Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:13

The presence of an acute phase response in experimental classical scrapie in lambs indicates a shift towards a pro-inflammatory status in the clinical terminal stages

Tilstedeværelsen av en akutt fase-reaksjon hos lam med eksperimentell klassisk skrapesjuke indikerer et skifte mot en pro-inflammatorisk tilstand i det kliniske endestadiet

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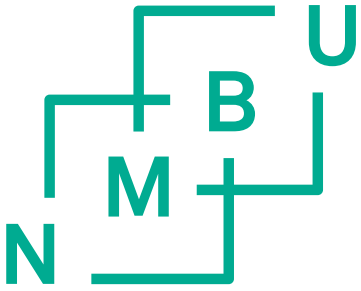

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Section of Small Ruminant Research

Sandnes 2021

Thesis number: 2021:13

ISSN: 1894-6402

ISBN: 978-82-575-1721-2

For the sheep when well is often times capricious and when sick does wish to die.
Anon

Acknowledgements

The present work was conducted at the Section of Small Ruminant Research (Sandnes) at the Norwegian University of Life Sciences (NMBU) between 2007 and 2013. The project was financed by the Research Council of Norway. I am incredibly grateful to have had this fantastic opportunity to undertake this work at NMBU and the Section at Sandnes.

First, I would like to express my very great appreciation to my main supervisor, Martha J. Ulvund (Professor emeritus, NMBU). Thank you for guiding me through these years, and for giving me much needed encouragement and support, inspiration, and valuable feedback. This had not been possible without you.

The support and guidance provided by my co-supervisors, Kjetil Bårdsen (NMBU), Anne Hjelle (IRIS), Olav Kvalheim (UiB) and Kerstin Skovgaard (DTU) are much appreciated. Thank you for your patience and sharing your invaluable knowledge and experience with me.

Advice and constructive recommendations given by the additional co-authors, Arne Arneberg (UiB) and Peter Mikal Helweg Heegaard (DTU) has been an invaluable help in improving the manuscripts.

Many thanks go to Eli Brundtland (NMBU), Mari Mæland (IRIS), Kai Erik Ueland (IRIS), Eivind Larssen (IRIS) and Karin Tarp (DTU) for their patience and help during the experiments. You all provided invaluable guidance and help to develop and improve my laboratory skills and the handling of large data sets.

I wish to acknowledge the support and help from the great staff at the Section of Small Ruminant Research at Sandnes, I have really enjoyed working with you.

I would like to offer my special thanks to Christian Helland (UiB) and his team at Haukeland University Hospital, Neurosurgery, for providing the best available medical care.

Special thanks go to my dear family for putting up with me and giving me the space and time to finish this work

Finally, I wish to thank the PhD committee for evaluating this thesis

"No man is an island"
- *John Donne*

Contents

Abbreviations

TSEs	Transmissible Spongiform Encephalopathies
CJD	Creutzfeldt Jakob Disease
vCJD	Variant Creutzfeldt Jakob Disease
BSE	Bovine Spongiform Encephalopathy
GSS	Gerstmann-Sträussler-Scheinker Diseases
FFI	Fatal Familial Insomnia
CWD	Chronic Wasting Diseases
TME	Transmissible Mink Encephalopathy
FSE	Feline Spongiform Encephalopathy
SELDI-TOF-MS	Surface Enhanced Laser Desorption Ionisation – Time of Flight – Mass Spectrometry
APR	Acute phase response
APP	Acute phase protein
PLS	Peripheral lymphatic system
CNS	Central nervous system
PNS	Peripheral nervous system
PrP^{C}	Cellular isoform of prion protein
PrP	Prion protein
PrP^{Sc}	Scrapie specific pathological isoform of prion protein
PRNP	Prion gene
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
PrP^{27-30}	The proteinase resistant core of prion protein
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
Hp	Haptoglobin
qPCR	Quantitative polymerase chain reaction (real-time)
SAA	Serum amyloid A
GPI	Glycophosphatidylinsotol
A	Alanine
V	Valine
R	Arginine
H	Histidine
Q	Glutamine
AMP	Adenosine monophosphatase
cAMP	Cyclic AMP
cDNA	Complementary DNA
DNase	Deoxyribonuclease
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
NMR	Nuclear magnetic resonance
ORF	open reading frame
bp	base pairs
AI	Artificial Insemination
NKS	Norwegian White Breed
NSG	Norwegian Association of Sheep and Goat Farmers

PMCA	protein misfolding cyclic amplification
GALT	Gut-associated lymphoid tissue
PPs	Peyer's patches
RAMALT	Recto-anal mucosa-associated lymphoid tissues
PSNS	Parasympathetic nervous system
SNS	Sympathetic nervous system
ANS	Autonomic nervous system
ENS	Enteric nervous system
DMNV	Dorsal motor nucleus of vagus
IMLC	Intermediolateral column
TBMs	Tingible body macrophages
PRND	Prion Like Protein Doppel Gene
SIRPα	Signal Regulatory Protein α
CDP	Chronic Demyelinating Polyneuropathy
PRNP$^{Ter/Ter}$	Naturally occurring nonsense mutation of prion protein gene terminating PrP synthesis
LPS	Lipopolysaccharide
IFN	Interferon
PBMCs	peripheral blood mononuclear cells

Summary

Classical scrapie in sheep is a transmissible and fatal neurodegenerative disease caused by the self-replicating and infectious prion protein, PrP^{Sc}, which is a conformational variant of the normal cellular prion protein, PrP^{C}. The prion protein is a highly conserved glycoprotein encoded by the *PRNP* gene and therefore within the same host both PrP^{C} and PRP^{SC} have the same unique amino acid sequence and they only differ in their three-dimensional folded structure. Specific mutations at codons 136, 154 and 171 of the *PRNP* gene leads to single amino acid substitutions, and the most common polymorphisms give rise to five possible alleles and 15 *PRNP* genotypes found in sheep. The different alleles are highly associated with levels of susceptibility to classical scrapie, where $A_{136}R_{154}R_{171}$ allele provides high genetic resistance and $V_{136}R_{154}Q_{171}$ allele results in highly susceptible animals. On the basis of this association between *PRNP* genotype and susceptibility, many EU MSs have implemented national breeding for resistance programme with the aim of increasing distribution of ARR allele and reducing the distribution of VRQ allele. For almost 20 years, the EU TSE regulation has required surveillance within each country to establish prevalence of prion diseases and the different *PRNP* genotypes. Classical scrapie has a widespread distribution and incidence rate fluctuates due to the complex interaction between prion and host factors, and prevalence can only be estimated by *ante mortem* testing through active and passive surveillance. Transmission between sheep occurs through direct and indirect contact, and PrP^{Sc} can remain infective in the environment for years. The most common route of infection is the oral route, and infected animals can excrete PrP^{Sc} through foetal membranes and fluids, saliva, urine, faeces, and milk. Pathogenesis is highly influenced by *PRNP* genotype, as animals of the most susceptible genotypes have the most effective uptake of PrP^{Sc} across small intestine followed by an extensive dissemination and involvement of the SLOs, and an early neuroinvasion with spread of PrP^{Sc} within the CNS. The susceptible genotypes will contribute the most to spread of infectivity and environmental contamination.

This work describes the results from experimental classical scrapie where homozygous VRQ lambs were inoculated orally at birth with homogenated brain material from either healthy sheep or from natural cases of classical scrapie. This resulted in a worst-case scenario type of classical scrapie with sudden onset of severe clinical signs at 22 wpi followed by a rapid deterioration and euthanasia at 23 wpi. Serum samples were collected at regular intervals and tissue samples from brain and liver were sampled at *post mortem* examination. Proteomic examinations of serum revealed a downregulation of several protein peaks during the pre-symptomatic incubation period in the scrapie affected group compared to the control group, and a shift to upregulation of protein peaks onwards from 22 wpi. Genomic examinations of serum samples showed a slight downregulation *IL1B* and *TLR4* at 16 wpi, followed by a change at 22 wpi with upregulation of genes encoding TLRs, C3 and APPs. Genomic examination of liver and brain tissues showed an alteration in gene expression of APPs in accordance with an APR. Serum analyses of different APPs showed increased levels of the positive APPs and a reduced concentration of negative APPs.

These findings are indicative of a shift from anti-inflammatory to pro-inflammatory systemic innate immune response that coincide with the onset of debilitating clinical disease. In neurodegenerative diseases, the innate immune response in the CNS has a key role in both onset and progression of disease and resolution of inflammation. The accumulation of PrP^{Sc} in the CNS has been associated with a chronic activation of the innate immune response, pro-inflammatory activation of microglia, neuroinflammation, and neurodegeneration.

The diseases phenotype registered in this work is a result of *PRNP* genotype, and time and dose of inoculation, which can occur naturally if the right circumstances are in place. New-born homozygous VRQ lambs from an infected dam can get infected at birth. These cases could develop a similar disease progression as described in this work, resulting in an efficient and fast uptake and widespread peripheral and central dissemination of PrP^{Sc}, and clinical disease at a young age. These cases would present as a diagnostic challenge and easily missed as classical scrapie. Due to their young age, these cases would not be sampled through active surveillance. If incubation period extends commercial lifespan, these lambs would be slaughtered for human consumption, and due to their *PRNP* genotype, prions would enter the food chain.

Control of classical scrapie can probably not be achieved by absence of infectivity, but absence of clinical disease is possible through breeding for resistance which will provide flock immunity to classical scrapie.

Sammendrag (summary in Norwegian)

Klassisk skrapesyke hos sau er en overførbar og dødelig nevrodegenerativ sykdom forårsaket av det selvrepliserende og smittsomme prionproteinet, PrP^{Sc}, som er en variant av det normale cellulære prionproteinet, PrP^{C}. Prionproteinet er et glykoprotein som er kodet for av *PRNP*-genet. Dette betyr at PrP^{C} og PRP^{SC} hos samme verten, har den samme unike aminosyresekvensen og det er kun den tredimensjonale strukturen som skiller dem. Spesifikke mutasjoner ved kodonene 136, 154 og 171 i *PRNP*-genet fører til substitusjoner av enkelte aminosyrer, og de vanligste polymorfismer gir opphav til fem mulige alleler, og 15 *PRNP*-genotyper hos sau. De forskjellige allelene er assosiert med nivå av mottakelighet for klassisk skrapesyke, og $A_{136}R_{154}R_{171}$-allel fører til genetisk resistens, og $V_{136}R_{154}Q_{171}$-allel gir høy mottagelighet. På bakgrunn av denne sammenhengen mellom *PRNP*-genotype og mottakelighet, har mange EU medlemsland innført nasjonale avlsprogram som har mål om å øke utbredelsen av ARR-allel, og samtidig en reduksjon av VRQ-allel. I snart 20 år har EUs TSE-regelverk krevd nasjonale overvåkingsprogram for å bestemme forekomsten av prionsykdommer og kartlegge utbredelsen av de forskjellige *PRNP*-genotypene. Klassisk skrapesyke er utbredt, men forekomsten vil variere med bakgrunn i det komplekse samspillet mellom prionprotein og vertsfaktorer. Prevalens kan estimeres gjennom *ante mortem* testing i forbindelse med aktivt og passivt overvåkingsprogram. Smitteoverføring mellom sau skjer ved direkte og indirekte kontakt, og PrP^{Sc} er smittsomt i flere år i miljøet. Den vanligste infeksjonsveien er gjennom oralt inntak, og dyr kan skille ut smittsomt PrP^{Sc} via fosterhinner og væsker, spytt, urin, feces og melk, og nivå er styrt av *PRNP* genotype.

Patogenesen er også sterkt påvirket av *PRNP*-genotype, og sau av de mest mottagelige genotypene har effektivt opptak av PrP^{Sc} over tynntarmen, etterfulgt av omfattende replikasjon og spredning av PrP^{Sc} til sekundære lymfeorganer og CNS via neuroinvasion.

Dette arbeidet beskriver resultatene fra eksperimentell klassisk skrapesyke der homozygote VRQ-lam ble inokulert oralt ved fødselen med homogent hjernemateriale fra enten friske sauer eller fra naturlige tilfeller av klassisk skrapesyke. Dette resulterte i en "Worst-case scenario" presentasjon av sykdommen med alvorlige kliniske symptomer allerede ved 22 uker, etterfulgt av en rask forverring og avliving ved 23 ukers alderen.

Proteomiske undersøkelser av serum viste en nedregulering av flere proteintopper i løpet av den pre-symptomatiske inkubasjonsperioden i skrapesyk-gruppen sammenlignet med kontrollgruppen. Fra 22 ukers alderen viste analysene en oppregulering av proteintopper. Genomiske undersøkelser av serumprøver viste en svak nedregulering av *IL1B* og *TLR4* ved 16 uker, etterfulgt av en oppregulering av gener for TLRs, C3 og APPs fra 22 uker. Genomiske undersøkelse av lever- og hjernevev viste endringer i genuttrykk for APP-er som er i samsvar med en APR. Serumanalyser av forskjellige APP-er viste økte nivåer av de positive APP-ene og en redusert konsentrasjon av negative APP-er. Disse funnene indikerer et skifte fra anti-inflammatorisk til pro-inflammatorisk systemisk immunrespons samtidig med alvorlig klinisk sykdom.

Den medfødte immunreaksjonen i CNS har en nøkkelrolle i både utbrudd og progresjon av nevrodegenerative sykdommer, men også oppløsning av betennelsesreaksjoner. Akkumulering av PrP^{Sc} i CNS har vært assosiert med en kronisk aktivering av det medfødte immunforsvaret og en pro-inflammatorisk aktivering av mikrogliacellene, nevroinflammasjon og nevrodegenerasjon.

Sykdomsfenotypen registrert i dette arbeidet er et resultat av kombinasjonen av *PRNP*-genotype, smittetidspunkt og smittedose. Lignende smittesituasjon kan oppstå naturlig dersom de rette omstendighetene er på plass. Nyfødte homozygote VRQ-lam fra en infisert søye kan bli smittet ved fødselen. Det er grunn til å tro at slike tilfeller kan få en lignende sykdomsutvikling som beskrevet i dette arbeidet. *PRNP* genotype og tidlig oral smitte gir effektivt og raskt opptak med påfølgende smittespredning til lymfevev og hjernevev, og klinisk sykdom i ung alder. Disse tilfellene byr på diagnostiske utfordringer og ville i de fleste tilfeller blitt oversett som klassisk skrapesyke. På grunn av deres unge alder, ville disse tilfellene ikke blitt inkludert for prøvetaking i forbindelse med overvåkingsprogrammet. Dersom inkubasjonsperioden går utover den kommersielle levetiden, ville disse lammene blitt slaktet til konsum, og på grunn av deres *PRNP*-genotype, ville prioner kommet inn i næringskjeden.

Kontroll av klassisk skrapesyke kan sannsynligvis ikke oppnås ved å eliminere smittestoffet, men fravær av klinisk sykdom er mulig gjennom avl for resistens, det ville gitt tilsvarende flokkimmunitet mot klassisk skrapesjuke.

List of papers:

Paper I:
Meling S, Kvalheim OM, Arneberg R, Bårdsen K, Hjelle A, Ulvund MJ.
Investigation of serum protein profiles in scrapie infected sheep by means of SELDI-TOF-MS and multivariate data analysis.
BMC Res Notes. 2013 Nov 14; 6:466.

Paper II:
Meling S, Bårdsen K, Ulvund MJ.
Presence of an acute phase response in sheep with clinical classical scrapie.
BMC Vet Res. 2012 Jul 17; 8:113

Paper III:
Meling S, Skovgaard K, Bårdsen K, Helweg Heegaard PM, Ulvund MJ.
Expression of selected genes isolated from whole blood, liver and obex in lambs with experimental classical scrapie and healthy controls, showing a systemic innate immune response at the clinical end-stage.
BMC Vet Res. 2018 Sep 12;14(1):281.

Introduction

Scrapie in sheep has been known to shepherds and the veterinary profession for centuries, and it has been characterised as the prototype of all prion diseases. The disease was observed and described already in 1732. Later, a German handbook on agriculture published in 1759 describes the clinical disease in details and recommends removal of the affected animal from the flock, and suggesting it to be served to the staff on the estate (Hörnlimann et al., 2007, chap. 1). Nevertheless, it was not until William J. Hadlow wrote a letter to the editor of the *Lancet* in 1959, that a connection was made between the human prion disease, Kuru, and scrapie (Hadlow, 1959). Today there are several prion diseases described in both humans and animals and they are all caused by small proteinaceous infectious particles; purified, described and termed "prions" by Stanley B. Prusiner in 1982 (Prusiner, 1982). A few years later it became known that the prion protein was indeed an endogenous protein encoded by the host itself, and both the scrapie associated (PrP^{Sc}) and the cellular (PrP^{C}) prion proteins were encoded by the same prion gene (*PRNP*) (Basler et al., 1986; Caughey et al., 1988b). This indicated that both PrP^{Sc} and PrP^{C}, within the same host, have the same amino acid sequence, and the difference between the two, is conformational only, and that this conformational conversion is an essential part of transmission and dissemination of PrP^{Sc} (Prusiner, 1998). It is believed that the identical amino acid sequence between the normal PrP^{C} and the pathological PrP^{Sc} explains the absence of a specific immune response, as PrP^{Sc} is considered as "self" by the host (Kasper et al., 1981, 1982).

Intensive research has revealed many interesting aspects of prion diseases, and we now know that the disease phenotype in sheep is influenced by several factors, such as prion strain, genotype and time, and dose of infection (Gonzalez et al., 2012). The increased attention to gene expression analyses has revealed changes in inflammatory gene expressions, both peripherally and within the central nervous system (CNS), and the involvement of the innate immune system (Gossner and Hopkins, 2015, 2014). Despite the absence of a specific immune response to PrP^{Sc}, the innate immune system can readily become activated by signals from associated damaged cells and tissues, regardless that the initial cause is perceived as "self". After oral infection, the lymphoreticular system (LRS) is involved in replication and dissemination of infectivity before PrP^{Sc} reaches the CNS, termed neuroinvasion. This neuroinvasion involves the same neural pathways within the autonomic nervous system (ANS) as the immune system uses to transmit immune messages between periphery and CNS (Dantzer, 2009; Tabouret et al., 2010; Keller et al., 2018).

This literature review presents a summary of the importance of *PRNP* genotypes, dose and time of infection, clinical presentation, and the accompanying innate immune response in orally acquired classical scrapie in sheep. The foundation behind this doctoral work is the unique experimental model resulting in a "worst-case scenario" of scrapie in sheep, which enables a remarkable opportunity to study the disease.

Sheep farming in Norway

There are currently about one million breeding sheep across 14 272 flocks in Norway, and sheep are kept in every county (Figure 2). Flock sizes vary from below twenty

ewes up to several hundred, and the average flock size is seventy-one ewes with a mean age of four and a half years.
(https://www.landbruksdirektoratet.no/no/statistikk/utvikling/antall-dyr/sauer).
The most common breed is the Norwegian White (NKS) which is a relatively new breed, with registered breed standards from 2000/01. The exact number of different breeds within the Norwegian sheep population is not known, but according to the Norwegian sheep recording system (NSRS), (Sauekontrollen), there are at least eighteen different breeds registered.

Currently 36.7 % of all flocks and 47.9 % of breeding ewes are registered in the NSRS, which provides access to information and data on the national flock, enabling good opportunities to monitor performance at individual flock level as well as the national population, within and between different breeds.

From mid-19th century until late-20th century, several hundred breeding animals of various breeds such as Cheviot, Suffolk, Oxford Down, Bluefaced Leicester and others were imported mainly from the United Kingdom (UK), to improve wool quality and carcass value in Norwegian short-tailed sheep. As a result of this crossbreeding, two distinctive new Norwegian breeds were recognized in 1924/25 in the south-western part of Norway; the Dala and Rygja breeds. Then again, from 1970s, the sheep were, further crossed with different breeds from other countries. The Norwegian White Breed (NKS) was established in 2000, and today it probably accounts for more than 70 % of the breeding ewes in Norway.

The national breeding program was initially established to ensure good availability of breeding rams with desirable traits and good estimated breeding values (EBV). The introduction of so-called ram circles in 1961, represented the beginning of organized national breeding programs. Each ram-circle, consisted of several individual flocks, they shared a small group of rams with high genetic merit which were mated with the best ewes in each flock. Performance data on offspring were registered in the NSRS and simple EBVs were in place from 1968. This system made it possible to further select the rams with highest nationwide genetic merit for artificial insemination (AI) use, which was in place by 1980. Ram circles and AI are today important in the national breeding program and plays an important role in selection and distribution of rams with high genetic merits for selected traits. Each year, ram lambs of a certain pedigree with a minimum breeding index (BI) of 125 can be showed to a team of fieldsmen and become approved test-rams for use in the ram circle.

The national breeding program for NKS started in year 2000, and data from more than 10 million individual sheep have since been registered in the NSRS. Ram circles and progeny testing are central in improving desirable traits within the breed. Young test-rams are selected from flocks within the ram-circle, and these are used in the ram-circle for one year. Progeny testing determine which of these test-rams qualify for further use as an elite-ram. The best elite-rams at national level are selected as AI rams to deliver good genetics to the whole national sheep population. Between 20 and 30 NKS rams are available for AI each year, and in 2017, a total of 32 514 doses were sold across all breeds. The use of AI is a popular and a very efficient method to spread genetic material throughout the national sheep population.

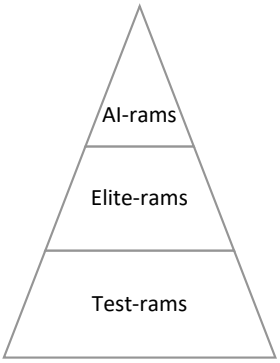

Figure 2. The selection process for test-rams, elite-rams, and AI rams

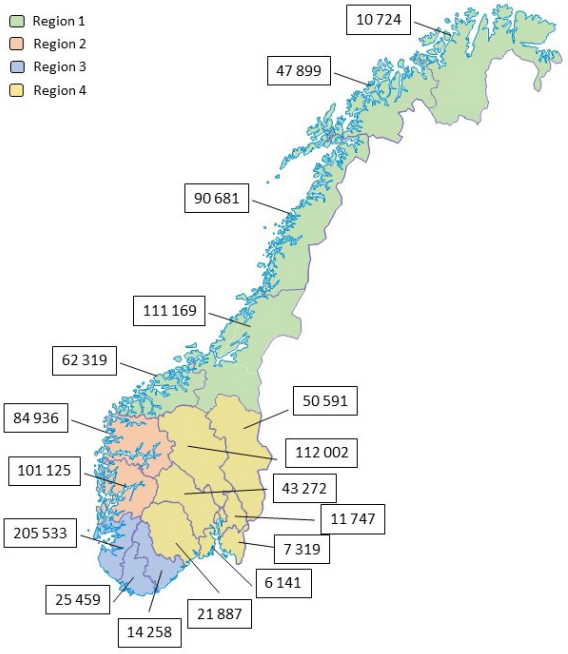

Figure 1. Distribution of sheep in Norway with total number of sheep in each county. The four sheep-regions are illustrated by different colours. About 30% of the sheep are situated in two counties south-western part of Norway: Rogaland (205 533) and Hordaland (101 125). Source: Norwegian Agriculture Agency.

Most sheep are kept indoors at relatively high stocking density throughout the winter months, from tupping in November until lambing in April. Sheep houses are both insulated and uninsulated, and the majority of them have slatted flooring. At lambing,

ewes are usually kept in separate lambing pens during parturition and the first few days of the neonatal period.

About 80 % of the sheep are sent away to common grazing areas in the mountains between June and September, where several different flocks co-graze in the same areas.

Since the 1990s, movement restrictions have been in place, and currently, Norway is divided into four distinct sheep-regions (Figure 2). Sheep are not allowed to move between these regions, and restrictions apply for moving sheep between counties within the same region. Generally, ewes are not allowed to move between flocks, but rams intended for breeding can move between flocks within same county within same region. Moving rams between counties within the same region requires movement license by a veterinarian.

Prion diseases.

Prion diseases, also called transmissible spongiform encephalopathies (TSEs), are progressive and always fatal neurodegenerative disorders affecting both humans and animals (Table 1). They are characterised by long incubation period, characteristic spongiform changes in the central nervous system (CNS) associated with neuronal loss and gliosis, and the absence of a specific immune response to the causal agent. The causal agent is an abnormal isoform of the normal prion protein, PrP, encoded by the prion protein gene, *PRNP* (Prusiner, 1982; Oesch et al., 1985). Although the mammalian PrP has a degree of conservation, variation between species and individuals exists due to mutations of *PRNP*, and these mutations affects level of susceptibility to prion infection (Mead, 2006; Colby and Prusiner, 2011). The TSEs are a unique group of diseases that can coexists in sporadic, inherited, and infectious forms. The discovery of the prions and the introduction of the protein-only-hypothesis stating prions being the only cause of these TSEs were not readily accepted, but every attempt to prove otherwise has since failed. It is now generally accepted that the causal agent of TSEs is a protein, the prion.

Diseases and variants	Species	Aetiology
Scrapie, Classical, Nor98	Ovine, Caprine	Infectious, spontaneous
Bovine Spongiform Encephalopathy (BSE)	Bovine	Infectious, spontaneous
Chronic Wasting Diseases (CWD)	Cervid	Infectious
Feline Spongiform Encephalopathy (FSE)	Feline	Infectious
Transmissible Mink Encephalopathy (TME)	Mink	Infectious
Camel Prion Disease (CPD)	Dromedary Camel	Unknown/Infectious
Kuru	Human	Infectious
Creutzfeldt-Jakob Disease; Iatrogenic (iCJD), Variant (vCJD), Familial (fCJD), Sporadic sCJD)	Human	Iatrogenic, infectious, hereditary, spontaneous
Gerstmann-Sträussler-Scheinker Diseases (GSS)	Human	Hereditary
Fatal Familial Insomnia (FFI), Sporadic Familial Insomnia (sFI)	Human	Hereditary, spontaneous

Table 1. The most known prion diseases in different species. All are caused by conversion of the host-encoded prion protein into a pathological form, resulting in progressive neurodegenerative disorders in different ways; spontaneous, infectious, iatrogenic, or hereditary through germline mutations.

Classical scrapie, the prototype of prion disease.

Scrapie is a progressive fatal neurodegenerative disease naturally occurring in sheep and goats, and it was the first prion disease to be described. Records show that it has existed in Europe for more than 250 years (Parry and Oppenheimer, 1983). Before the nature of the causal agent was known, scrapie was characterised as a slow infection, due to the long incubation period, transmissible and with a genetic component, as incidences in flocks followed a familial pattern (Dickinson et al., 1965). Records of scrapie in sheep from several countries show a diversity of disease phenotypes, with a long list of different clinical signs and variations in incubation time (Dickinson et al., 1965; Parry and Oppenheimer, 1983; Hörnlimann et al., 2007).

In Norway, sheep with clinical signs resembling scrapie was described already in 1890 and again in 1916. Locally, the specific disease was called "Grubbe-, gnave- or travesyken" (Kjos-Hansen and Holmboe, 1926). Scrapie in Norway was first diagnosed in two imported Suffolk rams in 1958 (Naerland, 1970), and then first diagnosed in indigenous sheep in 1981, after which the number of cases increased into the 1990s (Ulvund et al., 1996). The classical scrapie cases (unclassified and confirmed) were seen in a relatively confined area of Norway, and they were related to the import of sheep during the years between 1850 and 1960, Figure 3. Distribution of atypical scrapie cases, Nor98, seems much more random and scattered (Hopp et al., 2010).

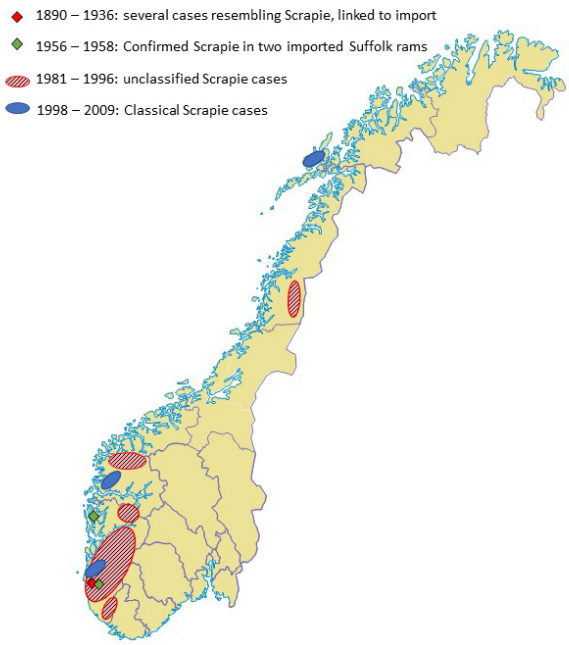

Figure 3. A map of Norway showing location of suspected and confirmed cases of scrapie in sheep. Modified after Ulvund et al. 1996 and Hopp et al. 2010 (Ulvund et al., 1996; Hopp et al., 2010).

The prion protein

The precise role and function of the normal cellular prion protein, PrP^{C}, remains an enigma, despite being one of the most studied proteins. A conformational variant of the normal protein, termed the "prion" by Prusiner in 1982, is the aetiological agent of prion diseases; it is self-replicating, infectious and transmissible (Prusiner, 1982). The prion protein, PrP^{C}, is a highly conserved glycoprotein encoded by the prion gene, *PRNP*, and it is referred to as the cellular prion protein, PrP^{C}, to differentiate this from the scrapie associated prion protein, PrP^{Sc}. Within the same host, both PrP^{Sc} and PrP^{C} are encoded by the same gene, and thus both have the same unique amino acid sequence, but they differ in their three-dimensional folded structure (alfa-helices and beta-sheets) (Oesch et al., 1985; Basler et al., 1986). As for all other proteins, the unique amino acid sequence is specified by the genes, and the sequence and properties of the amino acids influence the three-dimensional structure that enable proteins to carry out their biological function (Stryer, 1995, chap. 16). Some proteins are normally folded in a marginally stable form because they require some flexibility for protein folding, function, and removal. Specific mutations at codons 136, 154 and 171 of the *PRNP* gene leads to single substitutions of amino acids, which leave the prion protein more unstable and more susceptible to conformational changes. The misfolded PrP^{Sc} adapts a more stable form with more beta-sheets, in contrast to the PrP^{C} which contains more alpha-helices. The different isoforms of PrP have different physical properties and three-dimensional structure, and these difference arise from conformational changes involving this

transition from α-helices into β-sheets (Stahl et al., 1993; Govaerts et al., 2004). Nuclear magnetic resonance (NMR) spectroscopy of the globular domain of PrP^{C} from several mammalian species reveals a highly conserved architecture with three α-helices and two short anti-parallel β-sheets (Lysek et al., 2005; Kupfer et al., 2009). In contrast, the three-dimensional structure of PrP^{Sc} is not fully elucidated, due to technical and analytical difficulties, but available structural information give rise to two structural models which might coexist; parallel in-register intermolecular β-sheet (PIRIBS) architectures and the 4-rung β-solenoid model (Baskakov et al., 2019). The variation in prion protein stability and the related *PRNP* mutations at each of the three codons have been associated with the variations seen in length of incubation period (Goldmann et al., 1991). The PrP^{Sc} can act as a template and induce abnormal folding of PrP^{C} through direct contact, resulting in a cascade of protein misfolding and aggregation (Kupfer et al., 2009).

One of the first and central biochemical differences discovered between the isoforms of PrP is the level of sensitivity to proteinase K digestion (Bolton et al., 1982; McKinley et al., 1983). After proteinase K digestion of PrP, PrP^{C} is completely degraded, while PrP^{Sc} is detected as a resistant core which size is reduced to 27-30 kDa (PrP^{27-30}), which is found in infectious scrapie material (Meyer et al., 1986; Oesch et al., 1985). It is now clear, however, that not all disease associated PrP strains are protease resistant, and most naturally occurring prion strains are partially resistant to digestion (Benestad et al., 2003; Colby et al., 2010).

The study of the biochemistry and structure of the prion protein has revealed important information on PrP^{C} synthesis, location on cell surface within lipid raft and the tertiary structure with a C-terminal globular domain and an N-terminal flexible tail (Stahl et al., 1987; Riek et al., 1997; Zahn et al., 2000; Lysek et al., 2005). The N-terminal of PrP^{C} possesses the ability to bind several metal ions, especially copper, in the conserved octapeptide repeats (OR) regions, and this binding has been linked to both structural consequences and several different functional properties of PrP^{C}. Some of these appear conflicting and contradicting, such as shortening vs prolonging of incubation period and facilitating or inhibiting prion formation (David R. Brown et al., 1997; D. R. Brown et al., 1997; Wopfner et al., 1999; Brown and Harris, 2003; Walter et al., 2006, 2009; Pushie et al., 2011; Nguyen et al., 2019; Salzano et al., 2019). At least three cleavage sites have been identified and proteolytic cleavage events may have physiological and pathological effects (Lewis et al., 2016). The PrP^{C} is membrane bound and tethered to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor attached at the carboxy-terminus (Meyer et al., 1986; Stahl et al., 1987). It is reported that lymphocytes and lymphoid cell lines increase expression of surface PrP^{C} when activated, and thus propose a role of PrP^{C} in cell activation (Cashman et al., 1990).

The difficulty in revealing the true functions of PrP^{C} is intriguing as it is expressed in many different tissues and cell types, predominantly in cells at the interface between immune and nervous systems both centrally and peripherally (neurons and cells of the monocyte line) (Kretzschmar et al., 1986; Caughey et al., 1988a; Bendheim et al., 1992; Brown et al., 1990; Moser et al., 1995; Brown et al., 1998; Ford et al., 2002). In addition, the gene encoding PrP^{C} has been found in most vertebrates and key structural elements in the gene sequence are highly conserved between species (Premzl and Gamulin, 2007; Harrison et al., 2010). Several methods, with varying results have been applied to assess

PrP^C function, all of which have recently been reviewed (Wulf et al., 2017; Castle and Gill, 2017; Watts et al., 2018).

Several types of PrP gene knock-out models have been used to assess the function of PrP^C by registering "loss of function". The idea behind this is to observe for phenotypic alterations and consequences after *PRNP* ablation, and then assign these changes to the protein. The results must be used with some caution since proteins can have overlapping functions, and there may be some compensatory mechanisms. Mutations or total ablation of gene sequences may influence and mutate other genes, called passenger mutations, which may influence the phenotypic outcome (Vanden Berghe et al., 2015). Such functional link between *PRNP* and the gene encoding the doppel protein (*PRND)* in ataxic PrP-deficient mice has been discovered, and also between *PRNP* and the gene encoding the signal regulatory protein α (*SIRPα*) regarding phagocytotic rate of macrophages in PrP-deficient mice (Moore et al., 1999; Nuvolone et al., 2013). Knock-out models have resulted in a variety of proposed physiological roles of PrP^C, but unfortunately many have later been contraindicated (Wulf et al., 2017; Watts et al., 2018). However, an age-dependent demyelinating polyneuropathy (CDP) has presented in $PrP^{-/-}$ mice across different PrP^C-knockout mouse-lines suggesting that PrP^C binds to receptor on Schwann cells and elicit promyelinating response, thus proposing a role in peripheral myelin maintenance (Nishida et al., 1999; Bremer et al., 2010; Küffer et al., 2016; Nuvolone et al., 2016).

PRNP ablation has been performed with great success in mice, cattle and goats, but still without revealing the exact function of PrP^C (Richt et al., 2007; Zhu et al., 2009; Onodera et al., 2014). Initial results showed animals without any major phenotypic alterations, apart from being resistant to prion infection, and conclusions were made that PrP^C is not an essential protein for normal development and function (Büeler et al., 1992; Manson et al., 1994). $PRNP^{-/-}$ cattle were compared to wild-type cattle for 20 months through rigorous testing without significant difference detected between the two groups (Richt et al., 2007). Benestad et. al. reported in 2012 the discovery of apparently healthy Norwegian Dairy Goats lacking PrP^C due to a naturally occurring mutation located in codon 32, terminating the PrP^C synthesis prematurely. This allele in the *PRNP* was found at 11 % frequency, and two homozygous goats ($PRNP^{Ter/Ter}$) were identified which did not display any deviating phenotype and appeared healthy (Benestad et al., 2012). $PRNP^{Ter/Ter}$ goats showed a significantly longer duration of sickness behaviour, lower serum albumin concentration and increased expression of a number of genes downstream type I interferons (IFN) following intravenous lipopolysaccharide (LPS) challenge, concluding in a suggested role (loss-of-function) for PrP^C in modulating the innate immune response (Salvesen et al., 2017). Supporting these findings, gene expression studies of peripheral blood mononuclear cells (PBMCs) revealed that type I interferon-responsive genes were upregulated in $PRNP^{Ter/Ter}$ goats compared to normal $PRNP^{+/+}$ goats (Malachin et al., 2017).

