International School for Advanced Studies

Gene Expression Analysis of Whole Blood from Preclinical and Clinical Cattle Infected with Atypical Bovine Spongiform Encephalopathy.

CANDIDATE

Elena Xerxa

SUPERVISOR

Prof. Giuseppe Legname

Contents

List of abbreviation

BSE = bovine spongiform encephalopathy

cBSE = classical bovine spongiform encephalopathy

BASE = amyloidotic spongiform encephalopathy

UK = United Kingdom

CJD = Creutzfeldt-Jakob Disease

vCJD = variant Creutzfeldt-Jakob disease

TSEs = transmissible spongiform encephalopathies

PrP^{Sc} = scrapie prion protein

PrP^{C} = cellular prion protein

ORF = open reading frame

ER = endoplasmic reticulum

GPI = Glycosylphosphatidylinositol

HR = hexarepeat

OR = octapeptide domain

NMR = nuclear magnetic resonance

KO = knock-out

GSS = Gerstmann-Sträussler-Scheinker syndrome

FFI = fatal familial insomnia

PrP-CAA = PrP cerebral amyloid angiopathy

VPSPr = variably protease-sensitive prionopathy

iCJD= iatrogenic Creutzfeldt-Jakob disease

cCJD = classical Creutzfeldt-Jakob disease

sCJD = sporadic Creutzfeldt-Jakob disease

MHC = major histocompatibility complex

PK = proteinase kinase

TME = transmissible mink encephalopathy

FSE = feline spongiform encephalopathy

EUSE = exotic ungulate spongiform encephalopathy

CWD = chronic wasting disease

NHP= non human primates

CNS = central nervous system

NK = natural killer

DC = dendritic cell

FDC = follicular dendritic cell

HSC = hematopoietic stem cell

TCR = T-cell receptor

SCID = severe combined immunodeficient

GALT = gut-associated lymphoid tissue

PPs = Peyer's patches

FAE = follicle-associated epithelium

APC = antigens presenting cell

SLO = secondary lymphoid organs

MNP = mononuclear phagocytes

PMCA = Protein Misfolding Cyclic Amplification

ABSTRACT

BACKGROUND: Prion diseases, such as bovine spongiform encephalopathies (BSE), are transmissible neurodegenerative disorders affecting humans and a wide variety of mammals. Variant Creutzfeldt-Jakob disease (vCJD), a prion disease in humans, has been linked to exposure to BSE prions. This classical BSE (cBSE) is now rapidly disappearing as a result of appropriate measures to control animal feeding and monitoring. Besides cBSE, two atypical forms (named H- and L-type BSE) have recently been described in Europe, Japan, and North America. Here we describe the first wide-spectrum microarray analysis in whole blood of atypical BSE-infected cattle. Transcriptome changes in infected animals were analyzed prior to and after the onset of clinical signs. Some of the most significant differentially expressed genes (DEGs) were validated by quantitative real time PCR (RT-qPCR).

AIM: The aim of this study was to analyze the transcriptome changes in whole blood from atypical BSE-infected animals prior and after the onset of the clinical signs to understand the peripheral mechanisms of prion infection and to line out some candidate genes that could be further investigated as biomarker of the disease.

METHODS: Total RNA from whole blood samples from 8 intracranially BSE-challenged cattle (4 with H-type and 4 with L-type BSE) and 2 non-infected age- and sex-matched controls was isolated and subjected to the microarray analysis using the GeneChip® Bovine Genome Array (Affymetrix). In order to increase the animal cohort, RNA samples from four additional sex-matched control cattle were isolated using the same

protocol as the original study group and included in the final statistical analysis. Therefore, 24 RNA samples, divided in 8 preclinical (P1, P2, P4, P5, P7, P8, EP9 and P10), 8 clinical (S1, S2, S3, S4, S7, S8, S9 and S10), and 8 control (c2, c3, cP3, c5, cS5, cP6, cS6 and c9) samples, constituted our animal cohort. After the assessment and inspection of microarray quality controls (RNA degradation plot, RLE and NUSE plots) we identified one low quality control sample (cS5) and excluded it from the final statistical analysis. Gene probes with a p value ≤0.05 and fold-change ≥2 were considered to be differentially expressed. To confirm the microarray results, we performed RT-qPCR using SYBR® green assay (Bio-Rad Laboratories, Inc.) for a selected number of target genes. The RT-qPCR analysis was performed on 22 samples (7 control, 8 preclinical and 7 clinical animals). The normalization accuracy was improved by geometric averaging of multiple reference genes (GAPDH, RPL12 and ACTB) and using two inter-run calibrators to reduce inter-run variation.

RESULTS: The microarray analysis revealed a total of 101 differentially regulated probe sets (*p* value lower than 0.05 and changes in expression higher than 2-fold) in infected animals (clinical and preclinical) versus control group. In the clinical stage, a total of 207 probe sets showed significant alteration in expression levels compared to the control group. Interestingly, a pronounced alteration in the gene expression profile was also found in the preclinical stage, with a total number of 113 differentially expressed probe sets. A set of 35 differentially expressed genes was found to be in common between clinical and preclinical stages and showed a very similar expression pattern in the two phases. To further dissect gene expression alterations during the progression of the disease, we performed a statistical analysis to identify specific changes between the

clinical and preclinical stages (CvsP). Indeed, we found 235 DEGs, which were significantly enriched in pathways related to immune response. The comparison of all the analysis, revealed a 22-gene signature with an up/down-down/up pattern of expression, being differentially expressed in preclinical stage and then going back to control levels in the symptomatic phase. One gene, *SEL1L3*, was progressively downregulated during the progression of the disease. The identified genes belong to several pathways, such as immune response and metabolism, that may play an important role in prion pathogenesis. The RT-qPCR analysis confirmed the microarray results for six out of nine genes selected (XIST, CD40L, GNLY, PDK4, HBA2 and SEL1L3).

CONCLUSIONS: The present study has led to the identification of several gene expression changes in whole blood from atypical BSE infected cattle prior and after the manifestation of the pathology. Our findings suggest that it might be feasible to use whole blood RNA transcriptional profiles to distinguish between preclinical and clinical stages of prion infection. Overall, our study confirmed the differential expression of 6 genes (XIST, CD40L, GNLY, PDK4, HBA2 and SEL1L3), which may play several roles in atypical BSE pathogenesis and, possibly, in other prion infections. Even though further studies are required to investigate the specific involvement of all the identified genes in prion diseases, our data support the idea of a relationship of complicity and blindness between prion and the host immune system. As concluding remark, our study underlined the importance of utilizing whole blood, without any additional manipulation, as a source tissue for the development of a preclinical diagnostic test.

1. Introduction

Prion diseases are a group of fatal neurodegenerative disorders which affect humans and a wide variety of mammals. These pathologies are characterized by various neurological dysfunctions and share common histopathological features including neuronal cell loss, spongiform lesions in the brain, astrogliosis and accumulation of misfolded prion protein. Among the various neurodegenerative illnesses, prion diseases are peculiar in that their etiology, besides being sporadic and genetic, may also be infectious. Although such pathologies in humans are fairly rare (worldwide incidence of 1 case/million people every year), they have attracted great interest from the scientific community and general society for two main reasons: first, because of the bovine spongiform encephalopathy (BSE) epidemic in United Kingdom (UK) in 1992 and the growing body of evidence associating a new variant of Creutzfeldt-Jakob Disease (vCJD) with the consumption of BSE-infected meat and bone meal. Second, because of the unprecedented findings linked to the prion field which revolutionized some of the most important dogma in biology. Such findings include: 1) disease transmission through proteinaceous material, in the absence of any nucleic acid; 2) the presence of several different folding conformations associated with one protein, each with distinct biological properties; and 3) the transmission of biological information by the replication of protein conformation (Soto and Saborio 2001). Despite intense research, the molecular pathways leading to neurodegeneration in prion disease remain unknown and no effective treatments or ante-mortem diagnosis is currently available.

1.1 What is a prion

Prion is a term coined in 1982 by Stanley B. Prusiner to describe a “proteinaceous infectious particle“, which was identified as the causative agent of several neurodegenerative diseases in mammals, collectively known as prion diseases or transmissible spongiform encephalopathies (TSEs). According to the “protein only hypothesis” formulated by Stanley B. Prusiner (Prusiner 1982), a prion is composed entirely of protein material which is able to replicate and transmit infection without the need of nucleic acids. Prions are highly resistant to ultraviolet and ionizing radiation, procedures that can destroy pathogens containing DNA or RNA. Other peculiar characteristics of prions are their insolubility in detergents and their resistance to proteinases. These latter features were exploited by Prusiner and colleagues to isolate a protein of 27-30 kDa from the brain of prion-infected Syrian hamsters (Prusiner, Groth et al. 1984). This protein was called PrP^{Sc} (from **Sc**rapie **Pr**ion **P**rotein). The subsequent sequencing of PrP^{Sc} led to the identification of an endogenous gene (*PRNP* in humans and *Prnp* in mice) whose product was called cellular prion protein (PrP^{C}). Thus, this finding showed that PrP^{C} and its infectious isoform PrP^{Sc} share the same primary sequence but a different conformation.

1.2 PrP^{C} and PrP^{Sc} structure

As stated above, PrP^{C} and PrP^{Sc} are different in respect to several characteristics, such as solubility, proteinase K resistance and other features. Despite these differences, PrP^{C} and PrP^{Sc} share an identical primary structure whose sequence is coded by a gene found on chromosome 20 in humans, 13 in bovine and on chromosome 2 on mice. The entire open reading frame (ORF) of known mammalian and avian PrP genes is contained within a single exon. The two exons in, for example, Syrian hamster (SHa)PrP gene are separated by a 10-kb intron. The PrP genes of mouse, sheep, bovine and rat are constituted by 3 exons and the ORF lies entirely on exon 3 analogous to exon 2 of SHa(PrP) gene (Westaway et al. 1991, 1994, 1994). Similarly to (SHa)PrP gene, in humans the gene encoded by PRNP contains two exons and the ORF is contained entirely within exon 2. The mature transcript is translated into a 253 amino acid protein (254 in mice), which is post-translationally modified and shortened into a 209 amino acid-long protein (208 in mice). Limited proteolysis of PrP^{Sc} leads to the formation of PrP 27-30 fragment, which is composed of approximately 142 amino acids (**Fig. 1**).

