 DNA samples from individual animals were sequenced, however no SNP was detected.

4.3.1.2 Exon 2

With the primer pair Ex 2f-Ex 2r, one A/C SNP at position 110 (GenBank KC904791) was identified. Results of RFLP analysis with the restriction enzyme *Bse NI* (cuts at 5'–ACTGGN↓–3') however, were inconclusive. Fourteen of 17 digested PCR products from different sheep DNA samples still revealed the original band of 827 bp, which should have been completely digested in all variants. Results remained inconclusive after doubling enzyme concentration (results not shown).

4.3.1.3 Exon 2 to 3'-Untranslated Region (3'-UTR)

The third primer pair, PRND Ex 2fB and PRND 3UTRr, extended from exon 2 (codon 147) into the 3'-untranslated region (3'-UTR) and included part of the exon 2 region analysed with primer pair Ex 2f and Ex 2r (4.3.1.2). The authenticity of the A/C SNP at position 110 (GenBank KC904791) could also be tested with this primer pair, since it was located 89 base pairs after the first base of primer PRND Ex 2fB.

A 576 bp product was amplified with primer pair PRND Ex 2fB and 3'UTRr and PCR products from 15 sheep were sequenced (Figure 22). BLAST results are shown in Table 2A.

Initial mutation analysis revealed no SNP corresponding to position 110. However, a T/C polymorphism at position 262 of this fragment was identified (GenBank KC904791). This T/C SNP was only observed in one Scottish Blackface sheep and two Coburger Fox sheep, where all three were heterozygote T/C (Figure 23). All other sequenced DNA samples were homozygote T/T. This SNP is at base pair number 163 in the 3'-UTR region, corresponding to nucleotide position 2818 in the ovine complete cds (GenBank AF394223).

```
001 TGTGACTTTT GGTTGGAAAG GGGAGCAGGA CTTCAGGTCA CTCTGGACCA  Ex 2
051 GCCCATGATG CTCTGCCTGC TGGTTTTCAT TTGGTTTATT GTGAAATAAg
101 cttgcaggca agttggcagc cacagagatc aataggcaag caaaccataa  3'UTR
151 gcaagttatt ccagttcttc tcctctaacc ccaaacccca cgtgttctga
201 aggtaccaaa gaacagtgtg attgattctt tagcgcttga aatagcactc
251 ccaagtattc aYtcaggtgt ttgattatat ttgataaatg tgtgggtatc
301 aatcctctcc aggttctacc taaagttggc ttgttcatca ttgcattctc
351 aactctggtg tagcatctgg cccaccatat tatgcaataa atgtttggta
401 agcagataaa agaatgtgcc agggaccata ccaagcactt cacaatgctt
451 cctgacaact ctcagaggta ggtgtaataa gtgttattct cttggtatag
501 atgagaaaat tgaggctcca agaagtaaaa tagtaaagta gttagaaagt
551 atcagccata attcaaccct ggctta
```

Figure 22: Sequence obtained with primer pair PRND Ex2fB and PRND 3UTRr. Primers are underlined with exon 2 in capital letters and 3'UTR in lowercase. **Y** = T/C SNP. **a** = suspected A/C SNP, not observed in sequences attained with this primer pair.

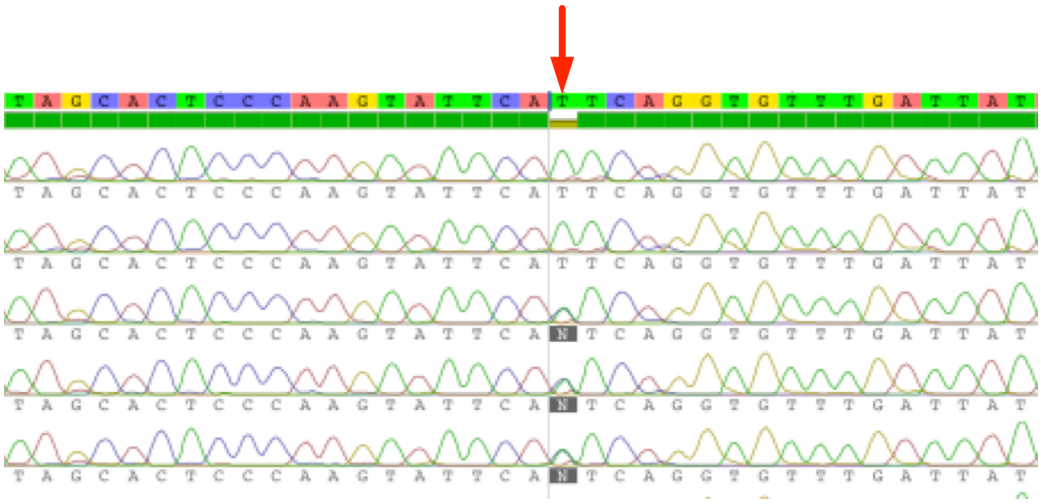

Figure 23: Chromatogram alignment of 7 PRND Ex 2fB sequences showing T/C SNP (arrow).

4.3.2 Demonstration of *PRND* T/C SNP by RFLP Analysis

An RFLP analysis was conducted with restriction enzyme *Dde I* (5'–C↓TNAG–3') (Figure 24). The T/C SNP identified is the first base (C) of the recognition site.

A total of of 259 animals were genotyped.

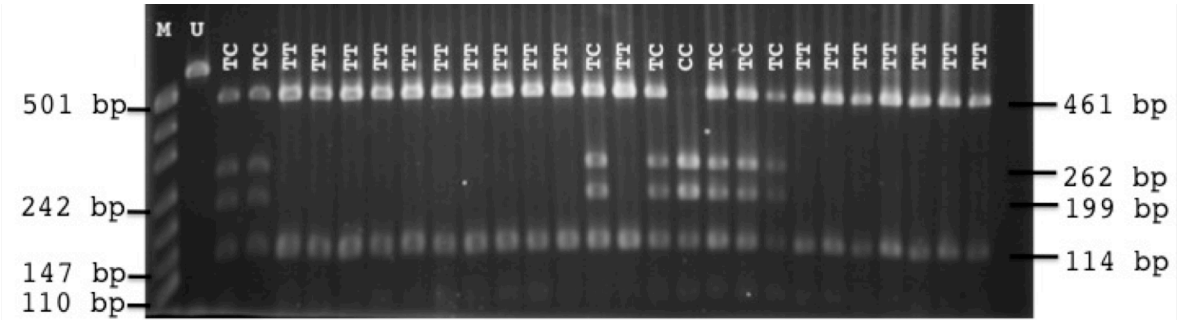

Figure 24: RFLP *PRND* T/C SNP. Agarose gel (1.5%) of PRND Ex 2fB and PRND 3UTRr PCR products digested with restriction enzyme *Dde I*. Restriction fragment lengths (bp) labelled on the right. Genotypes TT, TC and CC are shown. **M** = marker pUC19, fragment sizes are labelled on the left. **U** = undigested PCR product.

4.3.3 Genotype and Allele Frequencies of *PRND* T/C SNP and their Influence on Scrapie Susceptibility

Cumulative genotype frequencies were as follows: 0.4% CC, 7.3% TC and 92.3% TT. The cumulative allele frequency for C was 4.1% and for T 95.9% in all animals. No significant differences in genotype (Table 23) or allele (Table 24) frequencies were observed between the groups.

Table 23: *PRND* genotype frequency results for T/C SNP in classical and atypical scrapie groups. Total sheep shows total number of animals genotyped in each subgroup.

	PRND Genotype Frequency	Total Sheep (n)	**CC (%)**	**TC (%)**	**TT (%)**	p-value
classical scrapie	negative	74	0.0	6.8	93.2	0.65
	positive	56	0.0	8.9	91.1	
atypical scrapie	negative	73	1.4	8.2	90.4	0.55
	positive	56	0.0	5.4	94.6	

Table 24: *PRND* allele frequencies for T/C SNP between classical and atypical scrapie groups. Total sheep shows total number of animals genotyped in each subgroup

	PRND Allele Frequency	Total Sheep (n)	**C (%)**	**T (%)**	p-value
classical scrapie	negative	74	3.4	96.6	0.59
	positive	56	4.7	95.3	
atypical scrapie	negative	73	5.5	94.5	0.30
	positive	56	2.8	97.2	

4.3.4 HWE of Healthy Flock Mates

Observed and expected genotype frequencies according to Hardy-Weinberg equilibrium in the healthy cohort flock mates are shown below (Table 25).