Another approach in establishing function of PrP^C has been to investigate Doppel and Shadoo, the two other members of the prion protein family. These are both GPI-anchored glycoproteins, but Doppel, encoded by *PRND*, shares homology to the C-terminal domain, whereas Shadoo, encoded by *SPRN*, bears resemblance to half of the N-terminal of PrP^C (Watts and Westaway, 2007). Doppel is primarily expressed in testis, and at very low levels in the CNS. Shadoo is expressed in the brain and exerting

neuroprotective function, and proposed to have a modulatory role for PrP (Watts et al., 2007). Unfortunately, these studies of other members of the prion protein family have not revealed the functions of PrP^C (Paisley et al., 2004; Daude et al., 2012).

Functions of PrP^C could be inferred from investigations of other protein to which it binds and interacts. It has proven challenging to discriminate which interactions are of biological relevance, as PrP^C has displayed the ability to interact with several molecules and proteins (Onodera et al., 2020). Despite identifying many binding and interacting partners to PrP^C, understanding the role in prion diseases and function of PrP^C still remains unidentified. (Wulf et al., 2017; Castle and Gill, 2017; Watts et al., 2018).

In conclusion, knock-out models show that scrapie is not caused by a direct and straight forward loss of function, as PrP^C is not crucial for the resting health in higher organisms (Benestad et al., 2012). On the other hand, presence of the cellular normal PrP^C protein is absolutely essential for developing the disease, illustrated by the lack of disease in $PrP^{0/0}$ transgene mice (Büeler et al., 1993; Brandner et al., 1996).

Prion strain

Presence of different strains has been known since transmission studies began and different incubation periods and lesion profiles where noticed (Fraser and Dickinson, 1968). Strain-specific phenotype is maintained through a serial passage in the same host species, but strain adaption and selection can occur after interspecies transmission (Bruce et al., 1991; Bartz et al., 2000; Ghaemmaghami et al., 2013). Currently, several natural scrapie strains in sheep are known, and natural infection by more than one strain at the time can occur within the same animal (González et al., 2010; Thackray et al., 2011). Different strains within the same host will have the same amino acid sequence since it is encoded for and specified by the host *PRNP* genotype. The strain characteristics are thought to be determined by conformational stability, types and difference in biochemical properties (Peretz et al., 2001; Thackray et al., 2007; Colby et al., 2009; Ayers et al., 2011). There is a relationship between the conformational stability of prions and disease incubation period, and this conformational stability of the prion strain, depends solely on its inherent conformation and is independent of the PrP expression level of the host animal (Colby et al., 2009). Traditionally, the main criteria for differentiating between strains are incubation period and "lesion profile" i.e. intensity of spongiform degeneration in specific regions in the CNS at terminal disease (Fraser and Dickinson, 1968; Bruce et al., 1991). In addition, intensity and distribution of PrP^{Sc} accumulation, plaque formation and neural tropism and biochemical characteristics of the PrP^{Sc} provide further strain specific characteristics (Bruce, 2003). Classical scrapie include a range of different PrP^{Sc} strains, but Nor98 is a strain specific prion disease in sheep showing a completely different lesion profile and biochemical properties of PrP^{Sc} (Benestad et al., 2003).

Strain adaptation is seen after serial passages in a new species or hosts with a different PrP genotype. The limited amplification of PrP^{Sc} in homozygous ARR animals is believed to be the reason behind the limited capacity and opportunity for strains to adapt to ARR/ARR sheep (Lacroux et al., 2017). Transmission and inoculation studies confirm the importance of homology in amino acid sequence between PrP^C and PrP^{Sc}, and this thought to be the most important factor in species barrier (Goldmann, 2008).

Prion protein gene

Ovine PrP is encoded by a single gene, *PRNP*, which is located on chromosome 13 and consists of three exons (52, 98 and 4028 nucleotides in length) separated by two introns (2421 and 14031 nucleotides in length) (Oesch et al., 1985; Basler et al., 1986; Lee et al., 1998). The complete open reading frame (ORF) of 768 base pairs (bp) is located in exon 3 and gives rise to the primary protein product of 256 amino acids (Goldmann et al., 1990; Westaway et al., 1994).

Generally, any mutations in a specific gene will alter the amino acid sequence and may lead to a conformational change, abnormal function, and disease. A good example is a simple substitution of alanine (A) to valine (V) which will destabilize the α-helix and change to β-sheet, due to the hydrophobic properties of valine (Stryer, 1995). In sheep, numerous polymorphisms and allelic combinations in the *PRNP* gene have been discovered, and particularly polymorphisms at codons 136, 154 and 171 are associated with level of susceptibility, length of incubation period and scrapie pathogenesis (Goldmann et al., 1990; Belt et al., 1995; Bossers et al., 1996; Tranulis et al., 1999; Ersdal et al., 2005). The most common polymorphisms give rise to five possible alleles; ARR, AHQ, ARH, ARQ and VRQ, which then give 15 *PRNP* genotypes found in sheep (Table 4) (Goldmann et al., 1994; Belt et al., 1995). The allele $A_{136}R_{154}Q_{171}$ is considered to be the wildtype in sheep, and *PRNP* gene polymorphisms vary both within and between breeds (Hunter and Cairns, 1998; Tranulis et al., 1999; Hopp and Valheim, 2005). Genotypes with glutamine (Q) at codon 171 and the so-called "valine breeds" that encode for valine (V) at codon 136, show increased susceptibility (Goldmann et al., 1990, 1994; Westaway et al., 1994; Clouscard et al., 1995; Hunter et al., 1996; Tranulis et al., 1999; O'Doherty et al., 2002). Some breeds have limited genetic variation and lack certain polymorphisms, such as the Suffolk breed where the VRQ allele is rare, the Norwegian Pelt breed which does not have the ARR allele, and the Icelandic sheep which only have glutamine (Q) at codon 171 (Westaway et al., 1994; Hunter et al., 1997; Thorgeirsdottir et al., 1999; Hopp and Valheim, 2005). Homozygous VRQ *PRNP* genotype is rare in the general population, but often overrepresented in natural cases of classical scrapie (Tranulis et al., 1999; Baylis et al., 2002; Sviland et al., 2017). Out of 688 rams of various breeds tested in Norway in the period 2000 – 2015, only six were homozygous VRQ (Blichfeldt, 2019). In contrast, ARR allele is associated with genetic resistance to classical scrapie, and any increased frequency of ARR allele in the population will result in a considerable reduction in risk of disease (Baylis et al., 2004). Incidence of natural and experimental classical scrapie in sheep carrying at least one ARR allele is very much reduced, and only a few cases of naturally infected classical scrapie have ever been diagnosed in homozygous ARR sheep (Ikeda et al., 1995; Tranulis et al., 1999; Ersdal et al., 2005; Groschup et al., 2007). However, experimental intracerebral infection has resulted in induction of disease in homozygous ARR sheep, but with considerable longer incubation period and lower attack rate (Jeffrey et al., 2014; Lacroux et al., 2017).

Distribution of *PRNP* genotype alleles at codons 136, 154 and 171 in Norway.

In accordance with EU Decision 2002/1003/EC, a survey of *PRNP* genotypes in Norway was performed in 2005 on a representative number of animals from the main breeds

(Anon, 2002; Hopp and Valheim, 2005). The survey was undertaken by a collaboration between Norwegian Food Safety Authority (NFSA), Norwegian School of Veterinary Science (NSVS) and National Veterinary Institute (NVI), and the aim was to estimate the prevalence of *PRNP* gene allelic variation in Norwegian sheep breeds (Hopp and Valheim, 2005). A total of 714 animals (including AI-rams in 2003) were selected for this survey, and the results are presented in Table 2 (modified from Hopp and Valheim 2005).

Prevalence (%) of *PRNP* gene alleles (codons 136, 154 and 171) in 714 sheep of various breeds in Norway in 2005

Breed	Number of animals	**ARR** %	**ARQ** %	**AHQ** %	**ARH** %	**VRQ** %
NKS	154	43	33	9	9	6
White Spæl	47	14	64	20	0	2
Cheviot	51	31	32	21	1	15
Texel	51	29	35	9	20	7
Norwegian Pelt	52	0	68	15	0	16
Suffolk	50	19	66	2	1	12
Feral Sheep	53	23	54	16	0	8
Dala	50	49	28	9	4	10
Blæset	49	25	32	7	0	37
Fuglestad	51	30	32	27	2	9
Blackface	51	28	18	54	0	0
Old Norse	56	10	70	16	0	5
Total	714	28	43	16	4	10

Table 2. The prevalence of the different alleles is presented for each breed and across all samples (Total).

During the period 2003 – 2015, the *PRNP* genotype of 690 AI-rams from 20 different breeds was determined as they entered the national breeding program (Blichfeldt, 2019). The results are presented in Table 3.

Distribution (%) of *PRNP* gene alleles (codons 136, 154 and 171) in 690 AI-rams, during period 2003-2015.

Breed	Number of animals	ARR %	ARQ %	ARH %	AHQ %	VRQ %	AAH %	RQQ %	AHR %
NKS	335	42	36	5	9	8	0	0	0.1
White Spæl	107	8	69	0	15	7	0	0	0
Cheviot	29	47	26	2	12	14	0	0	0
Texel	9	56	17	28	0	0	0	0	0
Norwegian Pelt	29	0	62	0	3	34	0	0	0
Suffolk	7	50	50	0	0	0	0	0	0
Feral Sheep	27	6	69	0	19	4	2	2	0
Dala	8	56	31	6	0	6	0	0	0
Blæset	14	25	29	7	14	25	0	0	0
Fuglestad	9	22	50	0	11	17	0	0	0
Blackface	9	67	17	0	17	0	0	0	0
Old Norse	9	22	61	0	11	6	0	0	0
Rygja	13	42	38	4	8	8	0	0	0
Steigar	9	33	28	17	11	11	0	0	0
Grå Trønder	8	19	50	6	13	13	0	0	0
NorX	38	29	54	3	12	3	0	0	0
Coloured Spæl	22	9	77	0	11	2	0	0	0
Dorset	1	50	0	0	0	50	0	0	0
Charollais	5	30	60	0	10	0	0	0	0
Shropshire	2	0	25	0	75	0	0	0	0
Total	690	31	46	4	11	9	0	0	0

Table 3. Distribution of *PRNP* gene alleles in 690 AI-rams of different breeds and in total across all breeds in the period 2003 – 2015.

The tables show that the distribution of *PRNP* gene alleles from the 2005 survey (Table 2) is similar to the distribution among the AI-rams tested in the period 2003 – 2016 (Table 3). The Norwegian Pelt breed is in an unusual situation as there are no ARR alleles detected. Both the Norwegian Pelt and Blæset breeds have a relatively high prevalence of the VRQ allele, and this makes especially the Norwegian Pelt susceptible to scrapie. The overall prevalence of the different alleles in the main Norwegian breed, NKS, indicate that ARR and ARQ alleles are the most common, but the VRQ allele is still present in the population.

Distribution of *PRNP* genotypes grouped in accordance with British National Scrapie Plan (NSP) in Norwegian sheep population.

In accordance to Regulation (EC) No. 999/2001 Annex III, as amended by Regulation (EC) No 2245/2003, *PRNP* genotype was determined in 6988 healthy slaughtered sheep by NVI in the period between 2004 and 2016 (Anon, 2001; Benestad et al., 2010; Hopp et al., 2009, 2008, 2006; Sviland et al., 2017, 2016, 2015, 2014, 2013, 2012, 2011, 2007; Valheim et al., 2005). *PRNP* genotypes were determined and grouped in accordance to the British National Scrapie Plan (NSP) (Table 4) (Sviland et al., 2017). Through annual surveillance, the *PRNP* genotype has been determined in average 500 animals each year, and the annual distribution of NSP genotype groups are illustrated graphically below (Figure 4).

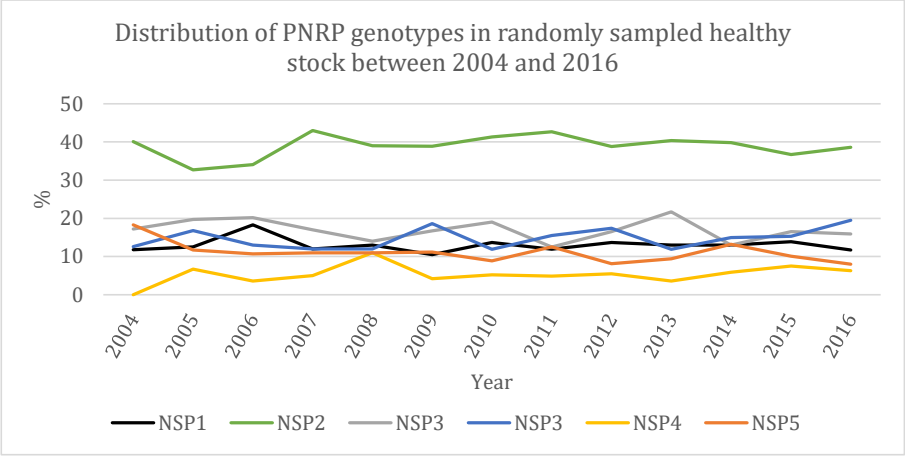

Figure 4. Graphical presentation of distribution of PRNP genotype according to NSP grouping each year between 2004 and 2016. Data used in the figure are from the annual reports from NVI between 2004 and 2016; Surveillance and control programmes for terrestrial and aquatic animals in Norway, The surveillance and control programme for scrapie in Norway. (Benestad et al., 2010, 2010; Hopp et al., 2009, 2008, 2006; Sviland et al., 2016, 2015, 2014, 2013, 2012, 2011, 2007; Valheim et al., 2005).

Annual surveillance of *PRNP* genotypes in randomly selected sheep within the population, provides good opportunity to monitor distribution of *PRNP* genotype over time and to detect any changes within the population. Norway has not adapted the breeding for resistance program to actively increase prevalence of the ARR allele and simultaneously reduce prevalence of VRQ allele, thus the evolution of *PRNP* genotype distribution is unforeseeable over time. The *PRNP* genotype of the breeding rams is not taken into consideration at flock level, nor at national level through the national breeding programme. Regardless, the distribution of different *PRNP* genotype NSP groups has been fairly stable without any marked changes during the surveillance period (Figure 4). NSP 2 has been the most common in the population, and NSP 1, NSP 4 and NSP 5 have been relatively stable, each group between 5 and 15 %.

Distribution of NSP groups in Norway and in 28 EU member states (MSs) are both presented in Table 4 and Figure 5. Due to the difference in breeding strategy, there is an obvious discrepancy between distribution of NSP groups in the EU MSs and Norway,

especially the most resistant and most susceptible NSP groups. The distribution in Norway is not unlike the situation in some MSs before implementing the breeding for resistance programme (EFSA Panel on Biological Hazards (BIOHAZ), 2014). Comparisons between distribution of NSP groups obtained from random samples in EU MSs in 2017 and Norway in 2016, show that the distribution of NSP 1 in EU MSs was 40.4 % while 11.7 % in Norway, and distribution of NSP 5 in EU MSs and Norway was 2.1 % and 8.0 %, respectively (European Food Safety Authority (EFSA), 2018; Sviland et al., 2017). Genotyping data from Cyprus is excluded as Cyprus systematically genotypes the breeding population and will, thus not be comparable here.

The vast majority of classical scrapie cases in sheep with known genotype (98.2 % in 2017), were in sheep with susceptible genotype groups (NSP3, NSP3O, NSP4 and NSP5), which is consistent with the pattern observed over the last ten years in the EU. Results from the MSs that carried out random genotyping of sheep in 2017 (excluding data from Cyprus) show that 26.5 % of the sheep population was susceptible to classical scrapie, compared to 49.7 % in the Norwegian population (European Food Safety Authority (EFSA), 2018; Sviland et al., 2017)

1	2	3	4	5	6	7	8
NSP	Allelic combination at codons 136, 154 and 171	Degree of resistance/ Susceptibility [1]	Surveillance of 6988 healthy stock 2004 – 2016. Random sampling %	690 AI-rams, all breeds (2003-2015) %	335 AI-rams NKS (2003-2015) %	Random sampling Norway in 2016 [2] %	Random sampling EU MSs* in 2017 [1] %
NSP1	ARR/ARR	Genetically most resistant	13.0	12.2	19.1	11.7	40.4
NSP2	ARR/ARQ, ARR/ARH, ARR/AHQ,	Genetically resistant	38.9	34.8	41.8	38.6	33.0
NSP3	ARQ/ARQ AHQ/AHQ, ARH/ARH, ARH/ARQ, AHQ/ARH, AHQ/ARQ	Genetically little resistant	16.8 15.1	21.9 14.8	11.0 12.8	15.9 19.5	16.1 4.6
NSP4	ARR/VRQ	Genetically susceptible	5.1	4.1	4.5	6.3	1.8
NSP5	ARQ/VRQ, ARH/VRQ, AHQ/VRQ, VRQ/VRQ	Genetically highly susceptible	11.2	12.3	10.7	8.0	2.1

Table 4. Distribution of *PRNP* genotypes in the sheep population in Norway and EU Member states (MSs). Genotype groups in accordance with the British National Scrapie Plan (NSP) (column 1); allelic combination at codons 136, 154 and 171 (column 2); degree of resistance or susceptibility to classical scrapie (column 3); results from surveillance through random sampling of sheep in Norway in the period 2004 – 2016 (column 4); genotyping of AI-rams from all breeds used in Norway during the period 2003 and 2015 (column 5); genotyping of AI-rams of the NKS breed used in Norway during the period 2003 – 2015 (column 6); genotypes detected through random sampling of sheep in Norway in 2016 (column 7); genotypes detected through random sampling of sheep in EU MSs (*excluding Cyprus) in 2017 (column 8).
[1] (European Food Safety Authority (EFSA), 2018)
[2] (Sviland et al., 2018)

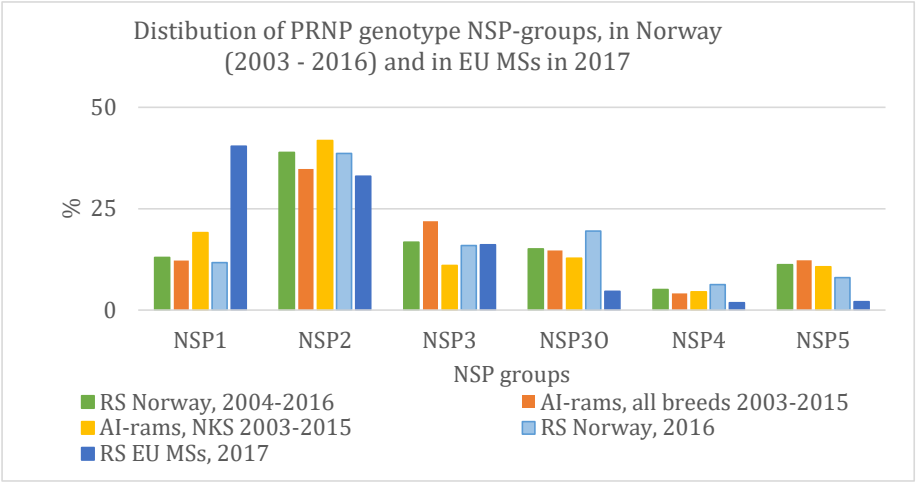

Figure 5. The bar diagram illustrates the distribution (%) of NSP groups in the genotyped sheep groups. Green: Random sampling (RS) of healthy stock during 2004 – 2016; orange: PrP genotyping of AI rams, all breeds, during 2003 – 2015; yellow: PrP genotyping of rams of NKS breed during 2003 – 2015; light blue: Random sampling of healthy stock in Norway in 2016; blue: Random sampling of healthy stock in EU MSs, excluding Cyprus, in 2017.

Distribution of VRQ and ARR alleles among the NKS AI-rams

Out of the 335 NKS AI-rams born between 2000 and 2014 with known *PRNP* genotype, none were homozygous VRQ, but 51 (15 %) rams were heterozygous VRQ, i.e. NSP4 or NSP5. Interestingly, at least 37 of these heterozygous VRQ rams were descendants after one particular Rygja ram born in 2000. Unfortunately, the *PRNP* genotype of this ram is not known, but *PRNP* genotype is known for 12 of his male offspring which have all been available through AI (Table 5). The Rygja ram has registered several hundreds to thousands of descendants, including breeding ewes and rams, and many of them have been used as test-rams, elite-rams, and AI-rams.

Offspring No.	1	2	3	4	**5**	6	7	8	9	10	11	12
Allele 1	ARH	ARH	ARH	ARR	**AHQ**	ARH	ARQ	ARQ	ARQ	ARH	VRQ	ARQ
Allele 2	VRQ	ARR	ARR	VRQ	**VRQ**	VRQ	VRQ	ARH	VRQ	ARR	ARR	ARH
NSP	5	2	2	4	**5**	5	5	3	5	2	4	2

Table 5. Distribution of allelic polymorphisms of *PRNP* gene in 12 male offspring of the Rygja ram

Information from genotyping these rams show that seven of them were heterozygous VRQ and belong to the genetically susceptible genotype groups NSP4 and NSP5 (Table 5). Furthermore, offspring number five went on to sire a long line of AI-rams, and the *PRNP* genotype of some of them are presented in Table 6. Ten of these were heterozygous VRQ and belong to NSP groups four and five.

Pedigree tree	***PRNP* Genotype**		
	Allele 1	**Allele 2**	**NSP Group**
Rygja ram……..	unknown	unknown	unknown
1. Offspring no. 5…	AHQ	VRQ	5
1.1. ……..	AHQ	ARR	2
1.2. ……..	AHQ	VRQ	5
1.3. ……..	ARR	VRQ	4
1.4. ……..	ARQ	VRQ	5
1.4.1. ……	ARR	ARR	1
1.4.2. ……	ARQ	ARR	2
1.4.2.1.	unknown	unknown	unknown
1.4.2.2.	ARR	ARR	1
1.4.2.3.	ARR	ARR	1
1.4.3. ……	ARQ	VRQ	5
1.4.3.1.	ARQ	VRQ	5
1.4.3.2.	ARQ	VRQ	5
1.4.3.3.	ARQ	VRQ	5
1.4.3.4.	ARR	VRQ	4
1.4.3.5.	ARQ	ARR	2
1.4.3.6.	unknown	unknown	unknown
1.4.4. ……	ARQ	VRQ	5
1.4.4.1.	ARQ	ARR	2
1.4.5……	ARR	ARR	1

Table 6. Overview over *PRNP* Genotype of a selection of descendants from the popular Rygja ram: offspring number five and his four sons, five grandsons and ten great-grand sons.

This popular Rygja ram and his descendants have displayed a desirable phenotype and high breeding indices, which have resulted in their widespread and nationwide use. It is claimed that "the ram is half the flock", and in this way the use of a heterozygous VRQ ram would, at random, pass down the VRQ allele to 50 % of his offspring. Extensive use of rams which are genetically resistant to scrapie would potentially contribute considerably to the "herd immunity" to classical scrapie in Norway, both at individual and national flock level.

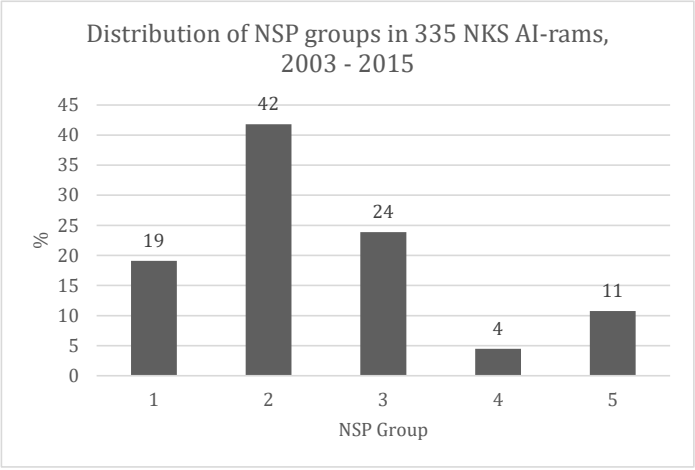

Figure 7. Distribution (%) of NKS AI-rams according to NSP Group Only NSP1 and NSP2 are considered to be resistant to classical scrapie and 61 % of these AI-rams belong to these groups. The remaining 3 % are classified as susceptible to classical scrapie.

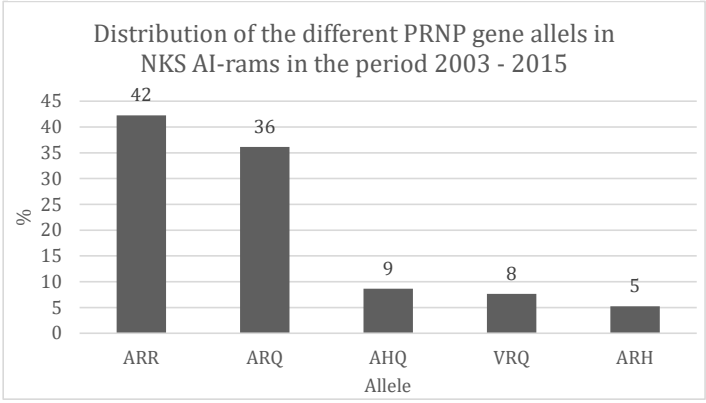

Figure 6. Distribution (%) of the PRNP alleles in NKS AI-rams show that ARR was the most frequent allele at 42 %, while VRQ allele was at 8 % in the period between 2003 – 2015.

Whether this distribution and ratio between ARR and VRQ alleles are adequate to provide some level of herd immunity in combination with other control measures, like movement restrictions and culling of scrapie affected flocks, has not been investigated, to my knowledge.

Epidemiology

Scrapie has widespread geographical distribution, still the true prevalence and incidence of classical scrapie is difficult to estimate. The incidence of classical scrapie will fluctuate both within and between breeds and regions due to complex interactions of strain and host factors, which will influence the length of incubation period and transmission of the agent (Prusiner et al., 1981, 1982; Bartz, 2016). Lack of preclinical screening tests and validated diagnostic tests in live animals, make studies of prevalence in a population impossible. Prevalence can only be estimated by *ante mortem* testing of uninfected and infected animals. The incidence rate will be based on confirmed cases, which will rely on reports of clinical signs of scrapie, and active and passive surveillance of sufficient numbers. Active surveillance and targeted sampling of flocks at risk are important in both determination of prevalence, and evaluation of success of control and eradication programmes. There is a degree of uncertainty related to the reliance of sheep owners to report cases with clinical signs of scrapie (Hopp et al., 2001). Clinical signs vary and may not be readily recognized by the untrained eye, and there may be commercial incentives to conceal the disease in fear of the consequences.

Transmission.

Infectivity is linked to PrP^{Sc}, and tissue deposition and distribution play an important role in natural transmission of scrapie (McKinley et al., 1983; Race et al., 1998). Transmission of the scrapie agent between individuals occurs through direct contact, or indirectly via the environment. Spread of classical scrapie between flocks occurs most likely through movement of preclinically infected sheep, but the first affected animal may still be a homebred animal (Hopp et al., 2001; McIntyre et al., 2008). Introduction of scrapie to Norway was probably through import of rams from England as early as 1890s, but was first confirmed in 1958 in two imported Suffolk rams (Naerland, 1970). After introducing infection to a flock, scrapie is on average detected 3.5 years later. By then, the infection would have had time and opportunity to spread to other animals and to the environment.

The scrapie agent persists in the environment for many years and retains infectivity under extreme conditions, and the environmental factors are difficult to control. The PrP^{Sc} is also resistant to boiling, to a variety of naturally occurring organic (psoralens) and inorganic (hydroxylamine) compounds, disinfectants, and to ultraviolet and ionizing radiation (Bellinger-Kawahara et al., 1987; Taylor et al., 1994). Infectivity is retained for at least 16 years in sheep-houses and at least three years in the environment (Brown and Gajdusek, 1991; Georgsson et al., 2006). Survival beyond this does not seem to have been studied.

Infectious prions may be shed/excreted continuously into the environment throughout the course of the disease through urine, faeces, saliva and nasal secretions (Ligios et al.,

2007; Safar et al., 2008; Bessen et al., 2010; Tamgüney et al., 2012). Secretion and/or excretion of the agent varies between *PRNP* genotypes, and thus, the level of environmental contamination and transmission depends on management practices and *PRNP* genotype profile of the population (McIntyre et al., 2008; Hagenaars and Windig, 2015). The level of excretion/contamination, i.e. the infective dose, influences the incubation period; the higher dose, the shorter incubation period (Prusiner et al., 1981, 1982).

The *PRNP* genotype profile will influence the rate of new infections in a population, due to infection will become established more readily in genetically susceptible animals. Higher frequency of the ARR allele in a population, regardless of management practices, will reduce the overall risk of scrapie (Ortiz-Pelaez and Bianchini, 2011). Experimentally induced scrapie in homozygous ARR sheep has not shown localized nor generalized PrP^{Sc} amplification and will therefore contribute relatively little to environmental contamination. Furthermore, oral and subcutaneous infection did not result in transmission of disease in genetically resistant animals (Jeffrey et al., 2014; Lacroux et al., 2017).

Management practices that influence the transmission dynamics within a flock are many, although there are differences between reports (Hopp et al., 2001; McIntyre et al., 2006; Hagenaars et al., 2018). Generally, *PRNP* genotype distribution within the flock, breed, lambing practices, flock size and replacement rate are some of the most important factors influencing the risk of scrapie within flocks (Hoinville et al., 2000; McIntyre et al., 2006). Sabatier et.al. developed a mathematical model for transmission dynamics within flocks and concluded that the *PRNP* genotype in a population would determine the size of the outbreak, but management practices would influence the type of the outbreak (Sabatier et al., 2004). Regardless of *PRNP* genotype profile of a flock, if management procedures allow heavy contamination of the environment, poor biosecurity and facilitate horizontal transmission, the outbreak will be difficult to restrict.

Under field conditions, transmission of the agent mainly occurs between individuals, direct horizontal transmission and/or via contaminated environment. The most likely route of infection is through oral uptake from environment and through ingestion of grass and soil, although uptake via scarification, mucous membrane and conjunctiva is possible (Carp, 1982; Gossner et al., 2006; Johnson et al., 2007; Hamir et al., 2008; Maddison et al., 2010; Pritzkow et al., 2015, 2018).

Vertical/maternal transmission between dam and offspring is possible, and the parturient and post-parturient periods are of importance (Andréoletti et al., 2002). There is an increased incidence of scrapie in offspring from naturally infected dams, and the risk of developing scrapie in offspring is not affected by scrapie status of sire (Hoinville et al., 2010). PrP^{Sc} has been detected in placenta from both experimentally and naturally infected homozygous VRQ ewes, consequently the placenta from scrapie-infected ewes can accumulate high amounts of PrP^{Sc} and become an important source of infection for the offspring, contribute to environmental contamination, and lead to higher infection rates (horizontal and vertical transmission) during the lambing period

(Pattison et al., 1972, 1974; Ersdal et al., 2005; Touzeau et al., 2006; González et al., 2012). Interestingly, the level of PrP^{Sc} accumulation in the placenta depends on the foetal genotype, and there is an absence of PrP^{Sc} in placentas carrying foetuses with at least one ARR allele. Thus, breeding from homozygous ARR rams would efficiently decrease the environmental contamination via placentas left by the lambing ewes (Tuo et al., 2002; Garza et al., 2017).

Transmission of infection *in utero* has long been debated and results are contradicting. The varied results are probably due to the importance of *PRNP* genotype of the foetuses and the anatomical and functional differences between the species studied (Andréoletti et al., 2002; Tuo et al., 2002; Foster et al., 2013). Making comparisons across species are difficult due to the species-specific anatomy of the placenta. The foetuses in sheep seemed to be separated and protected *in utero* from the PrP^{Sc} rich tissue and allantoic fluid of the infected dam by the PrP-free amnion surrounding the foetuses (Tuo et al., 2001). However, Garza *et al*, detected PrP^{Sc} in amniotic fluid of foetuses from naturally infected ewes by the highly sensitive PMCA technique, showing that *in utero* transmission can be possible under certain conditions and foetal *PRNP* genotypes (Garza et al., 2011, 2017). These findings were supported by Spiropoulos *et al* who reported the proof of PrP^{Sc} infectivity in foetal tissues such as umbilical cord and foetal mesenteric lymph node, however pointing out the importance of susceptible *PRNP* genotype of both foetus and dam, allowing peripheral distribution of PrP^{Sc} in dam during pregnancy (Spiropoulos et al., 2014).

The offspring is, nevertheless, likely to come in contact with PrP^{Sc} during and shortly after parturition when foetal membranes are broken (Tuo et al., 2001; Andréoletti et al., 2002). During the postnatal period, lambs can also become infected through colostrum and milk (Lacroux et al., 2008; Konold et al., 2013). Husbandry routines in some flocks may aid the transmission of PrP^{Sc} within the flock as colostrum from infected ewes could be administrated to other lambs if needed. There is evidence of scrapie transmission via milk, and at greater titres if the mammary gland is inflamed and with an accompanying high leucocyte count (Ligios et al., 2011; Konold et al., 2008, 2013).

Transmission of scrapie via germinal cells is not thought to be of importance, and semen and embryo transfer seem not to transmit the agent, although PrP^{C} has been demonstrated in semen from rams, transmission through artificial insemination and natural mating has not been confirmed (Foote et al., 1993; Wang et al., 2001; Gatti et al., 2002; Sarradin et al., 2008; Low et al., 2009).

The literature points to clear and strong evidence that infected animals, with *PRNP* genotypes that allow peripheral spread of PrP^{Sc} and infectivity, contribute greatly to environmental contamination and pose a serious threat of transmitting the disease to any offspring *in utero*, during birth and through colostrum and milk, both during preclinical and clinical stages of scrapie (Ryder et al., 2004; Konold et al., 2015). Thus, keeping susceptible genotypes in the population will have an impact on epidemiology and prevalence of classical scrapie (Andréoletti et al., 2002; Tuo et al., 2002; Konold et al., 2008; Foster et al., 2013; Garza et al., 2011, 2014, 2017).

Pathogenesis

Pathology and pathogenesis of classical scrapie are highly influenced by host and prion strain factors such as *PRNP* genotype and time and dose of inoculum, but the histopathological changes associated with all prion diseases remain consistent, although at varied distribution and intensity. These classical changes constitute a triad of histological lesions comprising of vacuolation of grey matter of the CNS, neuronal degeneration and gliosis, and in addition there is wide distribution of the diseases specific form of the prion protein, PrP^{Sc} (Hadlow, 1999; Jeffrey and González, 2007). The dissemination and accumulation of PrP^{Sc} peripherally and within the CNS, depend on site of entry, PrP^{Sc} strain, dose, *PRNP* genotype and host species (van Keulen et al., 1995, 1996; Somerville et al., 1997; Ersdal et al., 2003; Glatzel et al., 2003; Jeffrey and González, 2007; Otero et al., 2019). One ARR allele in sheep *PRNP* seem to prevent peripheral PrP^{Sc} accumulation (van Keulen et al., 1996, 2000). The dissemination and replication of PrP^{Sc} in cells and tissues, and the establishment and sustainment of infection, prerequisite the ability to express normal PrP^{C}, although uptake of PrP^{Sc} from gut seems to occur independently of PrP^{C} expression in intestinal epithelial cells (Prusiner, 1982; Prusiner et al., 1993; Büeler et al., 1993; Brandner et al., 1996; Blättler et al., 1997; Andréoletti et al., 2000; R. Heggebø et al., 2003; Ersdal et al., 2005; Marshall et al., 2018). The propagation of prions is thought to start with binding of PrP^{Sc} to PrP^{C}, which is expressed on a range of cells including neurons and LRS cells. This binding causes conformational conversion of PrP^{C} to PrP^{Sc}, resulting in increased β-sheet content, decreased solubility and generation of new binding sites for additional PrP^{C} on the growing PrP^{Sc} polymer (Pan et al., 1993).