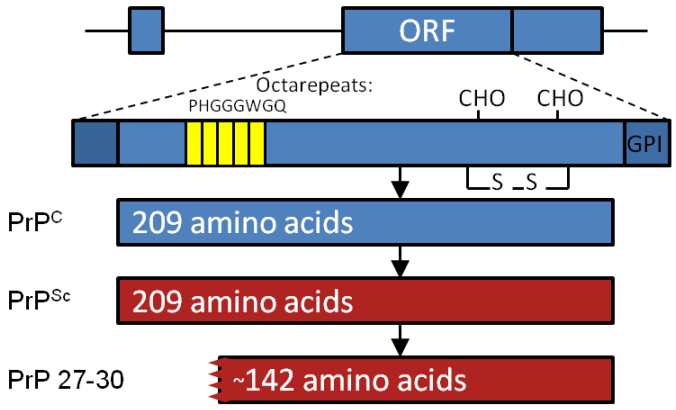


Fig. 1: *PRNP* gene and prion protein isoforms. PRNP ORF encodes for a protein of 254 residues that is post-translationally shortened to 209 residues, which constitute mature PrP^{C}. PrP^{Sc} shares the same primary structure with PrP^{C} but it is characterized by a different conformation. Limited proteolysis of PrP^{Sc} cleaves 67 amino acids from the amino terminus and produces PrP 27-30, composed of approximately 142 residues (adapted from (Colby and Prusiner 2011)).

PrP^{C} is constituted by an N-terminal unstructured region (residues 23-90) and a C-terminal globular domain (residues 90-231) (Riek, Hornemann et al. 1997, Zahn, Liu et al. 2000). The N-terminal endoplasmic reticulum (ER) signal peptide (residues 1-22) is post-translationally cleaved during the maturation of the protein as well as the C-terminal peptide 232-254, and it is modified by a GlycosylPhosphatidylInositol (GPI) anchor in position 231 that allows the insertion of the protein in the outer membrane (Riesner 2003). The flexible N-terminal “tail” contains two hexarepeats (HR, residues 34–39 and 45–50) and an octapeptide domain (OR, residues 51–91). Up to four copper ions can be associated to the octapeptide region, with the higher affinity for Cu^{2+} (Hornshaw, McDermott et al. 1995, Stockel, Safar et al. 1998, Whittal, Ball et al. 2000). In addition, two histidine residues able to bind copper are found in the 92–111 region (non-octapeptide copper binding site). A hydrophobic core at position 113–135, together with the non-octapeptide binding region (92-111), constitutes the central domain of prion protein. Residues 129-133 and 160-163 constitute two β-strands folded into a small antiparallel β-sheet. A first alpha-helix (H1) is present between the two β-strands (residues 144-153) while the other two alpha-helices (H2 and H3, residues 166-194, 200-226) form a two-helix bundle which is stabilized by a disulfide bond between Cys 179 and Cys 214 (Turk, Teplow et al. 1988). Finally, at position 180 and 196 a

consensus sequence for Asn-linked glycosylation is present, giving rise to the di-, mono- and unglycosylated PrP (Stimson, Hope et al. 1999) (**Fig. 2**).

A **B**

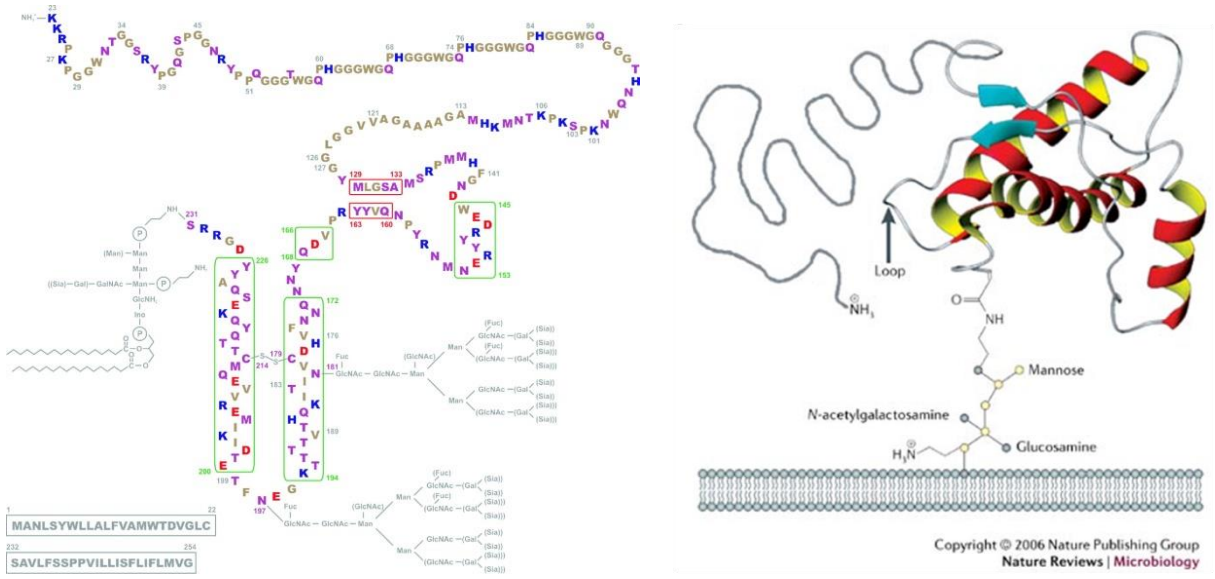


Fig 2. Amino acid sequence, post-translational modifications and 3D structure of prion protein. 1–22, post-translationally cleaved ER signal peptide; 34–39 and 45–50, 2 hexarepeats; 51–91, 5 octarepeats; 95-110, charged cluster; 113–128, conserved hydrophobic region; 121–231, hamster PrP^{C} C-terminal globular domain; 129-133, β-strand 1; 145–153/155, α-helix 1; 160–163, β-strand 2; 166–194, α-helix 2; 179/214, disulfide bridge; 180/181, N-glycosylation 1 in mouse/human; 196/197, N-glycosylation 2 in mouse/human; 200–226, α-helix 3; 231, GPI-anchor; 232–254, GPI signal peptide (cleaved). Positively charged amino acids (at pH 7) in red, negatively charged in blue, neutral in magenta, hydrophobic in brown (from Riesner D., 2003)(**A**). Tertiary structure of the cellular prion protein inserted into a lipid bilayer, as deduced from NMR spectroscopy, including the 'unstructured' N-terminal tail (grey) and the glycosyl phosphatidyl inositol (GPI) anchor. The loop connecting the second beta-sheet and the third alpha-helix is indicated by the black arrow. OR, octarepeat region (adapted from Aguzzi A. *et al*., 2006) (**B**).

Unlike PrP^{C}, PrP^{Sc} structure has not been elucidated yet, since its insolubility does not allow the access to structural analysis by nuclear magnetic resonance (NMR) or X-ray. Circular dichroism and infrared spectroscopy analysis have revealed that while PrP^{C} is constituted by a high content of α-helix (40% of the protein) and a small percentage of of β-sheet (3% of the protein), PrP^{Sc} instead is characterized by an higher content of β-sheet (45% of the protein) and less α-helix (30% of the protein) (Caughey, Dong et al. 1991, Safar, Roller et al. 1993). In electron micrographs, PrP^{Sc} is visible as fibrillar structures and staining with Congo red shows the typical fluorescence associated to amyloid fibrils.

1.3 Conversion of PrP^{C} into PrP^{Sc}

Studies on *Prnp* knock-out (KO) mice (also indicated as $Prnp^{-/-}$ mice) showed that the presence of endogenous PrP is necessary to sustain prion infection. Indeed, mice lacking PrP^{C} are resistant to infection and unable to generate infectious particles (Bueler, Aguzzi et al. 1993). These findings suggested that the prion replication process is based on the ability of PrP^{Sc} to replicate itself by transferring its toxic conformation to the host PrP^{C}. Molecular studies and *in vitro* prion replication analysis suggested the existence of a chaperone-like protein (designated as protein X), which could facilitate the structural conversion (Telling, Scott et al. 1995, Saborio, Soto et al. 1999). At present the nature of this protein remains unknown. Assuming the prion-only hypothesis, up to now two main models for PrP^{C} conversion into PrP^{Sc} have been

postulated (**Fig. 3**): the “template-assisted model” and the “nucleation model” (Cohen and Prusiner 1998, Aguzzi, Montrasio et al. 2001).

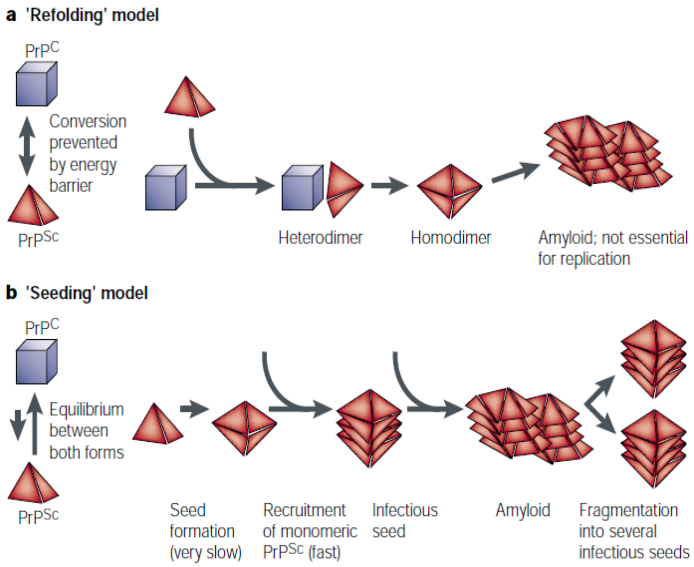


Fig. 3. Model for conversion of PrP^{C} into PrP^{Sc}. The template-assisted model, or refolding model, argues that PrP^{C} is separated from PrP^{Sc} by a high energy barrier, which prevents a spontaneous conversion. Exogenous administration of PrP^{Sc} acts as a catalyst and lowers the energy barrier. In addition, each prion strain acts as a unique template providing conformational constrains (**a**). According to the “seeding” model, the rate-limiting step is the formation of a seed: an oligomeric aggregate of PrP which is thermodynamically unstable and shifts the balance toward PrP^{Sc} formation (**b**). (Aguzzi et al., 2001).

According to the template-assisted model (or refolding model) a high-energy barrier might prevent spontaneous conversion of PrP^{C} into PrP^{Sc}. Therefore, a physical interaction between exogenous prion and endogenous PrP would allow the conversion process (**Fig 3a**). On the contrary, the nucleation or “seeding” model proposes a reversible thermodynamic equilibrium between PrP^{C} and PrP^{Sc} conformations, with PrP^{C} being more stable. Only the presence of several PrP^{Sc} molecules disposed into an ordered seed would shift the balance toward the PrP^{Sc} conformation. In this way new monomeric PrP^{Sc} molecules would be recruited to form oligomeric and fibrillary amyloid structures (**Fig. 3b**).