Table 25: Observed and expected *PRND* genotype frequencies in the classical and atypical healthy cohort flock mates.

	PRND	CC (%)	TC (%)	TT (%)	p-value
classical scrapie	observed	0.0	6.8	93.2	0.72
	expected	0.1	6.6	93.3	
atypical scrapie	observed	1.4	8.2	90.4	0.03
	expected	0.3	10.4	89.3	

4.3.5 Genotype and Allele Frequencies of *PRND* SNP 262Y in a Representative Selection of Hessian Sheep Breeds

PCR and RFLP analysis was conducted on DNA samples from 192 sheep representing breeds held in Hesse, Germany, with the primer pair PRND Ex 2fB and PRND 3UTRr. In this sample population, the CC genotype was observed exclusively in Coburger Fox sheep at a frequency of 6.25% (Table 26).

Table 26**:** Genotype and allele and frequencies of T/C SNP in Hessian sheep breeds.

Breed	Total Genotyped	TT (%)	TC (%)	CC (%)	T (%)	C (%)
Coburg Fox	32	68.75	25.0	6.25	81.25	18.75
Grey Horned Heath	32	100	0	0	100	0
Merino Land	31	96.8	3.2	0	98.4	1.6
Rhoen	32	100	0	0	100	0
German Blackheaded Mutton	31	96.8	3.2	0	98.4	1.6
Suffolk	16	100	0	0	100	0
Texel	16	100	0	0	100	0

4.4 Reticulon 4

4.4.1 *RTN4* Sequencing and SNPs

4.4.1.1 Exon 6 to Exon 8

Primer pair RTN4 Ex6f and RTN4 Ex8r amplified a fragment of approximately 950 nucleotides. For BLAST results see Table A2. New primers were then chosen from initial sequences attained for ACRS-RFLP analysis (Figure 25).

A G/A polymorphism was identified (Figure 26) within intron 7 at base pair Exon 7+127 or 62 bp before the start of exon 8.

```
001   TGAACGGCAT CAGgtaattt aactgtagat ttcagaatac aaacctcatt
051   ctcttatgtg gaacttagaa atgtcagtgc cagtttccaa aaccttatga
101   aaaacgagaa atgtggcagt tttaagcctt taatatgttg Raacacagtt
                                                  *
151   ctaagatttg ttagcc
```

Figure 25: *RTN4* incomplete exon 7 and intron sequence surrounding the G/A SNP (**R**). Capital letters represent exon 7 and lowercase letters represent intron 7. Primers designed for ACRS-RFLP are underlined, where * indicates mismatch base (a>t).

A second, C/T SNP was observed ten nucleotides downstream from the G/A SNP shown in Figure 25, at position 151. The homozygote TT genotype was not observed in the initial 11 animals sequenced. Nine of these animals carried the CC genotype, and two were heterozygote at this position (results not shown).

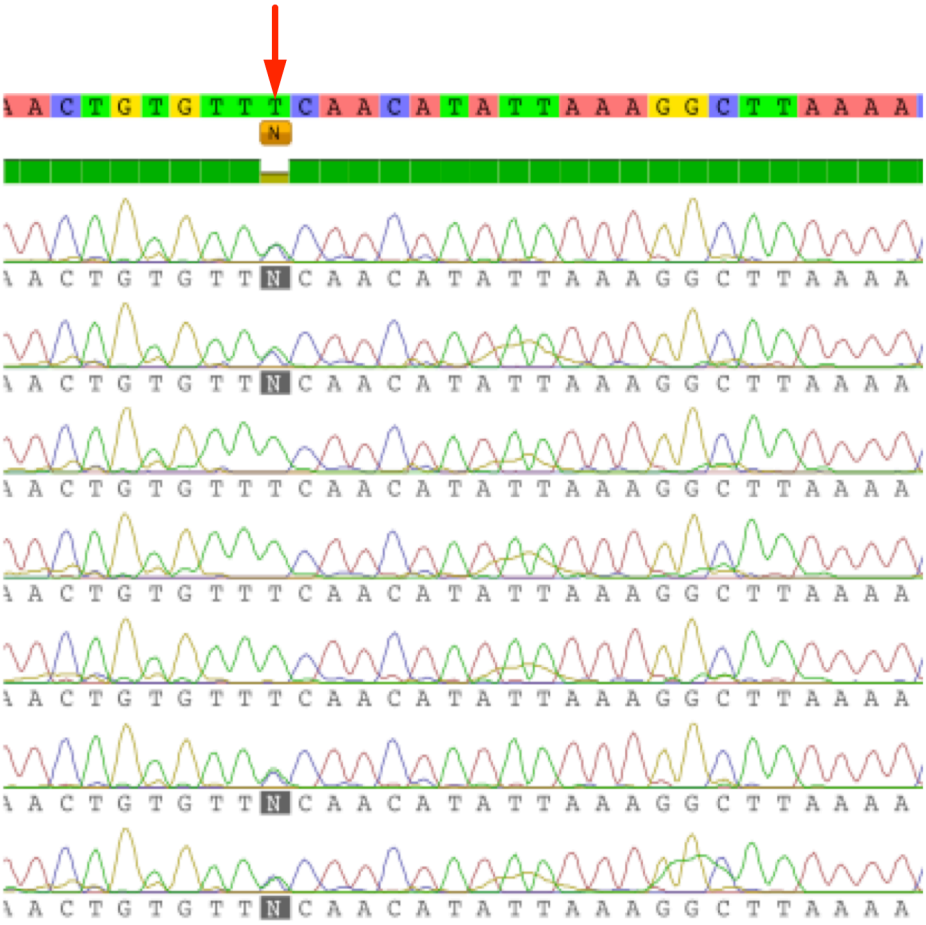

Figure 26: Comparison of seven reverse *RTN4* sequences, revealing corresponding T/C SNP, marked by arrow. Reverse sequences shown due to finer quality.

4.4.2 Demonstration of *RTN4* G/A SNP by ACRS-RFLP Analysis

An ACRS-RFLP digest with the restriction enzyme *Hinf I* (cuts at 5'–G↓ANTC–3') allowed a total of 563 animals to be genotyped (Figure 27). Restriction fragment sizes were 143 and 22 for the A allele and 165 for the G allele.

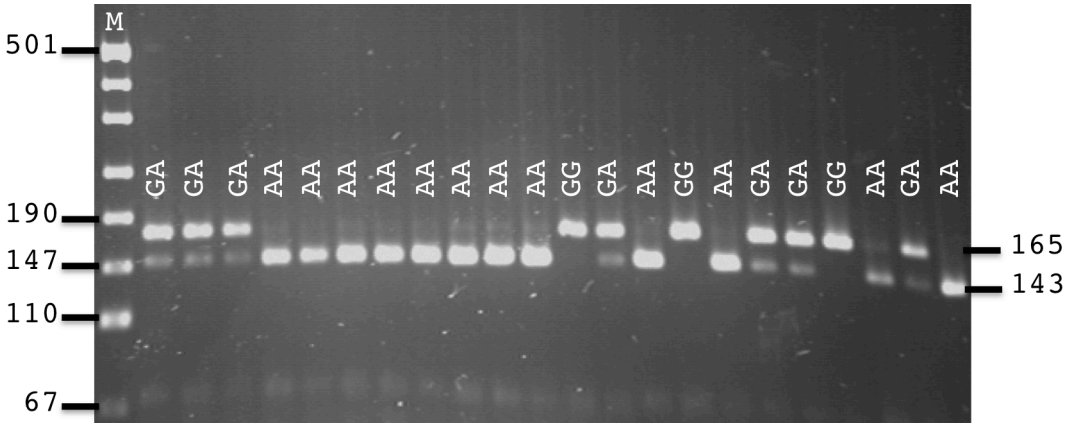

Figure 27: ACRS-RFLP for *RTN4*. Digest with *Hinf I* was run on 3.5% agarose gel. Genotypes shown for G/A SNP, where the G allele fragment is 165 bp and the A allele fragment lengths (labelled on right) are 143 bp and 22 bp (not shown). **M** = marker puc19, size fragments are labelled on left.