The most likely route of naturally acquired infection is uptake of prions via the alimentary tract, but infection may be acquired through scarification of mucosal surfaces in mouth, skin and conjunctiva, and prion disease has been experimentally transmitted between sheep through blood transfusion (Houston et al., 2008). After oral infection, prions are transported across the gastrointestinal epithelial lining to gain access to the Peyer's patches (PPs), which extend from submucosa and into mucosa and are part of the gut-associated lymphoid tissue (GALT) (Andréoletti et al., 2000; Press et al., 2004) . The replication and accumulation of prions within small intestinal GALT seem to be important and essential for the efficient subsequent neuroinvasion, much more than the large intestinal GALT (Donaldson et al., 2015). This transepithelial passage from gut lumen to PPs is facilitated by specialized enterocytes and highly phagocytic epithelial cells, termed M-cells, in the follicle-associated epithelium (FAE) which covers the luminal side of PPs (Heggebø et al., 2000; Kujala et al., 2011). These cell types are specialized in sampling, and also cooperate with dendritic cells (DCs) in transferring antigens and microorganisms from the gut lumen into the GALT, contributing to the mucosal immune response (Gebert, 1997; Lelouard et al., 2012). Developmental studies in ruminants and mice suggest that development and maturation of these M-cells occur in the late prenatal period and their functional number decrease with age, indicating they are fully functional and at their highest number at birth (Beyaz and Aşti, 2004; Kobayashi et al., 2013; Özbek and Bayraktaroğlu, 2019). This makes the young animal very efficient in PrP^{Sc} uptake from gut lumen, and results in increased susceptibility and reduced survival time when exposed to PrP^{Sc} at birth (Donaldson et al., 2016). Following

uptake, prions are believed to be carried by DCs and monocytes/tissue macrophages to the follicular dendritic cells (FDCs) in the germinal centres of B follicles the PPs and other secondary lymphoid organs (SLOs) (R. Heggebø et al., 2003; Huang et al., 2002). The surface area of ileal PP tissue, the lymphoid follicle density and the number of FDCs are at their highest in younger animals, and this is probably of crucial relevance in early pathogenesis (St Rose et al., 2006). The FDCs are probably the first site of prion conversion and PrP^{Sc} replication after oral exposure, and thus infection becomes efficiently established soon after exposure in young animals (McBride et al., 1992; Kujala et al., 2011; Marruchella et al., 2012). After uptake, PrP^{Sc} accumulates and replicates in GALT and other SLOs before it is detected in enteric nervous system (ENS) before neuroinvasion (Tabouret et al., 2010). The autonomic nervous system (ANS) supplies nervous input to all components of the immune system and this interface allows communication between the CNS and the immune system, and allowing regulation of neuroimmune processes (Elenkov et al., 2000; Kenney and Ganta, 2014). SLOs are predominantly innervated by sympathetic nervous system (SNS), but in the gastrointestinal tract, both sympathetic and parasympathetic nerves can directly synapse with the ENS (Elenkov et al., 2000; Nance and Sanders, 2007; Yoo and Mazmanian, 2017; Al-Shalan et al., 2019). By some, neuroinvasion is believed to initially occur retrograde along parasympathetic and sympathetic efferent nerve fibres in the gut wall where they are in close proximity to both ENS and immune cells (van Keulen et al., 2000; Defaweux et al., 2005). Studies of both experimental and natural cases of scrapie in sheep indicate that PrP^{Sc} spread from the ENS to the brain through nerve fibres of the vagal nerve to the dorsal motor nucleus of vagus (DMNV), and to the spinal cord segments T8-T10 via the coeliaco-mesenteric ganglion and the great splanchnic nerve to intermediolateral column (IMLC) (van Keulen et al., 2000; McBride et al., 2001; Ersdal et al., 2005; Tabouret et al., 2010). Subsequently, additional neuroinvasion can occur from other PrP^{Sc} positive lymphoid tissues along their sympathetic nervous innervation (Glatzel et al., 2001; Cancedda et al., 2014). As the disease progresses, PrP^{Sc} continues to disseminate, accumulate and replicate in lymphatic tissue and throughout the CNS (Tabouret et al., 2010; Wemheuer et al., 2011). Accumulation of PrP^{Sc} in lymphoid tissues is associated with changes in maturation of FDCs and alterations in tingible body macrophages (TBMs), but how these changes affect lymphoid tissue function or relate to clinical findings has not yet been fully elucidated (McGovern and Jeffrey, 2007). Gene expression studies of peripheral lymphoid tissue report repression of gene expression and an anti-inflammatory response in the early stages, and this may be related to both general progression of disease and later the accumulation of PrP^{Sc} in lymphoid tissues (A. Gossner et al., 2011; Filali et al., 2014; Gossner and Hopkins, 2015).

PrP^{Sc} can also be found at sites of chronic inflammation, where FDCs, TBMs and B lymphocytes are involved, and because they express PrP^{C}, replication and accumulation can occur at otherwise prion-free organs (Heikenwalder, 2005). Concurrent chronic inflammation, e.g. mastitis, nephritis and myositis, can potentially have an impact on peripheral spread of PrP^{Sc} that can increase excretion/secretion of prions and thereby contribute to spread and environmental contamination (Seeger, 2005; Ligios et al., 2005, 2011; Neumann et al., 2013)

After entering the CNS, PrP^{Sc} is widely distributed in all regions of the brain, but this is not necessarily accompanied by neural pathological changes and vacuolation. The PrP^{Sc} accumulation can be extracellular, cell membrane associated and intracellular. The extracellular/cell membrane PrP^{Sc} can be detected in the neuropil and associated with glial, ependymal and endothelial cells, while the intracellular accumulation is seen in neurons, astrocytes and microglia (Jeffrey and González, 2007). PrP^{Sc} is associated with cell membrane and cytoplasm of neurons, astrocytes, macrophages, and ependymal cells. Amyloid formation is associated with blood vessels and can be seen in vessel wall and perivascular. Morphological pathology, such as vacuolisation, gliosis and neuronal death, shows considerable variation and is greatly influenced by host and strain factors (Wood et al., 1997). Vacuolation is commonly found in the dorsal motor nucleus of the vagal nerve and is of diagnostic significance. Another characteristic finding, gliosis, vary and is not always accompanying vacuolation pattern. Significant cell loss is not always evident on routine histopathological examinations (van Keulen et al., 1995; Wood et al., 1997; Begara-McGorum et al., 2002; Ligios et al., 2002).

The actual pathophysiological pathways of neurodegeneration and neural dysfunction are not fully understood whether there is loss of function of PrP^{C} after conformational change from PrP^{C} to PrP^{Sc}, gain of function of PrP^{Sc}, or other involving factors. The direct and/or indirect toxic properties of PrP^{Sc} and the exact role of PrP^{C}, are debated as the correlation between morphological changes, PrP^{Sc} accumulation and clinical deficits are neither elusive nor consistent (Jeffrey and González, 2007; Aguzzi and Falsig, 2012; Hughes and Halliday, 2017). Gene expression studies have given additional insight into pathogenesis and disease progression, and there seem to be correspondence between pathological events and gene expression profile, although not prion disease specific (Hwang et al., 2009). Common for several of the gene expression analyses is the alterations in expression of genes associated with innate immunity and activation of glial cells within the CNS (Hwang et al., 2009; Gossner and Hopkins, 2014; Carroll et al., 2016, 2018). Activation of both astrocytes and microglia within the CNS can play a role in neurodegeneration (Pekny et al., 2014). Gene expression studies have revealed that PrP^{Sc} accumulation and prion disease associated pathology, show upregulation of genes associated with inflammation, immune response, autophagy and cell death, and regulation of transcription (Booth, 2004; Skinner et al., 2006; Hwang et al., 2009).

Although prions have been associated with circulating B cells in blood of sheep with scrapie, there is little evidence of haematogenetic spread of infection to CNS across the blood-brain-barrier (BBB), but circulating B cells may play a role in dissemination of prions between SLOs (McBride et al., 2001; Edwards et al., 2010; Dassanayake et al., 2011; Keller et al., 2018).

The clinical presentation of classical scrapie shows great variability, and it has been difficult to relate clinical symptoms to the classical triad of histological lesions; neuronal vacuolation, astrocytosis and neuronal loss. There are also discrepancies between PrP^{Sc} accumulation and clinical signs (Jeffrey and González, 2007). Nevertheless, the clinical signs are related to CNS involvement, as the accumulation of PrP^{Sc} peripherally has not been associated with any clinical signs. In natural scrapie, the accurate onset of clinical signs has been difficult to establish due to the insidious onset and the premonitory signs

are likely to pass unrecognised. Even in clinically affected sheep and cattle, there is no obvious link between clinical signs and location of histopathological changes and PrP^{Sc} accumulation in the CNS (Jeffrey and González, 2007). The clinical signs point towards a progressive neurodegenerative disease affecting both cerebellar and cerebral areas of the brain. The pruritus or neuropathic itch may be caused by a central lesion, when neurons or glia cells involved in pruritoception becomes damaged (Misery et al., 2014; Hachisuka et al., 2018).

Neurodegenerative diseases are characterised by selective dysfunction and progressive loss of synapses and neurons associated with pathologically altered proteins that deposit primarily in the human brain and spinal cord.

Clinical signs

As stated earlier, the scrapie phenotype depends on many host and prion factors, and combined they give rise to a great diversity of clinical presentation from sudden death to a slowly progressive disease with subtle neurological and behavioural changes (Gonzalez et al., 2012; Bartz, 2016). Due to the absence of a good and liable *ante mortem* diagnostic test, the diagnosis in the live animal relies on recognition of clinical signs by animal keeper and/or veterinary surgeon. A protocol was established in 2014 to aid *ante mortem* diagnostics based solely on clinical presentation (Konold and Phelan, 2014).

One of the most comprehensive records of clinical scrapie is from records on natural scrapie was made by Parry before the discovery of the prion and the important and highly relevant *PRNP* genotypes (Parry and Oppenheimer, 1983). Parry described scrapie as a slow progressive disease, invariably fatal, usually detectable between two and five years of age, and after three to six months progression. The clinical presentation showed vast individual variability. Nevertheless, disturbances were contained within five defined physiological systems; metabolism, motor function, sensation, behaviour and autonomic nervous control (Parry and Oppenheimer, 1983). Scrapie can also be diagnosed in so-called fallen stock without any previous symptoms registered by owner/shepherd. Even though sudden death can occur, it is most likely that the clinical signs have been missed (Detwiler, 1992).

Systems	Clinical sign	References
Metabolism	Inanition, wasting, weakness, ill-thrift, obesity, normal to good appetite	(Parry and Oppenheimer, 1983; Detwiler, 1992; Austin and Simmons, 1993; Ulvund et al., 1996; Austin et al., 1997; Ulvund, 1999; Detwiler and Baylis, 2003; Healy et al., 2003; Houston and Gravenor, 2003; Hörnlimann et al., 2007; Konold and Phelan, 2014)
Motor function	Fore and hind limb ataxia, dysmetria, clumsiness, tremors, abnormal posture, paresis, crouching stance, proprioceptive deficits	
Sensation	Compulsive rubbing, pruritus, nibble response, symmetrical wool loss, dirty wool	
Behaviour	Anxiety, apprehensive, confusion, hyperexcitability, nervous, aggressive, separate from flock, vagueness, altered mental status, hyperresponsive, restlessness	
Autonomic nervous control	Tachycardia, cardiac arrhythmia, alimentary mobility, abomasal dilatation/impaction	

Table 7. Overview of the most common clinical signs in classical scrapie in sheep.

The clinical picture will be a combination of signs listed in Table 7, and this is probably a result of influencing factors like *PRNP* genotype, breed, strain, environmental and other unknown factors. There seems to be geographical variation with dominating clinical signs in different countries. Pruritus seems to be a prominent sign in Norway, Spain and Italy, but not in Ireland, while hind limb ataxia seems to be a prominent sign in Ireland and Italy, and not in Norway (Hörnlimann et al., 2007). In fact, in an Irish study, pruritus was found to be negatively correlated to ataxia (Healy et al., 2003). In certain phenotypes, pruritus may be the first predominant presenting sign of classical scrapie (Houston and Gravenor, 2003).

Diagnosis, surveillance, and control

Scrapie became a notifiable disease in Norway in 1965, and the scrapie surveillance programme was first launched in 1997. The disease was first diagnosed in indigenous sheep in 1981 and was intermittently diagnosed until peak occurrence in 1996 when 32 scrapie positive flocks were detected and resulted in eradication of several hundred contact flocks. The number of annual cases then declined rapidly and the last flock diagnosed with classical scrapie appeared in 2009 (Sviland et al., 2018). In order to completely eradicate classical scrapie in sheep, Norway adopted the "stamping out" strategy in 1996 and did not implement the breeding for resistance strategy. In addition, movement restrictions were introduced to limit the unintended movement of potentially infected sheep, with extra caution of movements out of and within the four counties with a history of scrapie cases. Due to the structure of the Norwegian sheep industry, exceptions have been made regarding common grazing, ram sales and, in certain situations, breeding ewes (Anon, 2018).

"The TSE Regulation" (EC Regulation 999/2001) forms the legal base for almost all legislative actions on TSEs in the European union (EU) and Norway through the European Economic Area (EEA) Agreement (Anon, 2001). The purpose of the TSE legislation is to protect the health of consumers and animals, and to control and eradicate TSEs. The TSE Regulation refers to the "Manual for diagnostic test and vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE)" for

currently approved diagnostic methods, protocols and their purpose, Table 8 (International Office of Epizootics and Biological Standards Commission, 2018).

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals of populations post-vaccination
Agent identification						
Histo-pathology	n/a	n/a	-	+	-	n/a
IHC	n/a	n/a	++	+++	++	n/a
Western immune-blot	n/a	n/a	++	+++	++	n/a
Rapid tests	n/a	n/a	+++	+	+++	n/a

Table 8. Test methods available for diagnosis of scrapie and their purpose. From the Manual for diagnostic test and vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE) 2018. +++ = recommended method; ++ = suitable method; + = may be used in some situations, but costs, reliability, or other factors severely limit its application; – = not appropriate for this purpose; n/a = not applicable. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature, and the fact that they have been used widely without dubious results, makes them acceptable. IHC = immunohistochemistry.

Each country is committed to carry out an annual monitoring programme which is based on active and passive surveillance, and several EC Member States (MSs) have successfully implemented the voluntary breeding programme to select for resistance to TSEs in their ovine population. Genetic susceptibility is the basics behind the breeding for resistance program, with the aim of increasing the frequency of the ARR allele while reducing the prevalence of alleles associated with increased susceptibility (Anon, 2001). The distribution of the different *PRNP* genotypes will not reveal prevalence of classical scrapie, but *PRNP* genotype surveillance will monitor the susceptibility within the population and individual flocks. Flocks with more than 5.2 % frequency of the VRQ allele carry a high risk of being affected with clinical classical scrapie (Tongue et al., 2009).

There are a number of reports stating that the breeding program has good and rapid effect on the *PRNP* genotype, and as a result, decreased incidence of clinical classical scrapie cases (Nodelijk et al., 2011). The annual EFSA surveillance report for 2017 shows the development of the overall *PRNP* genotype distribution among countries that report results from random genotype testing. NSP1 (most resistant group) has increased from 27.8 % in 2008 to 50.6 % in 2015, and 81.8 % in 2017 for the 28 reporting MSs (European Food Safety Authority (EFSA), 2018). Random *PRNP* genotyping in EC MSs in 2017 showed that 26.5% of sheep population was susceptible to classical scrapie (groups NSP3-5), and this rose to 41.2 % in the four MSs that accounts for the highest caseload during 2017 (European Food Safety Authority (EFSA), 2018).

Following the EU Regulation (EC) No. 999/2001, as amended by Regulation (EC) No 2017/894, of May 2017, annual surveillance of *PRNP* genotypes will no longer be required. As a result of these changes, random genotyping is no longer performed in Norway. The initial objective of mapping scrapie susceptible sheep genotypes and identifying resistant sheep genotypes per country, had now been achieved. As of January 2018, *PRNP* genotype surveillance programme is replaced by requirements only applicable to countries with a breeding programme for resistance in place. The monitoring of *PRNP* genotypes in countries with breeding programmes will now be customized to each country to ensure detection of any changes in genotype prevalence, with high confidence. The aim of the breeding programme was to increase the frequency of the ARR allele in the sheep populations and reduce the incidence of classical scrapie, in order to prevent the entry of ovine prions into the food and feed chains. Following the reports of naturally occurring classical scrapie cases in homozygous ARR sheep, have been raised some concerns and the absolute resistance to classical scrapie in homozygous ARR genotypes should be asserted with caution. (Groschup et al., 2007; Jeffrey et al., 2014). Increasing the frequency of ARR alleles in the population is, however, still regarded as a safe and sustainable approach to control and eradicate classical scrapie, as PrP^{Sc} has minimal capacity to propagate in or adapt to homozygous ARR sheep (Lacroux et al., 2017).

Adequate active and passive surveillance of the population is necessary to achieve reliable knowledge of the epidemiological situation. According to the TSE Regulation, a certain number of sheep must annually be sampled through an active surveillance programme. In sheep populations exceeding 750 000 breeding ewes, such as Norway, a total of minimum 20 000 sheep must be sampled from the following three categories; 1. Slaughtered for human consumption, 2. Not slaughtered for human consumption i.e. fallen stock, 3. Culled animals in relation to disease eradication. The samples must come from animals over 18 months of age, randomly selected in the total population, while ensuring flocks exceeding 100 ewes being repeatedly monitored. Areas with low animal density (less than 10 % of the sheep population) without organised collection of dead animals, can be excluded from the program, given that the Commission is informed. The Norwegian Veterinary Institute estimates that 60 % of the fallen stock population dies in inaccessible and remote mountain and forest areas/pastures, thereby making active surveillance limited, and less animals are sampled than required by the TSE Regulation (Anon, 2001; Sviland et al., 2018).

The passive surveillance programme consists of testing animals identified as TSE suspect cases. The last cases of confirmed classical scrapie in Norway in 2006 and 2009 were detected through active surveillance (https://www.vetinst.no/overvaking/skrapesjuke-tse-sau).

PRNP genotype (codons 136, 154 and 171) must be determined in all positive cases in order to monitor the incidence of classical scrapie and Nor98 in relation to *PRNP* genotype. The EFSA report on surveillance for presence of TSEs in EU (28 Member States, MSs) and Iceland, Norway and Switzerland (non-Member States, non-MSs), states that in 2017, there were 832 classical scrapie cases in MSs (predominantly Greece, Spain, Italy and Romania) and one case in Iceland. *PRNP* genotype was known

in 780 cases of classical scrapie, and 766 of these were from the susceptible genotype groups; NSP3, NSP4 and NSP5 (European Food Safety Authority (EFSA), 2018). This agrees with findings from the past ten years (2008 – 2017), where over 90 % of the classical scrapie cases were from the susceptible genotype groups. None of the classical scrapie cases were registered in the resistant group (NSP1).

Only *post mortem* diagnostic tests are approved for the definitive and confirmatory diagnosis of scrapie (Table 8). The most sensitive diagnostic tests rely primarily on the presence and detection of PrP^{Sc} in the CNS. Typical histopathological and morphological changes are not on its own diagnostic and must be accompanied by demonstration of PrP^{Sc} in the CNS. Rapid tests are based on WB and ELISA methods, which are both quick and reliable. These methods allow large numbers of samples to be tested and are excellent for surveillance screening. Positive and inconclusive cases must be confirmed by any of the other approved methods such as histopathology, immunohistochemistry, immunoblotting or demonstration of characteristic fibrils by electron microscopy. The use of multiple diagnostic methods/criteria on accurately sampled CNS tissues are necessary to maintain a high degree of confidence in negative results.

Screening live animals for classical scrapie is possible by detecting the presence of PrP^{Sc} in biopsies from peripheral lymphoid tissues, such as the palatine tonsil, third eyelid lymphoid tissue and the recto-anal mucosa-associated lymphoid tissue (RAMALT) (Schreuder et al., 1998; O'Rourke et al., 2000; Espenes et al., 2006; Wolfe et al., 2007). However, preclinical accumulation of PrP^{Sc} in peripheral lymphoid tissues is not a constant feature of prion diseases. Sensitivity of PrP^{Sc} detection is dependent on age, time after infection, prion strains and *PRNP* genotypes (González et al., 2006, 2008; Thomsen et al., 2012). Recent research has shown that the large intestinal GALT is less important than the small intestinal GALT in the preclinical uptake, replication and accumulation of prions after oral acquisition, thus rectal biopsies can be sampled too early before PrP^{Sc} deposition, with a risk of false negative results (Donaldson et al., 2015).

New technology, like the ultrasensitive serial protein misfolding cyclic amplification (sPMCA), although not yet formally approved for statutory purposes, can give new opportunities by increasing the diagnostic sensitivity and potential strain discrimination (Gough et al., 2014; Wang et al., 2019).

Accurately knowing the status of classical scrapie in a sheep population or flock is challenging and depends on adequate surveillance (both passive and active) and knowledge of *PRNP* genotype distribution. Active surveillance is based on random sampling which assumes a homogeneous population where each animal has the same probability of becoming infected. Contrarily, the strong influence of *PRNP* genotype on susceptibility, makes the sheep population heterogeneous with different risk groups defined by the NSP groups. A negative test result from a susceptible sheep brings more evidence of scrapie freedom than a negative test result from a resistant sheep. Thus, failure to observe histological changes or detect PrP^{Sc} alone, does not confirm the absence of the disease, and knowledge of *PRNP* genotype of the tested sheep is necessary to interpret the results (Durand et al., 2009). The confidence level of detecting classical

scrapie depends on the prevalence of scrapie and the number of animals tested, but it will be highly influenced by *PRNP* genotype, as it is a major risk factor (Martinez et al., 2010). To substantiate disease freedom, a higher number of tests must be performed in a resistant population than in a susceptible population, therefore targeted testing of susceptible animals would possibly provide stronger confidence of detecting classical scrapie (Martinez et al., 2010).

Prion disease and immune system

The connection between the prion protein and the immune system is not fully understood, but the two are intertwined from time of infection through to the end stage of the disease. PrP^{C} is present in the midst of the cross-talk between the nervous and immune systems, in both cells of the nervous systems and on immune cells (Kretzschmar et al., 1986; Cashman et al., 1990; Aguzzi and Glatzel, 2000; Dürig et al., 2000; Herrmann et al., 2001; Steinman, 2004; Nance and Sanders, 2007; Bradford and Mabbott, 2012). Immune cells are activated from first exposure to prions, but the immune system fails to mount an inflammatory or adaptive immune response until the later clinical stages.

The nervous system and immune system have co-evolved and have both crucial roles in maintaining and controlling homeostasis and preventing harmful invasion, and thus bi-directional communication pathways between the two systems are necessary and essential (Sankowski et al., 2015). One of these communicating pathways is the hard-wire pathways where neurites innervate lymphoid tissues directly. Most lymphoid tissues are innervated by sympathetic nerves, and in addition, parasympathetic efferent nerves are involved in some immunological relevant organs. Recently another type of afferent connection between the lymphoid organs and CNS has been described, namely the wired antigen presenting cells (wAPCs) and the wired immune cells (wICs), which are innervated by free nerve endings, called a neural nexus (Wülfing and Günther, 2015; Wülfing et al., 2018; Hu et al., 2019). These neural nexuses can be found within lymphoid organs in defined tissue areas that function as “a gate”, i.e. antigen entrance, and thereby having a central role in the “first line of defence” (Wülfing et al., 2018). After oral exposure of prions, the infection is quickly associated with immune cells and established in Peyer’s patches and other SLO tissues containing wired cells and neural nexuses. Replication and dissemination of PrP^{Sc} in immune cells and tissues seem to be critical to peripheral and central prion pathogenesis and neuroinvasion (Glatzel et al., 2001; Bradford and Mabbott, 2012; Keller et al., 2018; Wülfing et al., 2018).

The innate immune system has a semi-specific protective role, representing the first line of defence, and it comprises of physical and chemical barriers and humoral and cell-mediated components (Riera Romo et al., 2016). The brain is said to be immune privileged or immunologically unique, implicating that immune responses are slow and tightly regulated to minimize the damaging effects of inflammatory responses. Nevertheless, both innate and adaptive inflammatory responses occur within the CNS (Amor et al., 2010). Upon recognition of pathogen/danger-associated molecular patterns (PAMPs/DAMPs) by pattern-recognition receptors (PRRs), the innate immune system elicits an immediate defence response; initiating an inflammatory response and alarming the nervous system of the imminent threat (Iwasaki and Medzhitov, 2015).

The complexity and diversity of recognition is vast, and the immune system must manage both harmful and benign substances. There are several classes of PRRs, and they vary in structure, location and specificity and respond to different PAMPs/DAMPs, but they share similar downstream signal transduction pathways, e.g. the activation of the NF-kB pathway. Toll-like receptors (TLRs) are one type of PRRs which typically promote production of proinflammatory cytokines when activated. Several TLRs are expressed on microglia, astrocytes, oligodendrocytes and neurons, and have they been implicated in neurodegeneration (Okun et al., 2009).

The complement system includes complement components and receptors expressed by astrocytes, microglia and neurons, and has been associated with proteinopathies in the CNS (Bonifati and Kishore, 2007).

In continuation of this immediate response, the innate immune system has a role in the activation and regulation of the long-lasting adaptive immunity (Iwasaki and Medzhitov, 2015).

Interestingly, both cellular and proteinaceous components of the innate immune system are associated, involved and activated during the uptake and dissemination of PrP^{Sc}; but despite reaching high levels of PrP^{Sc} in peripheral lymphoid tissues in pre-clinical stages, this does not seem to activate any inflammatory response. (Prusiner, 1982; Heikenwalder, 2005; Bradford and Mabbott, 2012; Michel et al., 2012). On the contrary, there is evidence of an anti-inflammatory response in lymphatic tissues in early stages (Gossner and Hopkins, 2015). This absence of both an initial inflammatory response and a subsequent adaptive immune response to PrP^{Sc}, is believed to be because immune cells are unable to properly present PrP^{Sc} as an antigen. Proposed explanations for this are that linear epitopes from PrP^{Sc} will have the same amino acid sequence as PrP^{C} and recognized as self, and proteases cannot effectively digest prions which will lead to accumulation of PrP^{Sc} in APCs and impairing their functions (Zabel and Avery, 2015). PrP^{C} is expressed by a variety of cells in many tissues and exposed epitopes on PrP^{C} are strongly tolerated by the immune system, and consequently, an efficient antibody response is difficult to generate (Grégoire et al., 2005). The identical amino acid sequence of all prions within the same host could make exposed epitopes indistinguishable and PrP^{Sc} will be recognised as "self". Any anti-self-reaction would potentially be detrimental to the host (Bendheim et al., 1992; Pradeu and Cooper, 2012; Zabel and Avery, 2015).

Microglia and astrocytes, which constitute the resident immune cells of the CNS, are important in maintaining homeostasis. They are part of the first line of defence and they become activated in the presence and accumulation of PrP^{Sc}, but initially without an inflammatory response (Betmouni et al., 1996). Microglia, the principal resident neuroimmune cells in the CNS, have several essential functions to provide neuroprotection through delicately balancing between anti- and pro- inflammatory status: sensing changes; promoting neuronal wellbeing and function (housekeeping); defence functions against modified-self and non-self-injurious agents (Hickman et al., 2018). As part of the Immune response in the CNS, activated microglia clear debris after myelin damage and remove necrotic cells, which aid repair and regeneration (Neumann et al., 2009; Amor et al., 2010). It is crucial for the brain to keep a suppressive immune

environment, and microglia and astrocytes take on an important role in limiting inflammation (Amor et al., 2010). Microglia become activated in prion diseases, as with other neurodegenerative diseases, and it has been proposed that a persistent neuroinflammatory response to PrP^{Sc} will eventually disrupt and dysregulate normal microglia function, eventually leading to an exaggerated proinflammatory response, neurotoxicity and neurodegeneration (Burguillos et al., 2011; Heneka et al., 2014; Chen et al., 2016; Hickman et al., 2018). It has become evident that the neuroinflammatory response initiated in prion diseases is not always associated with proinflammatory cytokine expression, but initially a neuroprotective anti-inflammatory response (Betmouni et al., 1996; Alibhai et al., 2016; Aguzzi and Zhu, 2017). The activation pathways are not yet fully elucidated but TLRs, complement system and altered expression of both anti- and proinflammatory cytokines have been described (Campbell et al., 1994; Burwinkel et al., 2004; Mabbott, 2004; Mitchell et al., 2007; Spinner et al., 2008; A. G. Gossner et al., 2011; Kang et al., 2016; Carroll et al., 2018). The TLRs 2 and 4 are upregulated during neurodegeneration and, when activated, they are assumed to promote a proinflammatory response (Okun et al., 2009).

Upon entering the CNS, prions disseminate and are eventually detected in various regions of the brain, yet the presence of PrP^{Sc} is not always accompanied by neurodegeneration (Alibhai et al., 2016). This shows that presence of abnormally folded proteins by itself is not enough to cause neuronal degeneration. In regions of the brain where presence of prions is not accompanied by neurodegeneration, microglia adapts or remain in an anti-inflammatory state; but in regions with neurodegeneration, the activated microglia take on a pro-inflammatory state with increased expression of pro-inflammatory genes, complement activation and antigen processing and presentation (Hwang et al., 2009; Crespo et al., 2012; Vincenti et al., 2015; Alibhai et al., 2016; Carroll and Chesebro, 2019). Gene expression studies are beginning to reveal which transduction pathways are involved, such as the rapid acting NF-κB (nuclear factor kappa-light-chain-enhancer of B cells), which is responsible for transcriptional induction of proinflammatory cytokines, chemokines and additional inflammatory mediators (Liu et al., 2017). This pathway is involved in the transcriptional control of acute phase gene expression (Bode et al., 2012). Several different reports indicate that this pathway is of importance in prion diseases (Carroll and Chesebro, 2019).

In summary, inflammatory processes seem to play an important role in neurodegeneration in a range of diseases, including prion diseases, although the sequence of events is not fully mapped out, microglial regulation of neuroinflammation and inflammatory mediators from innate and adaptive immune systems seem central in the balance between neurotoxicity and neuroprotection (Chen et al., 2016). The protective and defencing functions of microglia are disturbed in prion diseases, and Hickman et. al. propose that microglia initially attempt to clear PrP^{Sc} via scavenger receptors and TLRs, but the persistence leads to continuous activation and accumulation of PrP^{Sc} accompanied by decreased phagocytic capability and increased proinflammatory response, neurotoxicity and neurodegeneration (Hickman et al., 2018).

Neuroinflammation is not an isolated process within the CNS, there are bi-directional communications between the CNS and the periphery via neurotransmitters, peripheral nerves and cytokines (Czirr and Wyss-Coray, 2012; Ransohoff and Brown, 2012). There are many reports on how neuroinflammation is exaggerated by systemic inflammation, but also a small number of results suggesting that peripheral inflammation can become altered by signals coming from the CNS (Cunningham et al., 2009). In recent years, a few studies searching for peripheral biomarkers of neurodegeneration have identified biomarkers in peripheral blood that indicate an active systemic inflammatory response in Parkinson Disease (PD) and Alzheimer's Disease (AD). Neuroinflammation in these two diseases is accompanied by alterations in peripheral immune system, including elevated levels of proinflammatory cytokine in blood (Anna Boyko et al., 2017).

Acute phase proteins (APPs), complement system and cytokines are the humoral part of the innate immune response, and these allow for a systemic inflammatory reaction and changes distant to site of inflammation (Ceciliani et al., 2012; Riera Romo et al., 2016). This systemic reaction is characterised by behavioural, physiologic, biochemical and nutritional changes, in addition to the change in concentration of many plasma proteins, known as the acute phase proteins (APPs) (Gabay and Kushner, 1999; Ceciliani et al., 2002). Majority of APPs are synthesised and released by the liver, although there is extrahepatic expression in ruminant (Ceciliani et al., 2012). More than 40 APPs have been identified and their synthesis is regulated by circulating proinflammatory cytokines, and their plasma concentration changes by at least 25 % during inflammation. The APPs are divided into positive (increased plasma concentration) and negative (decreased plasma concentration), and major (10- to 100- fold), moderate (2- to 10-fold) and minor (< 2- fold) APPs (Tothova et al., 2014). Some of the APPs identified in ruminants are listed in Table 9. SAA and Hp are the most important in ruminants and concentrations are normally at a very low level in plasma, while other APPs like Cp are detectable in the healthy animal (Eckersall and Bell, 2010; Tothova et al., 2014).

Positive APPs	**Negative APPs**
Alpha1-acid glycoprotein	Albumin
Alpha-1 antitrypsin	Transferrin
Ceruloplasmin	Transthyretin
C-reactive protein	
Fibrinogen	
Haptoglobin	
Lactoferrin	
Serum amyloid A	

Table 9. Overview over the most known and identified positive and negative APPs in ruminants.
(Cray et al., 2009; Eckersall and Bell, 2010; Lepherd et al., 2011; Ceciliani et al., 2012; Wells et al., 2013; Tothova et al., 2014).

Co-infection with other pathogens and concurrent systemic inflammation have shown to have an impact on scrapie pathogenesis, including susceptibility, dissemination, and transmission. The close association between prions and immune cells results in increased prion uptake along with the immunological responses to pathogens. This

expands distribution of prion within the host to tissues not normally associated with PrP^{Sc} accumulation and increase PrP^{Sc} excretion (Heikenwalder, 2005; Ligios et al., 2005; Seeger, 2005; Donaldson et al., 2016). In situations with an ongoing immune response within the CNS, such as chronic neurodegeneration, an additional systemic infection and inflammation have resulted in an acute exacerbation and progression of the ongoing neurodegenerative disease (Cunningham et al., 2005; Perry et al., 2007; Cunningham et al., 2009; Perry, 2010; Perry and Teeling, 2013). It has also been shown that heavy infection of gastrointestinal parasites reduces the incubation period of scrapie (Gruner et al., 2004).

There is not much evidence of an adaptive immune response to prion diseases, although fluctuations in immunoglobulin G (IgG) have been seen in serum and CSF in clinically affected natural scrapie cases and experimental murine models (Collis and Kimberlin, 1983; Strain et al., 1984). Generation of anti-PrP antibodies in terminal stages under certain conditions cannot be excluded, and these would potentially be detrimental to the host due to the expression of prion protein on different cell types and tissues (Grégoire et al., 2005; Sassa et al., 2010).

Aim of thesis:

The main aim was to search blood and tissues for specific markers of scrapie, other than PrP^{Sc}. Search for other biomarkers of classical scrapie in sheep other than detection of PrP^{Sc} in tissues. The understanding of scrapie in young susceptible sheep.

Summary of papers:

Paper I

Investigation of serum protein profiles in scrapie infected sheep by means of SELDI-TOF-MS and multivariate data analysis.

Background: Classical scrapie in sheep is a fatal neurodegenerative disease associated with the conversion PrP^{C} to PrP^{Sc}. Much is known about genetic susceptibility, uptake, and dissemination of PrP^{Sc} in the body, but many aspects of prion diseases are still unknown. Different proteomic techniques have been used during the last decade to investigate differences in protein profiles between affected animals and healthy controls. We have investigated the protein profiles in serum of sheep with scrapie and healthy controls by SELDI-TOF-MS and LC-MS/MS. Latent Variable methods such as Principal Component Analysis, Partial Least Squares-Discriminant Analysis and Target Projection methods were used to describe the MS data.
Results: The serum proteomic profiles showed variable differences between the groups both throughout the incubation period and at the clinical end stage of scrapie. At the end stage, the target projection model separated the two groups with a sensitivity of 97.8%, and serum amyloid A was identified as one of the protein peaks that differed significantly between the groups.
Conclusions: At the clinical end stage of classical scrapie, ten SELDI peaks significantly discriminated the scrapie group from the healthy controls. During the non-clinical incubation period, individual SELDI peaks were differently expressed between the groups at different time points. Investigations of differences in -omic profiles can contribute to new insights into the underlying disease processes and pathways, and advance our understanding of prion diseases, but comparison and validation across laboratories is difficult and challenging.

Paper II

Presence of an acute phase response in sheep with clinical classical scrapie

Background: Work with experimental scrapie in sheep has been performed on-site for many years including studies on PrP^{Sc} dissemination and histopathology of organs and tissues both at preclinical and clinical stages. In this work serum was sampled at regular intervals from lambs which were infected immediately after birth and from parallel healthy controls and examined for acute phase proteins. In contrast to earlier experiments, which extensively studied PrP^{Sc} dissemination and histopathology in peripheral tissues and brain, this experiment is focusing on examination of serum for non-PrPSc markers that discriminates the two groups and give insight into other on-going processes detectable in serum samples.

Results: There was clear evidence of an acute phase response in sheep with clinical scrapie, both experimental and natural. All the three proteins, ceruloplasmin, haptoglobin and serum amyloid A, were increased at the clinical stage of scrapie.
Conclusion: There was evidence of a systemic measurable acute phase response at the clinical terminal end-stage of classical scrapie.

Paper III:

Expression of selected genes isolated from whole blood, liver and obex in lambs with experimental classical scrapie and healthy controls, showing a systemic innate immune response at the clinical end-stage.

Background: Incubation period, disease progression, pathology, and clinical presentation of classical scrapie in sheep are highly dependent on PRNP genotype, time and route of inoculation and prion strain. Our experimental model with pre-colostrum inoculation of homozygous VRQ lambs has shown to be an effective model with extensive PrP^{Sc} dissemination in lymphatic tissue and a short incubation period with severe clinical disease. Serum protein analysis has shown an elevation of acute phase proteins in the clinical stages of this experimental model, and here, we investigate changes in gene expression in whole blood, liver, and brain.
Results: The animals in the scrapie group showed severe signs of illness 22 weeks post inoculation necessitating euthanasia at 23 weeks post inoculation. This severe clinical presentation was accompanied by changes in expression of several genes. The following genes were differentially expressed in whole blood: TLR2, TLR4, C3, IL1B, LF and SAA, in liver tissue, the following genes differentially expressed: TNF-α, SAA, HP, CP, AAT, TTR and TF, and in the brain tissue, the following genes were differentially expressed: HP, CP, ALB and TTR.
Conclusions: We report a strong and evident transcriptional innate immune response in the terminal stage of classical scrapie in these animals. The *PRNP* genotype and time of inoculation are believed to contribute to the clinical presentation, including the extensive dissemination of PrP^{Sc} throughout the lymphatic tissue.