1.4 Prion diseases

1.4.1 Human TSEs

Human TSEs include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, fatal familial insomnia (FFI), PrP cerebral amyloid angiopathy (PrP-CAA) and variably protease-sensitive prionopathy (VPSPr). Unique among all the neurodegenerative disorders, prion diseases, in addition to being sporadic and genetic, can also be acquired. Acquired prion diseases arise from accidental exposure to prions through medical procedures, such as in iatrogenic CJD (iCJD), or from consumption of infected meat, as in variant vCJD and kuru. Sporadic TSEs are the most common, while genetic forms represent approximately 10% of the total number of prion disease cases (Mastrianni 2010). Genetic prion diseases are inherited in an autosomal dominant pattern and have a slightly earlier age at onset and a slower course. All mutations associated to genetic prion disorders are found on the *PRNP* gene. Recent findings indicate that the PrP genotype and the PrP^{Sc} type have a major influence on the disease phenotype (Gambetti, Kong et al. 2003).

1.4.1.1 CJD

CJD, also denoted as classical CJD (cCJD), is the most common human prion disease, although it is still rare. The sporadic forms of CJD (sCJD) are the most common, accounting for >80% of all human prion diseases, with an incidence of one per one million people every year. Familial CJD (fCJD) cases represent 5% to 15% of the total number of CJD cases and are all caused by mutation in the ORF of *PRNP*. Acquired

forms of CJD are constituted by iatrogenic CJD (iCJD) and variant CJD (vCJD). vCJD will be discussed in detail later in the text. Concerning iCJD, it account for less than 1% of all CJD cases. iCJD are due to accidental transmission via contaminated neurosurgical instruments, brains derivatives such as infected cadaveric human growth hormone (used prior to 1980, over 200 cases largely in France), corneal transplantation and dura mater graft (over 200 cases, largely in Japan). CJD pathology is characterized by rapidly progressive dementia, memory loss, personality changes, hallucinations, speech impairment, myoclonus, ataxia, seizures and changes in gait. Involuntary movements follow in most patients with these symptoms. The duration of the disease varies greatly. However, in most cases affected people die in the first year after the appearance of the main symptoms. Polymorphism at codon 129 (methionine or valine) and the type (type 1 or 2) of PrP^{Sc} have been recognized as major determinants of the clinical features and, as regards polymorphism at codon 129, as a genetic marker for susceptibility to CJD in the Caucasians (Mitrová, Mayer et al. 2005). Methionine homozygosity (M/M) has been identified in 70–74% of cases of sporadic sCJD (Laplanche, Delasnerie-Laupretre et al. 1994) and in 78.6% of patients suffering from fCJD carrying E200K mutation (Mitrova and Belay 2002).

1.4.1.2 vCJD

Foodborne transmission of BSE prions to humans was observed in the 1990s with the appearance of a new variant form of CJD (vCJD) (Will, Ironside et al. 1996). A link between vCJD and BSE was first hypothesized because of the association of these two TSEs in time and place. Further, it has been shown experimentally that BSE prions have strain characteristics identical to those of prion isolates from human cases of vCJD

(Bruce, Will et al. 1997). So far, 229 cases of vCJD have been reported worldwide (http://www.cjd.ed.ac.uk/documents/worldfigs.pdf). Although there is an overlap of clinical characteristics, vCJD cases are a distinct group respect to classical CJD. In contrast to the traditional forms of CJD, vCJD has affected younger patients (median age at death of 28 years, as opposed to 68 years) and it has a relatively longer duration of illness (median of 14 months as opposed to 4.5 months). Most people who have developed vCJD lived in the UK or were exposed to the BSE while residing in the UK. Past surgery or blood transfusion is not a risk factor. However, four cases were observed in patients who received blood from individuals that later were diagnosed as vCJD (Llewelyn, Hewitt et al. 2004, Peden, Head et al. 2004, Wroe, Pal et al. 2006) (see paragraph 1.9).

During the early stage of the disease, vCJD patients present psychiatric symptoms. The first neurological signs appear after 6 months, and include ataxia, cognitive impairment and involuntary movements. After a progressive neurological deterioration patients become mute, incontinent and bed-bound. Death is often due to intercurrent infection (Will 2003). M/M homozygosity was found in all tested cases of vCJD identified to date compared to about 39% of the general Caucasian population (Will, Ironside et al. 1996). A reduced frequency of HLA class II type DQ7 has been described in patients with vCJD but not in those with sCJD. The molecular basis of the protective association of DQ7 and vCJD is unknown. Class II molecules of the major histocompatibility complex (MHC) may have a direct role in disease pathogenesis, or a gene linked to the DQB locus may be involved (Jackson, Beck et al. 2001).

1.4.1.3 Gerstmann-Sträussler-Scheinker syndrome (GSS)

Differently from CJD, GSS is exclusively genetic. The precise incidence is unknown, but is estimated around 5 cases per 100 million inhabitants per year (Ribosa-Nogué, Pagonabarraga et al. 2016). It is characterized by dysarthria and slowly progressive cerebellar ataxia followed by dementia. In some cases loss of memory is the first sign of GSS. Extrapyramidal motor disturbances are common and they manifest as myoclonus, athetosis and rigidity. All GSS patients present widespread multicentric amyloid plaques in the brain. GSS is the slowest to progress among human prion diseases. Duration of illness can range from 3 months to 13 years. The most common GSS causal mutation consists in the substitution of a proline in position 102 of the ORF of PRNP to a leucine (P102L). Even if GSS is classified as transmissible disease, it has been shown that in patients carrying P102L mutation the pathology is transmissible in just 40% of the cases (Collins, McLean et al. 2001). The rest of the mutations are very rare and in the majority of the cases the associated GSS pathology cannot be transmitted in primates and rodents. A recent study published by Laura Pirisinu and colleagues, conclusively showed that GSS with P102L, A117V and F198S mutations can be transmitted efficiently and produces distinct pathological phenotypes in bank voles (Pirisinu, Di Bari et al. 2016).

1.4.1.4 Fatal Familial Insomnia (FFI)

As with GSS, FFI is almost invariably genetically determined, though sporadic forms have been described. Most genetic cases are linked to a mutation at codon 178 of PRNP causing substitution of asparagine to aspartic acid (D178N). This mutation is

found also in fCJD patients. However, FFI is associated with D178N mutation combined in *cis* with methionine at codon 129 (D178N–129M), whereas fCJD is linked to the presence of valine (D178N–129V) (Goldfarb, Petersen et al. 1992). The brain of typical D178N FFI patients shows a characteristic restricted degeneration, largely confined to the thalamus. The presentation of the disease varies considerably from person to person. The main symptom is a progressively worsening insomnia, which leads to hallucinations, delirium, loss of weight and dementia.

1.4.1.5 Kuru

Kuru came to the attention of medicine in the mid-1950s when remote parts of New Guinea were explored by Australian administrators. Clinical hallmarks of the disorder consist of cerebellar ataxia and body tremors, as well as uncontrolled laugh. The preclinical phase lasts between 5 to 20 years following initial exposure, while the clinical stage lasts an average of 12 months. It is now widely accepted that kuru was transmitted among members of the Fore tribe of Papua New Guinea via funerary cannibalism, becoming endemic amongst these populations since 1941 (Gajdusek and Reid 1961). Although the ultimate cause of kuru remains uncertain, a cannibalistic serial passage of a sporadic case of indigenous CJD remains the most plausible hypothesis (Gajdusek 1977).

1.4.1.6 PrP Cerebral amyloid angiopathy (PrP-CAA).

PrP-CAA is a rare form of genetic prion pathology characterized by neurofibrillary tangles and PrP-positive amyloid fibril deposits in arteries and arterioles walls. PrP-CAA, without co-localization of Aβ (deposition of Aβ in blood vessels is the most

common cause of CAA in humans), has thus far only been described in two patients with nonsense mutations at codon 145 (Y145X) or 163 (Y163X) of *PRNP* (Jansen, Parchi et al. 2010). Both stop mutations lead to the loss of the GPI anchor, required to attach the protein to the outer leaflet of the plasma membrane. This suggests that the GPI moiety might interfere with the ability of PrP to form amyloid fibrils and when it is absent PrP readily aggregates, resulting in cerebrovascular amyloid deposition (Chesebro, Trifilo et al. 2005, Revesz, Holton et al. 2009).

1.4.1.7 Variably Protease-Sensitive Prionopathy (VPSPr)

In 2008, a novel prion disease, initially referred to as protease-sensitive prionopathy, was reported in 11 patients. All of them had the 129VV genotype, and presented no mutations in the *PRNP* gene. Brain examination revealed a peculiar accumulation of micro plaques within the cerebellum and thalamus. The defining feature of this group of patients was the unusual biochemical properties of the abnormal PrP in the brain. The PrP^{Sc} in VPSPr was found to be much less resistant to protease digestion compared to PrP^{Sc} from sCJD, and presented a prominent low–molecular weight fragment of ≈8 kDa. Subsequently, 19 additional cases have been reported, including some in patients with 129MM and 129MV genotypes (Diack, Ritchie et al. 2014). Patients show psychiatric symptoms, speech deficits (aphasia and/or dysarthria), and cognitive impairment. In the 129VV genotype, which is present in around 65% of total VPSPr cases, the first clinical symptoms are mood and behavior changes, speech deficit and cognitive impairment, while 129MV and 129 MM genotype (≈23% and ≈12%, respectively) often present with parkinsonism followed by ataxia, myoclonus and progressive cognitive impairment. The common hallmark to all genotypes at codon 129 is the presence of moderate

spongiform degeneration comprising of vacuoles in the major cerebral regions (Zou, Puoti et al. 2010).

1.4.2 Animal TSEs

Animal prion diseases include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy (TME), feline spongiform encephalopathy (FSE), exotic ungulate spongiform encephalopathy (EUSE), chronic wasting disease (CWD) in cervids and spongiform encephalopathy of non-human primates. In the majority of the cases, animal TSEs derive from ingestion of contaminated feed or exposure to contaminated environment. In the following paragraphs a brief description of each animal TSE will be provided.