4.4.3 Genotype and Allele Frequencies of G/A SNP and their Influence on Scrapie Susceptibility

Cumulative genotype frequencies for all animals were as follows: 57.5% AA, 31.4% GA and 11.1% GG. The cumulative allele frequency for A was 71.7% and for G 28.3% in all animals. No significant differences were found in the genotype (Table 27) or allele (Table 28) frequencies when compared to scrapie status or type.

Table 27: *RTN4* genotype frequencies for G/A SNP within classical and atypical scrapie groups. Total sheep shows total number of animals genotyped in each subgroup.

	RTN4 Genotype Frequency	Total Sheep (n)	**GG (%)**	**GA (%)**	**AA (%)**	p-value
classical scrapie	negative	117	21.4	34.2	44.4	0.18
	positive	88	19.3	46.6	34.1	
atypical scrapie	negative	272	5.5	27.6	66.9	0.68
	positive	86	7.0	23.3	69.8	

Table 28: *RTN4* allele frequencies for G/A SNP within classical and atypical scrapie groups. Total sheep shows total number of animals genotyped in each subgroup.

	RTN4 Allele Frequency	Total Sheep (n)	**G (%)**	**A (%)**	p-value
classical scrapie	negative	117	39.8	60.2	0.60
	positive	88	42.3	57.7	
atypical scrapie	negative	272	22.1	77.9	0.39
	positive	86	19.1	80.9	

4.4.4 HWE of Healthy Flock Mates

Observed and expected genotype frequencies according to Hardy-Weinberg equilibrium in the healthy cohort flock mates are shown below (Table 29).

Table 29: Observed and expected *RTN4* genotype frequencies in the classical and atypical healthy cohort flock mates.

	RTN4	GG (%)	GA (%)	AA (%)	p-value
classical scrapie	observed	21.4	34.2	44.4	0.005
	expected	14.8	47.4	37.8	
atypical scrapie	observed	5.5	27.6	66.9	0.25
	expected	3.7	31.2	65.1	

4.4.5 Putative Location of Ovine *RTN4*

With the partial *RTN4* sequence obtained in this study, the sheep genome map (OAR v2.0, March 2011) was searched and *RTN4* located to OAR 3.

4.5 Vimentin

Primer pair VIM Ex 1f and VIM Ex 2r was chosen at the time of this study based on the alignment of ovine mRNA and bovine whole genome shotgun sequences (GenBank AF251147 and NW482313, respectively). This primer pair spanned intron 1, which was approximately 80 nucleotides in the bovine sequence (NW482313) and nonexistent in the corresponding human sequence (NT077569). A 307 base pair product was amplified and sequenced (GenBank KC904793; Figure 28) that showed 94% sequence similarity to human *VIM* mRNA (GenBank accession number NM003380.2) and 99% sequence similarity to *Bos taurus* whole genome shotgun sequence with GenBank accession number NW482313 (Table A2). In the case of the second alignment, intron 1 was missing in the ovine sequence attained in this study (Figure 28).

Mutational analysis showed only one C/T SNP at position 43 of the ovine *VIM* segment amplified (GenBank KC904793; Figure 29). However, due to BLAST results (Table A2) showing no part of intron 1, the PCR product amplified by this primer pair was assumed to be a pseudogene and no further analysis was undertaken.

Updated versions of *VIM* sequences have replaced both AF251147 and NW482313. A new bovine whole genome shotgun sequence for *VIM* (NC007311.4) showed that both

VIM primers from this study, Ex1f and VIM Ex2r, are found within the bovine *VIM* exon 1, which is homologous to exon 2 of the human *VIM* (NG012413).

```
001  GAGCTACGTG ACCACATCCA CCCGCACCTA CAGCCTGGGC AGYGCGCTGC
051  GCCCCTCCAC CAGCCGCACC CTCTACACCT CGTCCCCGGG TGGCGTGTAC
101  GCCACGCGCT CCTCGGCTGT GCGCCTGCGG AGCGGCGTGC CCGGCGTGCG
151  GCTGCTGCAG GACTCGGTGG ACTTCTCGTT GGCCGACGCC ATCAACACCG
201  AGTTCAAGAA CACCCGCACC AACGAGAAGG TGGAGCTGCA GGAGCTCAAT
251  GACCGCTTCG CCAACTACAT CGACAAGGTG CGCTTCCTGG AGCAGCAGAA
301  CAAGATC
```

Figure 28: *VIM* sequence from primer pair VIM Ex 1f and Ex 2r, which are underlined. **Y** (base pair 43) shows the position of the identified C/T SNP. Blue break line after position 52 indicates where intron 1 would be according to GenBank entry NW482313, which is now obsolete.

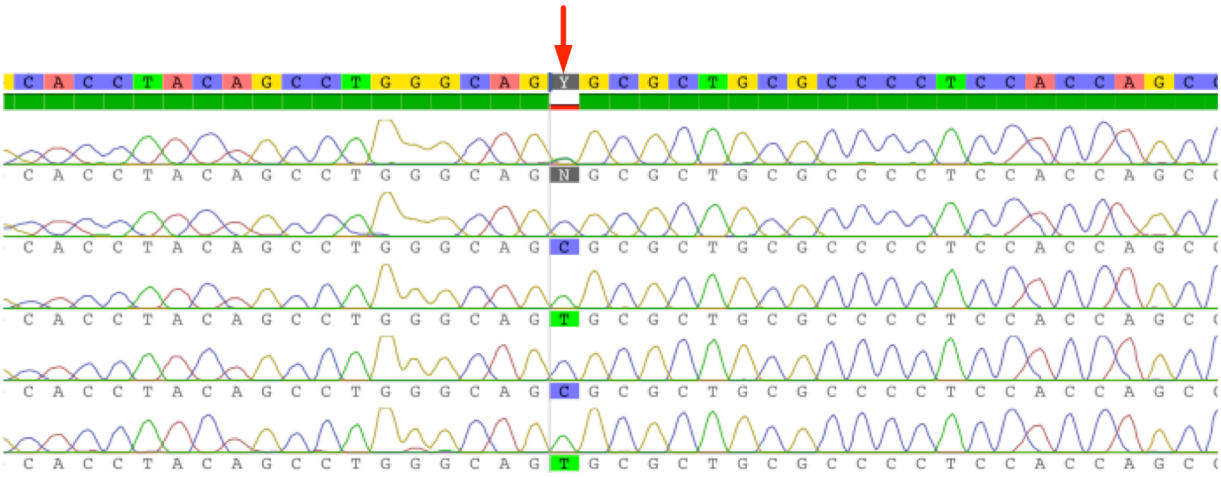

Figure 29: Chromatogram of five VIM Ex 1f - Ex 2r sequences showing C/T SNP, shown by arrow.

5 Discussion

The candidate genes analysed in this study have all been chosen according to gene characteristics meeting two or more of the following criteria: 1) genomic location within or near a QTL involved in scrapie incubation time; 2) differential expression between healthy and scrapie infected groups; and/or 3) evidence of a putative functional role in scrapie pathogenesis.

Single nucleotide polymorphisms in four of five candidate genes were tested as to their role in classical and atypical scrapie susceptibility in sheep. For each of these SNPs, a total of between 259 and 653 sheep DNA samples from the scrapie DNA bank were genotyped. The allele and genotype frequencies of each SNP found for the ovine genes *LAMR1*, *SCRG1*, *PRND* and *RTN4* were compared through association analyses in scrapie negative and scrapie (classical and atypical) positive sheep populations. The fifth candidate gene sequenced, *VIM*, was thought to be a pseudogene at the time. The *VIM* SNP identified was not further investigated with respect to classical or atypical scrapie status in sheep.

5.1 Selection of SNPs for Scrapie Association Analysis

Only one SNP each was found in the *SCRG1*, *PRND* and *VIM* candidate genes. Two possible SNPs for each of the genes *LAMR1* and *RTN4* were identified.