Methodological considerations

The methods used in this work are primarily described in papers I-III. Additional information and further considerations regarding methods are discussed in this section.

Ethical considerations

Scrapie is a serious and fatal disease of sheep, and although much knowledge and insight come from studying cell cultures and rodent disease models, the study of the disease in its natural host is both necessary and valuable. The guiding principles for ethical use of animals in research, the three Rs (Replace, Reduce and Refine) were applied to the experimental design (Russell and Burch, 1992). Replacement alternatives were not considered, as the aim of the study was to study scrapie *in vivo* in the natural host, sheep. Animal models are laborious and expensive and animal models must always be evaluated in an ethical perspective and improved whenever possible. Studying the disease in the natural host will always be advantageous compared to other laboratory animal models and cell cultures. Much effort was made to minimize the total number of animals used, and to establish strict sampling protocols and examine multiple samples from each animal. All the animals used in the experiment were closely monitored for signs of disease and discomfort. The experimental period ended at the first conclusive sign of clinical disease, and the animals were euthanized with a dose of pentobarbital administrated through the jugular vein, resulting in immediate unconsciousness.

The animal experiments were performed in compliance with ethical guidelines and approved by the Norwegian Animal Research Authority regarding the Norwegian regulation on animal experimentation (FOR-1996-01-15-23).

The three papers published were based on the same animal groups, and in addition, there were extensive clinical journals, video surveillance recordings, haematological analyses, diagnostic tests (western blot and immunohistochemistry) and histopathological examinations of brain sections. These are not specifically included in the papers but were merely work performed to confirm diagnosis and back up new findings.

Methods

The different methods used in this work are summarized in table 10, and the methods are described in the relevant paper.

Method	Paper I	Paper II	Paper III
SELDI-TOF-MS	**X**		
LC-MS/MS	**X**		
SDS-PAGE	**X**		
Latent variable (LV) projection multivariate statistical methods	**X**		
Principal component analysis (PCA)	**X**		
Partial least squares – Discriminant Analysis (PLS-DA)	**X**		
Non-parametric Discriminating Variable test (DIVA)	**X**		
Non-parametric Mann–Whitney U test	**X**		**X**
Non-parametric test, Wilcoxon signed-rank test		**X**	
T-test		**X**	
Enzyme-linked immunosorbent assay (ELISA), Phase™ Range Multispecies SAA kit		**X**	
Spectrophotometry, Phase™ Haptoglobin Assay kit		**X**	
Serum biochemistry		**X**	
RNA isolation			**X**
RNA quantification			**X**
RNA integrity estimation (RIN)			**X**
Primer design			**X**
cDNA synthesis			**X**
High-throughput qPCR (Fluidign BioMark HD)			**X**
Pre-processing and analysis of expression data (GenEx)			**X**

Table 10. Overview of the methods used in this thesis.

Animals, management, and experimental model

The animals used, inoculation material and time and route of infection were all aimed at creating a situation closest to natural transmission. The animals were recruited from the small research sheep flock, "scrapie-susceptible-flock" (SSF), which has been kept alongside the commercial sheep flock at NMBU, Section for small ruminant research at Sandnes. The SSF was established in the early 1990s, when scrapie became endemic in the south western parts of Norway. The *PRNP* genotype of all the ewes were determined in the commercial sheep flock, and the ewes with the most susceptible genotype became the initial breeding ewes of the SSF. Since then, this flock has been specifically bred for VRQ homozygosity. On average, the SSF has consisted of around 20 ewes, and the animals used in scrapie research have been recruited from this group. The SSF has been managed alongside the commercial flock, though kept in separate pens indoors and on separate pastures during the grazing season. It has been kept under disease surveillance, and routine diagnostic workups have been performed to make sure health and welfare standards were good. Despite having the most susceptible *PRNP* genotype, scrapie has never been diagnosed in animals used in control groups, nor in the SSF.

The inoculation material originates from brain homogenates from natural scrapie cases from 1995. The *PRNP* genotype of the donor animals were homozygous VRQ. The brain homogenate was divided into aliquots and stored at minus 70 degrees. It has not been

examined for strain type and there may be more than one prion strain type within the material.

The first inoculation study was performed in the late 1990s, and the experimental design has been refined since. The work described in this thesis results from inoculation of new-born lambs *per os* immediately after birth, before receiving colostrum. This "pre-colostrum model" was developed at the section to improve the experimental scrapie model to better mimic natural transmission, where infection occurs at or soon after birth. This model has shown to be very potent and efficient, resulting in a shortened incubation period and clinical disease within 26 weeks p.i.

During gestation, the ewes were housed alongside, but isolated from, the commercial flock, and subjected to the same management procedures. Approaching parturition, the ewes were continuously monitored, and as soon as lambing commenced, the ewes were moved to specially prepared research facilities, where the individual ewe was accommodated with her offspring for the length of the experiment. The new-born lambs were inoculated *per os* with homogenized brain material from either confirmed scrapie cases or healthy sheep.

Throughout the experimental period, the animals were inspected, examined, and sampled at regular intervals until clinical disease. The animals were euthanized and autopsied, and all relevant and planned samples were harvested according to procedure.

In paper II, serum samples from natural cases of scrapie, admitted to the section in the period between 1990 and 2005, were also used.

Sample selection

The main aim of this work was to study differences in blood between healthy and scrapie affected sheep from time of inoculation to the clinical end stage. Blood samples were collected at regular intervals into plain tubes, EDTA tubes and PAXgene Blood RNA tubes. All the samples were subjected to the same handling procedures throughout the experiment, and when appropriate, manufacturer's instructions were followed. Aliquots of serum and PAXgene Blood RNA tubes were stored at minus 70 degrees until further analysis. Blood samples were analysed using clinical biochemistry tests, proteomics, and genomics technologies.

Tissue samples from liver and posterior obex were sampled at standardized areas to ensure similarity in both cell types and numbers. The brain, and especially the obex, was used in different scrapie projects. It was carefully dissected, and multiple samples were collected for different diagnostic tests, histological examinations, and genomic analyses. The same autopsy protocol and sampling procedures were followed throughout the experiment. The size of the animals and high number of samples harvested carried the risk of delayed sampling and extensive tissue handling for certain tissues.

The "-omics" technologies

During the last decades, new technologies have emerged; the "-omics". These technologies are being used to study the functions, relationships, and actions of various

types of molecules found in cells, tissues, and bodily fluids. Hundreds, even thousands, of genes, transcripts, proteins, or metabolites can be analysed in a single analysis using the different -omics technology (Figure 8). These technologies provide the methods to study differences in DNA, RNA, proteins and metabolites between individuals and different groups, like healthy and diseased. Alongside this, bioinformatic tools have developed to manage and analyse the large sets of biological data retrieved, and to decipher vital information. The results contribute to the understanding of normal biological functions and mechanisms, and those underlying disease, and the definition of so-called "biomarkers". Biomarkers can be measured and evaluated as an indicator of normal biological or pathological processes. The identification of promising biomarkers is not without challenges. The ideal biomarker needs to be easy to quantify and measure, reproducible and specific, show little variation in the general population, and not be influenced by unrelated factors. It is not likely that one biomarker can fulfil these criteria, and therefore a panel of biomarkers is often presented as a tool in diagnostics, evaluation of disease progression and therapeutic effects.

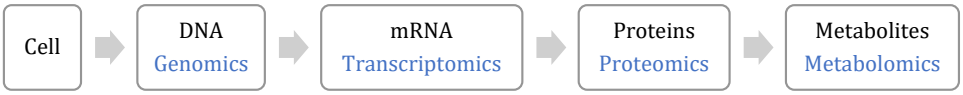

Figure 8. Schematic illustration of the different "-omics".

Genomics aim at mapping the entire DNA sequence of an organism, and since the first genetic map of a free-living organism was completed in 1995, thousands of genome sequences and genome information have been added to public databases such as Ensembl and NCBI/Genome (Fleischmann et al., 1995).

The synthesis of a protein results from the flow of genetic information through transcription of DNA to RNA and further translation of RNA to protein (Figure 9). The transcription of the coding regions (exons) of DNA into messenger RNA (mRNA) is the first stage of gene expression, and mRNA is the template for protein synthesis. A mRNA molecule is produced for each gene or group of genes that is to be expressed, and the total set of mRNAs produced by a cell or a population of cells at any point, can be defined as the transcriptome (Figure 8) (Stryer, 1995). Proteins are synthesised by translating the genetic information delivered by the mRNA into their unique amino acid sequences, which are specified by the genes. Studies have shown that transcription of DNA into mRNA not always followed by the translation into proteins, thus studies of changes in the proteome (proteomics) may be of more value (Anderson and Seilhamer, 1997). Proteins are the functional units and they play a crucial role in virtually all biological processes. The proteome represents the entire collection of proteins present in a living organism at any given time point. The proteome is therefore dynamic due to complex regulatory systems, making it more challenging to study, as compared to the static genome (Wilkins et al., 1996). Hence, fluctuations in gene expression levels can be determined by analysis of the transcriptome or proteome.

The serum proteome is extraordinarily complex and is a collection of those proteins that carry out their functions in the circulation, messenger proteins/peptides, proteins in transit and those that leak into the circulation as a result of tissue damage (Anderson

and Anderson, 2002). The few most abundant proteins, mainly albumin, constitute 95 % of the bulk mass, but they merely represent 0.1 % of the total number of proteins (Roche et al., 2006).

Gene expression (genomics and transcriptomics) in blood reflects the gene expression of blood cells. Comparison of genes expressed in blood to different tissues (including brain, liver, and spleen), revealed an overlapping of over 80 %. Circulating blood cells are in constant interaction with the entire body and changes in gene expression can reflect changes in different tissues (C.-C. Liew et al., 2006). Thus, analyses of alterations in serum gene expression can provide information on ongoing processes elsewhere in the body.

Figure 9. The flow of genetic information in normal cells.

Transcriptomics

Detection and quantification of the transcriptome can be achieved by reverse transcription of mRNA to generate complement DNA (cDNA), which then corresponds to the specifically expressed gene. Followed by reverse transcription polymerase chain reaction (RT-PCR), and real-time polymerase chain reaction (qPCR) methods to quantify the copy number of the specific cDNA. A combination of RT-PCR and high-throughput qPCR system (Fluidigm Biomark HD) was used to evaluate gene expression fluctuations in blood, liver, and brain in this present work (Paper III).

Extraction of RNA

Gene expression studies depend on high quality, purity and integrity of RNA samples, and degradation of RNA is the main problem in sample collection. Proper sample collection and handling, efficient RNA stabilisation and extraction, and good laboratory routines are essential to avoid compromising the RNA, and to facilitate accurate identification and quantification of transcripts (Fleige and Pfaffl, 2006). Retrieval of good quality RNA from brain tissue can be challenging, and total RNA quality can be influenced negatively by several factors involving tissue/sample retrieval and the following extraction process (Lipska et al., 2006; Buerlein et al., 2009). To standardise the collection and immediate processing of RNA, commercially available PAXgene Blood RNA Tubes kits and RNA*later* were used for blood and tissue samples, respectively (both by Qiagen). Total RNA was extracted using commercially available kits, PAXgene® Blood miRNA Kit for blood samples; RNeasy® Lipid Tissue Mini Kit for liver samples; and RNeasy® Lipid Tissue Midi Kit for brain samples. In addition, RNase-Free DNase Kit was used for digestion of any DNA present in the tissue samples.

Total extracted RNA was quantified by UV spectrometry at 260 nm and RNA integrity was evaluated (Agilent® 2100 Bioanalyzer with Agilent RNA 6000 Nano Kit) to ensure good quality and reliable results in the downstream analyses. Each sample was assigned

an RNA Integrity Number (RIN) ranging from 1 (worst) to 10 (best), which reflects the level of RNA degradation in the sample (Table 11). However, some claim that in brain tissue, the RIN reflects a decrease in full-length mRNA rather than integrity (Schroeder et al., 2006; Sonntag et al., 2016).

	Blood		**Liver**		**Brain**	
	ng/µl	RIN	ng/µl	RIN	ng/µl	RIN
Mean	53.8	8.7	572.3	7.9	160.4	6.7
Standard Error	2.3	0	52.2	0.1	15.5	0.1
Median	51.6	8.7	647.0	7.8	157.0	6.7
Standard Deviation	23.2	0.4	255.6	0.6	46.5	0.2
Range	130.9	2.1	963.0	2.1	144.0	0.6
Minimum	14.9	7.5	31.0	7.1	82.0	6.4
Maximum	145.8	9.6	994.0	9.2	226.0	7.0
Count	98.0	98.0	24.0	24.0	9.0	9.0

Table 11. Quantity and quality calculations of extracted RNA. Concentration (ng/µl) of total RNA and RIN numbers of all samples used in gene expression studies in Paper III (table from Additional file 1, Paper III)

The total RNA extracted from the sample reflects the cellular composition of the sample, and the cellular distribution will vary between samples from different tissues/bodily fluids, and between same type of samples but from different individuals. The transcriptome in whole blood reflects the gene expression of peripheral circulating leukocytes at time of sampling. Table 11 presents the concentration (ng/µl) and RNA integrity (RIN) of the extracted RNA from the individual samples from whole blood, liver tissue and brain tissue. The total RNA concentration was good for all sample types, although there were considerable individual differences. The concentration of total RNA was highest in liver tissues and lowest in whole blood. The mean RIN value was above the threshold for what is considered good (5) for all tissues, and mean RIN value for blood samples were above 8. These RIN values indicate good quality RNA for downstream analyses.

High-throughput qPCR

Real-time polymerase chain reaction, qPCR, is an advancement of the original PCR, and it is an excellent tool for cDNA/gDNA detection and quantification of fold differences between groups (Higuchi et al., 1992; Kubista et al., 2006; Forootan et al., 2017). The basic principles are amplifications of targeted DNA that binds to specific primers, and the reactions are driven by a thermostable DNA polymerase that synthesises the copy DNA. The PCR is performed by repeated temperature cycling where high temperature is applied to separate the double stranded DNA, followed by lower temperature, allowing primers to anneal to target area, and the cycle is finalized at temperature which is optimum for the polymerase that elongates the primers with synthesising new double stranded DNA. For each temperature cycle, the target DNA is doubled. A fluorescent reporter that binds to the double-stranded amplicon is added, and the relative quantification can be made by counting the number cycles required to reach a certain fluorescent signal threshold. The number of cycles required to reach this threshold is called the quantification cycle, Cq, and will reflect the initial amount of target DNA in the

sample. Cq for each target DNA can be compared between samples to calculate the fold change.

Expression of mRNA involves an initial step of reverse transcription (RT) of mRNA to cDNA before qPCR can be performed, and this method is termed RT-qPCR. Reverse transcription can introduce variation to the experiment, and cDNA must be produced accurately to correctly reflect the input number of mRNAs.

In this work, high-throughput qPCR was performed in 48.48 Dynamic Arrays using the BioMark thermocycler (Fluidigm Biomark HD platform). The 48.48 Dynamic Arrays enable 2304 separate and simultaneous qPCR reactions from 48 samples and 48 primers in one operation. This gives great advantages with high efficiency and reduced cost, and in addition reduced inter-run variations. One of the main disadvantages is that the annealing temperature cannot be optimized for each primer, thereby setting high demands to PCR efficiency and correlation coefficient for each individual primer. The RT-qPCR was performed using a strict protocol in accordance with manufacturer's instructions and recommended kits.

Two different primer pairs were designed to amplify different regions of each gene, and the correlation between these two was used to validate that the correct target gene was amplified. Sequences used for primer design were obtained from public database (GenBank, NCBI). BLAST search against *Ovis aries* genome was performed to confirm gene specificity for each primer sequence, and to show the absence of polymorphisms at the primer site.

Two separate technical replicates of cDNA were synthesised from each RNA sample using the QuantiTect reverse-transcription kit. Contamination of genomic DNA was eliminated by including a DNase step to both extraction of RNA and cDNA synthesis, and additionally, a non-reverse transcriptase control sample was included on each dynamic array.

The expression level of each gene was normalized against several reference genes (*RPLP0*, *PPIA*, *UBC*, *HPRT1*, *GAPDH* and *SDHA*) which were evaluated using GeNorm and NormFinder incorporated in the GenEx software.

RT-qPCR data was collected using the Fluidigm Real-Time PCR Analysis Software and expression data was pre-processed and analysed using GenEx Pro software (Paper III).

Proteomics

The study of the proteome can be challenging and time consuming due to the vast number of proteins present at any given time, but several techniques are now available for the separation, detection and identification of hundreds to thousands of proteins in a single experiment. Each of the available techniques has advantages and disadvantages, but reproducibility seems to be the biggest challenge across several different methods (Aslam et al., 2017). Based on different proteomic techniques, there are two approaches to reveal protein differences between groups, these are: identification-based biomarker discovery and pattern-based diagnostic approach (Veenstra et al., 2005). Differentially expressed proteins can be identified and thus provide new and important information that can increase our knowledge and understanding of underlying biological or

pathological processes (Aebersold and Mann, 2016). Examples of both approaches are presented in Paper I.

Pattern-based diagnostic approach

The surface enhanced laser desorption/ionization (SELDI)-time of flight (TOF)- mass spectrometry (MS) ProteinChip Array technology combines two powerful techniques: chromatography and mass spectrometry. SELDI-TOF-MS utilizes a selective protein extraction and retention technology on the surface of the different chromatographic chips, followed by direct analysis in a desorption/ionization mass spectrometer (Issaq et al., 2002). The method is fast, diverse, and versatile. Using different chromatographic surfaces and fractionation methods will give rise to multiple protein expression profiles, and visualization of the different subsets of the whole proteome within one sample. The ProteinChip Arrays can have various chemical or biological capture surfaces, each to capture a specific subset of proteins. Unbound proteins are washed away. A matrix/energy absorbing molecule is added to each of the eight spots on the ProteinChip, which results in a co-crystallized mixture of sample and matrix. The ProteinChip is then placed in the MS under high vacuum and irradiated with a nitrogen laser beam. The matrix absorbs the energy from the laser and the mixture is rapidly expanded into the gas phase. Ionised proteins are accelerated through an electric field down a flight tube to an ion detector (TOF tube). The mass-to-charge ratio (m/z) is determined by the software and is based on the flight time. Each ionised protein will be detected as a peak in the mass spectrum with its unique m/z value (SELDI peaks). Most ionised proteins generated are singly charged, thus each SELDI peak corresponds to a single protein/peptide with the m/z value equal to molecular weight (MW) (Poon, 2007).

To ensure sufficient data acquisition from the entire mass range, each ProteinChip is analysed with a spot protocol optimized for the low mass area (2-25,000 Da), which is the optimal MW region, and one protocol for the high mass area (> 20,000 Da). Before statistical analyses are performed, the acquired data are processed to reduce instrumental and handling artefacts, minimize variation within groups and maximizing variation between groups and improve peak detection (Bio-Rad, 2008). Despite good experimental design, laboratory protocols, sample handling and data processing, the technique is very sensitive to experimental variation and instrument settings, and, consequently, reproducibility is a major concern (Diamandis, 2004; Poon, 2007; Simpson et al., 2009). The second major disadvantage with this technology is that it is impossible to directly identify the protein peaks. Identification of SELDI peaks by alternative approaches is also challenging and time consuming (Diamandis, 2004; Ndao et al., 2010). Finally, analysis and interpretation of the complex dataset generated is challenging and require sophisticated multivariate statistical methods.

Data analysis to reveal the Discriminative Biomarker Pattern

Good bioinformatic tools are needed to identify promising biomarkers, either as a single protein marker, or as part of proteomic pattern profile. Various bioinformatic tools and statistical methods have been used to visualise the valuable information hidden in large proteomic data sets. Nevertheless, univariate statistical methods are widely used to consider one SELDI-peak at the time, to identify the most discriminate and significant SELDI-peaks between groups (Bio-Rad, 2008). When univariate methods are used, correlation between proteins within a biological sample will not be considered,

therefore various multivariate approaches, both supervised and unsupervised, have been suggested. In this work, supervised latent variable projection methods (LV) were used, and a binary response variable consisting of zeros (0) and ones (1) was added to assign group membership for each sample (Rajalahti et al., 2010). Furthermore, Partial Least Square – Discriminant Analysis (PLS-DA) was used to extract the variation in the data that separated the two predefined groups. The large number of PLS components required to describe the variation was then simplified and projected onto a single Target-Projected (TP) component, which represents the best discriminative axis in the PLS-DA modelled and cross validated spectral space (Kvalheim and Karstang, 1989; Kvalheim, 1990). The Selectivity Ratio (SR) value, indicating the ratio between explained and unexplained variation for each of the variables (SELDI-peaks) on the TP component, was estimated and hence the SELDI-peaks could be ranked according to their discriminatory ability (Tarja Rajalahti et al., 2009). This discriminatory ability of the SELDI-peaks (SR value) was plotting against the Mean Wilcoxon Rank Sum Rate to create the Discriminating Variable (DIVA) plot to visualize and quantify the probability of correct classification for each SELDI-peak (Figure 5, Paper I). The threshold for mean correct classification was set at 80 %, and the corresponding SR value could be read in the DIVA plot. Individual SELDI-peaks having an SR value above this threshold could be identified as a panel of proteins with good discriminating power and promising biomarkers (Figure 6, Paper I) (T. Rajalahti et al., 2009).

Identification-based biomarker discovery

The identification-biomarker discovery pathway involves separation of complex mixtures of proteins by gel electrophoresis or chromatography, before resolved proteins are identified by tandem mass spectrometry (MS/MS) (Veenstra et al., 2005). This approach is known as shotgun proteomics, which also refers to the use of so-called "bottom-up" proteomics approach. Complex protein mixtures are fractionated and digested by proteases into even more complex peptide mixtures, which are further separated into one or more dimensions by liquid chromatography (LC) before the peptides are subjected to MS/MS for analysis and characterization (Zhang et al., 2013). The resulting mass spectra of the peptides are matched to corresponding amino acid sequences in public protein databases, using specific software such as SEQUEST (Eng et al., 1994). The results depend on how efficient and complete the digestion of proteins were to reach a high amino acid sequence cover, and that the species-specific protein sequence is registered in the database.

In this work, LC-MS/MS shotgun approach was used to identify SELDI-peaks which were found to have good discriminating power, and only to be expressed in the scrapie samples. The same protein mixture as used in the SELDI-TOF-MS analysis was separated by gel-electrophoresis, and the resulting protein bands were stained for better visualisation and excision. The bands in the same MW region as the target SELDI-peak were excised and subjected to tryptic digestion before LC-MS/MS analysis. The resulting peptide sequences were searched with SEQUEST against even toed ungulate database available at NCBI, and SAA was identified by eight peptides (Figure 9, Paper I).

Clinical chemistry

Studies of serum or plasma proteins in relation to disease diagnostics have been performed for many decades, and several hundred different proteins have been identified by a variety of analytical methods (Anderson and Anderson, 2002). Routine

clinical chemistry tests to evaluate serum albumin-globulin rations, and measurements of serum APPs can be of great diagnostic value in various infectious and inflammatory conditions (Hurwitz and Whipple, 1917; Tothova et al., 2014).

Different analytical methods were used in Paper II to evaluate different serum proteins. The in-house clinical chemistry analysers, ABX Pentra and COBAS MIRA were used to measure serum total protein (TP), albumin (Alb) and ceruloplasmin (Cp). Serum haptoglobin (Hp) concentration was determined by the use of the Phase Haptoglobin Assay kit, and absorbance was read at 600 nm on a Multiskan GO Microplate Spectrophotometer using SkanIt Software. Serum amyloid A (SAA) concentration was determined by the use of the Phase Range Multispecies SAA ELISA kit, and absorbance was read at 450 nm on the Multiskan GO Microplate Spectrophotometer using SkanIt software.

The non-parametric Wilcoxon signed-rank test was used to calculate confidence interval and p-values for each of the protein tested at each sampling time. Significance level was set at $p < 0.05$.

Assessment of methods chosen and time of sampling

The methods used in this work were aimed at detecting discriminating biomarkers other than PrP^{Sc} in blood and tissues from the two experimental groups, healthy and scrapie infected. Study design, animal model and management, were all attempting to minimize differences between the groups that were not related to scrapie. Blood and serum samples were collected at two weeks intervals from birth until euthanasia to investigate changes in blood and serum during the incubation period and clinical disease. A thorough autopsy was performed on each of the animal included in the experiment, and tissue samples of liver and brain were collected for both histological examinations and genomic analyses.

Discussion

Scrapie is a fatal neurodegenerative disease naturally affecting sheep and goats. Although the prion protein and the disease have been extensively been studied for decades, there are still many unanswered questions. The function of PrP^{C} and the transition to PrP^{Sc}, and how this relates to histopathological findings and diversity of clinical presentation, still remains unclear. Nevertheless, the characteristic histopathological changes associated with classical scrapie are vacuolation of grey matter of the CNS, neurodegeneration and gliosis, and the presence of PrP^{Sc} is diagnostic (Jeffrey and González, 2007). Clinically, classical scrapie is known as a chronic, progressive, and invariably fatal neurodegenerative disease with typical onset of clinical disease at an average age of 3.5 years. The first clinical signs are subtle behavioural changes which progress into neurological signs characterised by ataxia and pruritus (Aitken, 2007). However, the disease phenotype is much more complex and strongly influenced by *PRNP* genotype, prion strain, and time and dose of infection (Gonzalez et al., 2012; Bartz, 2016). Morphological changes and PrP^{Sc} accumulation within the CNS do not necessarily correlate to the clinical deficits observed (Jeffrey and González, 2007).

The prion protein is one of the most extensively studied proteins and many different functions have been proposed without reaching conclusive results, in fact it has been proposed that PrP^{C} is not crucial for resting health in higher organisms (Watts et al., 2018; Benestad et al., 2012). Still, expression of PrP^{C} is absolutely necessary and essential for developing classical scrapie (Brandner et al., 1996). The actual pathophysiological pathways are not fully understood. Disease is associated with the conformational changes of PrP^{C} into PrP^{Sc}, and the following accumulation of PrP^{Sc}, but whether it is loss of function of PrP^{C}, gain of function of PrP^{Sc} or other involving factors are still debated (Aguzzi and Falsig, 2012).

The study of the relationship between the immune system and prion diseases is gaining interest as it has become clear that the innate immune system plays an important role throughout the development of classical scrapie (Mabbott, 2004; Bradford and Mabbott, 2012; Mabbott et al., 2018). PrP^{C} is expressed on immune and nervous cells, and the immune and nervous systems are thus central in the pathogenesis and dissemination of PrP^{Sc}. From an evolutionary point, absence of an adaptive and specific immune response to PrP^{Sc} would be beneficial to the host, and in murine studies, B cells have shown to be strongly tolerant to endogenous PrP^{C} (Grégoire et al., 2005). Any specific antibody response to PrP^{Sc} would probably also target PrP^{C}, as the linear epitopes of the two have the same amino acid sequence and would not be distinguishable. PrP^{Sc} and PrP^{C} within the same host have the exact same amino acid sequence, determined by *PRNP* genotype. Any specific anti-prion protein antibody production would harm two important body systems, the nervous and the immune, which would be detrimental to the host and undoubtedly contribute to the clinical disease, progression, and outcome.

The nervous and immune systems are connected, and they interact and communicate through direct contact via innervation of lymphoid tissues by ENS, ANS, wAPCs and wICs, and indirectly via cytokines, chemokines and other mediators in the blood flow (Sankowski et al., 2015; Wülfing and Günther, 2015). Cells which express PrP^{C} and are associated with uptake and dissemination of PrP^{Sc} are the same cell-types that communicate with the nervous system, such as cells of LRS, macrophages, M-cells and DCs (McBride et al., 2001; Weissmann et al., 2001; Ragna Heggebø et al., 2003; Michel et al., 2012; Matteoli and Boeckxstaens, 2013; Hu et al., 2019). Thus there is a direct passage between peripheral PrP^{Sc} and the CNS for neuroinvasion involving the two systems, and prions crossing the blood-brain-barrier play a less important role in disease progression (Bradford and Mabbott, 2012; Keller et al., 2018). Classical scrapie is a disease affecting the CNS, and even though the initial uptake and dissemination occurs peripherally, clinical disease first becomes evident when PrP^{Sc} accumulates in the brain. Gene expression studies have shown that an anti-inflammatory response dominates in peripheral lymphoid tissues during the asymptomatic early stages (Gossner and Hopkins, 2015). The results from the longitudinal study on changes in proteome-fraction in serum described in paper I, show significant under-expression of several protein peaks during the preclinical stages until 20 wpi (Table 2 in Paper I). The SELDI-TOF-MS method is a pattern-based approach, and the pattern observed in this study showed decreased protein expression in the scrapie group compared to the healthy controls. Although these results must be interpreted with some caution due the technical limitations of the methods used, the overall finding of a dampened state in the

scrapie group compared to healthy controls, corresponds well to results from gene expression studies in peripheral lymphoid tissue (Gossner and Hopkins, 2015). Unfortunately, the identification of these protein peaks cannot be determined by molecular weight only, and further analyses by different methods would have been necessary. Paper III presents the results of a gene expression study of the same sera as in paper I. Transcriptomic investigations showed a reduced expression in the scrapie group as compared to healthy controls at 16 wpi of the *TLR4* encoding PRR and the *IL1B* encoding the pro-inflammatory cytokine (Table 1, Paper III), also pointing to a repressed immune response during the pre-clinical stages. Paper II describes the analyses of APPs in serum samples of natural cases of classical scrapie in addition to analyses of the experimental group. The definition of APPs is that they change plasma concentration by at least 25 % during inflammation. None of the APPs analysed were significantly different between the two groups during the pre-clinical stages.

The results from extensive serum analyses throughout the asymptomatic incubation period did not indicate any pro-inflammatory nor active immune response. There are, however, indications of a dampened or anti-inflammatory response in serum which could reflect the systemic response to the replication, dissemination, accumulation and neuroinvasion of PrP^{Sc}. The presence of PrP^{Sc} in SLOs is apparently tolerated very well by the immune system, and it is not recognized as a DAMP/PAMP. It is unknown whether this apparently dampened response is due to strong tolerance to epitopes on $PrP^{C/Sc}$, impairment of immune cells by the accumulation of PrP^{Sc} becoming unable to carry out their full range of functions, or other factors. Affected sheep can apparently mount an immune response to other infections like mastitis and nephritis during the incubation period, and such infections can lead to accumulation of PrP^{Sc} in tissues not normally associated with PrP^{Sc} , and increased excretion of PrP^{Sc} through milk and urine (Ligios et al., 2005; Seeger, 2005).

Following neuroinvasion, PrP^{Sc} accumulates in various regions of the brain, and this accumulation is, not by itself, enough to cause neurodegeneration. Neurodegeneration is detectable when PrP^{Sc} is accompanied by an innate immune response, and this plays an important role in the onset and progression of clinical disease (Alibhai et al., 2016; Stephenson et al., 2018). Gene expression studies of tissue sections from posterior obex at time of euthanasia showed alterations in genes encoding four APPs (Table 3, paper III). Expression of the genes *Hp* and *Cp*, encoding positive APPs, were upregulated with 7.91- and 1.67-fold change, respectively. The genes *Alb* and *TTR* encoding negative APPs were downregulated with 1.64- and 1.61- fold change, respectively. This indicates the presence of an innate immune response with alterations in APPs locally in the obex at the clinical end-stage.

Many different cell types within the CNS contribute to the innate immune response, but the microglia are the principal resident innate immune cells and have diverse functions. Activated microglia are classed as either M1 or M2, depending on which chemokines and cytokines (pro-inflammatory or anti-inflammatory) they express, and their function can thus be either beneficial or detrimental (Stephenson et al., 2018) Protein aggregations in the CNS, as seen in prion diseases, have been associated with chronic activation of an innate immune response with microglia activation and increased

expression of pro-inflammatory cytokines and complement activation (Bradford and Mabbott, 2012; Amor et al., 2014).

Gene expression studies in liver tissue collected at the clinical end-stage also showed an alteration in expression of genes encoding different APPs (Table 2, paper III). There was increased expression of *SAA* (89.98 ± 46.32 fold change), *Hp* (542.96 ± 216.93 fold change) and *Cp* (1.98 ± 0.46 fold change) which encode important positive APPs in sheep. In addition, three negative APPs showed decreased expression, *TF* (-1.68 ± 0.1 fold change), *AAT* (-2.37 ± 0.05 fold change) and *TTR* (-2.03 ± 0.08 fold change). The liver is the main site for APP synthesis and expression, and this corresponds to the higher increase in expression of genes encoding the two main positive APPs in sheep, SAA and Hp, compared to brain tissue and serum.

Genomic, proteomic and biochemistry methods were used to analyse serum samples at both preclinical and clinical stages of the disease. Clinical signs were obvious first at 22 wpi, and this corresponds well to alterations in APP concentrations in blood from 22 wpi and onward, at both genetic expression (*SAA* and *LF*) and protein levels (Globulin, SAA, Hp and Cp) (Table 1, paper III and Figures 1-4, paper II). Proteomic analyses of serum samples showed a change from mainly downregulation of protein peaks to upregulation from 20 wpi (Table 2, paper I). The shift from downregulation to upregulation of proteins in serum was detected slightly earlier by the SELDI-TOF-MS, making this method more sensitive. The lack of identification of protein peaks makes interpretation of these findings difficult.

Serum from five natural scrapie cases were included and analysed for Tp, Alb, Glb, Hp and SAA, and results were compared to the experimental cases (Table 2, paper II). The clinical presentation of these cases varied, ranging from obvious and typical signs of classical scrapie (pruritus and nervous signs) to asymptomatic. PrP^{Sc} was detected in the brains, and vacuolisation with astrocytosis was recognised by histopathological examinations (Table 1, paper II). Interestingly, one asymptomatic case and one case which only displayed signs of pruritus, did not show increased concentration of SAA or Hp in serum. The three remaining sheep with nervous signs in addition to pruritus had increased serum levels of SAA and/or Hp. Even though the number of natural cases presented here is low and information is scarce, one can, nevertheless, think that severity and type of clinical signs can be reflected by the magnitude of the ongoing APR and the underlying neurodegeneration/neuroinflammation.

The immune and nervous systems have co-evolved and this have resulted in an intricate bi-directional dialog, such that any activation of an innate immune response anywhere will result in expression of pro-inflammatory cytokines which will have systemic influence causing sickness behaviour initiated in the brain and initiate synthesis of APPs, hepatically and extra-hepatically (Gruys et al., 2005; Dantzer, 2009).

Cytokines and chemokines are modulators and can act as messengers between periphery and the CNS to coordinate the local and systemic inflammatory response, and act on the brain to cause sickness behaviour (Dantzer et al., 2008). Serum is easy to access, and as blood cells can express much of the same genes as different tissues,

proteomic and genomic studies of serum can reflect changes in the CNS and other tissues (C. C. Liew et al., 2006).

Changes in microglia in prion diseases have been discussed since the early 1990s, and there have been evidence of activation of microglia preceding neuronal death and clinical symptoms, and association with PrP^{Sc} plaques (Guiroy et al., 1994; Williams et al., 1994a; Betmouni et al., 1996; Giese et al., 1998; Brown, 2001). Activation of microglia contributes to the increased expression of pro-inflammatory cytokines like IL-1, IL-6 and TNF-α detected in brain in the presence of PrP^{Sc}, however not uniformly throughout all regions of the brain (Williams et al., 1994b; Kim et al., 1999; Alibhai et al., 2016). Other gene expression studies of CNS have also revealed upregulation of genes associated with inflammation, immune response, autophagy and cell death in association to PrP^{Sc} accumulation and prion disease pathology (Booth, 2004; Skinner et al., 2006; Hwang et al., 2009).

Differential activation of the innate immune response to protein misfolding has been observed, and at least two distinct microglia responses occur during disease. One type where maintenance of homeostasis is attempted, and one where there is an up-regulation of an innate immune response with cytokines, activation of complement and antigen presenting/processing, and neurodegeneration being associated with the latter (Alibhai et al., 2016). In addition to increased expression of genes encoding APPs, expression of *TLR2*, *TLR4* and *C3* were up-regulated in serum from 22 wpi (Table 1, paper III). *IL1B*, gene encoding the pro-inflammatory cytokine IL-1B, was significantly up-regulated at 22 wpi, but down-regulated at 23 wpi, although not significantly (Table 1, paper III). These findings agree with the innate immune response detected in CNS, which is associated with neurodegeneration: activation of complement by C3 and increased antigen presenting/processing by the PRRs, TLR2 and TLR4 (Hwang et al., 2009; Okun et al., 2009; Crespo et al., 2012; Vincenti et al., 2015; Alibhai et al., 2016; Carroll and Chesebro, 2019).