1.4.2.1 Classical and Atypical BSE

Classical BSE (cBSE), commonly known as "mad cow disease", is a TSE found in cattle, which became epidemic in the UK in 1986-98. More than 180,000 cattle were infected and 4.4 million slaughtered during the eradication program. The pathology presents with tremors, gait abnormalities, difficultly in raising or walking, aggressive behavior, apprehension, and hyperreactivity to stimuli. PrP^{Sc} accumulation can be observed in brain tissues as early as 2 years post inoculation in experimental cases. The incubation period for BSE ranges from 2 to 8 years and most BSE cases have been found in 4 to 5 year old dairy cattle. Besides cBSE, in recent years two atypical forms of BSE have been identified in several European countries (Jacobs, Langeveld et al. 2007), Japan (Yamakawa, Hagiwara et al. 2003, Masujin, Shu et al. 2008), United States (Richt, Kunkle et al. 2007) and Canada (Dudas, Yang et al. 2010). The two

atypical BSE strains are denoted as H-type BSE and L-type BSE (also named bovine amyloidotic spongiform encephalopathy, BASE) (Casalone, Zanusso et al. 2004, Beringue, Andreoletti et al. 2007). The “H” and “L” identify the higher and lower electrophoretic mobility of the unglycosylated protease resistant PrP^{Sc} fragment, respectively (Biacabe, Laplanche et al. 2004) (**Fig. 4**).

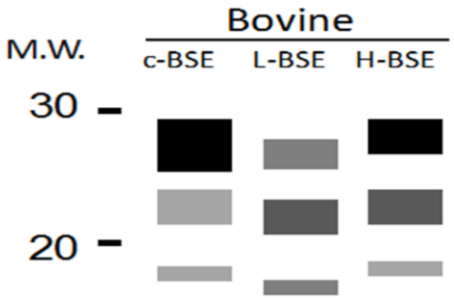


Fig. 4. PrP^{Sc} electrophoretic mobility after PK digestion in classical and atypical BSE. From the left: classical BSE, L-type BSE and H-type BSE banding in canonical western blot analysis after PK digestion (Gough, Rees et al. 2015).

Both atypical forms, but especially the L type, were further characterized by a smaller proportion of diglycosylated PrP^{Sc} compared to the proportion in cBSE isolates. In addition to the migration properties and glycosylation profiles, the H- and L-type isolates exhibited enhanced PK sensitivity at pH 8 compared to those of the cBSE isolates (Jacobs, Langeveld et al. 2007). So far, both atypical subtypes have been identified only in cattle that were at least eight years old (Tester, Juillerat et al. 2009). It has therefore been postulated that they represent a spontaneous TSE in cattle, although transmission through feed or the environment cannot be ruled out. Histopathological as well as immunohistochemical analyses show that atypical forms of BSE can be experimentally transmitted to transgenic mice and primates and clearly differ from cBSE isolates, with unique incubation periods, PrP^{Sc} profiles, and histological lesions (Buschmann, Gretzschel et al. 2006, Baron, Bencsik et al. 2007, Comoy, Casalone et al. 2008, Wilson, Dobie et al. 2013). However, some recent studies showed that H- and L-type

BSE prions may acquire cBSE–like properties during propagation in animals expressing homologous bovine prion protein (Torres, Andreoletti et al. 2011) or during inter-species transmission (Beringue, Andreoletti et al. 2007), respectively. Moreover, transmission experiments in transgenic mice and macaque have revealed clear indications for a higher zoonotic potential of this BSE form than classical BSE (Buschmann, Gretzschel et al. 2006, Comoy, Casalone et al. 2008, Kong, Zheng et al. 2008).

1.4.2.2 Scrapie

Scrapie is the ancient form of TSE and has sheep and goats as natural target species. The clinical features of this pathology are dependent on the prion strain and the animals' genetic background. The most common symptom is intense pruritus which leads to rubbing and scraping and subsequent loss of wool. This is associated to the characteristic nibbling response when pressure over the base of the tail or scratching to the dorsum is applied. Other symptoms could include behavioral changes, blindness, ataxia, incoordination, hyperexcitability and tremors. Incubation period is 2-5 years and death usually occurs within 2 weeks to 6 months after clinical onset. About 3-5% animals per affected flock may die annually (Jeffrey and Gonzalez 2007). In cases of atypical scrapie, pruritus is uncommon, while the major symptoms are ataxia and incoordination. Other signs include irritability, unusual alertness, restlessness, impaired vision, hyperesthesia, posture abnormalities, tremors, teeth grinding, and salivation (Benestad, Arsac et al. 2008). Several polymorphisms in *PRNP* have been associated to scrapie susceptibility in goat and sheep. For example, Q171R and A136V polymorphisms confer resistance and susceptibility to the infection, respectively. A three codon system based on A136V, R154H and Q171R/H polymorphisms has been

characterized with five alleles (ARQ, VRQ, AHQ, ARR and ARH) which can be combined to form 15 genotypes, i.e. ARR/ARR or VRQ/ARQ (Hussain, Babar et al. 2011). In turn, these 15 genotypes can be combined into five risk groups (R1-R5). The most resistant R1 genotype is ARR/ARR and the most susceptible R5 genotypes are VRQ/VRQ, VRQ/ARQ, VRQ/ARH and VRQ/AHQ. The remaining genotypes are of intermediary susceptibility (Imran and Mahmood 2011). This risk classification system was useful for many European states in selective breeding of sheep.

1.4.2.3 Transmissible Mink Encephalopathy (TME)

TME is a rare TSE of farmed mink. At clinical level the pathology is characterized by behavioral changes such as increased aggressiveness and hyperesthesia, depression, restlessness, and neglect in parental care and coat grooming. At the earlier disease stages, difficulty in eating and swallowing can also be observed. In the final stage of the disease, convulsions may occur; minks become somnolent and unresponsive, and can be seen to press their heads against the cage for hours. Inoculation of mink with various strains of the scrapie agent as well as inoculation with the classical BSE agent revealed the development of TME. Some studies suggested the atypical L-type BSE agent as the most likely candidate for being causative of TME (Baron, Bencsik et al. 2007).

1.4.2.4 Chronic Wasting Disease (CWD)

CWD is a TSE of captive and free-ranging members of the family Cervidae. Prevalence of the disease in affected herds may range from 0.1 to 50% or even 100%, sometimes. Chronic wasting is very common in affected cervids. Rough, dry coat and patchy retention of the winter coat in summer are also common. During the preclinical phase

some signs can be seen in affected cervids, such as lassitude, sudden death in deer after handling, a lowered head and drooping ears and behavioral changes such as fixed gaze and loss of fear of humans. Ataxia, head tremors, teeth grinding, hyperexcitability with handling and excessive salivation are some of the symptoms that can be observed during the clinical phase of the disease. Incubation time ranges from 16 months to 5 years. Death usually occurs 1 year after the onset of clinical signs (Imran and Mahmood 2011).

1.4.2.5 Exotic Ungulate Encephalopathy (EUE)

EUE is a TSE of exotic zoo ruminants of the family Bovidae. All infected animals died during the 1990s, with the last death occurring in 1998. The course of EUE and clinical symptoms varied, according to the species. It seems that these animals were infected through the consumption of meat and bone meal derived from BSE-infected cow. Indeed, similar neuropathological lesions and incubation periods were observed in mice inoculated with brain homogenates from EUE-infected ruminants and from BSE-infected cattle. Further, a similarity between the EUE and BSE strains in these mice was observed, supporting the hypothesis that EUE is caused by an infection with BSE prion.

1.4.2.6 Feline Spongiform Encephalopathy (FSE)

FSE is a TSE of domestic cats and captive wild members of the family Felidae. This disease was first reported in domestic cats within the United Kingdom in 1990. As most of the FSE cases occurred in parallel to the BSE epidemic, interspecies transmission via consumption of contaminated food was considered as causative of the disease. Indeed, mice infected with brain homogenate from BSE-infected cattle and from FSE-

infected cats developed a very similar pathology and presented similar prion strains (Eiden, Hoffmann et al. 2010). With a ban on the use of bovine spleen and central nervous system (CNS) tissue as pet feed, the FSE epidemic declined rapidly. The clinical manifestation of the disease includes severe behavioral changes (fear, aggressiveness or unusual timidity), depression, restlessness, abnormal gait and ataxia. In addition, excessive salivation, polyphagia, polydipsia and dilated pupils have been reported as symptoms of FSE. In the final stages of the disease convulsions and somnolence are common signs. Death occurs after 3-8 and 8-10 weeks of clinical onset of the disease in domestic cats and cheetahs, respectively (Sigurdson and Miller 2003).

1.4.2.7 TSE in Non Human Primates (NHP)

TSE in NHP was observed from 1996 to 1999 in Mayotte lemurs, mongoose lemurs and Rhesus macaque from a zoo and three primate facilities in France. As for other animal TSEs, also in this case the most probable cause of the infection was the ingestion of feed contaminated with BSE-prions. Indeed, lemurs experimentally inoculated with brain homogenates from BSE-infected cattle developed a TSE with profiles of neuropathological lesions similar to those seen in naturally infected lemurs and presented similar prion strains (Bons, Mestre-Frances et al. 1999, Imran and Mahmood 2011).

1.5 PrP^C physiological functions

PrP^C is a ubiquitous protein highly conserved among mammals and also expressed in other species such as fish, reptiles and birds. It is found predominantly in the CNS during embryogenesis and in adult life, but it is also highly abundant in the lymphoreticular tissues such as lymph nodes and spleen. In the CNS, PrP^C is prevalently expressed in neurons of the cerebellum, cerebral cortex, hippocampus, medulla oblongata, and thalamus. Its prevalent synaptic localization suggests an involvement in synaptic signaling and in the neurotransmitter system of the CNS (Collinge, Whittington et al. 1994, Sales, Rodolfo et al. 1998). Although most PrP^C is found in lipid rafts, some of the protein is transferred to clathrin-coated pits where is subjected to constitutive endocytosis and recycling (Naslavsky, Stein et al. 1997, Sunyach, Jen et al. 2003, Westergard, Christensen et al. 2007). The endocytic recycling pathway followed by PrP^C and its affinity for Cu^{2+} suggests a possible role in copper metabolism (Brown, Qin et al. 1997, Stockel, Safar et al. 1998, Millhauser 2007). For PrP^C many other possible biological functions have been suggested: cell adhesion, myelin maintenance, neurite outgrowth, antiapoptotic activity, and oxidative stress protection (Westergard, Christensen et al. 2007). Despite being one of the most intensively studied proteins, a definite role for PrP^C has not been clarified yet and constitutes one of the main challenge in prion biology. The possible functions of PrP in the immune system will be discussed in detail later in this text (see paragraph 1.7).