In the case of the *LAMR1* SNP (T/C) further analysed in this study, all three genotypes could be observed in the initial samples sequenced and thus appeared to be the better SNP to choose for association testing. The presence of all three genotypes within this sample population allowed this SNP to be tested for Mendelian inheritance in the sheep families. The second *LAMR1* SNP found (G/A) caused an unlikely glutamic acid (E) to glycine (G) change in the protein (Henikoff and Henikoff, 1992), and only two of the three possible genotypes were identified in initial samples sequenced. For this reason, it was not chosen for further scrapie association studies. This SNP may be an artifact of sequencing since this position is surrounded on both sides by two guanines, which may have led to increased and overlapping flourescence signal. In addition, the RFLP developed for genotyping for this SNP was insufficient. However, the possibility of an SNP at this position remains interesting since it is found within the putative binding site

for laminin (Qiao et al., 2009a). The homozygote GG genotype could represent a lethal mutation or be linked to one, which could explain the absence of GG homozygotes. The ovine, bovine and human translations for the *LAMR1* gene all display a glutamic acid at this position (GenBank: NP001098733.1, NP776804.1 and NP001012321.1). An amino acid change from a large, polar glutamic acid to a small, hydrophobic glycine would most likely disrupt the laminin binding site structure due to differences in side chain polarity, size and charge (Sunyaev et al., 2001; Taylor, 1986).

A possible second *RTN4* SNP (C/T) was also identified. In contrast to *LAMR1*, this SNP is located within an intron, so a lack of all three genotypes does not carry as much significance except when testing for Mendelian inheritance in the sheep DNA families within this study itself. Again, it was not clear if the SNP was real or if background signal from the surrounding thymine bases overshadowed the cytosine base in the Sanger sequencing fragments. For this reason, it was considered unsuitable for further testing of association with scrapie susceptibility.

5.2 Genomic Locations of SNPs Investigated

SNP occurrence rate is inversely proportional to genome sequence conservation rates (Allendorf et al., 2010). Of the SNPs identified in this study, two were located in exons but were synonymous. The *LAMR1* T/C SNP investigated is situated at the third nucleotide position of codon 232. The *VIM* C/T SNP described in this study is located at the first position of codon 43. Codon position three has been shown to have the highest rate of variability compared to codon positions one or two, due to the degeneracy of the amino acid code (Castle, 2011). Of the amino acids, 19 are degenerate at this position. The second nucleotide position in amino acid codons shows the lowest rate of variability, increasing the suspicion that the *LAMR1* G/A SNP is an artifact. This pattern is only observed in protein coding regions and not in adjacent but equally functional gene regions such as polyadenylation sites (Castle, 2011; Chasman and Adams, 2001).

In general, the rate of SNP occurrence over 100 bp into an intron is three times higher than SNP occurrence rates in protein coding exons (Chamary et al., 2006). However, the highest conservation and the lowest SNP rate can be found at splice sites, outside of protein coding regions. In humans and mice, the region from 5 to 20 bp after a stop codon shows a high SNP occurrence rate (Castle, 2011). Other studies have shown that miRNA binding sites in the 3'-UTR begin at least 15 bp after the stop codon (Grimson et

al., 2007). An area 30 to 17 bp upstream from polyadenylation sites in human and mouse shows high conservation and low variability, constituting the polyA signal site (Castle, 2011).

Since many SNPs within one gene or chromosome region can also be linked, initially an entire gene may not require sequencing and genotyping (Lunetta, 2008). Because of this, SNPs in noncoding regions may be just as informative as those in coding regions and should not be disregarded.

5.2.1 Synonymous SNPs in Coding DNA Sequences

Synonymous point mutations within exons or UTR's of scrapie candidate genes may also be important for scrapie pathogenesis. Point mutations in coding sequences may affect microRNA (miRNA) binding sites which can, in turn, regulate gene expression and protein levels (Urbich et al., 2008). In this way, even synonymous SNPs could be important for disease pathogenesis, as results from other studies have already implicated (Ebert and Sharp, 2010; Poliseno et al., 2010; Urbich et al., 2008). The *LAMR1* gene has been analyzed in 126 sheep and nine synonymous polymorphisms were identified (Marcos-Carcavilla et al., 2008). Although synonymous, two of these polymorphisms (position 198 in exon 4 and position 96 in exon 7) could affect putative miRNA binding sites, resulting in impaired translation and subsequently altered protein levels.

SNPs in 3'UTR regions of mRNAs can affect post-transcriptional regulatory processes affecting mRNA stability, movement or translation efficiency (Magee et al., 2010). Another study has demonstrated that SNPs in 3'UTR regions are associated with fatty acid composition of milk (Kgwatalala et al., 2009).

PRND is located in a QTL in mice found repeatedly to have a general effect in all TSE models, specifically the genetic cross (i.e. mouse lines used), routes of infection (intracranial or intraperitoneal) and TSE agent, implemented in various studies (Iyegbe et al., 2010; Lloyd et al., 2001; Lloyd et al., 2002; Stephenson et al., 2000). The *PRND* SNP investigated in this study is located 162 bp into the 3'-UTR region and is not involved in any known DNA or protein motif representing possible regulatory regions (de Castro et al., 2006). Previously investigated *PRND* SNPs in human, bovine and sheep have not shown significant association with prion disease (Balbus et al., 2005; Comincini et al., 2001; Jeong et al., 2005b; Mesquita et al., 2010). However, an association

between a SNP in the *PRND* 3'-UTR and sCJD was found in a Korean population, but not in European populations (Jeong et al., 2005a). It could be that this polymorphism is in linkage disequilibrium with a close locus involved in TSE susceptibility.

5.2.2 Intronic SNPs

SNPs occurring in introns also merit investigation as they may be located in functional and regulatory gene structures, such as transcription factor binding sites, putative splicing signal sites, or polyadenylation sites (Chamary et al., 2006; Manolio, 2010). A G/A SNP in intron 3 of the porcine *IGF2* gene has been shown to be the causal mutation within a QTL for muscle mass and fat deposit in pigs (Magee et al., 2010). The remaining three SNPs in the candidate genes *SCRG1*, *PRND* and *RTN4* investigated in this study are localized in introns but did not form parts of known regulatory structural motifs when analyzed *in silico* (http://www.uniprot.org/; http://prosite.expasy.org/). One study has identified highly significant SNPs for scrapie incubation time using heterogenous stock mice that were all located in introns or in the 3'-UTR region, but were without apparent functional significance (Lloyd et al., 2010). It has been estimated that only about 12% of SNPs associated with disease are located in protein-coding regions of genes. Approximately 40% are found in intergenic regions and the remaining SNPs are located in noncoding introns (Manolio, 2010).

5.3 *LAMR1* Pseudogenes and Susceptibility to Prion Disease

The initial attempt to target the ovine *LAMR1* gene resulted in the amplification of a pseudogene. The term pseudogene was coined to describe a truncated copy of a functional gene in the genome, but not thought to undergo transcription or translation (Jacq et al., 1977). Until recently, pseudogenes have been relegated to the 'junk DNA', the 98% of the mammalian genome not encoding functional genes. However, evidence exists that pseudogenes exhibit sequence conservation of open reading frames and have a slow rate of mutations in mammals and other complex organisms (Podlaha and Zhang, 2004; Rothenfluh et al., 1995; Sudbrak et al., 2003). The ENCODE (ENCyclopedia Of DNA Elements) Consortium has shown that the vast majority of large 'barren' DNA areas are indeed transcribed as non-protein-encoding RNA (ncRNA) in humans (Birney et al., 2007). In their analysis of a targeted 1% (29,998 kb) of the human genome, 124 processed and 77 non-processed pseudogenes were identified. It was estimated that at least 19% of these pseudogenes are transcribed.

Recent studies have ascribed certain pseudogenes biological roles in diseases such as cancer and diabetes (Chiefari et al., 2010; Kalyana-Sundaram et al., 2012; Poliseno et al., 2010). One study showed a significant association between the presence of an ovine *LAMR1* pseudogene and resistance to scrapie (Marcos-Carcavilla et al., 2008). The pseudogene was present in 68.6% of healthy sheep, but only in 45.4% in scrapie infected sheep, albeit in a small sample size (Marcos-Carcavilla et al., 2008). The role of pseudogenes, in particular *LAMR1* pseudogenes, in scrapie susceptibilty remains to be investigated.

5.3.1 *LAMR1* Pseudogenes

The full length functional *LAMR1* gene is a member of a multicopy gene family including many pseudogenes. The presence of these pseudogenes scattered throughout the mammalian genome has impeded molecular investigations of the functional gene. As implemented in this study, a simple method to avoid PCR amplification of pseudogenes from genomic DNA is to design the reverse primer from an intron region showing a high degree of similarity between species.