The periphery and the brain do not work separately or independently of each other, they communicate fast through neuroimmune connections. Alterations in serum transcriptome reflect changes in gene expressions in circulating blood cells and these changes may reflect gene expression alterations in affected tissue. Examination has revealed that over 80 % of genes expressed in any tissue, circulating blood cells were also able to express (C.-C. Liew et al., 2006).

Microglia are involved in maintenance of homeostasis in the CNS, including the regulation of innate immune responses. Pro-inflammatory activation of microglia leads to neuroinflammation which will be harmful to neuronal tissue, while anti-inflammatory activation can be beneficial for the recovery after neuronal injury. Activated microglia take on two different phenotypes, pro-inflammatory M1 and anti-inflammatory M2 (Xu et al., 2020).

The severity and time of molecular changes, pathogenesis and clinical signs in classical scrapie depend on *PRNP* genotype, time and dose of infection, and strain. The experimental design behind this work resulted in a worst-case phenotype of classical scrapie. The animals were of the most susceptible *PRNP* genotype, homozygous VRQ,

and they were inoculated with a high dose of natural scrapie material at birth before receiving colostrum. This experimental design facilitated efficient uptake from intestine in the young animal at time when gut cells, including M-cells and DCs, were most numerous and active. The *PRNP* genotype allowed for extensive proliferation, dissemination, and accumulation throughout SLO, which in turn facilitated early neuroinvasion via ANS.

The influence of host and prion factors on control in a population

Even before the discovery of the *PRNP* genotype's influence on susceptibility of classical scrapie, there were strong beliefs of a familial or even genetic disposition, as certain breeds and lines of sheep seemed overrepresented (Parry and Oppenheimer, 1983). This, and the strong persistence of infectivity in environmental contamination, make the epidemiology and control of classical scrapie complicated and challenging. Classical scrapie status and level of environmental contamination are greatly influenced by the properties of PrP^{Sc} and the prevalence of the different *PRNP* genotypes within the sheep population (Gonzalez et al., 2012; González et al., 2010). The VRQ allele contributes greatly to increased susceptibility, reduced incubation period and survival time, increased PrP^{Sc} accumulation within LRS and environmental contamination, and severity of clinical presentation (Bossers et al., 1996; Andréoletti et al., 2000, 2002; Tranulis, 2002; van Keulen et al., 2002; Jeffrey and González, 2007; Terry et al., 2011; Gough et al., 2012). Hence, the genetic composition, together with managemental procedures within a population, will influence time, size, and type of outbreak and disease phenotype observed (Sabatier et al., 2004).

The vast variation in disease phenotype, and that definitive diagnosis is only possible *post mortem*, makes it difficult to estimate true prevalence of classical scrapie. Control of the disease, by means of absence of clinical disease, is to a great extent possible by breeding for resistance, while eradication of disease including the causal agent might prove difficult due to persistence of infectivity and challenges around decontamination of farm environment (Brown and Gajdusek, 1991; Georgsson et al., 2006; Hawkins et al., 2015; Gough et al., 2019). There is a great deal of uncertainty how long infectivity persists, and control regulations in Iceland assume environmental infectivity to last for twenty years (mast, 2001).

It is widely accepted that high frequency of ARR allele in a population will make them more resistant to clinical diseases, while high frequency of VRQ allele will make them more susceptible (Ortiz-Pelaez and Bianchini, 2011). After introduction of infectivity into a sheep population, the *PRNP* genotype profile will have the highest influence on first appearance of clinical disease, rate of new infections and level of environmental contamination (Prusiner et al., 1981, 1982; Ligios et al., 2007; Safar et al., 2008; Bessen et al., 2010; Jeffrey et al., 2014; Lacroux et al., 2017). High frequency of ARR allele will delay first appearance of clinical disease, slow down rate of new infections and minimise environmental contamination, regardless of managemental practices (Tuo et al., 2002; McIntyre et al., 2008; Ortiz-Pelaez and Bianchini, 2011; Hagenaars and Windig, 2015; Garza et al., 2017).

Epidemiological data show that distribution of NSP groups will have a marked impact on number of classical scrapie cases, as over 90 % of the cases registered within EU belonged to susceptible NSP groups (European Food Safety Authority (EFSA), 2018). Due to this strong resistance of the homozygote ARR genotype to classical scrapie, the EFSA Panel on Biological Hazards compares the similarities regarding resistance between proportion of homozygote ARR and proportion immunized by vaccination in the population (EFSA Panel on Biological Hazards (BIOHAZ), 2014). The required minimum ARR allele frequency in a population to achieve a fading-out effect on classical scrapie depends on a number of factors, and so far the model has estimated that the minimum ARR allele frequency is in the range of 50 – 100 % (EFSA Panel on Biological Hazards (BIOHAZ), 2014). Data from those EU MSs that have implemented the breeding for resistance programme, show that both heterozygous and homozygous ARR frequency has increased. In The Netherlands (2005 – 2013), the ARR allele frequency increased from 38 % to 55 %, and ARR/ARR frequency increased from 17 % to 52 % (EFSA Panel on Biological Hazards (BIOHAZ), 2014). Data on distribution of *PRNP* gene alleles in Norway showed great variability between breeds, and for the NKS breed the ARR allele frequency was around 40 % and the ARR/ARR frequency was 19 % (Table 2-3) (Hopp and Valheim, 2005; Blichfeldt, 2019).

The required minimum ARR allele frequency required to achieve a fading-out effect in classical scrapie in Norway is not known, but our current frequency of ARR allele is similar to the level different countries experienced when the breeding programme was initiated. A study from The Netherlands shows that control and so-called "herd immunity" at national level can allow a low number of susceptible animals in the population, and this is important to genetic polymorphisms in some breeds (Nodelijk et al., 2011).

There have been some concerns about breeding for resistance could lead to a negative impact on economically important traits, reproduction, and health, but several studies have concluded that the presence of ARR allele was not associated with poorer performance (Moum et al., 2005; Man et al., 2007; Sawalha et al., 2007; Lipsky et al., 2008; Sweeney and Hanrahan, 2008; Sawalha et al., 2010). Still, some breeds may have a low frequency of ARR alleles, such as the short-tailed breeds (Table 3), and a stringent breeding for resistance programme would exclude rams with high genetic potential and increase the risk of inbreeding and loss of valuable genetic variation in future generations. This requires awareness and measures must be taken to counterbalance this (Townsend et al., 2005; Dawson et al., 2008).

Breeding from ARR/ARR rams makes a great difference on flock susceptibility and infection pressure and will over time shift the prevalence of NSP groups. It is estimated that on average replacement rates in commercial NKS sheep flocks range between 20 and 25 %, and if emphasis is made on using a ram belonging to NSP1 group, the resistance level would increase quickly. Unfortunately, *PRNP* genotype surveillance of the Norwegian sheep population shows that between 11 and 19 % of the sheep belong to NSP1 group (Table 4), and if breeding for resistance was implemented, sires with high genetic merit would potentially be disfavoured, and there would be loss of important genetic polymorphisms. Nevertheless, the EFSA Panel on Biological Hazards

recommends comprehensive use of ARR/ARR rams over three or four generations to generate a major shift in the genetic structure of the sheep population (EFSA Panel on Biological Hazards (BIOHAZ), 2014). If resistance was at adequate level in the population, new cases would be fewer and far between, and the disease would die out as the susceptible population was eliminated

Compared to EU MSs, the frequency of NSP5 in the Norwegian sheep population is relatively high (range 8 – 12.3 %) against 2.1 % in the EU MSs (Table 4). Investigations into the pedigree of a few of the most popular AI-rams revealed that many of them carried one VRQ allele (Tables 5-6). The national breeding programme, and the widespread use of AI-rams with at least one VRQ allele in several consecutive years, will create a shift in distribution of NSP groups towards a generally more susceptible population, and there should be awareness of such a fast shift in certain breeding flocks.

Controlling all TSEs in animals, including classical scrapie in sheep, has high importance within the EU, and the overall aim of the control programme is to prevent, control and eradicate TSEs in animals, thereby eliminating the risk of prions entering the food chain (Anon, 2001). A dilemma in establishing a control programme for classical scrapie is that the more resistant the population gets to classical scrapie, the harder it becomes to prove true freedom of disease. The aim of the control programme should state whether the aim is freedom of clinical scrapie and/or freedom of infectivity. The latter is challenging, as the presence of the agent in the environment is impossible to monitor. It is known to remain infective for many years, and in Iceland an area is defined as non-infected when scrapie has not been detected in the previous 20 years (Brown and Gajdusek, 1991; mast, 2001; Georgsson et al., 2006).

It is important to remember that *PRNP* genotype surveillance will monitor the level of susceptibility within the population and will not reveal prevalence of classical scrapie. Therefore, prevalence is estimated from identified classical scrapie cases. Identification and confirmation of classical scrapie in sheep can only be achieved *post mortem* due to the difficulties in making a diagnosis based on clinical presentation and the lack of a conclusive diagnostic test in the live animal. Estimation of prevalence in fallen stock, and in sheep population slaughtered for human consumption, is based on the surveillance programme, involving a representative selection of the animals over 18 months of age to be tested (Sviland et al., 2018). In 2018, 17 802 samples from sheep of unknown *PRNP* genotype were examined, and Nor98 was confirmed in eight cases, whereafter *PRNP* genotype was determined (Sviland et al., 2019). Confidence level in detecting scrapie through surveillance will require knowledge of *PRNP* genotype of the animals tested. A negative diagnostic test from a genetically susceptible sheep carries a higher level of confidence that scrapie is absent than a negative test result from a genetically resistant sheep. Infection is readily established in genetically susceptible sheep, and there is an extensive dissemination of PrP^{Sc} throughout lymphatic and nervous tissue, making this *PRNP* genotype most likely to show presence of PrP^{Sc} in the brain at time of sampling. Infection is rarely established in genetically resistant sheep, and experimental inoculation of ARR/ARR sheep has resulted in a very long incubation period (237 – 3011 days p.i.) and scarce deposition of PrP^{Sc} in brain in only those animals which developed symptoms (Lacroux et al., 2017). Age at sampling could also influence the sensitivity of

the control programme. Genetically very susceptible sheep (VRQ/VRQ) could succumb to classical scrapie at less than 6 months of age, as described in papers I-III, and the incubation period in sheep with one or two ARR alleles will in most cases exceed the commercial lifespan. Surveillance would gain higher confidence on detecting classical cases if the majority of tested animals were genetically susceptible, and at a broader age range.

Conclusion

Research on prion diseases has revealed many aspects, but this unique group of diseases still carry many secrets. We now have some knowledge about the complex interaction between strain factors and host factors, which results in varied and complex disease phenotypes. Confirmation of diagnosis *ante mortem* is one of the greatest challenges, and the diagnosis is only confirmed *post mortem* by the detection of PrP^{Sc}. Prevalence of classical scrapie is estimated through active surveillance, where a representative selection of sheep over 18 months of age are tested for presence of PrP^{Sc} in brain tissue.

The experimental setup described in this work created a "worst-case scenario" phenotype of classical scrapie in sheep. Homozygous VRQ genotype combined with pre-colostrum oral intake of a high infective dose resulted in onset of severe clinical disease at 22 wpi which progressed suddenly, and necessitated euthanasia at 23 wpi. The susceptible genotype and time of inoculation enabled the fast and effective uptake from gut, facilitating dissemination to SLOs and early neuroinvasion. The preclinical stage was accompanied by a downregulation of a serum proteomic profile, while the short clinical stage was accompanied by an innate immune response, an APR, and severe clinical signs. This systemic innate immune response was initiated within the CNS as a result of a shift from anti-inflammatory to pro-inflammatory status, with increased expression of pro-inflammatory cytokines, TLRs, activation of complement and resulting in increased synthesis of APPs, detectable in serum, and liver and brain tissues.

It is a probable hypothesis that this worst-case scenario of classical scrapie can occur naturally, and the *PRNP* genotype and time of inoculation are of the essence. If an infected dam of heterozygous VRQ genotype gives birth to homozygous VRQ offspring, there is a good chance that the offspring would be infected during or immediately after birth. Most lambs are finished by 160 days of age, and the infected lambs could either succumb to classical scrapie before that or be slaughtered for human consumptions without being tested for scrapie. The clinical presentation of these young animals is relatively unspecific and indicative of a systemic collapse and sickness behaviour associated with the APR, and they would most probably be misread and not diagnosed as classical scrapie.

Consequently, keeping the VRQ allele in the sheep population will lead to diagnostic challenges and a possible underdiagnosing and underestimating of the prevalence, as these cases will not be detected by the active surveillance programme where only sheep older than 18 months are tested. Infected sheep carrying one or two VRQ alleles may contribute to environmental contaminating during both preclinical and clinical stages and, because of their preclinical peripheral dissemination of PrP^{Sc} enabling prions to enter the human food chain.

Control of classical scrapie in Norway and EU is currently based on active surveillance, the number of clinical cases has been reduced, and the majority of infected animals are now detected in fallen stock or animals slaughtered for human consumption. The last reported case of classical scrapie in Norway was detected in fallen stock in 2009, and this was a 7-year old Texel sheep with ARH/VRQ genotype. Most cases detected within the EU have at least one VRQ allele.

Elimination of the VRQ allele in the sheep population would probably exclude several animals of high genetic merit, and there is currently no evidence that there will be negative consequences on desirable commercial/economic traits. Breeding towards a more genetically resistant population would create higher level of flock "immunity", reduce the risk of major outbreaks of classical scrapie to reappear, and reach freedom of disease. Reaching a relatively high prevalence of VRQ alleles in the population will have epidemiological implications, making the population vulnerable to classical scrapie, and an outbreak would be devastating for the sheep industry. In face of an outbreak, changing *PRNP* genotype will be impossible and there will be high cull numbers.

Future perspective

Even with small sample groups, this work has provided new and interesting insight in the pathogenesis of classical scrapie. The communication between the nervous and immune systems should be further explored in regard to neuroinvasion and how the immune system reacts within the CNS.

The innate immune system can initiate powerful responses, which can be both beneficial and detrimental to tissues, organs, and physiological functions. The innate immune response seen in the clinical stages of the classical scrapie can be compared to similar responses reported in other neurodegenerative diseases, and this area should be further explored to make connections to the clinical presentation and progression. Other genotypes should be explored to discover the intensity and onset of a pro-inflammatory innate immune response in relation to *PRNP* genotype.

Because the distribution of *PRNP* genotype has such a pronounced effect on classical scrapie disease phenotype, on confidence in diagnostic test results and prevalence in a population, additional work should be performed to further verify the importance of knowing *PRNP* genotype, as well as further explore the implications on protocol behind active surveillance.

The use of AI rams results in hundreds to thousands of offspring from a single ram. Thus, genetic material may spread efficiently throughout the national population. Further investigations are needed to see how this AI system may influence the distribution of *PRNP* genotypes in Norway. How AI can be used to produce a shift in genetic susceptibility to genetic resistance should be explored, as well as how distribution of different *PRNP* genotypes can influence flock immunity.

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http://www.biomedcentral.com/1756-0500/6/466

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RESEARCH ARTICLE Open Access

Investigation of serum protein profiles in scrapie infected sheep by means of SELDI-TOF-MS and multivariate data analysis

Siv Meling[1*], Olav M Kvalheim[2,3], Reidar Arneberg[4], Kjetil Bårdsen[1], Anne Hjelle[5] and Martha J Ulvund[1]

Abstract

Background: Classical scrapie in sheep is a fatal neurodegenerative disease associated with the conversion PrP^{C} to PrP^{Sc}. Much is known about genetic susceptibility, uptake and dissemination of PrP^{Sc} in the body, but many aspects of prion diseases are still unknown. Different proteomic techniques have been used during the last decade to investigate differences in protein profiles between affected animals and healthy controls. We have investigated the protein profiles in serum of sheep with scrapie and healthy controls by SELDI-TOF-MS and LC-MS/MS. Latent Variable methods such as Principal Component Analysis, Partial Least Squares-Discriminant Analysis and Target Projection methods were used to describe the MS data.

Results: The serum proteomic profiles showed variable differences between the groups both throughout the incubation period and at the clinical end stage of scrapie. At the end stage, the target projection model separated the two groups with a sensitivity of 97.8%, and serum amyloid A was identified as one of the protein peaks that differed significantly between the groups.

Conclusions: At the clinical end stage of classical scrapie, ten SELDI peaks significantly discriminated the scrapie group from the healthy controls. During the non-clinical incubation period, individual SELDI peaks were differently expressed between the groups at different time points. Investigations of differences in -omic profiles can contribute to new insights into the underlying disease processes and pathways, and advance our understanding of prion diseases, but comparison and validation across laboratories is difficult and challenging.

Keywords: Scrapie, SELDI-TOF-MS, PCA, PLS-DA, Target projection, LC-MS/MS, Serum amyloid A, Sheep

Background

Prion diseases, like scrapie in sheep, are often called Transmissible Spongiform Encephalopathies (TSEs). These are fatal neurodegenerative diseases in a variety of host species, including humans. They are all associated with the conversion of the normal host cellular prion protein, PrP^{C}, into the abnormal protease-resistant isoform, PrP^{Sc}. The PrP genotype influences susceptibility, incubation period and clinical presentation, the $V_{136}R_{154}Q_{171}$ allele being most highly associated with classical scrapie in sheep. To control and prevent spread of scrapie, genetic screening and breeding for resistance are widely used, and was implemented in the EU through Decision 2003/100/EC [1,2]. The PrP genotype is, however, neither a marker for definitive disease, nor the only genetic factor influencing prion diseases [3,4]. Despite the effort of reducing susceptibility, and monitoring and culling of ruminants, scrapie still exists [5,6].

As of today, much research into prion diseases has evolved around the prion protein itself through infection and dissemination studies, and relatively little has been done on other non-PrP^{Sc} disease processes. The most recent large scale survey on prevalent PrP^{Sc} in human appendix samples in Britain, suggests a higher prevalence of infection than formerly anticipated, in all human PrP genotypes, and these findings further necessitates focusing on various mechanisms in prion disease development and progression [7]. The variable incubation time, the complex epidemiology and different

* Correspondence: Siv.Meling@nvh.no
[1]Department of Production Animal Clinical Sciences, Section for Small Ruminant Research, Norwegian School of Veterinary Science, Kyrkjevegen 332-334, N-4325, Sandnes, Norway
Full list of author information is available at the end of the article

variables which may influence the clinical and pathological picture are increasingly important to elucidate [8-10]. Different -omic studies of tissues and body fluids, like serum, may potentially reveal markers that can contribute to unravel the intricate pathogenesis of prion diseases. Recently, several non-PrP^{Sc} proteins have been put forward as promising biomarkers for preclinical scrapie [11-15]. Identification of such non-PrP^{Sc} biomarkers may be crucial in future prion research.

The Surface Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (SELDI-TOF-MS) technology (Ciphergen Biosystems, Fremont, CA, USA) was designed to perform a mass spectrometry (MS) analysis of protein mixtures based on the mass-to-charge (*m/z*) ratio of the proteins, and on their binding affinity to the various chip surfaces. For a single charged protein, the molecular weight in Dalton (Da) usually corresponds well to the mass-to-charge (*m/z*) value, and the peak intensity corresponds well to the concentration in the sample. Different protein expression profiles may then be determined from these protein profiles by comparing the intensity of peaks of similar *m/z* value [16].

Proteins are good indicators of current cellular functions, and investigation into the serum proteome represents one direction in biomarker research [16]. One of the challenges in investigating the serum proteome is its complexity and the presence of high abundant blood proteins, particularly albumin. It is estimated that the high abundant proteins constitute 95% of the bulk mass of proteins, but they represent less than 0,1% of the total number of proteins [17]. These high abundant proteins may produce large signals and mask or interfere with the detection of other low abundant proteins [18]. To simplify the sample complexity, an up-front fractionation procedure is recommended in addition to the fractionation achieved by the chromatographic properties of the SELDI ProteinChip® Array technology [16,19,20].

Extracting crucial information from the retrieved mass spectrometry (MS) data can be challenging. These data often have a much higher number of variables compared to number of samples, they do not follow a normal distribution, there is heteroscedasticity and variables are highly correlated. For these reasons, much effort has been invested in finding reliable methods to assist the interpretation of such profiles. Machine learning methods represent one direction, and another is the latent variable (LV) approach where principal component analysis (PCA) is commonly used for unsupervised exploratory analysis of mass spectral data [21]. Partial least squares discriminant analysis (PLS-DA) is another method that utilizes the knowledge of group belonging to identify discriminating group data [22]. A problem with PLS-DA is that usually numerous latent variables are needed in order to achieve good discrimination between the groups and this can create interpretation problems. Following up with target projection (TP) method, the axis of best discrimination between groups can be achieved, and interpretation on a single predictive latent variable is obtained [23]. Rajalahti *et al.* developed a quantitative display called selectivity ratio (SR) plot for selecting biomarkers in spectral profiles. The SR plots provide both ranking and an objective measure of probability to guide the investigator in the selection process, resulting in a specific protein fingerprint profile that classifies unknown samples into controls or infected group [23,24]. It has been suggested that it is possible to classify samples based on multiple biomarker patterns, and therefore not constrained by the sensitivity and specificity of any single biomarker [16,20,25].

In this work, SELDI-TOF-MS technology was used in the analysis of pre-fractionated serum samples, and we describe the data processing steps and the following latent variable projection methods used to visualize the variation and highlight variables which separate the groups in question.

Results

Animals

At time of euthanasia, 23 weeks post inoculation (wpi), all the scrapie infected animals showed typical signs of scrapie, such as pruritus, ataxia, reduced live weight, weak coordination and poor wool quality. None of the animals in the control group showed any clinical signs of scrapie. Brain material from both groups and inoculation material used were examined by western blot (WB) for the presence of PrP^{Sc}, and results are presented in Figure 1.

SELDI-TOF-MS data processing and evaluation

Reproducibility of the SELDI-TOF MS analysis was evaluated on the basis of the calculated coefficient of variation (CV) of peak intensities and *m/z*. The pooled CVs (CVp) were calculated and results are in the same region as reported by others, and are shown in Table 1. CVp for mass accuracy across samples were all below 1%.

Data analysis of clinical end stage data

PCA analysis was performed on MS data from both end-stage study (ES) and longitudinal study (LS) on the basis of peak clusters derived from biomarker wizard feature (BW) included in the Ciphergen ProteinChip® Software, and score plots are presented in Figures 2 and 3 respectively.

The PCA analysis was used solely for visualisation purpose. The score plots in Figure 3 demonstrated that the healthy animals and infected animals segregated well at the clinical end-stage (23 weeks p.i.), but poorly during the asymptomatic incubation period. Principal component one (PC 1) describes most of the variation in

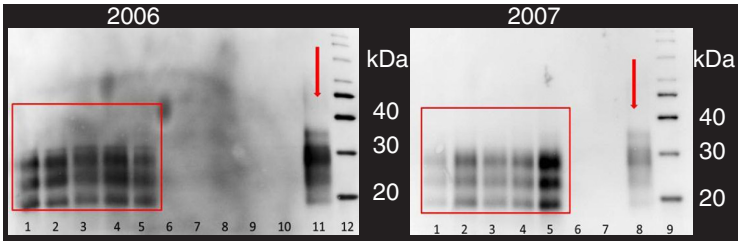

Figure 1 Detection of PrP^{Sc} by Western blotting. WB using P4 antibody of homogenated brain material from animals and inoculation material used in this experiment. Lanes 1–5 (2006 and 2007) represent the scrapie inoculated animals. Lanes 6–10 (2006) and 6–7 (2007) represent the control animals. The lanes indicated by the arrow represent inoculation material used in the scrapie groups. Molecular markers were placed in lanes 12 and 9. PrP^{Sc} was detected in inoculation material and in all the animals from the scrapie groups.

each data set, but how much of this variation is accounted for by scrapie is unknown, as this method does not take group belongings into account. Data sets from LS were not analysed any further with LV methods, due to the low number of peaks selected in BW, making these methods not suitable. The LS data was further analysed by the non-parametric Mann–Whitney U test for significant difference in individual peak intensity between the groups at each sampling time. The resulting peaks and their *m/z* value, significance level and fold change are listed in Table 2.

Only data from clinical end stage study was further analysed by PLS-DA using group classification as the dependent variable. Five (5) components were shown to possess predictive information according to cross validation. This model used 70.6% of the variables in the

Table 1 Coefficient of variation for peak intensities across samples (ES data) and quality control (QC) sample (LS data)

Sample ID	CVp%
1	20.5
2	14.8
3	12.6
4	22.6
5	23.4
6	28.0
7	16.9
10	15.6
11	26.1
12	25.4
13	14.1
14	14.8
15	28.7
19	19.4
20	16.8
21	15.8
22	18.4
23	28.6
24	28.6
QC	28.2

Statistical description of the CVp calculated for each of the individual sample and QC sample. All the calculated CVp's for peak intensities were below 28.7% which was in the same region as others have reported [19,25,28,29].

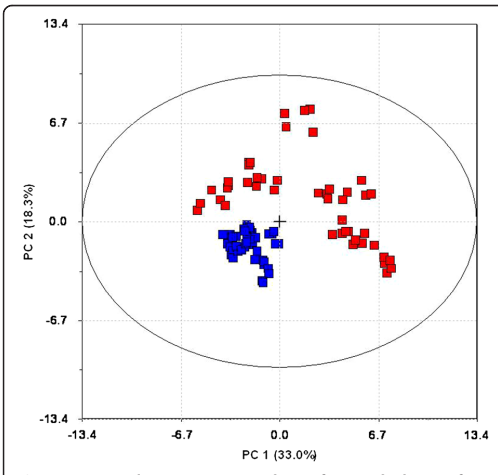

Figure 2 Principle component analysis of 38 peak clusters from end stage study (ES) data. Samples from scrapie affected animals are indicated in red, and healthy controls are indicated in blue. The first principal component explains 33% and second principal component explains 18.3% of total variation in data. Both these components visually separated the groups, and much of disease related variation contributed on first (PC 1) and second (PC2) principal components.

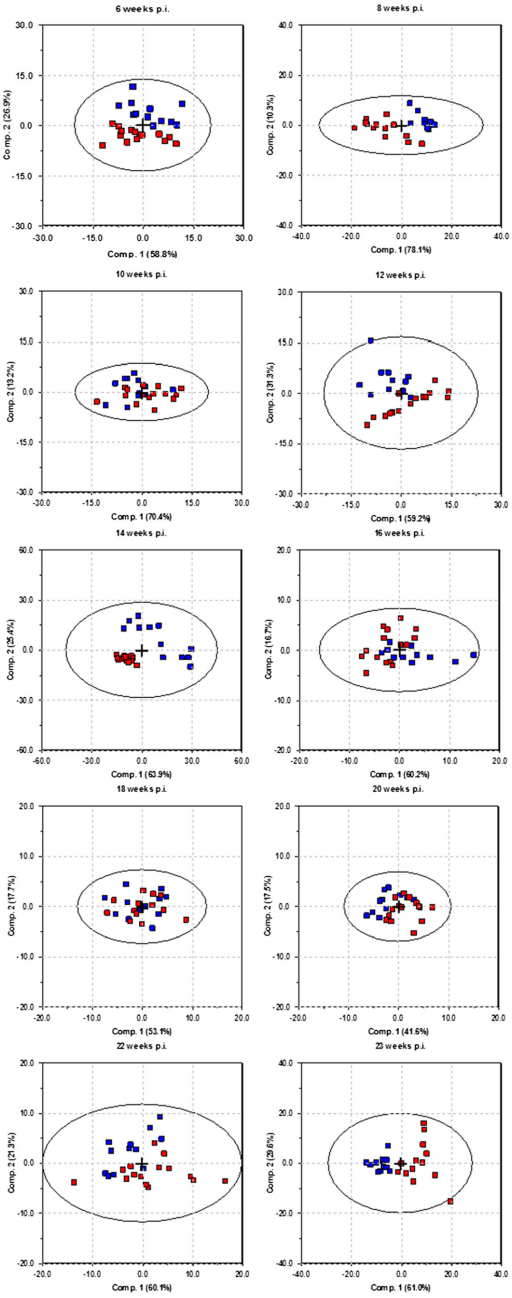

Figure 3 Principle component analysis of longitudinal study (LS) data. Samples from scrapie group are indicated in red, and samples from control group are indicated in blue. One PCA plot for each sample point; six, eight, ten, 12, 14, 16, 18, 20, 22 and 23 weeks of age/post infection.

Table 2 Significant peaks in the longitudinal study and fold change

	Weeks of age/post infection									
m/z	6	8	10	12	14	16	18	20	22	23
2030						↓(1,9)**	↓(1,7)*			
4395					↓(5,9)***					
4635		↑(1,9)***				↓(1,3)**		↑(1,3)*	↑(1,4)**	↑(2,1)***
5061										↑(2,0)**
5201					↓(4,4)**					
5695	↓(2,4)***	↓(1,7)**		↓(1,5)*						
5712	↓(2,2)***	↓(1,7)**							↑(1,5)*	
7542					↓(3,3)***					
8057					↓(5,7)**					
8509					↑(3,8)**			↑(4,3)***		↑(3,1)***
8625				↓(1,8)*						
8724										↑(3,2)**
8779					↓(7,8)***					
8796		↓(1,9)**		↓(2,6)***	↓(4,4)***					
8813		↓(1,7)**	↓(1,2)*	↓(2,5)***	↓(3,2)***					
9271		↑(1,9)**			↓(1,7)*	↓(1,4)*		↑(1,3)**	↑(1,4)*	↑(2,4)***
9478		↑(2,0)***			↓(1,6)*	↓(1,3)**			↑(1,5)*	
15073		↑(27,2)***			↓(6,5)***					
15278					↓(6,0)***					
16106		↑(19,7)***			↓(5,9)**					

Peaks found to be significantly under- and overexpressed in the scrapie group. Arrow indicates change in scrapie group relative to control group at different time points post infection (weeks of age/post infection). ↓ - under-expression, ↑ - over-expression, *m/z* – mass in Dalton, average fold change in expression is given in brackets. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

protein profile (explanatory variables) and explained 97.8% of the variance in group membership (response variable), indicating an excellent predictive model. This PLS-DA model was used as the basis for the TP model and the resulting TP scores are graphically presented in Figure 4, showing excellent discrimination between healthy controls and infected animals. The TP model uses only 19.7% of the variables in protein profiles to explain the same 97.8% of the variance in the group membership. This indicates that most of the variation in the mass spectral data was not related to the disease status, and therefore removed in the TP model. The two models are summarized in Table 3. By choosing 80% mean correct classification rate (MCCR) for the Mean Wilcoxon Rank Sum as the sensitivity threshold for selecting discriminating peaks, the Discriminating Variable (DIVA) plot indicated the corresponding Selectivity Ration (SR) threshold to be 0.41 (Figure 5). From this we were able to select ten variables, presented in the Selectivity Ratio Plot in Figure 6, with individual Wilcoxon classification rate (sensitivity) in the range of 82 – 95 per cent (Table 4). These ten peaks were used in a new PCA analysis for a visual impression of the distribution of animals on the basis of these ten peaks, Figure 7. As illustrated in this PCA Score Plot, the two groups were well separated along PC 1 which indicated that these ten variables were highly related to group differences, i.e. scrapie *versus* healthy. The intensity and standard deviation of each of these SELDI peaks represented by *m/z* value were plotted in a bar diagram and presented in Figure 8. From this we can see that all of these ten proteins were over-expressed at the clinical end stage of scrapie.

Protein identification

Serum Amyloid A (SAA) protein (gi1173354) was identified by eight peptides using high confidence filter, giving coverage of 45.54%, and SAA was only identified in the scrapie sample. The peptide sequence of SAA and the identified peptides are shown in Figure 9. SAA consists of 112 amino acids and has a theoretical molecular weight of 12 688 Da which corresponded well with one of the selected SELDI peaks with an *m/z* of 12 682. The data of this SELDI peak are presented in Table 5.

Discussion

In this study, we have evaluated the use of SELDI-MS-TOF data and latent variable methods to create and

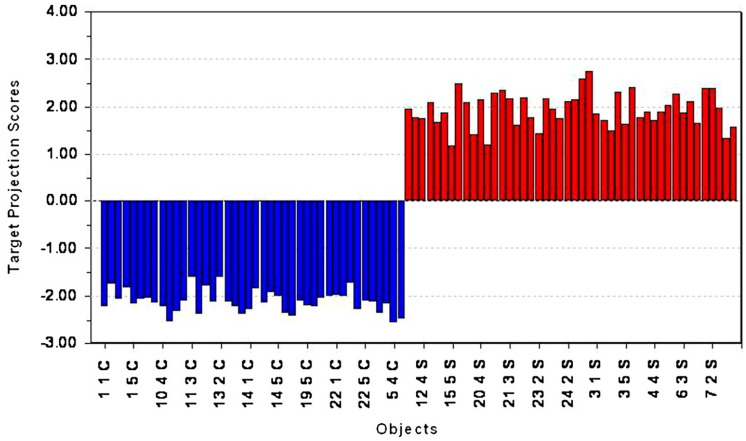

Figure 4 Target Projection score indicating grouping for each sample. All the scrapie samples (red) have a positive score value, and all the samples from healthy controls (blue) have a negative score value. The samples are indicated on the x-axis, and the target projection score on the y-axis. The TP model was able to separate the two groups with no misclassifications.

analyse serum protein profile data to discriminate healthy sheep from sheep with scrapie at various stages during the incubation period and at the clinical end-stage. Batxelli-Molina *et al.* discriminated sheep with early phase scrapie and healthy controls by the use of four SELDI peaks with sensitivity and specificity of 87.3% and 88.1%, respectively [11]. We were able to create a good predictive regression model only from the clinical end stage data, and based on ten peaks, to discriminate scrapie affected animals from controls with a sensitivity of 87.8%. One of these ten selected SELDI peaks had a relatively high intensity in the scrapie group and was barely detectable in the control group. This peak had a mass (*m/z*) of 12 682 Da and a mean sensitivity of 95%. Based on results from LS-MS/MS analysis of samples from both control groups and scrapie groups, this peak was identified as serum amyloid A (SAA). The finding corresponds well with our previously published data on quantitative measurement of SAA in serum samples from these animals [26].

A range of different univariate and multivariate data analysis methods and different software have been used for analysing SELDI spectral data [11,16,17,19,25,27-30]. We believe that multivariate methods based on latent variables are better suited, as these methods can handle data with more variables than observations and data which are noisy and highly collinear [22,31,32]. They provide a good tool for visualization of the data, detection of patterns and object classification. Latent variable models reduce dimensionality of the data and reveal the underlying concept and structure in them. These methods have been reported by others to produce good results from SELDI-TOF MS data [27]. However, due to the few peaks (variables) in datasets from the longitudinal study, we were not able to create a predictive model without increasing risk of over-fitting the regression model. We were not able to define valid components in the PLS-DA model and at the same time achieve satisfactory cross validation of data. Results from the longitudinal study were therefore only evaluated visually by the PCA method, and individual peaks were evaluated for significance through Mann Whitney U test. Although significant p-values were observed at each sampling time, these results should be interpreted with care due to poor reproducibility of the SELDI-TOF-MS analysis and the risk of false positives due to the "multiple comparisons problem" arising when a high number of peaks are independently compared between the

Table 3 Modelling results of both PLS-DA and TP predictive models before and after peak selection

Data	No. of spectra	No. of PLS comp	R2 (XPLS-DA)%	R2 (XTP)%	R2(y)%	% MCCR (DIVA)	SR limit	No. of selected peaks
C/S	88	5	70.6	19.7	97.8	80	0.41	10 (26%)
C/S[a]	88	4	91.1	48.6	87.8			

No. of spectra: 19 individuals in 3–5 replicates; R2 (XPLS-DA): 70.6% of total variance in X is explained in the PLS-DA model; R2 (XTP): 19.7% of total variance in X is explained in the TP model; R2(y): 97.8% of total variance in the response variable. y. is explained in both models; MCCR: mean correct classification rate; No. of selected peaks with percentage of total peak selection in brackets; C/S[a]: modelling results after reduction of subset to only include the selected peaks.