1.6 PrP^{Sc} pathological functions

In the prion field great effort has been made to understand the mechanisms of conversion and infectivity while much less attention has been posed on the mechanism by which prions cause the CNS pathology. How do prions exert their pathological effect? There are three main hypotheses explaining how the conversion of PrP^{C} into PrP^{Sc} may cause neurodegeneration in the CNS:

1) GAIN OF FUNCTION- According to this view, PrP^{Sc} would acquire new properties which are different from those related to the normal, physiological function of PrP^{C} (**Fig. 5A**). This toxic gain of function could be related to the activation of different interactors and signaling pathways or to the general impairment of cell homeostasis. For example, PrP amyloid fibrils may block the normal neuronal transport, interfere with axonal signaling or physically damage neuronal membranes (Ermolayev, Friedrich et al. 2009, Senatore, Restelli et al. 2013).
2) LOSS OF FUNCTION- This hypothesis postulates that upon conversion to or contact with PrP^{Sc}, PrP^{C} would lose all or some of its putative biological functions, thus resulting in neuronal damage and degeneration (**Fig. 5B**). However, several lines of experiments using PrP-KO prenatal or postnatal mice models showed a very slight effect on the phenotype and no features of prion pathology (Bueler, Fischer et al. 1992, Manson, Clarke et al. 1994, Mallucci, Ratte et al. 2002). Thus, loss of PrP^{C} function is not responsible, alone, for prion-induced neurodegeneration. The dominant inheritance of familial prion disease also belies the loss of function hypothesis. As an alternative, it is possible that the pathology caused by toxic gain of function would be exacerbated by a loss of function mechanism.

3) SUBVERSION OF FUNCTION- An intriguing possibility is that PrP^{Sc} subverts or alters the physiological function of PrP^{C}, rather than causing a complete loss of function. This effect could be produced by crosslinking of PrP^{Sc} to PrP^{C} and the subsequent activation of neurotoxic signaling pathways (Solforosi, Criado et al. 2004) (**Fig. 5C**). Consistent with this theory, experimental evidence has shown that expression of PrP^{C} in neurons is necessary to confer sensitivity to the infection (Brandner, Isenmann et al. 1996, Mallucci, Dickinson et al. 2003). GPI-anchoring appears to be required for such pathogenic mechanism, since anchorless prion protein results in infectious amyloid disease without clinical scrapie (Chesebro, Trifilo et al. 2005). How could PrP^{C} functions be modified? A possibility is that PrP^{Sc} fibrils and oligomers would induce aggregation of cell surface PrP^{C}, thereby generating a neurotoxic rather than a neuroprotective signal. Alternatively, PrP^{Sc} may bind specific PrP^{C} domains thereby altering the signaling properties of the latter (Westergard, Christensen et al. 2007).

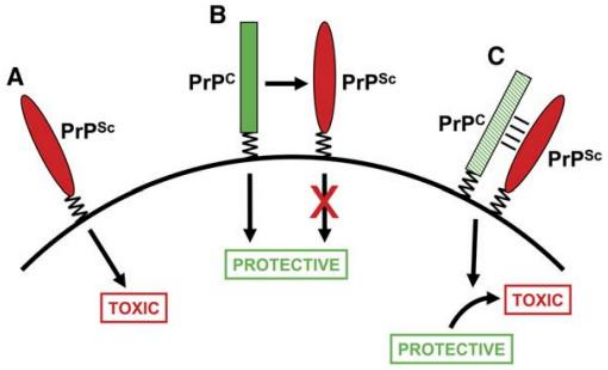


Fig. 5. Models for the cellular toxicity of PrP^{Sc}. Toxic gain-of-function: PrP^{Sc} possesses a novel neurotoxic activity that is independent of the normal function of PrP^{C} (A); Loss-of-function: PrP^{C} possesses a normal, physiological activity that is lost upon conversion to PrP^{Sc} (B); Subversion-of-function: The normal, neuroprotective activity of PrP^{C} is subverted by binding to PrP^{Sc}. From (Harris and True 2006).

1.7 PrP^C and the immune system

For obvious reasons the majority of the research in the prion field has been focused on the pathophysiology of PrP in the CNS. However, PrP^C is a protein expressed throughout the organism and it is selectively enriched and developmentally regulated in other cell types besides neurons (Linden, Martins et al. 2008). Importantly, lymphoid and myeloid tissues represent the second compartment, after the brain, where PrP^C is most abundantly expressed (Ballerini, Gourdain et al. 2006). A substantial amount of PrP^C has been found in human T and B lymphocytes, natural killer (NK) cells, platelets, monocytes, dendritic cells (DCs) and follicular dendritic cells (FDCs) as well as in hematopoietic stem cells (HSCs) (Dodelet and Cashman 1998, Barclay, Hope et al. 1999, Burthem, Urban et al. 2001, Li, Liu et al. 2001, Thielen, Antoine et al. 2001, Isaacs, Jackson et al. 2006, Zhang, Steele et al. 2006). In addition, the immune system plays a fundamental role in prion pathology, since a substantial amplification of the infectious agent takes place within lymphoid compartments in early stages of the diseases (Aguzzi, Heppner et al. 2003, Linden, Martins et al. 2008). This process is dependent at least in part on expression of PrP^C by certain immune cells (Brown, Stewart et al. 1999). However, as for the CNS, PrP^C seems to be not strictly required for the normal physiology of the immune system. Indeed, no gross abnormalities were detected in PrP-KO mice, both in the numbers and maturation of the immune cells and in the expression of important cell surface antigens (Linden, Martins et al. 2008). In particular, PrP null mice displayed a normal number of dendritic, T CD4+, T CD8+ and B cells and had normal expression of MHC class I and II molecules (Bueler, Fischer et al. 1992, Kubosaki, Nishimura-Nasu et al. 2003, Ballerini, Gourdain et al. 2006, Zhang,

Steele et al. 2006). This suggests that immunomodulatory functions of PrP^C are either subtle, or may be relevant only in particular contexts. Expression of PrP^C during myeloid ontogenesis has been reported by Zhang and colleagues, who found a role for PrP in the self-renewal properties of long-term repopulating HSCs (Zhang, Steele et al. 2006). In another study, it was shown that the maturation of human CD34+ into CD15+ granulocytes is associated to a downregulation of PrP^C expression in these cells (Dodelet and Cashman 1998). In a similar manner, PrP^C levels are downregulated in mature neutrophils compared to CD43+ Gr-1+ granulocyte precursors from mouse bone marrow (Dodelet and Cashman 1998). On the contrary, maturation of DCs and monocytes leads to PrP^C upregulation, (Durig, Giese et al. 2000, Burthem, Urban et al. 2001, Ballerini, Gourdain et al. 2006), even if a causal effect was excluded by subsequent experiments on PrP null mice (Ballerini, Gourdain et al. 2006, Linden, Martins et al. 2008). PrP^C has been proposed to down-regulate phagocytosis by macrophages (de Almeida, Chiarini et al. 2005, Wang, Zhao et al. 2014) but also to be required for the macrophage invasion by Brucella abortus (Watarai, Kim et al. 2003), although this finding has been challenged (Fontes, Alvarez-Martinez et al. 2005). Regarding lymphoid ontogenesis, there are contrasting results related to the involvement of PrP^C. Maturation of T and B lymphocytes in mice has been associated to downregulated levels of PrP^C while in human and sheep its expression on mature blood and lymphoid cells remained high (Politopoulou, Seebach et al. 2000). PrP^C expression was found to be increased during human NK cell differentiation, particularly in CD56+ CD3+ NK T cells (Durig, Giese et al. 2000). Concerning the mature T cell subset, PrP^C has been found at slightly higher levels in CD8+ compared to CD4+ cells and in

CD45RO+ memory compared to CD45RA+ naive T lymphocytes (Durig, Giese et al. 2000, Politopoulou, Seebach et al. 2000, Li, Liu et al. 2001, Isaacs, Jackson et al. 2006). Hence, PrP^C may be more important in certain types of functionally differentiated lymphocytes that operate in particular immune environments. In addition to the maturation status of various cell types, PrP^C expression also depends on their activation status. In this regard, an upregulation of PrP upon activation of T cells, monocytes, and DCs has been found (Durig, Giese et al. 2000, Li, Liu et al. 2001, Martínez del Hoyo, López-Bravo et al. 2006). Also, treatment with anti-CD3 antibodies or concanavalin A (Con A) leads to PrP^C upregulation and cell proliferation (Cashman, Loertscher et al. 1990, Kubosaki, Nishimura-Nasu et al. 2003). Con A-induced proliferation was prevented by anti-PrP^C antibodies (Cashman, Loertscher et al. 1990). Subsequent studies confirmed the upregulation of PrP^C following stimulation with multivalent lectins (Mabbott, Brown et al. 1997, Li, Liu et al. 2001, Ballerini, Gourdain et al. 2006) and showed reduced mitogen-induced proliferation in PrP-null murine splenic lymphocytes (Mabbott, Brown et al. 1997, Linden, Martins et al. 2008). Interestingly, following stimulation with ConA, which requires the T-cell receptor (TCR) complex, but not after stimulation with phorbol ester plus ionomycin, which bypasses the TCR, supernatants of transfected PrP^C-expressing splenocytes contained higher amounts of both IL-2 and IL-4, compared to PrP-null splenocytes (Bainbridge and Walker 2005, Linden, Martins et al. 2008). Such finding suggests that signalling via elements of the TCR may be required to induce changes in PrP expression. Contrary to T lymphocytes, upregulation of PrP^C was not observed in activated B cells treated with lipopolysaccharide (Kubosaki, Nishimura-Nasu et al. 2003). In human T cells PrP^C has been found to co-localize

and/or co-immunoprecipitate with ganglioside GM3 (Mattei, Garofalo et al. 2002), and to a lesser extent GM1 (Hugel, Martinez et al. 2004) and with Fyn, Reggie-1, and Lymphocyte-specific tyrosine kinase P56lck (Lck) (Mattei, Garofalo et al. 2004, Stuermer, Langhorst et al. 2004). PrP^C association with Zeta chain associated protein 70 kDa (Zap70) was also observed following activation with anti-CD3 and anti-CD28 (Mattei, Garofalo et al. 2004). However, although PrP^C accumulates at sites of T cell-DC contact during MHC-peptide stimulation, it does not co-localize with CD3, LFA-1 (leucocyte function antigen 1), CD43, LAT (linker for activation of T cells) or Thy-1 (thyroid hormone 1) (Ballerini, Gourdain et al. 2006, Isaacs, Jackson et al. 2006). Thus, if PrP^C plays a specific role within the immune synapse or if its association to the signaling complex involved in T cell activation reflects only a non-specific clustering effect is still under debate.

1.8 PrP^{Sc} and the immune system

In all TSEs the pathogenic and ultimately lethal effects of prion infection are confined to the neuronal tissues of the brain. However, there are many crucial steps occurring in lymphatic organs which precede prion neuroinvasion. Three main evidences support the involvement of peripheral lymphoid tissues in prion propagation:

1) Accumulation and early infectivity of lymphoid tissues before clinical symptoms and infectivity in the CNS (Hilton, Fathers et al. , van Keulen, Schreuder et al. 1996, Andréoletti, Berthon et al. 2000).