In the human genome, 63 *LAMR1* pseudogenes have been identified as well as 45 in the mouse genome, with evidence of at least one in humans and two in mice being transcribed (Asano et al., 2004; Balasubramanian et al., 2009; Fernandez et al., 1991; Jackers et al., 1996a; Richardson et al., 1998). Of those mapped, the pseudogenes are distributed over 3 chromosomes in the mouse (MMU 2, 4 and 11) and over 6 chromosomes in human (HSA 1, 6, 9, 13, 20 and X).

Eleven *LAMR1* pseudogenes have been identified and mapped in the ovine genome (Van den Broeke et al., 2010). Six of the eleven ovine pseudogenes are transcribed. Transcribed pseudogenes can produce small interfering RNAs (siRNAs) that are capable of regulating expression of the functional genes or other genes (Khachane and Harrison, 2009). Since one of these pseudogenes may play a role in resistance to scrapie (Marcos-Carcavilla et al., 2008), the ovine *LAMR1* family is still worthy of further investigation in regard to their role in scrapie susceptibility. A study examining SNPs in a human *LAMR1* gene and their role in sporadic CJD did not find any association with disease susceptibilty (Yun et al., 2011). However it was not clear in the study if the SNPs were located in the gene or in a pseudogene.

5.3.2 *RARB-THRB* Locus in Mice Contains a *LAMR1* Pseudogene

A genome-wide association study incorporating a human GeneChip® covering 100K and 500K SNPs in patients identified an SNP in the region of the *RARB-THRB* genes (HSA 3) conferring increased risk to vCJD and iCJD (Mead et al., 2009). Consequently these genes were genotyped in a follow up study using 1052 mice phenotyped for scrapie incubation time (Grizenkova et al., 2010). The *RARB-THRB* locus is conserved in mice and is mapped to MMU14 (17.2 Mb and 18.49 Mb, repectively). Although all polymorphisms identified (Grizenkova et al., 2010) were synonymous, one SNP in the *RARB* gene (A>C in exon 3) and one SNP in the *THRB* gene (G>A in exon 6) were chosen for genotyping. Both showed a highly significant association with scrapie incubation time in mice. Since these SNPs displayed the same mouse strain distribution, it was inferred that they were linked which prevents elucidation of which gene and/or structure in this region might contribute to the effect on scrapie incubation time.

This region has never been shown to contain a QTL for scrapie incubation time in mice. However, the most recent mouse genome map (Build 37.2) predicts a *LAMR1* pseudogene (Gene ID 100416372) located between *RARB* and *THRB*. On human chromosome 3 (Map Build 37.3), a ribosomal protein L31 pseudogene (24.47 Mb) is located between these two genes. The functional *LAMR1* gene is also located downstream on HSA 3. Using sequences from both human and bovine *RARB* and *THRB* genes to BLAST (Altschul et al., 1997) the ovine Genome Assembly v2.0 (www.livestockgenomics.csiro.au) database, both genes locate to OAR 26. However, no ovine *LAMR1* pseudogene has been mapped to this chromosome (Van den Broeke et al., 2010). From this positional evidence, it cannot be excluded that the mouse *LAMR1* pseudogene at this locus may be involved in modulating scrapie incubation time.

5.4 Genotype Frequencies

No significant differences were found in the genotype frequencies between classical and atypical scrapie status groups for the SNPs investigated. Some trends can be observed when comparing genotype frequency results attained to expected frequencies according to Hardy-Weinberg equilibrium (HWE) (Hardy, 1908; Weinberg, 1908). In human case control studies, testing for Hardy-Weinberg equilibrium as a quality control step in control groups is controversial (Gomes et al., 1999; Teo et al., 2007). However, if population

prevalence of the phenotype is low, HWE testing should be conducted on the controls (Lunetta, 2008).

Genotype frequencies in both atypical and classical control groups for *LAMR1* (Table 19) and *SCRG1* (Table 22) do not support HW proportions. In the positive atypical scrapie group, the *LAMR1* genotype frequencies show a shift to heterozygote status (Table 17). This was observed only for the atypical sheep flocks, many of which consist of Merino Land (17%) or Merino Land cross sheep. Further genotyping of additional samples is required to enable interpretation of this trend.

PRND genotypes in the atypical scrapie control group are in HW equilibrium, despite the low frequency of the minor allele, and in HW disequilibrium in the classical scrapie control group (Table 25). Sample size was quite small for *PRND* and the minor allele displayed a low frequency, requiring genotyping of more samples to interpret these results. Low frequency of the minor allele decreases sensitivity of HWE calculation and may inflate error rates (Hosking et al., 2004; Lunetta, 2008).

The *RTN4* genotype frequencies in the control group for classical scrapie are in Hardy-Weinberg equilibrium but in HW disequilibrium in the control group for atypical scrapie (Table 29). Again, this may be due to differences in breeding populations, since classical scrapie tends to occur in 'Suffolk-type' flocks and atypical scrapie in 'Merino-type' flocks. However, most flocks in this study (74%) are of mixed breed. Population stratification could confound results in that *RTN4* may be linked to a trait under the influence of genetic selection in some sheep breeds.

5.5 Limitations of the Study

5.5.1 Choosing Candidate Genes from Scrapie Incubation QTL Studies

At the time this study was conducted, QTL studies were identifying large genomic regions that were linked to prion disease incubation times in mice (Lloyd et al., 2002; Moreno et al., 2003; Stephenson et al., 2000). These genomic stretches could contain numerous candidate genes as well as putative gene regulatory sites. Inherent to candidate gene approaches as well as QTL mapping are many limitations. Identifying QTLs for prion disease incubation times is dependent on scrapie routes of admission (intracranial versus intraperitoneal), genetic background of the chosen host as well as the choice of scrapie agent. These effects are manifested in the consistency of studies

identifying a variety of different QTLs, which ultimately may not be relevant to naturally occurring infections (Iyegbe et al., 2010).

QTL studies cannot exclude the existence or involvement of *PRNP* regulators or epigenetic effects (i.e. genomic imprinting, maternal effects) (Georges et al., 2003). Environmental factors do not tend to alter genotype and therefore would not confound SNP association studies (Lunetta, 2008). However, environmental effects should not be underestimated in comparing results from different QTL studies (Georges et al., 2003; Whitelaw and Martin, 2001). Gene interactions, epigenetic effects and complex disease mechanisms of scrapie cannot be implicitly considered in a candidate gene approach. It is especially difficult to test single candidate genes in a disease like scrapie, since incubation phenotypes are most likely the product of multiple gene effects, where only the collective heritability may be cumulatively significant (Iyegbe et al., 2010).

However, common QTLs have been identified in studies employing different TSE strains, different genetic backgrounds and/or different routes of infection (Iyegbe et al., 2010; Lloyd et al., 2001; Lloyd et al., 2002; Manolakou et al., 2001; Moreno et al., 2003; Moreno et al., 2010; Stephenson et al., 2000) which supports a genetic influence independent of the *PRNP* locus (Iyegbe et al., 2010). The candidate genes *VIM*, *SCRG1* and *RTN4* investigated in this study are in QTLs repeatedly identified in different studies. The gene *VIM* is located in or very near QTLs identified in four separate studies (Iyegbe et al., 2010; Lloyd et al., 2001; Lloyd et al., 2002; Manolakou et al., 2001). The *VIM* SNP identified in this study could still be investigated with respect to scrapie susceptibility.

5.5.2 Sample Size

Small sampling sizes are a limitation in this study, weakening statistical outcomes and their interpretations. Sample size is limited by the low scrapie prevalence in Germany. In this study, the highest number of individuals genotyped in any one group was 304 sheep. A large sampling size of at least 500 is required to identify small effects of polymorphisms on disease phenotype at sufficient power (Long and Langley, 1999). Candidate genes themselves are, individually, expected to have a very small effect on scrapie susceptibility. *PRNP* alone has been estimated to account for 79% of genetic variation involved in scrapie susceptibility and the remaining variance due to polygenic influence (Diaz et al., 2005). Because of the large influence of *PRNP* on scrapie susceptibility, effects of *PRNP* codons on scrapie susceptibility in the German sheep

population have been determined using the same sample DNA bank as incorporated in this study (Lühken et al., 2007).