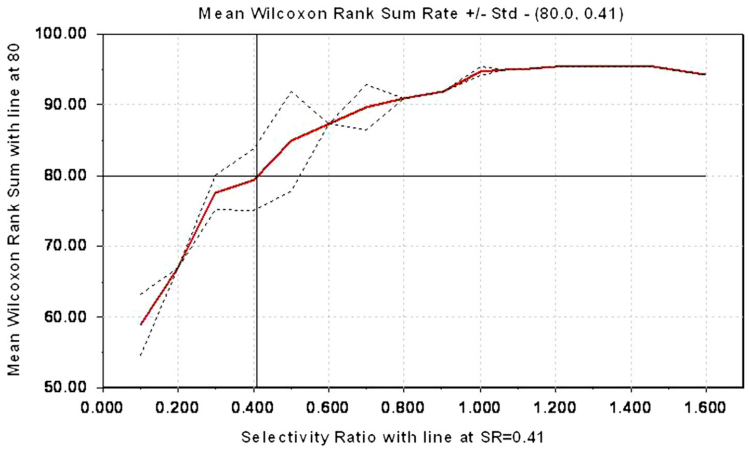

Figure 5 DIVA plot. A DIVA plot of the TP model with the red solid line indicating mean Wilcoxon classification rate and standard deviation (dashed line), and SR values on the x-axis. Horizontal line indicates the chosen 80% MWCR, and the vertical line indicating the resultant SR threshold of 0.41.

two groups. PCA is a powerful technique for data visualization, but it is an unsupervised method including all variance in the data into the analysis, and does not use any a priori information regarding group membership [32]. Much of this variance may also be due to other non-scrapie related differences between the animals such as sex, age, genetics, sampling time and individual physiological factors. Important biomarker patterns in serum proteome may be buried under such major differences and by using methods taking group membership into account, disease relevant differences may become clear. We have illustrated this by using PLS-DA to analyse ES data, where the model focuses on maximum separation of the two groups, in contrast to maximum variation in the PCA model [22,33]. PLS-DA model gives rise to large numbers of PLS components required to describe the majority of the variation in the data, and by combining these PLS components into a single TP component, which represents the direction in the multivariate predictive space with strongest relation to the response, interpretation

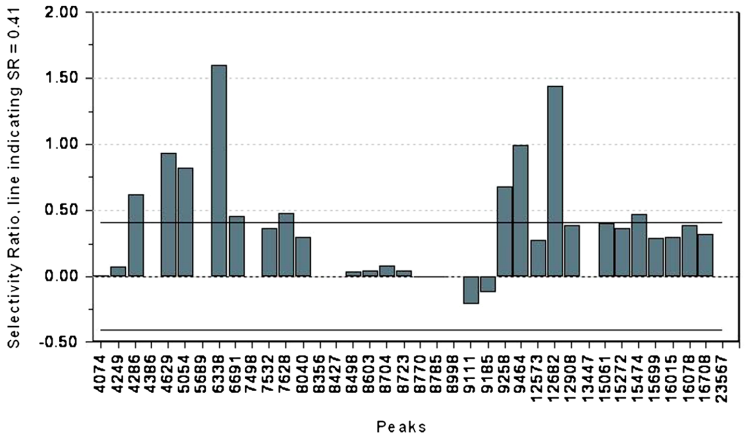

Figure 6 Selectivity Ratio plot for all peaks in the model. A bar chart of all the peaks (x-axis) used in the model and their calculated SR value (y-axis). The two horizontal lines indicate the SR threshold with an absolute value of 0.41. Ten peaks have a SR value above this threshold.

Table 4 Selectivity ratio value, Wilcoxon classification rate and univariate p-value for each of the selected variable

Variable (*m/z*)	SR	% Wilcoxon Classification Rate	Mann–Whitney U test p-value
4286	0.62	92	0.00E + 00
4629	0.94	94	0.00E + 00
5054	0.82	92	0.00E + 00
6338	1.60	94	0.00E + 00
6691	0.46	95	0.00E + 00
7628	0.48	84	3.87E-08
9258	0.68	87	1.50E-09
9464	0.99	95	0.00E + 00
12682	1.44	95	0.00E + 00
15474	0.46	82	1.78E-07

becomes easy [34,35]. The information with no correlation to group membership has then been removed, and the TP score vector displays the discriminative information between the two groups on a single scale. This is illustrated and summarised in Table 3, where we show that total variance in data used to describe the predictive model was reduced to 19.7% in the TP model, from 70.6% in the PLS-DA model. The TP model also provides a quantitative measure of each original variable's contribution to the discrimination between groups, but as peaks with large variance and little correlation to group membership may dominate over peaks with little variance and high correlation to group membership, this could not directly be used to select interesting peaks [34]. The selectivity ratio (SR) for each variable on the TP component is directly related to each variable's ability to predict group membership and this was used to select variables in the model [23,24].

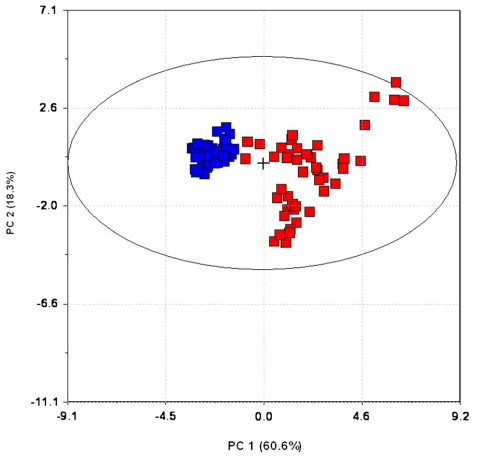

Figure 7 PCA plot of distribution of the two groups using only ten peaks. The ten peaks selected from the analysis were used in a PCA plot and there was good visual separation of the groups with PC1 accounting for about 60% of the variation in the dataset based on these ten peaks.

As described by Rajalahti *et al.*, a sensitivity level, or correct classification rate, for a set of peaks can be chosen individually for each data set and this is done statistically by the non-parametric Wilcoxon Rank Sum test. Completely random classification with equal number of samples in each group then gives a correct classification rate of 50%, and correct classification of all the samples will have a CR of 100% [23,24]. Setting the sensitivity threshold must balance the risk between selecting false biomarkers and missing important ones. In this study, we chose a mean sensitivity level/correct classification rate of 80% for the selected variables which gave a selectivity ratio (SR) value of 0.41, this is illustrated in the DIVA plot in Figure 5. Further on, this SR value was applied to all the variables in the Selectivity Ratio plot, Figure 6, and ten SELDI peaks qualified for selection by having a SR value above this threshold.

For two-group comparisons, like in this work, receiver operating characteristics (ROC) curves could be used to compare the sensitivity and specificity of a biomarker candidate at different cut-off values for peak intensity [36]. But as correct classification rate is identical to the sensitivity in a binary classification it will give us the same picture, only that the DIVA plot expands into the multivariate space.

The ten selected SELDI peaks were used in a PCA plot in Figure 7 to illustrate how well they separated the two groups in question along the PC 1. Figure 8 illustrates the intensity of these ten peaks in the SELDI spectra, and the increased expression in the scrapie group compared to the control group is probably related to the clinical status of the animals.

One of these peaks, with the *m/z* of 12682 Da, was identified by LC-MS/MS as serum amyloid A (SAA), which is a major acute phase protein (APP) in sheep. It has been quite common to identify acute phase proteins as discriminating biomarkers between groups of affected and not affected individuals, as these are highly sensitive reactants produced in response to an insult [18]. They are, however, not very specific, although different insults may produce different patterns of acute phase response (APR). Many of the reported diagnostic SELDI peaks have been found to be acute phase proteins, and are described in several reviews [11,19,37,38]. SAA is primarily induced by pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, which are released by a variety of cells including activated tissue macrophages and blood

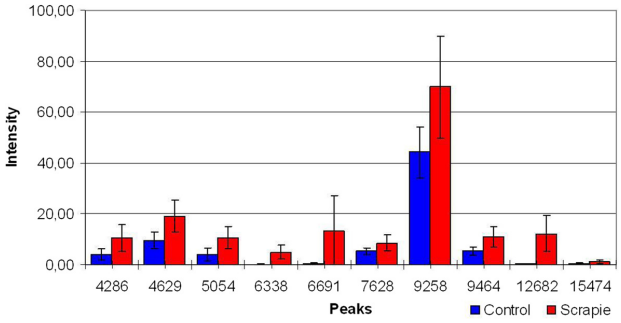

Figure 8 The mean peak intensity and standard deviation for the ten selected peaks. A bar chart of the ten selectee peaks, with *m/z* on the x-axis and intensity on y-axis. Scrapie samples were indicated in red, and controls were blue. Standard deviation for each mean was indicated on each of the bars.

monocytes in response to injury [39,40]. Sheep with natural scrapie, and mice with experimental scrapie, show reactive astrocytosis and microglia activation and increased cytokine expression in the brain at the time of clinical signs and neuropathological changes [41-43]. These cytokines can cross over into the blood and initiate a systemic APR with increased synthesis of APPs from hepatocytes, such as SAA [44]. Coe et al. reported an increased level of serum amyloid P in plasma of mice with scrapie as evidence for systemic inflammatory response to scrapie [45]. Batxelli-Molina *et al.* identified transthyretin as being under-expressed in sheep with clinical scrapie [11]. Transthyretin is a negative APP expressed at lower levels during an APR along with the other negative APPs. Although identification of APPs as biomarkers of disease has not been considered significant, we believe that identification of any protein, regardless of specificity that significantly differs between scrapie affected and healthy controls, will contribute to novel information of underlying pathological processes of scrapie. The long incubation period, large variety in clinical presentation, as well as lack of direct link between neuropathology, PrP^{Sc} dissemination and clinical presentation, create the need for new knowledge of underlying processes at all stages of scrapie. Identification of discriminating proteins will contribute in this matter.

The SELDI-TOF-MS may be an excellent tool for protein profiling due to its high throughput, but, as this work has shown, there are too many technical limitations resulting in lack of peak identification and poor reproducibility to make this the technique of choice in the search for specific biomarkers. The challenges and limitations associated with SELDI-TOF-MS are nicely reflected by the poor reproducibility between our longitudinal and end point studies, and the low number of peaks detected at some time points, like 10 and 18 weeks. The method failed to detect the peak with *m/z* 12 kDa at both ES and LS, even though this peak separated the groups well and had high intensity in the ES study. Even though there are a number of peaks found to be significantly under- and overexpressed in the scrapie group compared to the control group in the LS data, the findings are of limited value, as long as the peaks are not identified as specific proteins which can elucidate specific pathological pathways of processes. It is also uncertain whether these individual peaks are separate proteins, several peaks can represent the same protein with different charges or modifications. We also noticed that there were large differences between the different time points, even though all the samples included in the LS were run randomly at the same time. This could be due to introduction of variables during handling and pre-processing of samples, especially from the initial

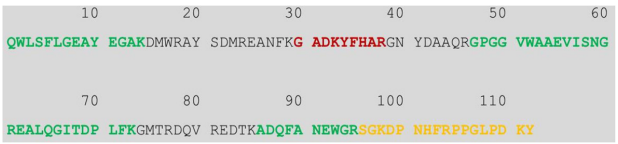

Figure 9 SAA sequence and the identified peptides. Here is the total ovine Serum Amyloid A protein sequence and peptides identified by LC-MS/MS are highlighted. The peptide in red was identified with low confidence, the peptide in yellow was identified with medium confidence, and the peptides in green were identified with high confidence.

Table 5 Results from data analysis for SELDI peak with *m/z* of 12682

Peak *m/z*	SR	Mean Intensity – Control	Standard Deviation – Control	Mean Intensity – Scrapie	Standard Deviation – Scrapie	% Wilcoxon Classification Rate	Mann–Whitney U test p-value
12682	1.44	0.24	0.16	12.20	7.21	95	0.00E + 00

fractionation step. The difference in number of peaks detected in each group could be due to suspected variation in quality and quantity in the FT fraction. As pointed out also by Van Gorp *et al.*, many promising studies on discriminating SELDI peaks have been published, but few follow-up papers on peak identification and validation have been published [46]. Barr *et al.* actually proposed a protein fingerprint for TSE infection in blood [47].

To create a proteomic profile able to detect sheep infected with scrapie during the incubation period with high sensitivity and specificity, rigorous testing of a large number of animals would be necessary, in addition to eliminating variability through sample handling and analytical procedures. In addition to scrapie, other neurological diseases would have to be similarly mapped. The reproducibility and validity of discriminating proteomic profiles would need to be confirmed across different laboratories and animal groups, including different genotypes, scrapie strains and age groups. One of the major limiting factors of SELDI proteomic profiles is the lack of direct comparisons of SELDI peaks based solely on *m/z*. Differences in experimental set-up from animal model to data analysis result in poor reproducibility in number of peaks detected, peak height and *m/z*, making the resultant peak list incomparable [48]. Comparison of SELDI data from different sample sets, different runs on the same or across SELDI-TOF-MS instrument(s) have resulted in considerable variation in number of discriminating peaks [37]. Comparisons made across different studies may also be misleading, as one protein species can generate about ten major peaks and many minor satellite peaks due to chemical reactions that may take place during the sample preparation and analysis. Proteins with approximately the same mass will show up with overlapping peaks, and spectra obtained with different machine settings can look different [49]. Our results also confirm this problem, as the samples set for LS and ES were prepared and analysed on two different occasions, and we were not able to reproduce the exact same results in the end point data sets. The relatively high CVp seen for peak intensity both within and between runs, indicate that slight changes in peak intensity between groups may not indicate an actual difference between groups, and thus careful interpretation of results was necessary. This problem may be overcome by considerably increasing the number of animals in each group. Results across different age-groups were not compared, as natural changes in protein profiles related to age changes may overshadow the difference due to disease status. We worked with very similar groups to enhance differences relating to scrapie, and minimize differences related to pre-analytical factors like age, sex, production status and genotype. The variance attributed to pre-analytical factors was also minimized by one normalization step before peak selection, and not two as proposed by Poon (2007), due to the risk of introducing "false" differences between profiles by this renormalization [11,19,27,50]. The difficulty in identification of proteins that correspond to the SELDI peaks is, as mentioned earlier, another major limiting factor, as also mentioned by Batxelli-Molina *et al.* and much effort should be made to identify these discriminating proteins, especially those which are significantly different between the groups [11].

Conclusion

In conclusion, on the basis of the experimental infection model used, including route of infection and PrP genotype of the animals, we believe that the results in this study are relevant to the study of several aspects of naturally infected classical scrapie cases. Choosing peaks/proteins in biomarker research based solely on p-values from univariate models may, however, result in a number of false markers, and latent variable methods are much more suitable for these types of data. Such methods are simple to use for non-statistical users, and interpretation is made easy as results are visually well presented. This article describes one approach, from animal model to data analysis, and the resulting selection of significant protein peaks and creation of a predictive model. The results show that it is possible to use data from SELDI-TOF-MS in combination with multivariate data analysis to discriminate scrapie affected sheep from healthy controls. We identified one peak, or one discriminating protein, to be serum amyloid A (SAA), in the scrapie affected animals at the end stage. However, the practical application of this predictive model is restricted due to the limiting factors of SELDI-TOF MS. The multiple detected differences between these groups might, therefore, have been more completely illustrated by other -omic methods. Studies on differences in proteomic profiles between healthy and scrapie infected sheep will, undoubtedly, provide novel insight into the underlying pathogenic and pathological events. However, as long as these discriminating protein peaks remain unidentified, the pathological and clinical relevance of the actual proteins in relation to scrapie remains unknown.

Our conclusion is therefore that there is a need for sensitive and specific bioassays using identified biomarkers, obtained by –omic methods, which can be utilized by various research groups across experiments.

Materials and methods

Animals

A total of 19 lambs over two consecutive years (2006 and 2007) were included in this study, all having the same PrP genotype, homozygous $V_{136}R_{154}Q_{171}$ (Table 6). Lambs were inoculated orally with 1 gram homogenated pooled brain material from either healthy sheep or confirmed cases of classical scrapie immediately after birth and before any ingestion of colostrum and then grouped (control or scrapie group) according to inoculation material. Inoculation brain material used in both groups was tested for PrP^{Sc} by WB (Figure 1). The lambs were left with their mothers in confined isolated boxes under similar conditions and feeding regimes. All the lambs used were born within a time period of 15 days. At post mortem examination, the obex area of the brain from each animal was sampled for detection of PrP^{Sc} by WB (Figure 1). Animal experiments were approved by the Norwegian Animal Research Authority.

Serum samples

Serum samples used in this work were drawn every two weeks from six weeks post infection (p.i.) until euthanasia in 2007 for the longitudinal study (LS). Serum samples at time of euthanasia from both 2006 and 2007 were used for the end-stage study (ES). Serum samples were allowed to clot at room temperature for a minimum of 30 minutes and maximum 60 minutes, and then processed. Serum was pipetted in aliquots and frozen at minus 80 degrees within two hours of sampling. All the samples were subjected to the same handling procedures throughout the experiment.

Serum fractionation

Serum samples were fractionated prior to SELDI-TOF MS analysis, using strong anion exchange fractionation kit, ProteinChip® Q Spin Columns (Bio-Rad), containing Q ceramic HyperD F sorbent. Before application to columns, proteins were denatured by addition of 150 μl 9 M Urea 2% Chapters 50 mM Tris–HCl pH 9 (U9) buffer to each of the 100 μl of serum samples, this followed by an additional 250 μl 1 M Urea 0,2% Chapters 50 mM Tris–HCl pH 9 (U1) buffer. The 500 μl serum mixture was added to the columns, and incubation time was set to 30 minutes at 4 degrees on a rotator to ensure complete mixing of serum mixture and column sorbent. Each sample was fractionated into six fractions (FT/F1, F2, F3, F4, F5 and F6). Flow through (FT) fraction was captured directly after sample incubation, and the consecutive fractions were captured after adding washing buffers with decreasing pH, starting at pH 9 and ending at pH 3 when capturing F5. The last fraction, F6, was captured after a wash with an organic buffer. The different fractions were aliquoted, and stored at – 80°C soon after capture until further analysis.

SELDI-TOF MS analysis

A Weak cation exchange array (ProteinChip® CM10 Array, Bio-Rad) in combination with high stringency buffer, 50 mM HEPES pH 7.0 as binding and washing buffer was used to analyse the flow through (FT) fraction in this work. Each FT fraction was diluted 1:10 with binding buffer before application to array, and each individual LS and ES sample was applied randomly onto the array in three and five replicates, respectively. The matrix, ProteinChip® Sinapinic Acid (SPA) Energy Absorbing Molecules (EAM), was applied before the SELDI-TOF-MS analysis. The arrays were prepared and handled according to manufacturer's instructions. The arrays were analysed on the Protein Biology System II (PBS-IIc) with autoloader (Bio-Rad Laboratories) using Ciphergen ProteinChip® Software Version 3.2.1. (ProteinChip® Software) with the integrated Biomarker WizardTM (BW) cluster analyses software [51]. Each chip was analysed with a spot protocol optimized for the low mass area (LM) between 2 and 25 kDa, and spectra were collected using an average of 130 laser shots. ES and LS samples were prepared and analysed separately. The BW feature of the ProteinChip® Software was used for peak clustering in the range of interest (2 kDa – 25 kDa).

Data processing

Spectral data was processed to reduce instrumental and handling artefacts, minimize variation within groups and maximize variation between groups, and improve peak detection. Spectra were named and organised into groups according to age at sampling and group belonging (control

Table 6 Overview over samples, animals, genotype and age of sampling at end stage of disease

Sample ID	Year	Group	Genotype	Sex	Age in weeks
1, 2, 10, 11, 19	2006	Control	VRQ/VRQ	Male and Female	24 – 25
5, 13, 14, 22	2007	Control	VRQ/VRQ	Male and Female	25
3, 4, 12, 20, 21	2006	Scrapie	VRQ/VRQ	Male and Female	23
6, 7, 15, 23, 24	2007	Scrapie	VRQ/VRQ	Male and Female	23 – 24

and scrapie). Data were processed using ProteinChip® Software [51]. This process involved four steps; calibration, baseline subtraction, filtering and noise reduction and normalization (TIC). Finally, peak selection was performed by BW. Data processing was performed following recommendations described by Bio-Rad [36]. The collected peak data was exported into Microsoft® Office Excel 2003 and Sirius Version 8.1 (Pattern Recognition System AS, Bergen, Norway) for further data analyses. The spectra were evaluated for intra-cassette and inter-cassette reproducibility by calculation of the coefficient of variation (CV) for both peak intensity and peak mass (*m/z*). The CV for ES data set was calculated for each of the samples based on peak information in each of the five replicates, and CV for LS data set was calculated from peak information in a quality control (QC) sample that was repeatedly run with the samples.

A calibration equation was created using the calibration feature in the ProteinChip® Software and standards containing peptides and proteins of known mass (ProteinChip All-In-One Peptide/Protein Standard, Bio-Rad), which were run parallel to the samples. One equation for each data set, ES and LS, was calculated and applied to all the spectra in each of the respective study.

The shape of the baseline of each spectrum was examined and the baseline feature was used to subtract baseline. Fitting width was set to two times (2×) expected peak width. The noise range was set to 2 kDa to exclude matrix attenuation range from the analysis, and end was set to 100% of spectrum size.

The baseline and noise reduced spectra were normalized using the Total Ion Count (TIC) Normalization feature in the ProteinChip® Software, which normalizes each spectrum to equal sum detected signal under the curve in the region of interest. Each group, based on age and group belonging was normalized separately. The resulting normalization factor created for each spectrum was inspected and evaluated. Spectra with normalization factor above mean + 2 standard deviations were excluded from further analysis.

Peak clusters were generated using the BW function in the ProteinChip® Software to detect peaks of similar mass across the spectra. Peaks were detected using the following settings; first-pass detection with signal-to-noise ratio > 5, with cluster completion using a second-pass with signal-to-noise ratio > 2. The peaks needed to be present in at least 20% of the spectra (giving a presence in at least half of each group). A mass difference of 0.3% was allowed. Peak cluster information was exported to Excel for further analysis.

Data analysis

Univariate

The data were tested for difference in relative peak intensity between the two groups using the non-parametric Mann–Whitney U test included in the BW and Sirius software. The fold change in intensity was calculated as the mean peak intensity control/mean peak intensity scrapie for significantly down-regulated peaks, and vice-versa for up-regulated peaks. For all tests, the significance level was set to $p < 0.05$.

Multivariate

Latent variable projection methods (LV) were used to analyse the SELDI-TOF-MS data. Both ES and LS data was analysed by principal component analysis (PCA) to visually evaluate the distribution of the data irrespectively of group belonging. Only ES data were further analysed using other LV methods. A group membership variable was defined, assigning "0" to all the samples in the control group, and "1" to all the members in the scrapie group. Partial least squares – Discriminant Analysis (PLS-DA) and target projection method (TP) were then used to evaluate the data distribution according to group membership. For all analyses, the spectral variables were standardized to unit variance, thereby preventing variables with high variance to dominate the data analysis. A non-parametric Discriminating Variable test (DIVA) was used to connect Selectivity Ratio (SR) value to the discriminatory ability of the variables, quantified as the probability of correct classification. Each variable got a correct classification rate (CR), i.e. how well each variable separated the two groups in question. The SR value was plotted against the Mean Wilcoxon Rank Sum Rate to obtain the DIVA plot.

Cross validation was used for ES data to optimize the LV models with respect to predictive performance. Different procedures for cross validation have been developed [52]. The ES data were split into four groups, constructing one PLS model for each group, one group was used as validation set and the others as training sets. The number of PLS components was chosen as the one giving the first minimum in prediction error.

Protein identification

One ES sample from each of the groups was prepared and processed for protein identification. Thirteen μl of the FT fraction were mixed with 6 μl 4× LDS, 2.5 μl 10× DTT. The sample mixture was heated to 60°C for 15 - minutes. 2.5 μl IAA (60 mM) was added to the mix and

Table 7 High and medium confidence peptide filter settings

Charge (z)	XCorr Score High confidence	XCorr Score Medium confidence
1	1.2	0.7
2	1.9	0.8
3	2.3	1.0
> = 4	2.6	1.2

let to incubate for 15 minutes at room temperature and in the dark before loading on a 16% ClearPAGE gel (C.B. S. Scientific, USA). The gel was run at 150 V for 85 - minutes. After electrophoresis the gel was stained with Gelcode Blue Safe Stain (Pierce, USA) for 1 hour and de-stained overnight with ultrapure water. Three protein bands in the region of 9 and 14 kDa bands on the gel were excised and subjected to tryptic digestion using OMX tube devices (OMX, Germany) following the manufacturer's protocol.

Tryptic peptide samples were sent to International Research Institute in Stavanger (IRIS), Mekjarvik, Norway, and protein identification was done according their standard operating procedure. The protein identification was performed by LC-MS/MS analysis using an UltiMate 3000 dual pump nanoflow HPLC system (Dionex, Sunnyvale, CA, USA) connected to a linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap XL, Thermo Fisher Scientific, Waltham, MA, USA). A sample volume of 5 μl from each sample was loaded onto a trapping column (Acclaim PepMap100 C18, 5 μm, 300 μm I.D. × 5 mm length, Dionex) at a flow rate of 2 μl/min in 0.1% formic acid (VWR) in MilliQ water (Elga) for clean-up and pre-concentration. Peptides were separated in the analytical column (Acclaim PepMap100 C18, 3 μm, 75 μm I.D. × 15 cm length, Dionex). The mobile phases for the analytical separation consisted of 0.1% formic acid in 2.5%/97.5% acetonitrile/water (A) and 0.1% formic acid in 80%/20% acetonitrile/water (B) and were pumped with a flow of 300 nL/min. The peptides were separated on the analytical column using a linear gradient from 5 to 60% B in 165 min after a 10 min delay post injection. The gradient was then run to 100% B in 10 min and held there for 30 min to wash the columns. A total run time of 256 min was used, including the washing step and 30 min re-equilibration of the columns. A PicoTip emitter (SilicaTip, New Objective) with a 10 μm tip and without coating was used as an ESI interface. The electrospray voltage was set to 1 kV, and no sheath gas was used. The mass spectrometer was used in positive mode. Full scans were performed in the Orbitrap in the *m/z* range from 200 to 2000, and data-dependent MS/MS scans performed in the linear ion trap for the five most abundant masses with $z \geq 2$ and intensity ≥10000 counts. Dynamic exclusion was used with 3 min of exclusion after fragmentation of a given *m/z* value four times. Collision-induced dissociation (CID) was used with a collision energy of 35% and with activation Q setting of 0.400 and activation time of 30 ms for MS2. The mass spectrometer was tuned daily and calibrated weekly using the calibration solution recommended by Thermo Scientific.

Each LTQ-Orbitrap raw file was analysed using the Proteome Discoverer 1.0 (Thermo Fisher Scientific). Protein identifications were performed with the SEQUEST algorithm searching against even toed ungulate database available at NCBI with trypsin as digestion enzyme, and allowing for maximum two missed cleavage sites. Carbamidomethyl (C) was set as a static modification, and oxidation (M) as a dynamic modification. Precursor ion and fragment ion mass tolerances were set to 10 ppm and 0.8 Da, respectively. Results were filtered for minimum 2 peptides and using a high and medium significance XCorr Score adjusted for peptide charges (z), Table 7.

Abbreviations

TSEs: Transmissible spongiform encephalopathies; PrP^{C}: Normal cellular prion protein; PrP^{Sc}: Scrapie prion protein; SELDI-TOF-MS: Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; PCA: Principal component analysis; PLS-DA: Partial least square discriminant analysis; TP: Target projection; SR: Selectivity ratio; LC-MS/MS: Liquid chromatography tandem mass spectrometry; TIC: Total ion current; CV: Coefficient of variation; Da: Dalton; BW: Biomarker wizard; PC: Principal component; MWCR: Mean Wilcoxon classification rate; DIVA: Discriminating variable; z: Charge; LDS: Lithium dodecyl sulphate; DTT: Dithiothreitol; IAA: Iodine acetamide; LV: Latent variable; WB: Western blot; wpi: Weeks post inoculation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SM carried out the proteomic studies, statistical data analysis, participated in protein identification and drafted the manuscript. OMK participated in the design of the study, statistical data analysis and helped to draft the manuscript. RA participated in the statistical data analysis. KB participated in the design of the study, carried out parts of the protein identification and helped to draft the manuscript. AH participated in the design and performance of the proteomic studies. MJU participated in its design and coordination and helped to draft the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We are grateful to Eli Brundtland for excellent help with the serum samples, and to Mari Mæland, Kai-Erik Uleberg and Eivind Larsen for sharing their knowledge and expertise on SELDI-TOF-MS analysis. This study was supported by grant NRC 178268 (2006–2010) funded by the Research Council of Norway.

Author details

[1]Department of Production Animal Clinical Sciences, Section for Small Ruminant Research, Norwegian School of Veterinary Science, Kyrkjevegen 332-334, N-4325, Sandnes, Norway. [2]Department of Chemistry, University of Bergen, N-5007, Bergen, Norway. [3]Faculty of Health Studies, Sogn og Fjordane University College, N-6800, Førde, Norway. [4]Department of Innovation, Telemark Technological R&D Centre, Kjølnes ring 30, N-3918, Porsgrunn, Norway. [5]International Research Institute of Stavanger (IRIS, Biomiljø), P.O. Box 8046N-4068, Stavanger, Norway.

Received: 29 October 2013 Accepted: 4 November 2013
Published: 14 November 2013

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doi:10.1186/1756-0500-6-466
Cite this article as: Meling *et al.*: Investigation of serum protein profiles in scrapie infected sheep by means of SELDI-TOF-MS and multivariate data analysis. *BMC Research Notes* 2013 **6**:466.

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Meling et al. BMC Veterinary Research 2012, 8:113
http://www.biomedcentral.com/1746-6148/8/113

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Presence of an acute phase response in sheep with clinical classical scrapie

Siv Meling*, Kjetil Bårdsen and Martha J Ulvund

Abstract

Background: Work with experimental scrapie in sheep has been performed on-site for many years including studies on PrP^{Sc} dissemination and histopathology of organs and tissues both at preclinical and clinical stages. In this work serum was sampled at regular intervals from lambs which were infected immediately after birth and from parallel healthy controls, and examined for acute phase proteins. In contrast to earlier experiments, which extensively studied PrP^{Sc} dissemination and histopathology in peripheral tissues and brain, this experiment is focusing on examination of serum for non-PrP^{Sc} markers that discriminates the two groups, and give insight into other on-going processes detectable in serum samples.

Results: There was clear evidence of an acute phase response in sheep with clinical scrapie, both experimental and natural. All the three proteins, ceruloplasmin, haptoglobin and serum amyloid A, were increased at the clinical stage of scrapie.

Conclusion: There was evidence of a systemic measurable acute phase response at the clinical terminal end-stage of classical scrapie.

Background

Scrapie is a fatal neurodegenerative disease of sheep, and is one of the diseases in the group called Transmissible Spongiform Encephalopathies (TSEs), where PrP^{Sc}, an abnormal form of the normal cellular PrP^{C}, is believed to be the infective agent [1]. Other TSEs are Creutzfeldt-Jakob disease (CJD) in man, Bovine Spongiform Encephalopathy (BSE) in cattle and Chronic Wasting Disease (CWD) in deer, to mention a few. TSEs were at first regarded as neurodegenerative diseases without an inflammatory component, but the characterisation of a marked functional activation of microglial cells and identification of increased cytokine expression in the affected areas of the brain, points towards a localised inflammatory response [2]. The PrP^{Sc} and its dissemination and related histopathology have been, and is still, extensively studied in several different animal models, especially in murine models. Scrapie affected sheep (both experimentally and naturally infected) with the most susceptible PrP genotype, VRQ/VRQ and VRQ/ARQ, have a preclinical PrP^{Sc} dissemination in peripheral lymphoid tissue throughout the body, and this has proven useful in preclinical diagnosis by immunohistochemistry (IHC) and Western Blot (WB) of sheep of such PrP genotypes [3-8]. Less susceptible PrP genotypes have shown to have a less predictable lymphatic tissue involvement, thus increasing the risk of false negative lymphoid biopsies [9,10]. During the last decade there has been an increasing interest in the search for other non-PrP^{Sc} disease related biomarkers for TSEs by the use of different -omics techniques, especially in the search for preclinical diagnostic markers that could be used for diagnostics in the live animal. In the search for non-PrP^{Sc} markers of scrapie much work has been done in screening mostly brain, but also peripheral lymphoid tissue and serum in different murine models, for different gene expressions, especially different cytokines which have been detected both at mRNA and protein levels [11-16]. Not all of these murine models show the same results, and Tribouillard-Tanvier et al (2009) suggest that this may be attributable to the animal model used [15]. Recently, Huzarewich et al (2010) published a review on different potential disease markers detected in the application of

* Correspondence: siv.meling@nvh.no
Norwegian School of Veterinary Science, Department of Production Animal Clinical Sciences, Section for Small Ruminant Research, Kyrkjevegen 332-334, N-4325 Sandnes, Norway

-omics to prion biomarker discovery [17]. In the search for a non-PrP^{Sc} marker of prion diseases it is quite noticeable that many of the significant genes and proteins are linked to the activation of microglia in the brain, and the processes in and around the innate immune response including the acute phase response.

Measurements of serum or plasma proteins in relation to diagnostics have been performed for many years, and particularly acute phase proteins (APPs), which increase or decrease in response to a number of inflammatory insults as part of the acute phase response (APR) [18]. The APR is part of the innate immune system and first line of defence. It is the organism's first response to tissue injuries, infections, stress and inflammation, and is characterised by a local reaction at site of injury followed by a number of systemic reactions including changes in the concentration of acute phase proteins (APPs) [19-21]. The majority of serum proteins are synthesised in the liver, including the APPs, which are part of the APR. The APR is thought to be beneficial to the animal in restoring homeostasis, and the response is tightly controlled by negative feedback loops, as the APR itself can cause harm if it comes out of control.

Measurements of APPs in serum/plasma can be used to assess the innate immune system's systemic response to infection, inflammation, trauma and other pathological injuries; they have even been suggested as markers for overall herd/flock health in farm animals by detecting subclinical conditions. The APPs are mainly synthesised in hepatocytes, but also extrahepatically, and they are induced by cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor-necrosis factor-alpha (TNF-α) released mainly from macrophages, monocytes and astrocytes at site of lesion [22,23]. Measurements of APPs have shown to be useful as diagnostic and prognostic markers in several conditions in addition to assessing response to treatment, as their levels correspond to tissue damage. APPs lack specificity, but are highly sensitive indicators of inflammation and tissue injury [24].

Serum amyloid A (SAA), haptoglobin (Hp) and Ceruloplasmin (Cp) have been described as important APPs in sheep, with SAA and Hp being the major APPs in this animal species [22,23,25]. Increased plasma and serum levels of these proteins have been associated with a variety of diseases and conditions, and neither SAA nor Hp are usually detected at all in healthy sheep. Hp and Cp have been shown to increase significantly in experimental *Mannheimia haemolytica* infection, and remain elevated for ten days before returning to pre-infection levels [26]. Cp is an effective antioxidant, also in the CNS, protecting neural cells from oxidative stress, which plays a crucial role during CNS injury, when free iron and reactive oxygen species (ROS) increase [27].

Moderate increases in SAA and Hp have been detected in subclinical infections, and, on this basis, it has been suggested that measurements of these two APPs can become useful in determining health status in flocks. As there is an association between APP levels and the severity of disease it has also been proposed that APPs can be used as prognostic indicators [25,26,28-33].

This work is part of a larger study to investigate non-PrP^{Sc} markers in serum from experimentally infected sheep. In previous work by Ersdal et al. the inoculation was performed between 46 and 61 days of age, while this later model, described by Ulvund et al, the lambs are inoculated at birth [34,35]. This later model has a relatively short incubation period where the animals show clinical disease already at 4–5 months of age, and the distribution of PrP^{Sc} is extensive in the brain at clinical end stage. Initial proteomic analysis of serum from these sheep at the terminal clinical end stage revealed serum amyloid A (SAA) levels in the scrapie group to be significantly differently expressed from the healthy group, and by latent variable methods, was found to discriminate affected from healthy sheep with 95% correct classification rate (data not shown). To our knowledge, this represents the first verification of a measurable APR in sheep with both experimental and naturally occurring classical scrapie. The APR was mainly measured by Hp, SAA and Cp levels in serum.

Results

Experimental classical scrapie model

This experimental model of classical scrapie has been reported elsewhere [34,35]. All the animals in the scrapie group developed clinical signs of classical scrapie within time of euthanasia at 23 weeks of age/post infection. Already at four months of age, very subtle clinical signs of pruritus and wool eating were detected; though only on video surveillance. Clear clinical signs of disease were first obvious during the last week before euthanasia, and clinical presentation deteriorated quickly with noticeable pruritus, wool changes, ataxia, depressed mental status and recumbency. Very few macroscopic changes were recorded during post mortem examination, apart from redness and abrasions of skin in areas of pruritus and wool loss. The brain from all the animals in both groups were examined for PrP^{Sc} by immunohistochemistry (IHC) and WB, and only the animals in the scrapie group were positive (data not shown).