2) Removal of local draining lymphoid tissue blocks subsequent circulation to other lymphoid organs and neuroinvasion (Glaysher and Mabbott 2007, Glaysher and Mabbott 2007).

3) Effect of targeted immune impairments on host susceptibility to peripheral inoculation (Aguzzi, Heppner et al. 2003).

In most documented infectious forms of TSEs, prions must transit through the lymphoreticular compartment where they spread and multiply without any apparent clinical symptoms (Aucouturier and Carnaud 2002). Prions can escape immune surveillance, colonize and hijack immune components, replicate in the lymphoreticular compartment and finally gain access to the CNS. Of note, severe combined immunodeficient (SCID) mice proved to be partially resistant against combined intracerebral and intraperitoneal inoculation of BSE cow brain homogenate (Brown, Stewart et al. 1997). On the contrary, SCID mice intracerebrally infected with a mouse-passaged scrapie strain, ME7, produced 100% incidence of the disease (Fraser, Brown

et al. 1996). Such results suggest that the peripheral immune system may also give a major contribution in interspecies transmission and species-barrier phenomenon. Neuroimmunological phenomena also play an important role in the final stages of the disease (Aguzzi, Barres et al. 2013). For all these reasons, it is of the utmost importance to understand the peripheral mechanisms of prion infection and to identify the cell subsets and the genes of the immune systems which are mainly involved in prion pathogenesis.

1.8.1 Prion entry sites and their spread within the host

Entry sites of prions are crucially dependent on the route of infection. Prion inoculation via peritoneal, cutaneous, or venous routes typically leads to lymph node and spleen invasion first, followed by that of intestine mucosa and spinal cord, and then the brain (Aucouturier and Carnaud 2002). As for oral infection, which is the most relevant route for acquired prion disease in nature, the first area to be colonized is the gut-associated lymphoid tissue (GALT). Studies in natural or experimental scrapie (Maignien, Beringue et al. 1999, Sigurdson, Williams et al. 1999, Andréoletti, Berthon et al. 2000, Beekes and McBride 2000, Heggebø, Press et al. 2000), in sheep and mice infected with BSE (Somerville, Birkett et al. 1997, Maignien, Beringue et al. 1999, Foster, Parnham et al. 2001) or in deer with CWD (Sigurdson, Williams et al. 1999) indicated a main involvement of the ileal Peyer's patches (PPs). PPs are aggregated lymphoid follicles surrounded by a particular epithelium, the follicle-associated epithelium (FAE) (Jung, Hugot et al. 2010). Located within the FAE of PPs are microfold cells (M cells), a subset of epithelial cells specialized for transepithelial transport of luminal macromolecules and

particulate antigens toward the lamina propria, where there are resident antigens presenting cells (APCs). M cells have been shown to play a major role in prion uptake, since their depletion blocked prion oral infection in C57BL/6 mice infected with ME7 scrapie prions (Donaldson, Kobayashi et al. 2012). In addition, the rapid accumulation of prions in PPs after intra-gastric inoculation and the positive correlation between the number of PPs and susceptibility to prion oral infection both argue in favor of FAE of the PPs as the most plausible prion entry site (Beekes and McBride 2000, Prinz, Huber et al. 2003).

After crossing the FAE, prions spread — possibly through a cell-mediated mechanism – within the lymphatic system and then into the brain. The mesenteric lymph nodes are colonized first and the infection proceeds in other secondary lymphoid organs (SLO), the sympathetic nervous fibers and myenteric ganglia. Prion accumulation and replication within SLO has been found mainly in natural scrapie (Pattison and Millson 1960), CWD (Sigurdson, Williams et al. 1999), TME (Hadlow, Race et al. 1987) and vCJD (Hilton, Fathers et al. 1998). Therefore, prions causing such pathologies are indicated as lymphotropic. In some cases, prions may invade the brain directly, without spreading in the lymphoid tissues (neurotropic prions) (Mohri, Handa et al. 1987). However, no strict distinction can be made between the two forms of the pathology since evidence indicates that the extent of lymphotropism of a prion is the result of a combination of the prion strain, inoculation route, the host species and the gene sequence encoding the prion protein (Aguzzi, Nuvolone et al. 2013). In sharp contrast with what happens in the brain, the spreading of prions through the lymphoid tissues is not associated with major morphological or functional alterations (Aucouturier and

Carnaud 2002). The finding that prions invade the lymphoreticular system long before the CNS opens prospects regarding preclinical diagnosis and prevention of brain colonization. Interestingly, direct intracerebral inoculation also results in early infectivity of spleen and lymphoid organs (Lawson, Furness et al. 2010). The spleen is one of the main organs involved in peripheral prion replication and infectivity (Wadsworth, Joiner et al. 2001, Daude 2004). Genetic asplenia or splenectomy prior to, or shortly after, a peripheral scrapie challenge significantly extended the survival of mice (Clarke and Haig 1971, Mabbott, Farquhar et al. 1998). However, the contribution of the spleen in prion spread seems to be important only in the first phases of the diseases, since studies of splenectomy at different times after peripheral infection showed that survival was not extended once the infection had already reached the spinal cord (Kimberlin and Walker 1989). Fractionation of splenocytes and experiments using sublethal ionizing irradiation — which depletes mitotically active haematopoietic cells but not mitotically quiescent stromal cells — indicated that most infectivity is present in stromal compartment of the spleen rather than hematopoietic one (Lavelle, Sturman et al. 1972, Fraser and Farquhar 1987).

1.8.2 Role of Follicular Dendritic Cells (FDCs)

As FDCs are derived from stromal perivascular precursor cells (Krautler, Kana et al. 2012) and express high levels of PrP^{C}, they were thought to represent a candidate for peripheral prion replication. The FDC are tissue-fixed and non-phagocytic cells localized within B cell follicles of lymphoid tissues. It has been found that FDCs are important peripheral sites for PrP^{Sc} accumulation (Kitamoto, Muramoto et al. 1991) and that

FDCs-specific PrP^C expression alone is sufficient to sustain prion infection in the spleen (McCulloch, Brown et al. 2011). Formation and maintenance of mature FDCs during the germinal center response require the presence of B cells expressing membrane-bound lymphotoxin-α1/β2 (LTα1β2) (Myers, King et al. 2013). Treatment of mice with soluble lymphotoxin-β receptor resulted in the disappearance of mature FDCs from the spleen and to the abolishment of splenic prion replication and retarded neuroinvasion after intraperitoneal scrapie inoculation (Montrasio, Frigg et al. 2000). In the following years, it has emerged that the positioning of FDCs within the spleen controls prion neuroinvasion: ablation of the chemokine receptor CXCR5 juxtaposes follicular FDCs to major splenic nerves, and accelerates the transfer of intraperitoneally administered prions into the spinal cord (Prinz, Heikenwalder et al. 2003). However, ablation of TNFR1 (Tumor necrosis factor receptor 1) signaling prevents prion pathogenesis in spleen but not in lymph nodes, despite the absence of FDCs (Prinz, Montrasio et al. 2002). The latter result suggests that, despite the primary role of FDCs, other cell types contribute to prion amplification in lymph nodes and probably in the spleen.

1.8.3 Role of Dendritic Cells (DCs)

The most plausible cells involved in the spread of prion from the FAE are DCs, which migrate through the lymphatic system and contribute to prion propagation inside the organism (Aguzzi, Nuvolone et al. 2013). DCs are potent APCs which act as a bridge between the innate and adaptive immune systems. Once activated, they migrate to the lymph nodes where they interact with T and B lymphocytes to initiate an antigen-specific immune response or induce tolerance (Banchereau, Briere et al. 2000). In contrast to

FDCs, DCs derived from bone-marrow precursors, are migratory cells and have phagocytic functions. They are present in those tissues that are in contact with the external environment, such as skin (where there are specialized DCs called Langerhans cells) or inner layers of intestines, stomach, etc. A subset of DCs is present also in the blood. They do not have dendrites and are less mature but still they perform several functions such as antigen presentation, chemokine production and INFα secretion. Since DCs play a variegate array of functions within the immune system, they can interact in diverse ways with prions and contribute in several contrasting manners to prion pathogenesis. Some studies revealed that DCs may help in the protection of the host against infection by sequestering and destroying the prions (Luhr, Wallin et al. 2002, Mohan, Hopkins et al. 2005, Rybner-Barnier, Jacquemot et al. 2006). Others suggests that prions may exploit DCs as a Trojan horse to enter and spread from the infected sites to the lymphoid tissues (Huang, Farquhar et al. 2002, Raymond, Aucouturier et al. 2007, Sethi, Kerksiek et al. 2007, Cordier-Dirikoc and Chabry 2008). An important role for DCs in peripheral prion spread was highlighted by Raymond and colleagues in 2007. They showed that depletion of CD11c+ DCs *in vivo* impaired prion accumulation in GALT and spleen and reduced the susceptibility to orally administered prions (Raymond, Aucouturier et al. 2007, Aguzzi, Nuvolone et al. 2013). DCs may also be relevant in the subsequent transfer of prions from the periphery to the CNS by connecting the immune and peripheral nervous systems (Mabbott and Bradford 2015). In a study published by Christelle Langevin and colleagues, it has been shown that bone-marrow-derived DCs (BMDCs) are able to transfer PrP^{Sc} to cerebellar neurons by direct cell–cell contact via tunnelling nanotubes (Langevin, Gousset et al. 2010).

1.8.4 Role of B lymphocytes

A 1997 study published by Klein and colleagues revealed an important and unexpected role for B cells in prion diseases. Using a panel of immune-deficient mice, the authors found that only defects in B cell maturation and activation prevented the development of clinical scrapie. Moreover, they found that involvement of B lymphocytes was independent from the specificity of their receptors and from the activation of FDCs (Klein, Frigg et al. 1997). This discovery was followed by a plethora of follow-up studies. Surprisingly, it was found that PrP^C expression on B lymphocytes is not required for prion neuroinvasion (Klein, Frigg et al. 1998). However, experiments performed on chimeric *Prnp*$^{-/-}$ mice which were reconstituted with wild-type bone marrow or fetal liver cells, showed that PrP^C expression is required in other cell type to sustain the replication of prions in the spleen (Blattler, Brandner et al. 1997, Kaeser, Klein et al. 2001). Thus, these data indicate that prion replication in the spleen requires a PrP^C-expressing cells of stromal origin that depends, directly or indirectly, on B cells, without regard to PrP^C expression in the latter (Aguzzi, Nuvolone et al. 2013).