Sensitivity of Hardy-Weinberg deviation calculation is reduced when the minor allele frequency is less than 5%, which can occur when sample size is too small (Hosking et al., 2004). This was the case for *PRND*. In large sampling sizes, genotype frequency results deviating from HWE can indicate population stratification or genotyping mistakes (Lunetta, 2008; Marquard et al., 2009). Genotypes for a single locus in large, randomly mating populations should display HWE (Hardy, 1908; Weinberg, 1908). However, one study has shown that SNPs displaying deviation from HWE as well as a low minor allele frequency may be susceptible to false negative associations (Chan et al., 2009). Chan et al. (2009) also showed no relation between deviations in HWE and false associations for SNPs with a minor genotype frequency of less than 0.9%.

5.5.3 Population Stratification

In this study, for each scrapie positive sheep, a phenotypically similar sheep from the same flock was chosen as a control to minimize the effects of population stratification. Healthy flock mate sheep were matched in age and breed where possible. For the *PRND* data set, healthy flock mate sheep were also matched at *PRNP* codons 136, 154 and 171 or risk level group. Hardy-Weinberg equilibrium is a consequence of random mating in a population free of mutation, natural selection or genetic drift (Hardy, 1908; Lunetta, 2008; Weinberg, 1908). In populations under the influence of non-random mating, population stratification occurs resulting in significant differences in allele frequencies between subpopulations (Kang et al., 2010). Population stratification in livestock could be the result of genetic and artificial selection or genetic sampling or drift (Ma et al., 2012). In a sheep flock, there is likely to be a high degree of related individuals in a randomly chosen sample set, which can be a source of false-positive associations (Devlin and Roeder, 1999; Ma et al., 2012). Also, breeding for resistance to scrapie may compound population stratification in European sheep flocks. Conversely, the amount of genetic diversity within a flock is less than that between flocks.

5.5.4 Errors in Determining Scrapie Status of Individual Sheep

Determining scrapie case or control status of the study animals is a challenge due to long incubation times. The ovine DNA bank incorporated in this study is one of the most extensive for classical and atypical scrapie. Long incubation periods and previous

difficulties in diagnostic methods (i.e. differentiating between classical and atypical scrapie) may lead to possible discordances in the data. Keeping extensive records on individual flock animals has not been required from flock owners and is logistically difficult. A sheep may be infected with scrapie but show no clinical signs at the time of blood or tissue collection, therefore being labelled as a healthy cohort flock mate. The resulting genotype from this sheep for a specific gene that may have an influence on susceptibility could then skew results or at least reduce significance calculations. Although every effort is made to stay in touch with sheep flock owners and update the database, it cannot be excluded that such cases occur. Larger sample sizes could reduce the effect of such errors. The development of an economical *in vivo* test for classical and atypical scrapie that could identify preclinical cases would help to resolve classifying case or control sheep (Orrú et al., 2011; Rubenstein et al., 2010; Terry et al., 2009).

5.5.5 Genotyping Errors

The SNPs identified in this study have been shown to follow Mendelian inheritance patterns in family pedigrees, which supports their existence. Manual genotyping of smaller data sets as in this study can be advantageous to automatic genotyping in very large data sets attained with the use of high density oligonucleotide array-based chips. Error rates due to automatic genotyping can become more inflated as sample size increases (Marquard et al., 2009). Problematic are gross genotyping errors in extremely large data sets, as can happen with high density oligonucleotide array-based chips where genotyping is automated. Automated calling procedures have been shown to potentially falsify or inflate association studies results due to automated genotyping errors (Clayton et al., 2005; Teo et al., 2007). In this case, calculation of HW deviations can detect genotyping errors (Teo et al., 2007) but also delete SNPs that may actually contribute to disease phenotype (Fardo et al., 2009).

Genotyping errors within case control populations can occur through sample mishandling, DNA contamination, the presence of pseudogenes or errors within the high-throughput genotyping process (Hosking et al., 2004; Pompanon et al., 2005). In this study, DNA sample plates were created using a pipetting robot, which should have prevented DNA contamination between wells. Previously sequenced control DNA samples (3.1.2) included in the scrapie DNA sample plates used for RFLP analyses prevented errors in manually obtained genotypes.

Genotyping errors decrease the power of certain statistical tests for association, including Chi^2 test (Gordon et al., 2002). Errors can occur more frequently in certain methodologies, however some SNPs in Hardy-Weinberg disequilibrium (HWD) still can reveal no obvious reason for deviation (Hosking et al., 2004). Previously, HWE was used to detect errors in genotyping with the result that many SNPs likely to have been associated with disease phenotype were excluded from further investigation (Cox and Kraft, 2006; Leal, 2005). More recent studies have shown that HWD is not a sufficient indicator for minor genotyping errors and even SNPs in HWD could be retyped with another method and further investigated with regard to phenotype association (Fardo et al., 2009; Marquard et al., 2009; Wittke-Thompson et al., 2005). Such SNPs have been shown not to increase the number of false positive associations in population-based studies (Fardo et al., 2009). In this study, HWE results most likely do not reflect genotyping errors, rather reflect breeding practices and differences within and between herds.

5.6 Information Databases

Studies such as this are highly dependent on information available in public access databases such as NCBI and GenBank (http://www.ncbi.nlm.nih.gov/), the later of which which may contain erroneous sequence information gathered by methods aimed at high throughput but low precision. Primers for the candidate gene *VIM* were based on the alignment of the bovine, ovine and human sequences available at the time of this study. All of these sequences are now obsolete and have been replaced with updated sequences. However, at the time, comparison of the sequenced gene segment attained for *VIM* to those in the GenBank in 2007 indicated that the amplified product was a pseudogene due to the apparent absence of introns, thus no further analysis was undertaken. Assumming the updated records are correct (NC007311.4, NG012413 and NC019470), the *VIM* primer pair implemented in this study is most likely targeting the gene, since no record of a human, mouse, bovine or ovine *VIM* pseudogene exists to date.

On the other hand, sequences attained in studies such as the one presented here may utilize the vast information gathered since the initial construction of a reference ovine genome (ISGC et al., 2010). Using the sequences attained for the candidate genes, putative locations for *SCRG1* and *RTN4* could be determined. A search of the *O. aries*

genome assembly (Version 2.0) with the *SCRG1* sequence attained in this study places the ovine *SCRG1* gene on OAR2. In the mouse, *SCRG1* is physically linked to the gene *SAP30* (Dron et al., 2000) and a BLAST with the bovine *SAP30* mRNA sequence (NM001191124.1) also locates this gene to OAR 2. This region of OAR 2 is homologous to the region on mouse chromosome 8 containing a QTL for scrapie and BSE resistance (Moreno et al., 2003) as well as the mouse *SCRG1* gene. The sequence for *RTN4* attained in this study localizes the gene to OAR 3. The same search was conducted with the bovine *RTN4* mRNA sequence (NM001113221.1) due to the short sequence segment attained in this study. Both searches place ovine *RTN4* on OAR 3. Such results support the authenticity of the ovine sequences attained in this study.

5.7 Ovine SNP GeneChip

The Illumina Ovine SNP50 BeadChip was released in January 2009 by the International Sheep Genome Consortium (ISGC). DNA samples from a panel of 9 genetically separate sheep were sequenced and aligned to identify SNPs, of which approximately 54,241 were chosen for the SNP chip. The SNP chip covers the ovine genome with an average of one marker per 46 kb. This development has made it more efficient to identify SNP loci segregating with disease phenotypes (Becker et al., 2010; Zhao et al., 2011). These loci are more localized, sometimes scanning only 6 Mb (Zhao et al., 2011), thereby reducing the genomic region of interest and consequently, the number of candidate genes.

The genetic causes of dwarfism in Texel sheep (Zhao et al., 2012), rickets in Corriedale sheep (Zhao et al., 2011) as well as location of the horns locus in Australian Merino sheep (Dominik et al., 2012) have since been discovered with the help of the ovine SNP chip. Other applications for the SNP chip is to determine genetic division among and between breeds (Kijas et al., 2012), minimizing inbreeding (Garcia-Gamez et al., 2012) as well as determining population stratification or a minimum marker panel to be used in genomic selection (Hayes et al., 2012).