Total protein and albumin measurements and globulin calculation

The mean total protein (TP) concentration in both groups increased steadily from six weeks of age (woa) until a relatively high peak at 18 woa (control group, mean ± SEM, 79.5 ± 0.87 mg/ml) and at 20 woa (scrapie

group at 80.2 ± 1.91 mg/ml). TP level in the control group then decreased to reach 68.1 ± 1.71 mg/ml at 24 woa. TP level in the scrapie group also declined after peaking, but later increased again at euthanasia at 23 woa (77.2 ± 3.53 mg/ml), creating a significant difference between the groups ($p < 0.05$). This significant difference in TP level was in majority due to the higher globulin level in the scrapie group (49.9 ± 2.3 mg/ml) compared to the control group (40.2 ±1.5 mg/ml).

Globulin levels in both groups increased and decreased in the same fashion as TP levels, and the fluctuation in TP levels seemed to be the result of globulin changes. This was not the case at 14 woa, where there was a significant difference in TP levels between the groups ($p < 0.05$). Coinciding with this, there was a significant decrease in albumin concentration ($p < 0.05$) in the scrapie group (23.4 ± 0.8 mg/ml) compared to the control group (27.5 ± 0.9 mg/ml). Changes in these proteins over time are presented in Figure 1.

Acute phase protein responses in experimental classical scrapie

SAA and Hp concentrations were from undetectable to very low in the majority of samples during the study. Analytical sensitivity of the two kits used was 0.3 μg/ml for SAA and 0.005 mg/ml for Hp (according to the manufacturer). One control animal had detectable SAA response at ages 10, 12 and 18 woa, and increased Hp measurements at 10 and 14 woa. Two scrapie animals had increased levels of both SAA and Hp at 10 and 14 woa respectively. No clinical signs of disease were detectable at any of these incidences in either group. From 20 woa, SAA and Hp levels increased in the scrapie group, while remained undetectable in the control group. Measurements in serum from one of the animals in the scrapie group had only a moderate level of SAA (2.69 μg/ml) at 22 weeks and undetectable level of end stage, and Hp measurements at the same times where at the levels measured for the control group.

The four animals in the control group were sampled ten times each, and out of these 40 individual samples, only three had detectable SAA levels, and all the three samples were from the same animal (ranged 0 to 32.3 μg/ml). Of the 50 samples from the scrapie group, 39 had undetectable SAA levels, two animals had isolated incidences of elevated SAA levels at a subclinical stage of scrapie at 10 (1.54 μg/ml) and 14 (18.46 μg/ml) woa. From 20 weeks of age, there was a tendency for SAA levels to increase more dramatically until time of euthanasia (ranged 0 to 222.8 μg/ml). The individual SAA measurements are shown in dot plots representing each time point sampled in Figure 2.

Hp levels in the control group were low throughout the sampling period with a mean value of 0.11 ± 0.01 mg/ml, and the mean level in the scrapie group up until 22 weeks of age was similar at 0.10 ± 0.02 mg/ml. Thereafter the haptoglobin level increased steeply to reach a mean value of 3.0 ±1.17 mg/ml. Hp measurements are presented individually in a dot plot at each time point in Figure 3.

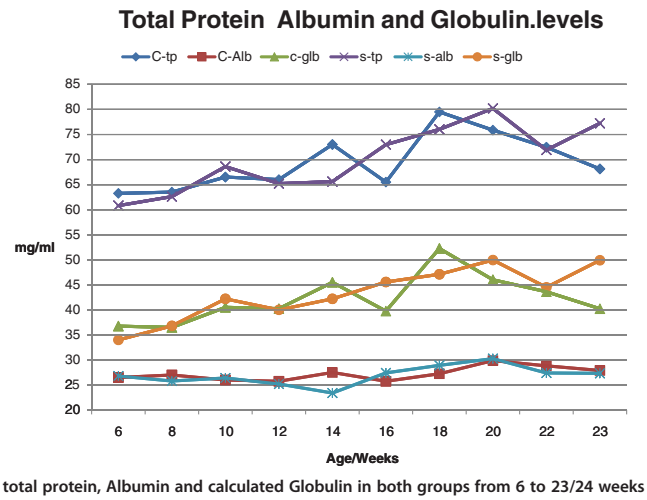

Figure 1 Serum levels of total protein, Albumin and calculated Globulin in both groups from 6 to 23/24 weeks of age. Total protein level in control group (♦ blue) and scrapie group (× purple), Globulin levels for control group (▲ green) and scrapie group (● orange), and albumin levels for control group (■ red) and scrapie group (∗ turquoise). Each time point is presented as the mean values for each group at each time of sampling.

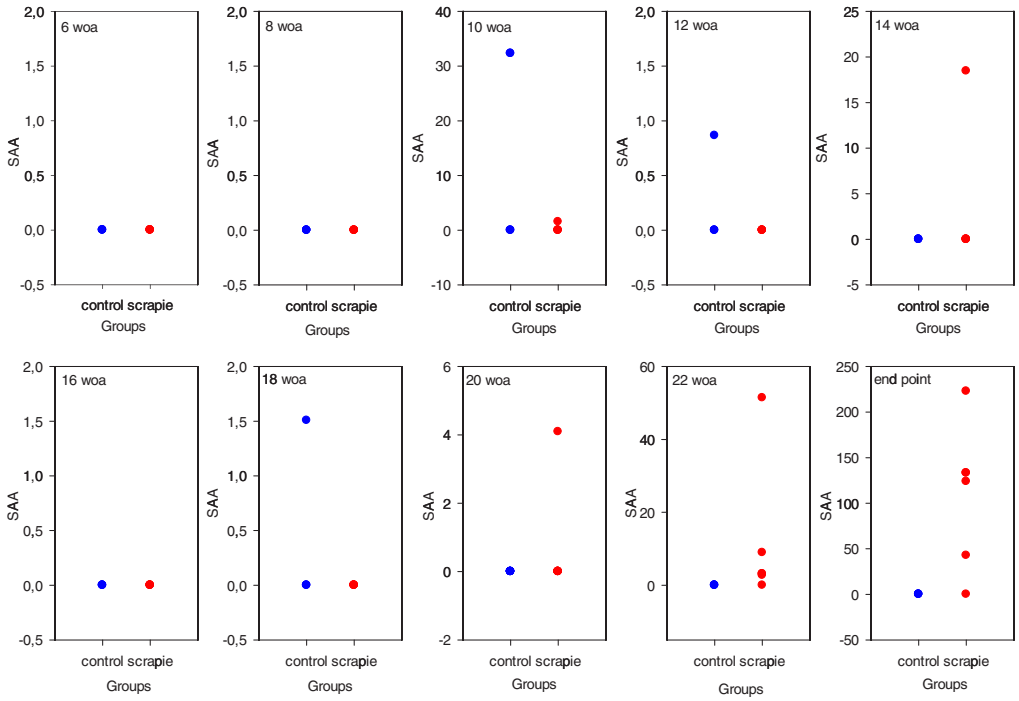

Figure 2 Comparison of difference in SAA levels in serum between healthy controls and scrapie infected sheep from six to 23/24 weeks of age. Each of the individual five scrapie samples (red dots) and four control samples (blue dots) are presented individually as a single dot to indicate the measured serum amyloid A (SAA) level which is presented on y-axis in μg/ml. At most time points, individual samples in both groups were below the detection range of the SAA kit, and thus the dots appear on top of each other as one single dot at 0.0 μg/ml.

Cp levels were slightly higher in the scrapie group throughout most of the studied period, but the most prominent difference was detectable from 22 weeks of age and onwards. During the last fortnight, the Cp levels in the control group decreased, while there was a sharp increase in the scrapie group, resulting in a significant difference ($p < 0.05$) between the two groups at time of euthanasia (31.7 ± 6.7 vs. 17.6 ± 2.0). Figure 4.

Natural cases of classical scrapie

All the natural cases of classical scrapie were confirmed by histopathological examination of the brain by typical lesions as well as detection of PrP^{Sc} [3,35]. Four out of these five animals had shown pruritus for more than three weeks, two of these developed severe neurological signs before euthanasia, and one animal was without any visible clinical signs (Table 1). TP levels in these five sheep ranged from 57.6 to 93.8 mg/ml (70.9 ± 7.1 mg/ml). Albumin levels were relatively stable for all five sheep at 22.6 ± 0.8 mg/ml, and slightly lower in one sheep (19.6 mg/ml). Calculated globulin levels ranged from 34.5 to 70.1 mg/ml (48.2 ± 7.3 mg/ml). Hp concentration was compared to the experimental control group and not found to be statistically different ($p > 0.05$). One animal had a very high level of Hp at 7.15 mg/ml, while the remaining four had a mean of 0.18 mg/ml. All the animals had detectable SAA levels, ranged from 0.43 to 273.3 μg/ml, with significant difference from the control group ($p < 0.05$). Results for all the measured serum proteins are presented in Table 2.

Evaluation Hp and SAA kit performance

The coefficients of variation for both kits are presented in Table 3. Intra-assay CV for Hp and SAA was 4.6% and 10.6%, respectively, and inter-assay CV for Hp and SAA was 0.9% and 9.1%. The limitations in these measurements were that the inter-assay CV was based on only two separate plates and the first plate mean was calculated from only one duplicate. The second mean was calculated from the same duplicate samples as used for intra-assay CV. To achieve a better estimate of the inter-assay CV, more samples and assays would be

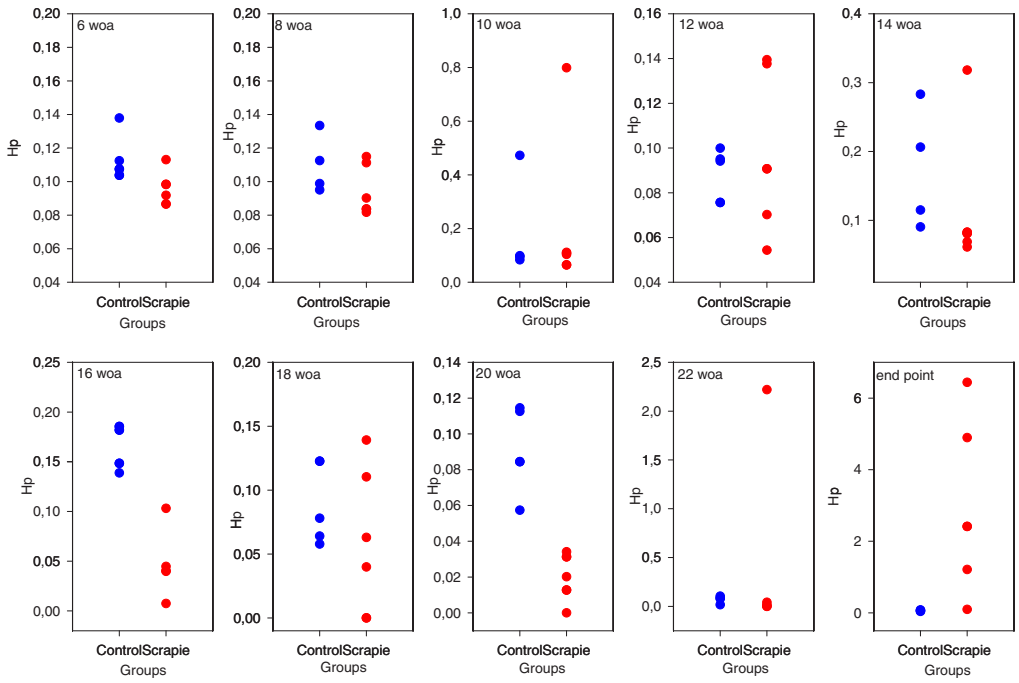

Figure 3 Comparison of difference in Hp levels in serum between healthy controls and scrapie infected sheep from six to 23/24 weeks of age. Scrapie (red dots), controls (blue dots), haptoglobin (Hp) levels measured in mg/ml indicated on y-axis and groups are presented on the x-axis. Each sample is individually plotted (four control samples and five scrapie samples), but, at some time points, the dots appear as one single measurement due to very similar values and the scale accommodates the scrapie measurements, as they show more variation and a higher levels.

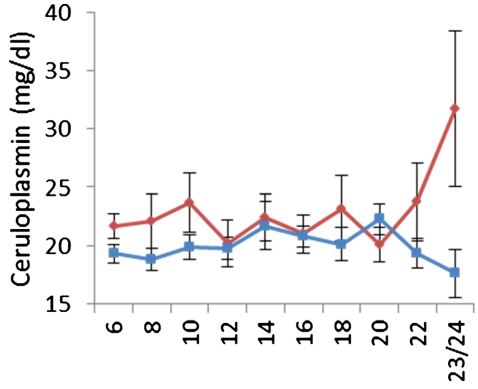

Figure 4 Comparison of ceruloplasmin (Cp) between healthy controls and scrapie infected sheep from six to 23/24 weeks of age. Scrapie (♦ red dotted line), controls (■ blue), Ceruloplasmin (Cp).

needed. The intra-assay CV was based on similar numbers of samples as presented by the manufacturer.

Discussion

In this work, we describe increased levels of certain APPs in sheep with experimental and natural cases of clinical classical scrapie. Such rise of these APPs in sheep has not earlier been published in classical scrapie. Regarding protein degradation during storage, serum samples from experimental scrapie had been stored in aliquots at −70 °C, and the sera from natural cases had been stored at −20 °C. All the samples had been stored for more than one year, and one of the natural cases was from 1994. The deteriorating effects on protein concentration in these samples are unknown, but it has been shown that albumin and total proteins levels remain stable for at least eight months in canine serum at −20 °C [36] and recently Gislefoss et al. reported non-significant differences of albumin in samples stored for 25 years, two years and one month [37]. Based on these examinations, the results achieved in this work were regarded to

Table 1 Presentation of the natural cases of classical scrapie

ID	Admitted	Sampled	Age	Genotype	Clinical presentation	Brain	Brain PrP^{Sc}
S1	10.02.94	14.02.94	6	VRQ/VRQ	Pruritic, nervous, tremors	Vacuolisation with astrocytosis	Positive
S2	20.03.96	07.05.96	5	VRQ/VRQ	Pruritic, ataxia, nervous, altered mental state	Vacuolisation with astrocytosis	Positive
S3	27.08.98	16.09.98	7	ARQ/VRQ	Pruritus with hyperkeratosis	Vacuolisation with astrocytosis	Positive
S4	14.07.99	15.09.99	2	ARQ/VRQ	Normal	Vacuolisation with astrocytosis	Positive
S5	14.02.01	23.02.01	7	VRQ/VRQ	Pruritic	Vacuolisation with astrocytosis	Positive

Overview over admission dates and time of euthanasia, age in years, PrP genotype, clinical presentation at time of euthanasia and pathognomonic histopathology of the brain of the five natural classical scrapie cases. PrP^{Sc} was identified by immunohistochemistry on sections of the brain as described by Ersdal et al. (2005) [3]

be reliable and indicative of the true protein status in the samples. Cp measurements were based on its oxidative activity, which decreases over time in storage, and therefore Cp was not measured in the naturally occurring classical scrapie cases.

As age has a major effect on the concentration of many serum proteins, it was difficult to find reference ranges based on animals in the age range of 6 weeks to six months of age [17,38-41]. Total serum protein has a wide dynamic range, thereby making analysis difficult to interpret when comparing to reference ranges developed by others. In addition, the protein levels may also be influenced by breed, management systems and analytical methods, and available reference ranges usually do not give either of these influential factors. Therefore, it may be misleading to uncritically compare results with any reference range listed. The animals used in this project were kept under very strict management system, and all the animals were managed similarly, they are of the same breed and age. Due to reasons mentioned above, the serum proteins were compared between the groups in question at same age, breed and feeding regime and management.

The total protein and albumin profile changed over time in a similar fashion to what was described by Kaneko [42], although the total protein levels in both groups were slightly to moderately higher than those reported for lambs younger than 12 months. Albumin levels were clearly lower. As albumin and globulins make up most of the total serum protein, globulin levels were higher than indicated by others [38-41]. There was a general increase in total protein from birth, combined with a minimal change in albumin and a marked increase in globulins with advancing age, reaching high levels at 18 – 20 weeks of age. At birth, the total protein is relatively low, but increase rapidly after ingestion of colostrum and maternal immunoglobulins. As globulins declined due to normal turnover, the animals begin to synthesize their own immunoglobulins, and reach adult levels of albumins and globulins in young adulthood. Total protein levels reached above average adult levels as a result of a minor decrease in albumin and progressive increase in globulins [42]. From 20 weeks of age, there were significant differences in TP levels between the groups, with a marked increase in the scrapie group. This increase was mainly due to a significant increase in the globulin fraction. As the APPs and immunoglobulins are part of the globulin fraction, this increase could be the result of increased synthesis of APPs, as seen for the three positive APPs tested in this work: Hp, SAA and Cp. Several studies have failed to describe a significant increase in immunoglobulins at clinical classical scrapie, especially scrapie-specific IgG [43-45].

Table 2 TP, Alb, Glb, Hp and SAA measurements in the five natural classical scrapie cases at time of euthanasia

ID	Tp (mg/ml)	Alb (mg/ml)	Glb (mg/ml)	Hp (mg/ml)	SAA μg/ml
S1	64.2	23.7	40.5	0.29	273.30
S2	80.5	19.6	60.9	7.15	1.31
S3	93.8	23.7	70.1	0.18	8.55
S4	58.2	23.7	34.5	0.16	0.43
S5	57.6	22.5	35.1	0.07	0.43
C				0.06	-

Level of different proteins in serum from five cases of naturally occurring classical scrapie. Hp and SAA levels were compared to measurements of the oldest animals in the control group. The "-" indicates that measured levels were below detection range of test.

There are very few available reference ranges for APPs in sheep, but recently Lepherd *et al.* [39] published good reference ranges for SAA and Hp in young sheep. Although these reference intervals were from a different breed and sheep kept under different management systems, they were in the same age range and seemed appropriate in that manner. Skinner and Roberts [32] evaluated Hp levels in a variety of conditions, both infectious and non-infectious, in addition to healthy sheep, and concluded that Hp values above 0.2 g/l were "positive". It is also interesting to see that 7% of the randomly selected healthy sheep had a positive Hp value, which could indicate the presence of subclinical conditions. In other studies, levels of SAA below 2 μg/ml (range 0 to 29.4) have been reported in clinical healthy sheep [33,39]. APPs are often reported as undetectable to very low levels in healthy individuals, and subclinical conditions would often lead to a temporary elevation before

Table 3 Intra- and inter-assay coefficient of variation of Hp and SAA kits

	Intra-assay CV		Inter-assay CV		
	n	Average % CV	Mean of means	SD of means	% CV of means
Hp	7	4.6	11.62	0.10	0.9
SAA	13	10.6	22.28	2.04	9.2

CV: Coefficient of variation; n: number of duplicate samples; SD: Standard Deviation; Hp: haptoglobin; SAA: Serum amyloid A.

decreasing to resting levels. Thus the moderate increase in SAA and Hp seen approximately half-way through the incubation period in two animals in the scrapie group, and one control, could be due to a subclinical condition. As no clinical signs of disease were detected in any of the animals at this time, it is difficult to rule in or out any specific conditions, including scrapie. There is a possibility that the increase in SAA and Hp in the two scrapie infected animals at 10 and 14 weeks of age could relate to the expected PrP^{Sc} dissemination through peripheral lymphoid tissue that is seen about half-way through the incubation period in this experimental model. This would need further testing to confirm. Batxelli-Molina et al. (2010) reported transthyretin to discriminate scrapie affected sheep from healthy sheep, both during early asymptomatic phase and later during the symptomatic phase, although only significant at the late stage [46]. Transthyretin is a negative APP, and thus decreased levels would be an expected finding along with increased levels of positive APPs during an APR. The clear and significant increase in SAA, Hp and Cp measured at the clinical end stage indicate a detectable APR at this stage of classical scrapie, both in the experimental and the natural cases. This coincides well with the pathological changes seen on histopathological examination of the brain and the onset of severe clinical disease seen at this late stage in this experimental model. APPs levels are reported to be directly related to severity of both tissue damage and level of inflammation, i.e. they are specific markers for tissue damage and inflammation [25]. Pro-inflammatory cytokines, like interleukin 6 (IL-6), interleukin-1 (IL-1) and tumor-necrosis factor α (TNF-α), are released by damaged tissues locally and into the circulation, where more inflammatory cells are activated. These responses result in production of more cytokines and inflammatory mediators circulating in the blood acting on different target organs/cells leading up to a systemic reaction with alteration in synthesis of APPs in the liver and activation of the hypothalamic-pituitary-adrenal (HPA) axis [30]. This stimulation will lead to increased production of cortisol by the adrenal glands, which has a negative feedback effect on this axis. This stimulation of the HPA axis could contribute to the hypercorticism with increased cortisol, 20β-dihydrocortisol and cortisone detected in plasma and urine of sheep with subclinical and clinical scrapie that has been reported with a certain discriminating level [47-49]. Cytokines induce production of adrenocorticotrophic hormone (ACTH) which in turn stimulates synthesis of the corticosteroid, cortisol [50,51]. This is an important regulatory feature in inflammatory and cytokine responses, as adrenalectomized rodents show increased mortality in experiments involving injection of bacterial lipopolysaccharide, IL-1 or TNF-α. After administration of glucocorticoids, these mice survived. Thus by regulating cytokine production and action, HPA axis contributes to modulation of the inflammatory response [52].

SAA and Hp are characterised as major acute phase proteins (APPs) in sheep and these are known to increase with severity of tissue damage and level of inflammation. Interestingly, the SAA response in these experimental cases of scrapie are higher than levels reported for mulesing and caseous lymphadenitis (CLA) [29,31], and more in the region of experimental mastitis [33]. It is tempting to think that the APP levels in the naturally affected animals were related to extent of histopathological changes in the brain, as the two animals with clear neurological signs had highest levels of APP. The others with much lower levels of APP presented with pruritus as the main clinical sign, although all of them had pathological changes in the brain. It is also worth noticing the clear increase in the globulin fraction of the TP in three out of the five natural cases which was outside the reference range [42]. Relating these results to clinical findings, these three sheep were presented with the most severe clinical signs. Albumin level in the worst clinically affected sheep were even lower than the others, perhaps due to the on-going APR. This leads to the thought that the systemic effects of scrapie was related to severity of clinical signs and could be measured in serum by SAA, Hp and Cp levels. Measuring APP levels in serum may provide objective information about the extent of the on-going process, magnitude and duration may reflect the severity [22]. Even though the APR is a non-specific response, it is a feature of clinical scrapie, and due to the high sensitivity of the APR, measurements of APPs could be useful in evaluation of the underlying pathological processes and the systemic involvement of scrapie.

APPs respond rapidly to insults and the majority peak about one day after the initial insult before returning to

resting levels within a week due to the fact that most APPs have half-life of only 24 to 48 hours [25]. This means that some important time points may have been missed. Even though this experimental model gives fairly predictive and consistent development of classical scrapie and clinical signs at around the same time, individual differences may result in individual APP expression profiles. A different sampling strategy with more frequent blood sampling could detect more subtle changes in the APP profile of scrapie affected sheep throughout the incubation period.

The exact underlying cause or the actual triggering factors behind the detectable increase in APPs seen in this study remains unknown, but this response coincides well with the known accumulation of PrP^{Sc} in the brain, astrocytosis and vacuolar changes seen at this late clinical stage in VRQ/VRQ animals [3,4,34]. Several murine models of classical scrapie show that pro-inflammatory cytokines like IL-1, IL-6 and TNF-α, have increased expression at both mRNA and protein levels in brain, peripheral lymphoid tissue and serum at the terminal end stage [11,12,14,15]. Microglia produce pro-inflammatory cytokines like IL-1, IL-6 and TNF-α upon activation, and these are stimulators of the production of most APPs [19,22,53]. Newsom et al. (2011) reported recently quite an extensive list of proteins with altered expression in brain, lymphoid tissue and serum both pre-clinically and clinically in a murine model [14]. Volkel et al. (2001) investigated plasma samples from patients with CJD and found significant increase in both C-Reactive Protein (CRP) and Interleukin-6 (IL-6) compared to healthy controls, and concluded that these could be assessed for cell damage and inflammation in similar fashion to other markers[54]. Coe et al. (2001) showed in an experimental scrapie murine model increasing plasma levels of serum amyloid P with the onset and progression of clinical signs [55]. There exist differences in expression patterns both in the brain and peripheral in the different murine models, and as postulated by Newsom et al, this could be attributable to the animal model used [14]. There are only a couple of records of APPs being of significance in scrapie in sheep, serum transthyretin and urinary α-1Antichymotrypsin have been found to discriminate healthy from scrapie infected both at pre-clinical and clinical stage of scrapie [46,56].

Conclusion

Based on the results from these different murine models and the two reports on sheep, our results are of great relevance as we described increased levels of the major APPs in sheep which to our knowledge has not been published in relation to scrapie in sheep. It is also interesting to see that in an experimental model as the one used here, where PrP^{Sc} dissemination, pathological changes and onset of clinical disease are expected to show little between individual differences, there is a great variation in quantitative expression of the APPs.

Due to the low number of animals in this study and the lack of disease specificity of the acute phase response, these findings will need to be further evaluated in larger groups to establish the expected dynamic range of APPs in classical scrapie and other neurological and non-neurological diseases of sheep. Comparable conditions of interest are listeriosis, cerebrocortical necrosis, focal symmetrical encephalomalacia and tick born fever and encephalitis.

Methods

Experimental classical scrapie model

Nine Norwegian Rygja lambs with the same PrP genotype (homozygous $V_{136}R_{154}Q_{171}$) were inoculated through a stomach tube with 1 gram of pooled brain material immediately after birth and before ingestion of colostrum as described by Ulvund et al [34]. Four lambs in the control group were inoculated with brain material originating from healthy scrapie-free sheep of the same genotype and PrP^{Sc} negative, and five lambs were inoculated with brain material originating from confirmed cases of classical scrapie. The lambs in both groups were kept with their dams for the whole time length of the project until euthanasia at 23–25 weeks of age. As the control group was born about two weeks before the animals in the scrapie group, animals in the control group were 25 weeks old at euthanasia, while the scrapie group were only 23 weeks old. Each dam and offspring were kept isolated in confined units without any contact with other animals, and the scrapie group was under video surveillance. Both groups were kept under similar conditions and feeding regimes. Animal experiments were approved by the Norwegian Animal Research Authority.

Blood sample collection

Non-fasting blood samples were collected into 10 ml plain tubes (Terumo Venoject®) every fortnight from six woa until euthanasia at 23/25 weeks of age, for a total of ten times. Blood samples were allowed to clot at room temperature for a minimum of 30 minutes and maximum 60 minutes before processing. Serum was pipetted in aliquots and frozen at −80 °C within two hours of sampling. All the samples were subjected to the same handling procedures throughout the experiment. Serum was used for biochemistry, Hp and SAA concentration analysis.

Natural cases of classical scrapie

During the early 1990s and until 2005, several cases of suspected classical scrapie from farms in the surrounding area were received at the Norwegian School of

Veterinary Science, Section for Small Ruminant Research, for diagnostic purposes. The cases were admitted for clinical examination, blood sampling, genotyping and diagnosis. Sometimes these sheep were kept under video surveillance for some time while in isolation, before euthanasia and histopathological examination of the brain. Blood sampling was performed at least 48 hours after transport. Serum samples from five of these cases, stored at - 20 °C, were used for protein analysis. Table 1.

Protein measurement

Serum total protein was measured with ABX Pentra Total Protein (TP), ceruloplasmin (CP) and albumin (Alb) with ABX Pentra Albumin CP, both kits from Horiba ABX Diagnostics (Montpellier, France). The oxidase activity of Cp in serum was determined using a colorimetric enzyme assay where oxidation of p-phenylenediamine dihydrochloride was measured and thereby estimating the Cp concentration in mg/dl. All three measurements were adapted for COBAS MIRA Plus equipment (Roche Diagnostics, Basel, Switzerland) and routinely performed in the laboratory.

Globulin concentration was calculated by subtracting albumin concentration from total protein measurement.

Serum Hp concentrations were determined manually using the Phase™ Haptoglobin Assay kit (Tridelta Development Limited, County Kildare, Ireland). All the samples were run in duplicates, and the mean of each duplicate was used to determine final concentration. The analyses were run according to manufacturer's instructions and immediately read at 600 nm on a Multiskan* GO Microplate Spectrophotometer using SkanIt Software 3.2, both from Thermo Fisher Scientific (Waltham, MA, USA). Samples with signal greater than the highest standard were diluted and re-run until all the signals fell within the linear part of the standard curve.

SAA concentrations were determined using Phase™ Range Multispecies SAA ELISA kit (Tridelta Development Limited, County Kildare, Ireland). Sera were initially diluted 1:100 in Diluent buffer, and samples with signals greater than the highest standard were further diluted and re-run until all the signals fell within the linear part of the standard curve. Some samples were diluted 1:1000. The only automated part of the procedure was the washing steps, using the ELx50 Microplate Strip Washer (BioTek Instruments, Inc., Winooski, VT, USA). The absorbance was read on the Multiskan* GO Microplate Spectrophotometer using SkanIt Software 3.2 at 450 nm using 630 nm as a reference.

Statistical analysis

The results were adjusted for age such that the groups were compared at the same animal age. At end point comparison, the lambs of the scrapie group were 23 weeks old and the control group was 24 weeks of age.

Significant difference ($p < 0.05$) of protein measurements between the two groups at each sampling was determined by calculation of the 95% confidence interval for the difference in population medians. A non-parametric test, Wilcoxon signed-rank test, was used for calculation of confidence interval and p-values, as normal distribution of data could not be assumed due to small sample size. SigmaPlot Version 12.0 (Systat Software Inc., Erkrath, Germany) and Microsoft Excel 2010 were used.

Reference range used and evaluation of assay performance

As no reference ranges for any of the proteins evaluated here were available for Norwegian sheep breeds, and especially not for lambs in the age range of six to 24 weeks, the level of proteins in the scrapie group was evaluated in reference to the control group. The protein levels (TP, Alb and Glb) in the natural cases were evaluated in relation to reference ranges provided by Kaneko [42], this due to the great age difference to the experimental control group. HP and SAA levels were compared to the experimental control group at 24 woa.

Assay performance was evaluated by intra- and inter-assay coefficients of variation (CV). These were calculated by dividing the standard deviation by the mean of the same set of measurements and thereafter converting it into percentage. The intra-assay CV for SAA was calculated from 13 duplicate samples by averaging the CV (%) for each of the individual duplicates. Intra-assay CV for Hp was calculated in the same manner, based on seven duplicates. Inter-assay CV was calculated from the average CV value from two assays run six months apart. One assay was the same as used for intra-assay CV calculations and the other only contained one duplicate sample.

Abbreviations

TSEs: Transmissible Spongiform Encephalopathies; PrP^{C}: Normal Cellular Prion Protein; PrP^{Sc}: Scrapie Prion Protein; APP: Acute Phase Protein; APR: Acute Phase Response; TP: Total Protein; Alb: Albumin; Glb: Globulin; Cp: Ceruloplasmin; Hp: Haptoglobin; SAA: Serum Amyloid A; BSE: Bovine Spongiform Encephalopathy; CJD: Creutzfeldt-Jakob Disease; HPA: Hypothalamus-Pituitary-Adrenal; CLA: Caseous Lymphadenitis; IL: Interleukin; TNF: Tumor Necrosis Factor; ACTH: Adrenocorticotropic Hormone; ROS: Reactive Oxygen Species; woa: Weeks of age; IHC: Immunohistochemistry; WB: Western Blot; CV: Coefficient of variation; SD: Standard Deviation; SEM: Standard Error of Mean.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We are grateful to Eli Brundtland, Siri Hamre and Wenche Okstad for excellent help with serum handling and protein analysis, and to Cecilie Ersdal for sharing her expert knowledge and thoughts on scrapie in sheep

with us. This study was supported by grant NRC 178268 (2006–2010) funded by the Research Council of Norway.

Authors' contributions

SM carried out the protein measurements, statistical data analysis and drafted the manuscript. KB participated in the design of the study, sample preparation and helped to draft the manuscript. MJU participated in the design and coordination of the study, sample collection and helped to draft the manuscript. All authors read and approved the final manuscript.

Received: 20 March 2012 Accepted: 17 July 2012
Published: 17 July 2012

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doi:10.1186/1746-6148-8-113
Cite this article as: Meling *et al.*: Presence of an acute phase response in sheep with clinical classical scrapie. *BMC Veterinary Research* 2012 **8**:113.

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Meling et al. BMC Veterinary Research (2018) 14:281
https://doi.org/10.1186/s12917-018-1607-9

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Expression of selected genes isolated from whole blood, liver and obex in lambs with experimental classical scrapie and healthy controls, showing a systemic innate immune response at the clinical end-stage

Siv Meling[1*], Kerstin Skovgaard[2], Kjetil Bårdsen[1], Peter Mikael Helweg Heegaard[2] and Martha J. Ulvund[1]

Abstract

Background: Incubation period, disease progression, pathology and clinical presentation of classical scrapie in sheep are highly dependent on *PRNP* genotype, time and route of inoculation and prion strain. Our experimental model with pre-colostrum inoculation of homozygous VRQ lambs has shown to be an effective model with extensive PrP^{Sc} dissemination in lymphatic tissue and a short incubation period with severe clinical disease. Serum protein analysis has shown an elevation of acute phase proteins in the clinical stages of this experimental model, and here, we investigate changes in gene expression in whole blood, liver and brain.

Results: The animals in the scrapie group showed severe signs of illness 22 weeks post inoculation necessitating euthanasia at 23 weeks post inoculation. This severe clinical presentation was accompanied by changes in expression of several genes. The following genes were differentially expressed in whole blood: *TLR2, TLR4, C3, IL1B, LF* and *SAA,* in liver tissue, the following genes differentially expressed: *TNF-a, SAA, HP, CP, AAT, TTR* and *TF,* and in the brain tissue, the following genes were differentially expressed: *HP, CP, ALB* and *TTR.*

Conclusions: We report a strong and evident transcriptional innate immune response in the terminal stage of classical scrapie in these animals. The *PRNP* genotype and time of inoculation are believed to contribute to the clinical presentation, including the extensive dissemination of PrP^{Sc} throughout the lymphatic tissue.

Keywords: Classical scrapie, Innate immune response, qPCR, Whole blood, Liver tissue, Brain tissue, Sheep

Background

Prion diseases are a group of diseases with common pathology of the central nervous system including neuronal degeneration, vacuolation and gliosis [1]. The causative agent is an infectious protein, called the prion (PrP^{Sc}), which is an abnormal isoform of the cellular prion protein, PrP^{C}, found in many cell types in the body [2]. Prion diseases are also named Transmissible Spongiform Encephalopathies (TSEs), and scrapie is the TSE that affects sheep and goats. Sheep can become infected early in life, around and soon after birth, from infectious prions in foetal fluids and membranes and bodily secretions like colostrum/milk and faeces [3–6].

Susceptibility to classical scrapie in sheep is highly dependent on polymorphism at codons 136, 154 and 171 of the PrP gene (*PRNP*). The five possible alleles, ARR, AHQ, ARH, ARQ and VRQ, give rise to 15 combinations found in sheep, and these are also linked to the different presentations of classical scrapie [7]. These genotypes differ widely in their susceptibility to scrapie, from extreme susceptibility in homozygous $V_{136}R_{154}Q_{171}$ genotype, to very low susceptibility in homozygous $A_{136}R_{154}R_{171}$ [8–10]. The PrP genotype not only influences the degree of susceptibility, but also the course of

* Correspondence: sivmeling@lyse.net
§Corresponding author
[1]Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Sandnes, Norway
Full list of author information is available at the end of the article

disease, incubation period and clinical picture [7, 11]. This genetic susceptibility is the basis behind the breeding for resistance program successfully implemented in the EU as per EC Regulation 999/2001, where only ewes carrying at least one ARR allele and no VRQ allele, and rams of ARR/ARR genotype, are bred from [12]. Several member states have implemented breeding programs to select for resistance to TSEs in their ovine population, resulting in a marked increase in ARR frequency and a decreased VRQ frequency. There are now reports of a shift in *PRNP* genotypes in national flocks after years of breeding from low susceptibility genotypes [13–16]. Thus, the prevalence of classical scrapie at population level decreased as highly susceptible genotypes are eliminated.

Presentation and progression of disease are influenced by *PRNP* genotype, PrP^{Sc} strain, and route of inoculation. Lateral transmission via the oral route is the most important and commonest route of infection, and in a flock where scrapie is endemic, new-born lambs become infected at birth from their infected dam and/or from the contaminated environment [17]. After oral infection, prions seem to be taken up across the gastrointestinal mucosa by M-cells, and dendritic cells (DCs) and possibly macrophages are involved in the spread to the lymphoreticular system (LRS). Follicular dendritic cells (FDCs) within the LRS, are the site of prion replication, but the extent and speed of lymphatic involvement is dependent on *PRNP* genotype [18]. Sheep carrying the least susceptible ARR allele show minimal PrP^{Sc} deposition within the LRS, while carriers of the VRQ allele are associated with extensive PrP^{Sc} deposition [19–24]. When lambs with the most susceptible genotype are orally challenged with scrapie infection, PrP^{Sc} is detected very early in lymphoid tissues associated with the gastrointestinal tract, and is followed by dissemination of PrP^{Sc} to other, non-gastrointestinal, lymphoid tissues [24–26]. Due to this early PrP^{Sc} accumulation in lymphatic tissue in certain genotypes, the European Food Safety Authority considers young stock, less than six months of age and from TSE infected flocks, as potentially highly infectious [27].