1.8.5 Role of T lymphocytes

Experiments performed in 1971 by Clark and Haig showed that genetic athymia or thymectomy do not extend the survival of mice peripherally inoculated with prions (Clarke and Haig 1971). On the other hand, constitutive or acquired lymphocyte deficiency was shown to impair prion replication following peripheral, but not intracerebral, inoculation of rodent-adapted prions (Kitamoto, Muramoto et al. 1991,

Lasmézas, Cesbron et al. 1996, Aguzzi, Nuvolone et al. 2013). Taken together, these results indicate that lymphocytes may be important for trafficking prions within lymphoid organs but not as major reservoir of infectivity. There is some experimental evidence linking T lymphocyte with prion pathology, even though its role in the pathogenic process is still far from being elucidated. In a very recent study, it has been found that PrP^{Sc} is present in the blood from preclinical infected sheep in association with monocytes and T lymphocytes (Dassanayake, Madsen-Bouterse et al. 2016). In another study, the existence of a CD4+ T cell-mediated immunity against foreign PrP^{C} was shown, which may be involved in the species-barrier phenomenon. The authors argue that in a natural situation the infectious materials consist of both PrP^{Sc} and PrP^{C}, therefore they assume that an immune response against PrP^{C} will also affect PrP^{Sc} (Stoltze, Rezaei et al. 2003). Finally, T lymphocytes may have a role in CNS prion pathology, since CD4+ and CD8+ T lymphocytes infiltrates have been found in murine and human brains infected with TSE (Lewicki, Tishon et al. 2003).

1.8.6 Innate immune system in prion disease

The innate immune system is the first defense of the organism against invading pathogens. It is evolutionally older with respect to the adaptive immunity and unlike the latter it does not maintain immunological memory. Cells of the innate immune system recognize a set of generic molecules present on pathogens, collectively known as pathogen-associated molecular patterns (PAMPs). Following activation, these immune cells release inflammatory mediators, such as cytokines and chemokines, which in turn attract and activate other cells of the adaptive and innate immunity. Innate immunity has

been shown to play a dual role in TSEs, both abetting and contrasting prion pathogenesis (Zabel and Avery 2015). Hereunder some of the components of innate immunity as they relate to prion pathology will be discussed.

1.8.6.1 Complement system

It has been proposed that the first active response to prion infection may be the complement system, which is activated during the early phases of TSE pathogenesis (Kovacs, Gasque et al. 2004). The complement system is composed of numerous small blood proteins that are generally synthesized in the liver but also by tissue-resident mononuclear phagocytes (MNPs), epithelial cells and stromal components such as FDCs (Bradford and Mabbott 2012). The complement system, including C1q and C3, can opsonize PrP^{Sc} and aid for its targeting to lymphoid follicles (Mabbott 2004). In particular, it has been shown that C1q enhances the uptake of disease-associated PrP by classical DCs (Flores-Langarica, Sebti et al. 2009). Regarding a possible role for the complement system in CNS prion pathogenesis, at present there is little evidence supporting this hypothesis. Absence of C1qa, C2 or C3 did not alter the host response to intracerebral inoculation (Klein, Kaeser et al. 2001) and mice lacking C3 and C4 presented no deficit to prion pathogenesis after aerosol exposure to prions (Haybaeck, Heikenwalder et al. 2011).

1.8.6.2 Macrophages and monocytes

Macrophages, together with dendritic cells, monocytes and Langerhans cells, are part of a heterogeneous group of hematopoietic-derived phagocytes known as mononuclear

phagocytes (MNPs). By virtue of their diversity, MNPs may play variegated roles in prion pathogenesis. In fact, they may either destroy TSE agents or contribute to their spread within the lymphoid system. In literature, a body of evidence exists showing that macrophages are able to phagocytize and degrade scrapie and BSE prions (Carp and Callahan 1981, Sassa, Inoshima et al. 2010, Sassa, Yamasaki et al. 2010). Furthermore, TSE pathogenesis is accelerated in macrophage-depleted mice and scrapie accumulation is increased in the spleen (Beringue, Demoy et al. 2000). On the other hand, macrophages may be also involved in prion delivery from M cells to lymphoid tissues (Takakura, Miyazawa et al. 2011). Finally, with regard to monocytes (the precursors of both DCs and macrophages), they may be involved in prion spread during the preclinical phase of TSE infection. Indeed, a recent study identified the scrapie agent associated to monocytes in blood from naturally affected sheep (Dassanayake, Madsen-Bouterse et al. 2016).

1.8.6.3 Granulocytes

Granulocytes are white blood cells characterized by the presence of granules within the cytoplasm, which contain toxic material or inflammatory substances such as antimicrobial agents, enzymes, hydrolases, histamine, prostaglandins or nitric oxide. Granulocytes are classified as a) basophils, b) eosinophils, c) neutrophils, and, d) mast cells. Basophils represent only about 0.5 to 1% of circulating white blood cells. They are involved in inflammatory reactions during immune response, as well as in the formation of acute and chronic allergic diseases. At present there are no reports linking basophil activity and prion disorders. Mast cells are very similar in both appearance and function

to the basophils. During the early response to a generic infection they release heparin and histamine. Mast cells are able to migrate from blood to brain (Silverman, Sutherland et al. 2000) and they do express high levels of PrP^{C} which can be shed upon their activation (Haddon, Hughes et al. 2009). Notwithstanding, due to the non-inflammatory nature of prion diseases outside the CNS, it is unlikely that mast cells may play a major role in peripheral TSE pathology. In some case it has been observed that factors, such as C3a and Chemokine (C-C motif) ligand 3 (CCL3), can lead to mast cell activation without degranulation (Gilfillan and Tkaczyk 2006), which may occur in TSE infection. In the CNS, mast cells exert neuromodulatory functions and their trafficking into the brain can be triggered behaviorally (Silver, Silverman et al. 1996). Thus, in the late phases of the infection, mast cell could be able to provide the CNS with a copious amount of PrP^{C} as substrate for conversion (Bradford and Mabbott 2012). As for eosinophils, they are more abundant in the blood with respect to the basophils, representing about 1–6% of circulating leukocytes. They do not express PrP^{C} (Barclay, Houston et al. 2002). Although amyloid deposits themselves are eosinophilic, there is some evidence to indicate the presence of eosinophils within inclusions of both central and peripheral amyloid plaques (Iwasaki, Mori et al. 2014). Finally, the neutrophils has been found to be inhibited by both native PrP and PrP^{27-30} (Miragliotta, Fumarulo et al. 1990).

1.8.6.4 Natural killer (NK)

NK is a type of cytotoxic lymphocyte mainly involved in protection against viral infection and tumor formation via the expression of granzyme and perforin. NKs differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils and thymus, where they enter

into circulation. They are different from the natural killer T cells (NKT), in that they do not express TCRs nor CD3 or immunoglobulins. On the contrary, they usually express CD16 (FcRIII) and CD56 (also known as NCAM**)** in humans. Recent research highlighted the important functions played by NKs, which go beyond their cytotoxic role in innate immunity. Indeed, NKs have been found to be involved in self-tolerance and immunological memory. NK cells are typically stimulated via interleukins, 2, 12, 15 and 18 and chemokine (C-C motif) ligand 5 (CCL5) as well as down-regulation of MHC class I molecules following viral infection (Bradford and Mabbott 2012). Overall evidence in literature indicates that NKs have little influence on the pathogenesis of prion diseases. Indeed, KO mice which did not express perforin did not show any difference in prion pathology compared to the wild-type controls (Klein, Frigg et al. 1997). In a recent study published by Castro-Seoane and colleagues, high titers of prions were found for the first time associated to NK cells (Castro-Seoane, Hummerich et al. 2012). Thus, the fact that NKs have not been reported to respond to prion infection could be the consequence of immune-escape mechanisms adopted by prions. However, at present there is little evidence for such a theory.

1.8.6.5 Platelet

Platelets, also called thrombocytes, are cells without a nucleus that circulate within the blood and form clots in the case of damaged vessels and bleeding (hemostasis). They are fragments of cytoplasm derived from the megakaryocytes. Once they recognize an injury, they become activated and aggregate. Formation of a platelet plug is associated with activation of the coagulation cascade with resultant fibrin deposition and linking. It

has been suggested that platelets may have a role in prion spread since some studies showed that, within the blood, they express the highest amount of PrP^C (Barclay, Hope et al. 1999, MacGregor, Drummond et al. 2000). In particular, it has been shown that in platelets PrP^C is associated to alpha granules, together with proteins such as CD62 (also known as P-selectin), cluster of differentiation 36 (CD36), transforming growth factor β (TGFβ), and insulin-like growth factor 1 (**IGF-1)** (Starke, Harrison et al. 2005). Activated platelets release PrP^C initially in small quantities on microvesicles and subsequently at higher levels on exosomes. (Perini, Vidal et al. 1996, Robertson, Booth et al. 2006). The role of PrP^C in platelets, as well the role of platelet-derived exosomes, is at present unknown. One hypothesis is that exosomes release is a general mechanism for the transport of proteins and, consequently, a vehicle for pathogen transmission, including prions, between cells (Fevrier, Vilette et al. 2004, Février, Vilette et al. 2005, Porto-Carreiro, Fevrier et al. 2005). Therefore, it has been suggested that platelet-derived exosomes could potentially act as an important source of PrP^C for prion replication (Robertson, Booth et al. 2006). In line with this hypothesis, sheep bioassay experiments revealed that platelets, in combination with B lymphocytes, harbor the highest level of prion infectivity within blood (Dassanayake, Schneider et al. 2011).