Diseases such as scrapie, which may involve the interactions of different genetic loci, are more difficult to study, but SNP array chips may allow a more exact identification of candidate gene chromosomal regions. Similarly, most economically important traits in livestock depend on multiple genetic loci, such as milk yield in dairy cows (Guo et al.,

2012; Mai et al., 2010; Pryce et al., 2010), growth traits in beef as well as dairy cattle (Bolormaa et al., 2011) or egg production and quality in layer hens (Liu et al., 2011).

None of the SNPs reported in this study are included on the Illumina Ovine SNP50 BeadChip. The location of the nearest chip SNP to those examined in this study are presented in Table 30. Three of the SNPs on the Illumina Ovine SNP50 BeadChip are located in structures belonging to the candidate genes *LAMR1*, *SCRG1*, and *RTN4* investigated in this study (Table 30). With the exception of the *PRND* and *VIM* SNPs, the remaining SNPs investigated in this study are located relatively close to an SNP on the chip. For the SNPs in *LAMR1*, *SCRG1* and *RTN4* from this study, the chip would not necessarily provide additional information with the same scrapie sample population since the investigated and chip SNPs are most likely linked due to their assumed proximity. However, linkage disequilibrium between SNP and QTL may not be consistent between different flock populations, resulting in higher false discovery rates (Bolormaa et al., 2011).

There are also disadvantages to using SNP chips to investigate traits resulting from cumulative small effects of QTL, such as scrapie susceptibility. Expecting that QTL other than the *PRNP* locus will have small, additive effects on scrapie susceptibility, testing less than 1000 sheep results in low power of GWAS. Increased sample numbers decrease false discovery rates in GWAS studies incorporating SNP chips (Bolormaa et al., 2011). Ideally, SNP associations with scrapie susceptibility should be confirmed in different breeds or populations. Again, some SNPs could also differ in the strength of their linkage disequilibrium when tested in different sheep breeds thus weakening power of associations. Conversely, a QTL may have pleiotropic effects on more than one unrelated trait or two linked QTLs may each affect different traits leading again to high false discovery rates. Two large populations of the same breed, but under the influence of divergent selection, could reduce the effect of these problems. This could mean, for example, implementing samples from scrapie-affected Merino or Suffolk populations from different European countries where no recent genetic mix has occurred.

Table 30: Locations of SNPs investigated in this study compared to those closest on the Illumina Ovine SNP50 BeadChip. Δ indicates bp difference in location, + downstream and – upstream; ‡ not within structure belonging to candidate gene listed

Gene	Chromosome	Positions* (bp) and Structure		ΔDistance (bp)
		Present study	Illumina Gene Chip	
LAMR1	OAR 19	12 621 707 *Exon 6*	12 614 512 *Intron 4*	– 4 264
SCRG1	OAR 2	106 202 228 *Intron 1*	106 202 276 *Intron 1*	+ 48
PRND	OAR 13	46 252 184 *3'-UTR*	46 825 711 ‡	+ 573 527
RTN4	OAR 3	68 943 382 *Intron 7*	68 948 322 *3'-UTR*	+ 4 940
VIM	OAR 13	30 747 665 *Exon 1*	30 710 419 ‡	– 37 204

* based on Map OARv3.1 (www.livestockgenomics.csiro.au) from October 2012

5.8 Future Considerations

The genes investigated in this study remain interesting candidates for scrapie susceptibility in sheep. The primer and sequence information could be used to test these candidate gene segments in other sheep and goat populations. Combining scrapie DNA banks from diferent countries would simultaneously increase sample numbers while offsetting other effects such as population stratification.

It would be justifiable to test for SNPs linked to scrapie susceptibility using the ovine SNP chip and the scrapie DNA bank in this study. However, results must be carefuly compared to those of other sheep populations to identify true associations. In addition, focus should not concentrate only on the effect of candidate genes, but also on the role of their regulatory elements and pathways. Genome locations of such elements could also vary even more than those of genes, creating more complexity and affecting the power of association studies. Pseudogenes may also be investigated as to their role in scrapie susceptibility and/or pathogenesis. A *LAMR1* pseudogene has already been implicated in modulating scrapie susceptibility (Marcos-Carcavilla et al., 2008). Knowledge of cellular regulation through protein homeostasis affected by various mRNA processing pathways and regulatory mechanisms (i.e miRNA or non coding RNA) continues to increase, permitting application to scrapie susceptibilty and pathogenesis. The interactions of genetic factors contributing to scrapie susceptibility in sheep, as in all multifactorial traits, appear highly complex. A single method to identify these genetic factors may not capture this complexity, rather a combination of different approaches may be necessary. Still, informative genetic material such as the DNA data bank incorporated in this study is essential.

6 Summary

Certain polymorphisms in the prion protein gene (*PRNP*) gene have been shown to modulate classical and atypical scrapie susceptibility in sheep. Sheep carrying the ARR/ARR genotype seem more resistant to classical scrapie whereas the VRQ haplotype greatly increases classical scrapie susceptibilty. Classical scrapie has been reported in one ARR/ARR sheep and with the advent of active surveillance in the EU, not only have two other ARR/ARR sheep with classical scrapie been identified, but also atypical scrapie was shown to be more prevalent than expected. Haplotypes increasing susceptibility to atypical scrapie, such as AHQ or AF141RQ, contrast with those of classical scrapie. It has been estimated that *PRNP* is responsible for approximately 79% of the genetic influence of *PRNP* on classical scrapie susceptibility.

The goal of the study was to identify single nucleotide polymorphisms (SNPs) in candidate genes for scrapie susceptibility and test them for association with both classical and atypical scrapie susceptibility in sheep. Five candidate genes, *LAMR1*, *SCRG1*, *PRND*, *RTN4* and *VIM*, were chosen due to their locations within or near mouse quantitative trait loci (QTL) for scrapie susceptibility. The ovine candidate genes were then amplified and sequenced. Sequences attained for *LAMR1*, *SCRG1*, *PRND*, and *VIM* were submitted to GenBank.

A synonymous C/T *LAMR1* SNP analysed is located in Exon 6 at the third nucleotide position of codon 232. For the *SCRG1*, *PRND* and *RTN4* genes, a G/A SNP (Intron1), a T/C SNP (3'-UTR) and a G/A SNP (Intron 7) could be identified. None of these mutations were found to be located in a functional or regulatory site within noncoding regions. For each candidate gene, one SNP was tested for possible association with scrapie status using DNA samples from an established scrapie DNA bank held at the Institute of Animal Breeding and Genetics, Justus-Liebig-University, Giessen, Germany.

Genotyping was accomplished using restriction fragment length polymorphism (RFLP) or amplification-created restriction site (ACRS)-RFLP analyses. For the *LAMR1*, *SCRG1* and *RTN4* SNPs, DNA samples from up to 106 classical scrapie positive sheep as well as up to 137 healthy flock mates were genotyped. In the atypical scrapie group, DNA samples from up to 93 atypical scrapie positive sheep and up to 304 healthy flock mates were genotyped. Since *PRND* is located near the *PRNP* gene on ovine chromosome

(OAR) 13, positive and negative DNA samples were matched for *PRNP* genotype. For the *PRND* SNP, 56 DNA samples each from both classical and atypical scrapie positive sheep were genotyped. Negative controls consisted of DNA samples from 74 healthy flock mates from classical scrapie herds and 73 healthy flock mates from atypical scrapie herds. For each of the SNPs investigated in this study, no significant differences in allele or genotype frequencies were observed for classical or atypical scrapie positive groups when compared to healthy flock mate control groups.

Although one SNP was identified in the ovine *VIM* gene, sequence information available at that time indicated a pseudogene had been amplified. Since, updated sequence information for bovine *VIM* shows that the synonymous C/T SNP identified in this study is located at the first position of codon 43.

No significant associations between candidate gene SNPs and scrapie status could be shown. Due to the low numbers of animals genotyped for the association studies, these genes cannot be excluded as candidate genes for scrapie susceptibility in sheep.

With the development of the Illumina Ovine SNP50 BeadChip, it would be possible to use the chip to identify SNPs associated with scrapie susceptibility. However, sample size needs to be increased in order to identify genetic loci that have modest effects on scrapie susceptibility in sheep. This could be accomplished by combining various scrapie DNA banks from different EU countries.