The pathogenesis and progression of TSE in different species is still not fully understood, and sheep with classical scrapie is a good model to study the disease mechanisms. Pre-colostrum inoculation of lambs at birth is an effective model that has shown to produce severe clinical disease after a relatively short incubation period [28]. One can say, our model produces a "worse-case scenario" of prion disease, where the genotype, donor material and time of infection are optimal. Ersdal and co-workers reported a study of PrP^{Sc} uptake and dissemination in an earlier experimental model, where older lambs where orally inoculated, with the same VRQ-prion donor material, between 46 and 61 days of age [22]. Later, this experimental model was repeated, only this time the lambs where inoculated at birth, and produced a much shorter incubation period and more severe clinical disease [28]. Both these models revealed an early and strong dissemination of PrP^{Sc} in lymphatic tissue in combination with relatively moderate cytopathogenic changes in the CNS in VRQ/VRQ animals. We have earlier published our findings of an acute phase response in lambs inoculated at birth, evident at serum protein level [29]. In the present study, transcriptional changes of the above lambs were (further) studied. This article describes the expression of a selection of genes in RNA isolated from whole blood, liver and posterior obex of healthy and scrapie affected lambs.

Many genes are expressed by leukocytes in the blood as a systemic response to pathology in peripheral tissues. Injured and diseased tissues and organs will alter the gene expression profile in blood leukocytes and in the liver, which is the main producer of acute phase proteins associated with an innate immune response. Several studies of gene expression in circulating blood cells have revealed expression profiles characteristic for a wide variety of diseases [30–32]. Gene expression studies of whole blood can thus improve our knowledge of disease related processes regardless of which tissue is primarily affected. White blood cells have an important role in the immune system, and, although scrapie is known to not produce a specific immune response per se, cells of the monocyte line seem to play an important role in the uptake from the gut and dissemination of PrP^{Sc} to lymphoid organs and further into the CNS resulting in gliosis and astrocytosis [20, 22].

Collection and stabilization of total RNA from whole blood using PAXgene Blood RNA Tubes (BD Biosciences) offer a robust and simple method that minimize sampling-to-sampling differences. PAXgene technology capture the RNA profiles at the time of collection of all cell types in whole blood, including peripheral blood monocytes, lymphocytes, erythrocytes/reticulocytes, granulocytes and platelets [33].

The aim of this study was to investigate how scrapie infection influences the gene expression profile of selected genes in whole blood during the preclinical incubation period and through to the terminal clinical end stage. Further, the gene expression patterns were studied in liver and posterior obex at the terminal end stage.

Methods

Animals

The animals and experimental model used in this project were described in detail previously, and all animal procedures were approved by the Norwegian Animal Research Authority [28, 29]. Briefly, nine lambs were inoculated by stomach tube, at birth and before colostrum intake, with pooled brain material from either healthy,

scrapie free sheep (control group) or from confirmed cases of classical scrapie (scrapie group). All the lambs originated from the commercial sheep flock belonging to the Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Production Animal Clinical Sciences, Section of Small Ruminant Research, 4325 Sandnes, Norway. Within this flock, a number of animals have selectively been bred to maintain the homozygous $V_{136}R_{154}Q_{171}$ *PRNP* genotype. The control group consisted of two twins (four lambs) and the scrapie group consisted of triplets and twins (five lambs). The lambs and their dams were housed individually until euthanasia at 23 weeks of age. All the animals were inspected daily, and the scrapie group was under video surveillance. Euthanasia was performed by an intravenous injection of sodium pentobarbital, using the jugular vein.

Blood and tissue collection and RNA isolation

Blood samples were collected at regular intervals from the jugular vein into appropriate blood tubes, depending on the requirement for the different analyses. Whole blood (2.5 mL) was collected at 12, 14, 16, 20, 22 and 23 weeks post inoculation (wpi) in PAXgene Blood RNA Tubes (Qiagen/BD Company). All the samples were handled according to manufacturer's instructions and stored at minus 70 °C until analysis.

Brain and liver tissues were removed immediately post mortem and placed in RNA*later* (Qiagen) prior to storage at minus 70 °C. Total RNA was extracted and purified from stabilized blood samples manually, using PAXgene® Blood miRNA Kit (Qiagen/BD Company) according to manufacturer's instructions using the protocol for manual purification. The RNA eluate was immediately stored at minus70°C.

Total RNA was isolated from 20 to 25 mg tissue sections, lysed and homogenized, from the posterior obex region in the brain and from the liver using RNeasy® Lipid Tissue Midi Kit and (Qiagen) and RNeasy® Lipid Tissue Mini Kit (Qiagen), respectively, including digestion of DNA using RNase-Free DNase kit (Qiagen).

Total RNA was quantified by UV spectrometry at 260 nm using a NanoDrop ND-1000 spectrophotometer (Saveen and Werner AB, Limhamn, Sweden). In addition, for each sample 260/280 and 260/230 ratios were provided, enabling RNA sample purity estimations. RNA integrity was evaluated using the Agilent® 2100 Bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany), according to manufacturer's instructions. Each total RNA sample was assigned an RNA Integrity Number (RIN) ranging from 1 to 10. The mean and standard deviation of all the RIN values were calculated and are shown in Additional file 1.

Target genes

Two different primer pairs were designed to amplify different regions of each gene to ensure high quality results. Sequences used for primer design were obtained from public database (GenBank, NCBI) and are listed together with reaction conditions in Additional file 2. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and synthesized at TAG Copenhagen A/S (Copenhagen, Denmark). BLAST searches were performed to confirm gene specificity of the primer sequence, and to show the absence of polymorphisms at the primer site. Whenever possible, only sequences from *Ovis aries* were used, if not, sequences specific for *Bos taurus* or *Capra ibex* were used. Further, NetPrimer software available at http://www.premierbiosoft.com was used to obtain a rating value for each primer pair.

High-throughput qPCR

Two separate technical replicates of complementary DNA (cDNA) were synthesised from each RNA sample using 300 ng of total RNA using the QuantiTect reverse-transcription kit (Qiagen®/BD Company), according to the manufacturer's instructions. All samples were diluted, with a dilution factor of 0.13, in low EDTA TE-buffer (VWR, APLIA8569.0500) in a total solution of 20 μl.

Custom-designed primers (TAG Copenhagen, Copenhagen, Denmark) were diluted to 100 μM. For preamplification and qPCR, each primer pair, consisting of forward and reverse primers was further diluted in low EDTA TE-buffer (VWR, APLIA8569.0500) to reach a final concentration of 20 μM. Then a primer mix was created from 5 μl of each 20 μM primer pair and low EDTA TE-buffer in a 500 μl, 200 nM solution. A PreAmp mix, consisting of 5 μl TaqMan PreAmp Master Mix (Applied Biosystems, PN 4391128) and 2.5 μl 200 nM primer mix, was added to 2.5 μl of each cDNA sample. This mix was incubated under thermal cycling conditions as follows; initially held at 95 °C for 10 min, followed by 16 pre-amplification cycles of 15 s at 95 °C and 4 min at 60 °C, before returning to 4 °C. Before storage at − 20 °C, each sample was diluted 1:4 in low EDTA TE-buffer. The liver samples were optimised by a second run, L2; using a 1:10 dilution.

High-throughput qPCR was performed in 48.48 Dynamic Arrays using the BioMark thermocycler (Fluidigm Corporation, CA, USA) following the manufacturer's protocol. The 48.48 dynamic array enables 2304 separate and simultaneous qPCR reactions from 48 samples and 48 primers in one operation. Each 48.48 dynamic array was prepared with control line fluid and primed in an IFC Controller MX (Fluidigm Corporation, CA, USA) while assays and samples were prepared. The Assay

master mix (2.75 μl), consisted of 2.5 μl loading reagent (85,000,800 Sample and Assay Loading kit, Fluidigm) and 0.25 μl low EDTA TE-buffer, were combined with 2.3 μl 20 μM of each specific primer pair before loading each corresponding assay inlets on the 48.48 dynamic array. The Pre-sample mix (4.5 μl), consisting of 3 μl TaqMan Gene Expression Master mix (Applied Biosystems, PN 4369016), 0.3 μl DNA binding dye (100–0388, Fluidigm), 0.3 μl EvaGreen (VWR, BTIU31000) and 0.9 μl low EDTA TE-buffer, and 1.5 μl preamplified cDNA sample were added to each corresponding sample inlets on the 48.48 dynamic array. The 48.48 dynamic array was then returned to the IFC Controller to load samples and assays into the Integrated Fluidic Circuit of the dynamic array. The 48.48 dynamic array was placed in the BioMark HD instrument for qPCR with a ten-minute hot start phase at 95 °C followed by 35 cycles of denaturing at 95 °C for 15 s and annealing/elongation at 60 °C for 1 min. Melting curves were generated after each run to confirm the presence of a single PCR product (from 60 °C to 95 °C, increasing 1 °C/3 s). Non-template controls, non-reverse transcriptase controls and three interplate calibrators were included on each dynamic array if data from multiple dynamic arrays were analysed.

Data collection and pre-processing

RT-qPCR data was collected using Fluidigm Real-Time PCR Analysis Software Version 3.0.2 (Fluidigm Corporation, CA, USA). Melting curves and non-template control (NTC) were used to monitor non-specific amplification and sample contamination. Non-reverse transcriptase controls were used to assess potential genomic DNA contamination. Each sample was examined to confirm a single PCR product, and lack of such product resulted in the rejection of that quantification cycle (Cq) value.

Expression data were pre-processed and analysed using GenEx Pro Version 5.3.6 (MultiD Analyses AB, Goteborg, Sweden). Pre-processed data were separated into four data subsets (blood, obex, liver1 and liver2) according to type of sample and time of analysis, and data analysis was performed with GenEx Pro Version 5.3.6 in the following order: 1. Initial visual evaluation of melting curves and amplification curves; 2. Efficiency correction. PCR amplification efficiency was established by the means of calibration curves and determination of the slope (S) of the log-linear portion. The calibration curve was constructed from dilution series (4 × 4) using a pool consisting of 1 μl of each sample tested, and diluted into following relative concentrations: F1 0.333, F2 0.067, F3 0.0133 and F4 0.00267. Both slope value (m) and correlation coefficient R^2 for each assay was determined using the above. Efficiency value (E) was calculated as follows: $E = 10^{1/m} - 1$, and used for the efficiency correction of each primer assay individually in GenEx. Any assay with E values less than 0.8 or higher than 1.1, and R^2 values less than 0.95, were discarded and excluded for further analysis; 3. Inter-plate calibration (IPC): based on the three interplate calibrators; 4. Normalization to reference genes. Six different genes (*RPLP0*, *PPIA*, *UBC*, *HPRT1*, *GAPDH* and *SDHA*) were evaluated using GeNorm and NormFinder incorporated in GenEx; 5. Averaging technical cDNA replicates. Before cDNA technical replicates were averaged, the data set was inspected for level of variation between replicates. A maximum of 20% of difference in Cq between the two replicates was accepted.; 6. The relative expression of each sample analysed, was calculated using the mean Cq value of the control croup as a base line. Any sample with higher Cq (lower expression) than mean Cq of control group, get a value below 1, and any sample with lower Cq (higher expression) than mean Cq of control group, get a value above 1. This was done for each primer assay individually; 7. Finally, the data was converted into logarithmic scale, using $\log_2$ transformation for all statistical analysis. Fold change was calculated by scaling the relative expression data. A scaling factor was calculated based on the average relative expression (fold change) in the control group was scaled to 1. Individual expression data was multiplied by the scaling factor, giving individual fold change. Average fold change and standard error of the mean (SEM) was calculated for each group/primer assay. When relative expression (RE) was below 1, it was translated to a negative fold change value using $-2^{-(\log_2 RE)}$, i.e. a relative expression of 0.8, gave a fold change of − 1.2.

Statistical analysis

The data were analysed separately using statistical features of GenEx software (MultiD). The pre-processed data from the qPCR analyses was separated into subsets of data, based on time and type of sampling. The non-parametric statistical model, Mann-Whitney U Test, was used to calculate *p*-values between scrapie and control groups in each of the data subsets, and significant p-value was set to $p < 0.05$. Due to the small group size, p-values should be interpreted with some caution. Genes were considered as differentially expressed if they showed a fold change of ≥1.5 and reached significant p-values.

Results

Animals

Animals in the control group remained healthy without any clinical symptoms for the full experimental period. No obvious clinical signs of illness were visible in the scrapie group until 22 wpi, although subtle signs of unspecific ill-thrift were observed in two animals from 19 wpi. By the end of 22 wpi., all the animals in the scrapie group showed signs of disease, such as weakness, dullness and

lethargy. Two animals in the infected group displayed an accelerated worsening of clinical signs to terminal recumbency and severe depression. This sudden change, over a few days, made euthanasia necessary. Brain tissue from all the animals in both groups was examined for PrP^{Sc} by immunohistochemistry (IHC) and Western Blot (WB), and only the animals in the scrapie group were found PrP^{Sc}-positive (Fig. 1). There were moderate vacuolation of neuropil and neurons with marked distribution of PrP^{Sc} deposits in the CNS, and PrP^{Sc} was found to be widely distributed in lymphoid organs (spleen and retropharyngeal lymph nodes), consistent with previous findings [28].

Gene expression analysis in blood

Statistically differentially expressed genes, fold change and *p*-value are summarized in Table 1. The magnitude of the fold change at each time points, reflects the gene expression alterations relative to the mean level of the control group scaled to 1. Of the 10 target genes analysed after pre-processing, only six were significantly differently expressed in the scrapie group compared to the control group at any time point. No genes were differentially expressed at 12 nor 14 wpi. *IL1B* was differentially expressed at 16 and 22 wpi, and *TLR2, TLR4, C3* and *SAA* were differentially expressed at 22 and 23 wpi. *LF* was only differentially expressed at 23 wpi. Apart from *IL-1B* which was downregulated at 16 wpi, all the others were upregulated. Expression levels of *TLR9, IL1RN, IL8* and *LR/LAMR1* were not significantly altered at any of the time points. (Table 1).

Gene expression analysis in liver at 23 wpi

Due to the small group size, only descriptive analyses were performed. Differentially expressed genes of the scrapie group relative to the control group with a fold change of ≥1.5 can be seen in Table 2. The fold change value is relative to the mean expression level in the control group, set as value 1. Of the 12 target genes analysed after pre-processing, seven genes were expressed with fold change ≥1.5 (Table 2). Three genes were upregulated (*SAA*, *Hp* and *Cp*) and four were downregulated (*TNFa*, *AAT*, *TF* and *TTR*).

Gene expression analysis in brain at 23 wpi

Gene expression differences in brain tissue are listed in Table 3. Out of 12 target genes analysed after pre-processing, only four were expressed with fold change ≥1.5. *Hp* and *Cp* were upregulated, and *ALB* and *TTR* were downregulated.

Discussion

Classical scrapie is known to present with a variety of clinical signs, and clinical presentation is dependent on *PRNP* genotype, PrP^{Sc} strain, infective dose and age of host at infection. Our experimental model resembles natural infection and illustrates the "worst-case" scenario which can occur naturally when the right conditions are present. Clinical signs of disease in the scrapie group was first observed at 19 wpi. The symptoms aggravated quickly, and by 23 wpi, two lambs had reached the terminal stages. The very short incubation period was dominated by

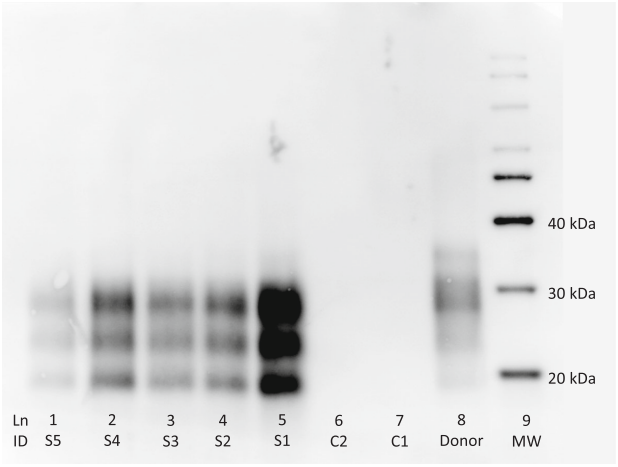

Fig. 1 Western Blot image. Western immunoblot using P4 antibody for the detection of PrP^{Sc} in equal amount of homogenated brain tissue from animals and inoculation material used in the experiment (TeSeE Western Blot, Bio-Rad). Lanes 1–5 represent the five animals in the scrapie group, and lanes 6 and 7 represent two animals in the control group. Lane 8 represents inoculation material (donor) used in the scrapie group and a molecular marker was placed in lane 9. PrP^{Sc} was detected in inoculation material and in all the animals from the scrapie group

Table 1 Mean fold change and *p*-values of selected genes in whole blood at three time points

Gene function	Gene name		Whole blood					
			16 wpi		22 wpi		23 wpi	
			FC ± SEM	p	FC ± SEM	p	FC ± SEM	p
Pattern Recognition Receptor	*TLR2*	Toll-like receptor 2	1.11 ± 0.04	0.73	1.96 ± 0.28	0.02	1.98 ± 0.54	0.03
	TLR4	Toll-like receptor 4	−1.41 ± 0.05	0.41	2.14 ± 0.42	0.02	2.00 ± 0.42	0.03
Complement Component	*C3*	Complement component 3	1.57 ± 0.22	0.19	3.04 ± 0.91	0.02	4.74 ± 2.32	0.02
Interleukin	*IL1B*	Interleukin-1β	−1.71 ± 0.03	0.02	1.66 ± 0.16	0.02	−1.24 ± 0.15	> 0.05
Acute Phase Protein	*SAA*	Serum Amyloid A	1.07 ± 0.35	0.73	5.00 ± 1.49	0.02	21.99 ± 18.03	0.03
	LF	Lactoferrin	1.05 ± 0.35	1.00	4.64 ± 2.64	0.06	13.70 ± 10.35	0.02

Mean fold change (FC) with standard error of the mean (SEM) and p-value of differentially expressed genes in whole blood in the scrapie group. The mean fold change value is relative to the mean of the control group which is scaled to 1, at each of the different times (weeks) post inoculation

sudden progressive weakness, followed by rapid worsening and necessitating euthanasia at an early age. Hence, the observed clinical presentation is not typical for classical scrapie known to present in older animals, which is characterised by a slow and long incubation period with signs of progressive neurodegeneration associated with dissemination and accumulation of PrP^{Sc} within the CNS. Although this experimental scrapie model attempts to eliminate many of the factors that generate disease variation, individual variation still occurred. Individual progression of clinical disease and expression profiles in blood and tissues differs between animals. The APR is fast, and the level of inflammatory mediators, such as cytokines, changes continuously. Cytokines are multifunctional (both pro-inflammatory and anti-inflammatory) intra-cellular and inter-cellular signalling molecules, operating in a very complex signalling network to initiate and fine-tune the immune response [34–38]. Their levels are tightly controlled within a narrow range by inhibitory mechanisms and antagonists, whilst APPs can increase quite dramatically depending on type of protein [39, 40].

In the present study, we have investigated the expression of a selection of genes in blood approximately half-way through the incubation period, at 12–16 wpi, and at the clinical end-stage, at 22–23 wpi. Expression levels of selected genes were also investigated in liver and brain tissues, harvested at post mortem examination. In summary, the mean expression of six genes were significantly altered in the scrapie group compared to the control group. We were not able to calculate statistical significance in mean expression levels in brain and liver tissues, but we believe that a difference in mean expression levels greater than 1.5 fold change is of biological relevance and will be discussed. Despite our best efforts, only four genes in the brain tissue were differently expressed in the scrapie group, compared to the control group. These findings were unexpected as all the animals in the scrapie group were clinically affected and were positive for PrP^{Sc} in the brain on both IHC examination and WB (Fig. 1).

Our overall results, presented in Fig. 2, indicate an innate immune response, where genes involved in pattern recognition, complement system and acute phase proteins, are significantly altered in blood at the clinical stages (22 and 23 wpi). Mean gene expression levels of brain and liver tissues in the scrapie group show ≥1.5 fold change of several APPs. These findings coincide well with our earlier reports on increased serum levels of APPs, and identification of SAA as being a discriminating protein by proteomic analysis of serum from the scrapie group [29, 41].

Prion disease and the innate immune system have previously been reviewed by Bradford and Mabbott, and activation of cells within the innate immune system is critical to peripheral and central prion pathogenesis [42].

Table 2 Fold change of selected genes in liver tissue

Gene function	Gene Name		Fold change ± SEM
TNF super family	*TNFa*	Tumor Necrosis Factor alfa	−1.77 ± 0.12
Acute phase protein	*SAA*	Serum Amyloid A	89.98 ± 46.32
	TF	Transferrin	−1.68 ± 0.10
	Hp	Haptoglobin	542.96 ± 216.93
	Cp	Ceruloplasmin	1.98 ± 0.46
	AAT	Alpha-1 antitrypsin	−2.37 ± 0.05
	TTR	Transthyretin	−2.03 ± 0.08

Mean hepatic gene expression and SEM of the scrapie group at 23 wpi with fold change ≥1.5 relative to the mean expression in the control group scaled to 1

Table 3 Fold change of selected genes in brain tissue

Gene function	Gene Name		Fold Change ± SEM
Acute Phase Protein	*Hp*	Haptoglobin	7.91 ± 2.39
	Cp	Ceruloplasmin	1.67 ± 0.73
	ALB	Albumin	−1.64 ± 0.06
	TTR	Transthyretrin	−1.61 ± 0.17

Mean fold change and SEM in brain tissue in the scrapie infected group at 23 wpi relative to the expression in the control group

Gene network analyses of brain tissue from prion infected mice show that increased expression of C3, PRRs (including TLR2), and other receptors involved with PrP^{Sc} recognition and uptake are one of the first transcriptome changes [43, 44]. TLR2 is one of the, at least, ten members of the ovine TLRs family which are membrane-bound PRRs [45]. Our results show significantly increased *TLR2* and *TLR4* expression in blood from 22 wpi, thus, indicating an increased transcription of these receptors in circulating blood cells. Both these TLRs are situated on the cell surface and recognize a variety of microbial components, and activation will trigger expression of several genes and signalling pathways involved in the immune response [37, 46, 47]. Activation of different TLRs leads to specific gene expression profile patterns, and the specific signalling pathways in prion diseases are not yet fully revealed. TLR2 and TLR4 signalling pathways share a common MyD88-dependent pathway which results in production of inflammatory cytokines. In addition, TLR4 stimulation can also initiate a MyD88-independent pathway [46]. Prinz et al. demonstrated that $MyD88^{-/-}$ mice were highly susceptible to prion infection, and thus pathogenesis and neuroinvasion are not solely dependent on the MyD88-dependent pathway [48]. Spinner et al. showed that scrapie pathogenesis occurs more rapidly in mice mutant of TLR4 signalling and concludes that this may be due to a reduced innate response to PrP^{Sc} [49]. The TLRs may be involved in binding PrP^{Sc} and initiating the innate immune response [42, 48–52]. Alongside the TLRs, there is a significant increased expression of C3 in blood at both 22 and 23 wpi. Binding of PrP^{Sc} to complement components plays an important role in getting the agent to lymphoid tissue and early accumulation in FDCs [53]. PrP^{Sc} interacts directly with C1q and thereby activates C3 through the classical pathway [54–56]. The complement system, in general, sense and react quickly to danger signals and can potentially initiate a strong inflammatory response, which needs to be tightly balanced between activation and inhibition [54].

Binding to and activation of PRRs lead to the production of pro-inflammatory cytokines that function both protective and degenerative. Increased levels of pro-inflammatory cytokines are associated with the systemic inflammatory response seen in prion infection and other neurodegenerative diseases [57, 58]. Cytokine antibody array analysis of scrapie-infected Tg338 mice shows significant alteration in expression of interleukins in spleen, mesenteric lymph node, brain and serum at both preclinical and clinical stages of infection [59]. We analysed several different interleukins, but only the expression *IL1B* was significantly changed in blood in the scrapie group; first downregulated at 16 wpi and then upregulated at 22 wpi. IL-1 beta is a powerful pro inflammatory mediator, and increased levels initiate a negative feedback loop [35, 39].

Translation and release of proinflammatory cytokines initiate a wide range of systemic inflammatory effects, including the synthesis and release of APPs mainly from the liver, but also extrahepatically [36, 38, 47, 60]. The APPs are classified as positive or negative depending

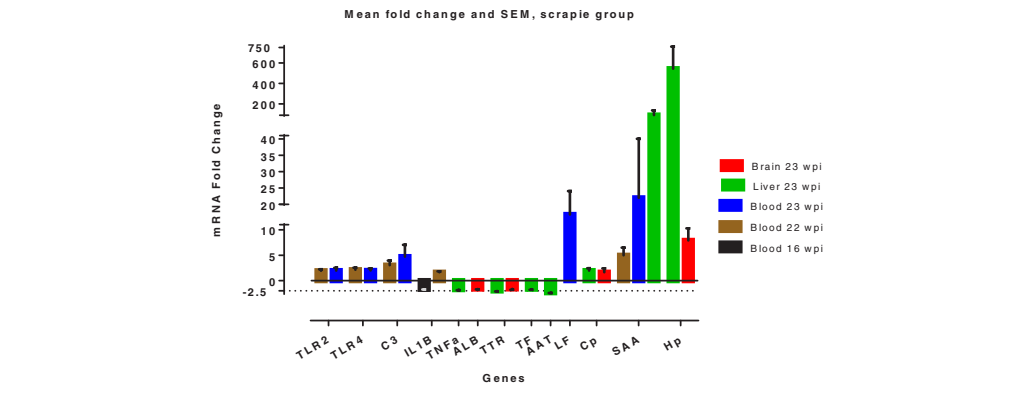

Fig. 2 Bar diagram presenting average fold change and SEM in the scrapie group of selected genes. The fold change value is scaled, in each data set, to the mean fold change value of the control group equals 1 (one)

whether their serum concentration increases or decreases during the APR [36, 47, 61, 62]. Our results show changes at transcription levels of both negative and positive APPs at the end stage. *ALB*, *TTR* and *TF* are negative APPs, and these have decreased expression in brain and liver. *ALB* is the major negative APP and synthesis is down-regulated during the APR. Transthyretin is a transport protein in serum and CSF, and one of its functions is inhibition of interleukin-1 production by monocytes and endothelial cells, and a decrease may, thus be pro-inflammatory [36]. *TTR* expression is reduced during the APR and the protein has been seen to decrease in serum in late stage scrapie in sheep [63, 64].

Of the positive APPs, *LF*, *Cp*, *SAA* and *Hp* have increased expression at clinical end stage, while *AAT* was down regulated. Alpha-1 antitrypsin (AAT) is a circulating serine protease inhibitor (serpin) and an acute phase protease, and it is classified as a minor positive acute phase protein in ruminants [64]. It is synthesised and secreted from the liver, and it plays an important role in the control of the inflammatory response [47, 65]. Serpinopathies and AAT deficiency have been associated with protein misfolding diseases [65, 66]. We found a decreased transcription of *AAT* mRNA in the liver in the scrapie group compared to the control group.

Gene expression of lactoferrin (LF), another minor positive APP, was increased by a 13.7-fold in blood in the scrapie group at 23 wpi. LF is a multifunctional glycoprotein with great iron-binding affinity and it is produced by mucosal epithelial cells [67]. It is involved in regulation of iron absorption and immune responses, and it has been suggested that LF has multifunctional antiprion activities [68]. Neutrophils are the source of lactoferrin in serum [69].

Ceruloplasmin (Cp) is a moderate APP with important biological functions. It is the major copper-carrying protein in blood, it plays a role in iron homeostasis and has oxidase activity in the CNS [47, 70–72]. Cp is mainly produced in the liver, and we detected an increased transcription in liver tissue in the scrapie group at 23 wpi. This coincides well with our previous finding of increased serum levels of ceruloplasmin at 23 wpi [29].

Serum amyloid A (SAA) and haptoglobin (Hp) are major APPs in sheep and increased serum levels are associated with a number of diseases. These proteins are primarily synthesised by hepatocytes, but other tissues and cell types are able to produce these two locally, though at a much lower level [47]. Our results show an increased transcription of both genes in the scrapie group at the clinical end stage. *SAA* gene expression in blood was significantly increased at both 22 and 23 wpi, with an average fold change of 5.00 and 21.99, respectively. In liver tissue, the mean expression level was increased by an average 89.98 fold change in the scrapie group. These results indicate that the increased level of SAA in in serum, reported earlier, originates both from increased synthesis in the liver and in the blood.

Hp showed increased gene transcription in both liver and brain tissue, with a mean fold change of 542.86 and 7.91, respectively. This indicates an increased synthesis of Hp hepatically and extrahepatically within the brain in the scrapie group at 23 wpi.

There was considerable variation in relative expression of individual gene expression within the scrapie group, indicated by the SEM values (Tables 1-3). One animal stands out in gene expression fold change in liver and blood, and on clinical presentation (individual data not shown). We believe there is relationship between the magnitude of the systemic acute phase response and clinical presentation in these animals. The acute phase response leads to a number of behavioural, physiological, biochemical and nutritional changes [36]. The brain recognizes proinflammatory cytokines as molecular signals of sickness, and metabolic and behavioural changes are initiated. These changes are collectively called “sickness behaviour” [73]. These peripherally released cytokines communicate and act on the brain via a fast neural pathway through afferent neurons (especially the vagus nerve), and via a slow humoral pathway [37]. In this sense, the immune- and nervous systems are in close contact. The afferent branches of the vagus nerve are involved in transmitting signals of inflammation to the brain via cytokine receptors [37]. Dendritic cells and macrophages are present in close association to vagal nerve fibres and they are important in the immune-brain communication [74]. Dendritic cells and macrophages express membrane TLRs and can produce proinflammatory cytokines upon activation, and at the same time, these cell types play a role in peripheral prion pathogenesis by interacting with PrP^{Sc} [42, 75]. Immune signals and PrP^{Sc} neuroinvasion may share common neural pathway transmitting immune messages and PrP^{Sc} infectivity, respectively, from periphery to the brain, where DCs have an important role [37, 76]. The innate immune system and the nervous system are, thus closely linked through common hormonal and neuronal routes. The ANS and CNS enhance and dampen the inflammatory response, and regulation through negative feedback loops are important. Any interference may have deleterious consequences. Recently, Salvesen et al. suggested that PrP^{C} is one of the modulators of the innate immune response, and loss of PrP^{C} prolonged sickness behaviour and stimulated pro-inflammatory activity [77].

Similar experimental scrapie models in sheep show PrP^{Sc} accumulation in peripheral lymphatic tissue and gene expression changes about half-way through the incubation period, while the animals appear asymptomatic [20, 22, 24, 43]. We believe the situation in the scrapie group was similar, an asymptomatic incubation period

was followed be a short clinical end stage characterised by progressive apathy, dirty wool and passiveness. By 23 wpi two sheep were recumbent, weak, incapacitated and responded poorly to external stimuli. Sheep are very good at hiding behaviour changes as indicators of disease, thus presence of clinical signs cannot always be relied upon [78]. Sickness behaviour can be both beneficial and disadvantageous to the animal, and it can, to some extent, be overruled by fear and mothering behaviour [73, 79]. Why the long asymptomatic incubation period, despite the ongoing accumulation of PrP^{Sc} in LRS and CNS and gene expressions alterations, is not fully understood. Many of the genes involved are part of the innate immune system, and it is known that a prolonged and excessive inflammatory response can have negative or even lethal effects [34, 37, 80].

The particular disease phenotype seen in present study, is the result of the interaction between donor material, dosage and time of inoculation and recipient genotype, also described by González et al. [81]. The inoculation at birth before access to colostrum results in an effective and fast uptake of PrP^{Sc} across the susceptible new-born gut, similarly described by Tabouret et al. [24, 28]. These lambs have a characteristic dissemination of PrP^{Sc} throughout peripheral lymphatic tissues well in advance of PrP^{Sc} detection in the brain. The early and progressive development of clinical signs is unusual and have only been described earlier in a few rare cases of natural scrapie by Ulvund, although not at such a young age [82]. The histopathological examinations of the brain from these pre-colostrum inoculated lambs revealed only moderate cytopathological changes, with a mild to moderate gliosis with few vacuolated neurons. This is in somewhat in contrast to an earlier experimental model, described by Ersdal et al., where lambs of the same genotype (VRQ) were orally inoculated with brain tissue homogenate at ages between 46 and 61 days of age (mean 55.6 days).

Conclusion

We believe our experimental model produces a "worst-case" scenario which can occur in natural scrapie settings. Homozygous VRQ offspring from subclinically infected ewes, can become infected at birth and develop clinical scrapie similar to the cases described here. In naturally occurring scrapie, such animals will pose a threat to other susceptible sheep through environmental contamination due to high infectivity. The young age and atypical clinical presentation of these cases can result in misdiagnosis and not be recognisable as scrapie. Further work is needed to investigate the connection between the innate immune system and prion diseases, and whether the severe response we observed in our model can be linked to loss of modulatory function of PrP^{C}.

Additional files

Additional file 1: Quantity and quality calculations of extracted RNA. Descriptive statistical presentation of quantitative and qualitative properties of the extracted RNA from blood, liver tissue and brain tissue. (DOCX 13 kb)

Additional file 2: Primers and reaction conditions. Gene functional class, gene abbreviation, gene name, gene bank entry and primer sequences used in the real time qPCR analysis. Calculated PCR efficiency and correlation are also listed. (XLSX 28 kb)

Abbreviations

AAT: Alfa-1 antitrypsin; ALB: albumin; ANS: Autonomic nervous system; APP: Acute Phase Protein; APR: acute phase response; C3: complement component 3; cDNA: complementary DNA; CNS: Central nervous system; Cp: Ceruloplasmin; CSF: cerebrospinal fluid; DCs: Dendritic cells; DNA: Deoxyribonucleic acid; FC: fold change; FDCs: follicular dendritic cells; Hp: Haptoglobin; IHC: immunohistochemistry; IL1B: Interleukin-1-beta; LF: Lactoferrin; LRS: Lymphoreticular system; mRNA: Messenger ribonucleic acid; NTC: No template control; PNS: Peripheral nervous system; PRNP: Prion protein gene; PrP^{C}: Normal cellular prion protein; PrP^{Sc}: Abnormal isoform of prion protein; PRRs: pattern recognition receptors; RIN: RNA integrity number; RNA: Ribonucleic acid; RT-qPCR: Reverse transcription quantitative real-time polymerase chain reaction; SAA: Serum amyloid A; SEM: Standard errors of mean; TF: Transferrin; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TLRs: Toll-like receptors; TNFa: Tumor necrosis factor alfa; TSE: Transmissible spongiform encephalopathy; TTR: Transthyretin; WB: western blot; wpi: weeks post inoculation

Acknowledgements

The authors gratefully thank Eli Brundtland for assisting in sampling of material and PRNP-genotyping, and Karin Tarp for excellent help and guidance on sample preparation and RT-qPCR analysis.

Consent to publication

Not applicable.

Funding

This study was supported by grant NRC 178268 (2006–2010) funded by the Research Council of Norway, and Johan Haaland's Legat.

Availability of data and materials

Data and materials supporting the conclusions are contained within the manuscript. Any additional data are available upon request. RIN values and calculated are shown in Additional file 1. Primer sequences are listed together with reaction conditions in Additional file 2.

Author's contributions

MJU, SM and KB designed the study, and SM, MJU and KB performed the experiments. KS participated in designing primers and laboratory procedures and pre-processing of the expression data. SM designed primers, carried out laboratory procedures, performed the statistical analyses and drafted the manuscript. MJU participated in the interpretation of analytical data and in drafting the manuscript. PMHH coordinated the analyses in Denmark. All authors have critically read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests.

Ethics approval

The animal experiment was performed in compliance with ethical guidelines and approved by the Norwegian Animal Research Authority (FOTS id852) with reference to the Norwegian regulation on animal experimentation (FOR-1996-01-15-23).

Publisher's Note

Author details
[1]Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Sandnes, Norway. [2]Department of Biotechnology and Biomedicine, Technical University of Denmark, Kemitorvet, 2800 Lyngby, Denmark.

Received: 20 March 2018 Accepted: 31 August 2018
Published online: 12 September 2018

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ISBN: 978-82-575-1721-2
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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