7 Zusammenfassung

Bestimmte Polymorphismen des Prionenproteingens (*PRNP)* besitzen einen Einfluss auf die Empfänglichkeit für klassische und atypische Scrapie beim Schaf. Träger des ARR/ARR Genotyps scheinen mehr Resistenz gegenüber klassische Scrapie zu besitzen, wohingegen der VRQ Haplotyp die Empfänglichkeit erhöht. Allerdings wurde in einem Schaf mit ARR/ARR Genotyp klassische Scrapie nachgewiesen, und mit der Einführung der aktiven Überwachung in der EU sogar in zwei weiteren Schafen mit ARR/ARR Genotyp. Die aktive Überwachung hat zudem gezeigt, dass die Prävalenz von atypischer Scrapie höher ist als erwartet. Haplotypen wie z.B. AHQ und $AF_{141}Q$, welche die Empfänglichkeit für atypische Scrapie erhöhen, wirken sich im Gegensatz hierzu unterschiedlich auf die Empfänglichkeit für klassische Scrapie aus. 79% der Empfänglichkeit für klassischer Scrapie werden dem *PRNP* Gen zugeschrieben, die übrigen 21% anderen, unbekannten Faktoren.

Das Ziel dieser Studie war es, SNPs (single nucleotide polymorphism) in Kandidatengenen für Scrapie Empfänglichkeit zu identifizieren, und diese auf Assoziationen sowohl mit klassischer als auch atypischer Scrapie zu überprüfen. Hierfür wurden 5 Kandidatengene, *LAMR1*, *SCRG1*, *PRND*, *RTN4* and *VIM* aufgrund ihrer Lokalisation in Maus QTLs (quantitative trait loci) für Scrapie Empfänglichkeit ausgewählt. Anschließend wurden diese Gene im Schaf amplifiziert und sequenziert. Die ermittelten Sequenzen von *LAMR1*, *SCRG1*, *PRND*, und *VIM* wurden elektronisch bei der GenBank eingereicht.

Der C/T *LAMR1* SNP konnte im Exon 6 an der dritten Position von Kodon 232 lokalisiert werden. Für die *SCRG1*, *PRND* und *RTN4* Gene wurden entsprechend ein A/G SNP (Intron 1), T/C SNP (3'-UTR) und ein G/A SNP (Intron 7) identifiziert. Keiner dieser drei SNPs war in einem funktionellen oder regulatorischen Bereich innerhalb der nicht-kodierenden DNA lokalisiert.

Jeweils einer der für *LAMR1, SCRG1*, *PRND* und *RTN4* identifizierten SNPs wurde anschließend mit Hilfe der Scrapie DNA Bank (Institut für Tierzucht und Haustiergenetik, Justus-Liebig-Universität, Giessen) auf eine Assoziation mit Scrapiestatus getestet.

Die Genotypisierung erfolgte mittels RFLP (restriction fragment length polymorphism) oder ACRS (amplification-created restriction site)-RFLP Analyse. Für die *LAMR1*, *SCRG1* und *RTN4* SNPs wurden DNA Proben von bis zu 106 auf klassische Scrapie positiv getestete Schafe sowie bis zu 137 gesunden Herdenmitglieder zur Genotypisierung verwendet. Für die atypische Scrapie Gruppe wurden DNA Proben von bis zu 93 positiv getesteten Schafen und bis zu 304 gesunden Herdenmitgliedern zur Genotypisierung verwendet. Da *PRND* stromabwärts vom *PRNP* Gen auf dem ovinen Chromosom 13 lokalisiert ist, stimmten Scrapie positive und negative Proben hinsichtlich ihrer *PRNP* Genotyps überein. Für den *PRND* SNP wurden von jeder klassischen und atypischen Scrapie positiven Gruppe jeweils 56 Proben genotypisiert. Als Negativkontrolle dienten Proben von 74 Herdenmitglieder bei klassischer Scrapie und 73 Herdenmitglieder bei atypischer Scrapie. In Hinblick auf Empfänglichkeit für klassische oder atypische Scrapie konnten keine signifikanten Assoziationen der Allel- oder Genotypfrequenzen gefunden werden.

Ein SNP wurde in dem *VIM* Gen identifiziert. Zum Zeitpunkt dieser Studie deutete die Sequenzinformation daraufhin, dass ein Pseudogen amplifiziert wurde. Neue Sequenzerkenntnisse zeigten allerdings dass der synonyme C/T SNP auf der ersten Position des Kodons 43 lokalisiert ist.

Obwohl keine Assoziationen nachgewiesen worden sind, bleiben die untersuchten Kandidatengene interessant in Hinblick auf Scrapieempfänglichkeit beim Schaf. Aufgrund der niedrigen Probenzahlen kann die Relevanz dieser Kandidatengene nicht ausgeschlossen werden.

Zukünftige Studien können inzwischen auf den Illumina OvineSNP50 Gen Chip zurückgreifen, um die Identifizierung Scrapie assoziierter SNPs zu ermöglichen. Dies erfordert allerdings eine ausreichende Probenzahl, um auch Assoziationen von Genorten mit mäßigem Effekt auf Scrapie Empfänglichkeit nachzuweisen. Eine Möglichkeit, um die Probenzahl zu erhöhen, wäre die Verwendung von Proben aus Scrapie DNA Banken von verschiedenen EU Ländern.

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9 Appendix

Table A1: Characteristics of ovine classical scrapie and atypical scrapie.

	Classical Scrapie	Atypical scrapie
Average Age of Onset	2-5 years of age, ∅ 3.1	≥ 5 years
Incubation time	≥ 1 year	?
Clinical signs	behavioral changes, ataxia, pruritus, recumbency, loss of body condition	ataxia, loss of body condition
Epidemiology	horizontal and vertical transmission (orally); ≥1 animal/flock	no evidence of natural horizontal or vertical transmission; only 1 animal/flock
Most susceptible *PRNP* haplotypes	$V_{136}R_{154}Q_{171}$	$A_{136}H_{154}Q_{171}$ $A_{136}F_{141}R_{154}Q_{171}$
PrP^{Sc} distribution in CNS	medulla oblongata	cerebellum and cerebral cortex
Histopathology	vacuolation of neurons, neuropil	vacuolation
PrP^{Sc} distribution in periphery	LRS, PNS, placenta	not detected
Rapid test method	ELISA	ELISA
Confirmatory test method	WB with mAb P4	WB with mAb P4
PrP^{Sc} glycoprofile	mAb 6H4: 3 bands 19-30 kDa mAb P4: 3 bands 19-30 kDa	mAb P4: multiple band pattern, lowest band ≤ 15 kDa

∅=average; LRS= lymphoreticular system; TBM: tingible body macrophages; FDC: follicular dendritic cells; PNS= peripheral nervous system; mAB= monoclonal antibody; WB= Western blot; ELISA: Enzyme Linked Immunosorbent Assay

Appendix

Table A2: Genbank Accession No. is first BLAST result for candidate gene sequences. Parameter describes BLAST program search optimizations. Degree of similarity listed as percentage.

Gene	Genbank Accession No.	Parameter	Sequence Similarity (%)	Comment
LAMR1 **Ex5f - In6r**	NW_930073 *Bos taurus* whole genome shotgun	discontinuous megablast	86%	includes complete introns 5 & 6
		megablast	94%	includes intron 5 and first 17 bases of intron 6
LAMR1 **Ex5f - In6r**	NM_001105263.1 *Ovis aries* mRNA	megablast	> 99%	exon 5 and exon 6
SCRG1 **Ex1f - In1rB**	NT_016354.19 *Homo sapiens* genomic contig	megablast	89%	including intron 1
PRND **Ex2f B - 3'UTRr**	AY_017311.1 Ovis aries complete coding seq	megablast	100%	exon 2 including 3'UTR region
RTN4 Ex7-In7	AY_102285.1 *Homo sapiens* complete sequence	discontinuous megablast	76%	including intron 7; exon 7 100% similar
	NM_001145178 *Ovis aries* mRNA	discontinuous megablast	100%	exon 7 only
VIM **Ex1f - Ex2r**	NG_012413 *Homo sapiens* RefSeq	megablast	94%	sequence in exon 2
	NC_007311 *Bos taurus* whole genome shotgun	megablast	99%	sequence in exon 1

megablast = highly similar sequences; discontiguous megablast = more dissimilar sequences

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