1.9 Prion and blood

Recent evidence has emerged that PrP^{Sc} can be detected in the blood of humans and animals affected by TSEs. Indeed, it is nowadays a matter of fact that prions can be transmitted through blood or blood derivatives (Andréoletti, Litaise et al. 2012, Aguzzi, Nuvolone et al. 2013). Propagation of prions through blood has been showed by Mathiason and colleagues, who found that CWD can be transmitted via blood transfusion in deer (Mathiason, Powers et al. 2006). Importantly, it has been shown that prion disease can be transmitted by whole blood transfusion during the symptom-free phase of experimental BSE infection (Houston, Foster et al. 2000, McCutcheon, Alejo Blanco et al. 2011). It seems that not only white cells, but all blood components (red cells, plasma and platelets), can transmit prion infection (McCutcheon, Alejo Blanco et al. 2011). These reports highlight the importance of finding a preclinical test for vCJD, since blood transfusion from asymptomatic vCJD affected patients may represent a risk of spread of vCJD infection among the population, especially in UK. At present, the prevalence of vCJD infected and asymptomatic individuals in the BSE-exposed population remains extremely uncertain (Lacroux, Comoy et al. 2014). According to statistical modeling and retrospective studies performed on appendix and tonsil samples, the number of vCJD-infected asymptomatic individuals ranges from 900 (statistical modelling; Clarke et al., 2007) to 3800 (screening of anonymous appendix and tonsil tissue archives; Hilton et al., 2004). A more recent study on 32400 appendix samples from patients born between 1941 and 1984, indicated a vCJD prevalence even higher, equal to 1 over 2000 in this age cohort (Gill, Spencer et al. 2013). Recent modeling studies predict a prevalence of vCJD between 84 and 3000 cases in the years

2010 to 2179 (Garske and Ghani 2010, Bishop, Diack et al. 2013). In addition, the possibility of silent carrying by vCJD infected individuals was raised by studies on human PrP transgenic mouse models which indicated that the BSE agent can colonize lymphoid tissues without involving the CNS and causing clinical disease (Béringue, Herzog et al. 2012). So far, four cases of vCJD transmission between human patients have been identified following blood transfusion from asymptomatic donors who subsequently developed vCJD (Llewelyn, Hewitt et al. 2004, Peden, Head et al. 2004, Wroe, Pal et al. 2006). Three of them developed clinical disease and the fourth died of other causes but was positive for markers of the infection. Case-control studies were not able to predict or anticipate these unlucky events (Collins, Law et al. 1999). Many attempts have been made to identify prions in blood and to develop a preclinical test for TSE detection (Wadsworth, Joiner et al. 2001). Studies in rodent models suggest that the levels of infectivity in blood are very low, with buffy coat fractions containing 2 or 10 intracerebral median lethal dose ($LD_{50)}$ units per mL and 100 LD_{50} units per mL during the asymptomatic and the symptomatic phases of disease, respectively (Brown, Rohwer et al. 1998, Edgeworth, Farmer et al. 2011). Whole blood from mice contained as little as 10 LD_{50} units in 1 ml (Cervenakova, Yakovleva et al. 2003). Blood fractions from a single vCJD affected patient revealed an infectious titer of 4.45 LD per mL (Douet, Zafar et al. 2014). These ranges are several orders of magnitude lower than the sensitivity of conventionally used immunoassays. Furthermore, PrP^{C} background in blood is higher with respect to any other tissue (Edgeworth, Farmer et al. 2011). Over the past decade several approaches have been tested to develop a blood-based diagnostic test for prion diseases. Protein Misfolding Cyclic Amplification (PMCA) has been used to detect PrP^{Sc}

in buffy coat fractions of rodent and sheep blood (Castilla, Saa et al. 2005, Saa, Castilla et al. 2006, Thorne and Terry 2008). Human platelets have been used in PMCA experiments as substrate for the amplification of PrP^{Sc} from variant and sporadic CJD brain homogenates (Jones, Peden et al. 2007, Jones, Peden et al. 2009). In 2014 Lacroux and colleagues identified appropriate conditions and substrates for highly efficient and specific *in vitro* amplification of vCJD/BSE agent using PMCA. With this approach they could identify three out of the four tested vCJD affected patients and no false positives were observed in 141 healthy controls (Lacroux, Comoy et al. 2014). However, as expressed in the paper of Tattum and colleagues, the technical limitations associated with PMCA, such as the time scales involved, substrate availability and suitability and increasing evidence for the spontaneous generation of protease resistant PrP *de novo,* suggest that PMCA is unlikely to provide the sole technology for a prion blood screening assay (Tattum, Jones et al. 2010). Besides PMCA, other approaches have been tested, such as magnetic bead-based immunoassay coupled with laser-induced fluorescence spectrofluorometry (Kim, Wang et al. 2005), sandwich conformation-dependent immunoassay with capturing antibody with and without PK digestion (Bellon, Seyfert-Brandt et al. 2003, Tattum, Jones et al. 2010) and counting of single prion particles bound to a capture-antibody surface (Birkmann, Henke et al. 2007). Further, optimized solid-state capture matrix coupled with direct immunodetection of the surface-bound material was tested by Edgeworth and colleagues (Edgeworth, Farmer et al. 2011). Real-time quacking-induced conversion (RT-QuiC) assay of cerebrospinal fluid and was used to distinguish with high sensitivity CJD from non-CJD condition (Orru, Groveman et al. 2015). Miele and colleagues used

differential display analysis of cDNAs to identify transcripts that were differentially expressed during the course of prion diseases (Miele, Manson et al. 2001). With this unbiased 'transcriptomics' approach they could identify a downregulated transcript coding for erythroid differentiation-related factor (EDRF), a protein mainly found in erythroid precursors—a cell lineage that nobody would have suspected to be associated with prions. Such discovery opened the doors to a genomic approach to prion diagnostic.

2. Aims

The aim of this study was to identify common transcriptome changes in whole blood from atypical H- and L- type-infected animals prior and after the onset of the clinical signs. This approach would allow the identification of candidate genes that could be further investigated as biomarkers of the disease. In addition, our study could contribute to shed light on the peripheral mechanisms of prion infection.

3. Results

3.1 Preparation and selection of samples for microarray analysis.

To investigate if gene expression alterations were present in blood from atypical BSE-infected cattle (clinical and preclinical), we performed microarray experiments using Affymetrix GeneChip® Bovine Genome Array. Frozen blood samples collected from preclinical and clinical infected cattle (from Konold's study group: Konold et al 2012) were sent in dry ice from the Animal Health and Veterinary Laboratories Agency (AHVLA, UK) to the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (IZSPLV, Turin, Italy), where RNA isolation was performed in a BSL3 facility. A first trial set of RNA samples was purified and eluted in 100 µL of RNase free water. These samples were frozen and sent to SISSA (Trieste, Italy) for subsequent analysis. Their concentration and quality was assessed by capillary electrophoresis. We observed quite a low RNA concentration for the majority of the samples as well as a low RNA Integrity Number (RIN) value for many of them. These samples did not satisfy the minimal requirements for proceeding to the microarray analysis. Therefore, we tried to improve the extraction protocol for RNA isolation and we obtained new purified samples from our collaborators at IZSTO. Such samples were eluted in 50 µL of RNase free water instead of 100 µL in order to increase the overall concentration. Non-Stick RNase-free Microfuge Tubes (Ambion) were used to collect the samples. Furthermore, an additional step of DNase I treatment was included in the RNA extraction protocol to reduce DNA contamination and increase the overall RNA quality. We found that the

integrity of this new sample set was considerably improved. However, their concentrations were still very low (a minimum concentration of 33 ng/uL is required for the microarray protocol). At this point, we decided to concentrate the samples by using a Labconco CentriVap concentrator. Following this procedure the concentration of the samples increased significantly and the quality was maintained satisfactory for the majority of them (**Table 1**).

Table 1. List of RNA samples isolated from blood of infected and control animals.

Sample name	Sample type	Concentration (ng/µL)	Concentration after Centrivap protocol (ng/µL)	RIN Value after Centrivap protocol
P1	Preclinical H type BSE	15	77.2	5.5
P2	Preclinical H type BSE	21	125.4	6.8
cP3	Control (preclinical)	18	78	6.6
P4	Preclinical L type BSE	12	146.8	6.5
P5	Preclinical L type BSE	19	56	5.5
S1	Clinical H type BSE	19	111.4	7.1
S2	Clinical H type BSE	15	146.6	7.9
S3	Clinical L type BSE	38	237.2	N/A
S4	Clinical L type BSE	7	48.4	N/A
cS5	Control (clinical)	13	40.0	7.8
cP6	Control (preclinical)	10	51.9	6.6
P7	Preclinica H type BSE	16	98.8	7.2
P8	Preclinica H type BSE	8	58.4	6.7
P9	Preclinica L type BSE	12	69.4	2.2
P10	Preclinica L type BSE	20	116.0	6.4
cS6	Control (clinical)	10	63	8.4
S7	Clinical H type BSE	15	86.5	7.5
S8	Clinical H type BSE	15	108.2	6.8
S9	Clinical L type BSE	17	100.5	8.7
S10	Clinical L type BSE	15	86.4	7.8

Since the sample size of controls was quite limited and much smaller with respect to that of the infected animals (only four control samples *vs* sixteen infected samples) we decided to include additional sex-matched negative controls in our study. These cattle were aged from 12 to 37 months and derived from a different herd compared to the Konold's study groups. Again for these samples the RNA concentration was quite low, and therefore we proceeded with the concentration at Centrivap. After this procedure the final amount of RNA per volume was satisfactory for the majority of them and the high integrity was maintained (**Table 2**).

Table 2. Additional negative control samples.

Sample name	Sample type	Concentration (ng/µL)	Concentration after Centrivap protocol (ng/µL)	RIN Value after Centrivap protocol
c1	Control	11	35	7.4
c2	**Control**	**23**	**65**	**7.1**
c3	**Control**	**29**	**88,9**	**6.7**
c4	Control	19	59.1	7.6
c5	**Control**	**11**	**49.4**	**7.2**
c6	Control	14	27.7	7.6
c7	Control	13	21.6	7.6
c8	Control	15	23.9	7.4
c9	**Control**	**27**	**83.8**	**7.2**

In order to have an equal sample size for preclinical, clinical and control population, we selected four control samples to be included in the microarray analysis. Such samples were selected on the basis of their concentration and quality (RIN value) and are

highlighted in bold in **Table 2**. Thus, we had 8 preclinical, 8 clinical and 8 control samples (4 from the Konold's study and 4 from a different herd) which were subjected to the microarray hybridization. This analysis had to be performed in 3 different moments due to the capacity of the microarray chip scanner. Therefore, we divided our population in three sample sets which were subjected to the microarray hybridization and staining protocol in different moments. Each sample set was composed of a balanced number of control, preclinical and clinical samples in order to reduce technical biases due to inter run variability. The sample sets are indicated in **Table 3**.

Table 3. Sample sets which were subjected to the microarray hybridization and staining protocol in different moments.

	Preclinical sample	Clinical sample	Control sample
First sample set	P2	S2	cP3
	P7	S7	cS5
	P8	S8	c3
Second sample set	P4	S3	c9
	P5	S4	c5
	P9	S9	c2
Third sample set	P10	S10	cS6
	P1	S1	cP6

Microarray hybridization for the first and third sample set were repeated twice since some technical problems occurred. The first sample set (composed of sample P2, P7, P8, S2, S7, S8, cP3, cS5 and c3) was characterized by low signal intensities compared to the rest of the arrays (**Figure 1A**). Probably, a problem during the staining step of the

microarray protocol occurred. Therefore, we repeated the staining for such samples and could obtain intensity signals comparable to the rest of the arrays (**Figure 1B).**

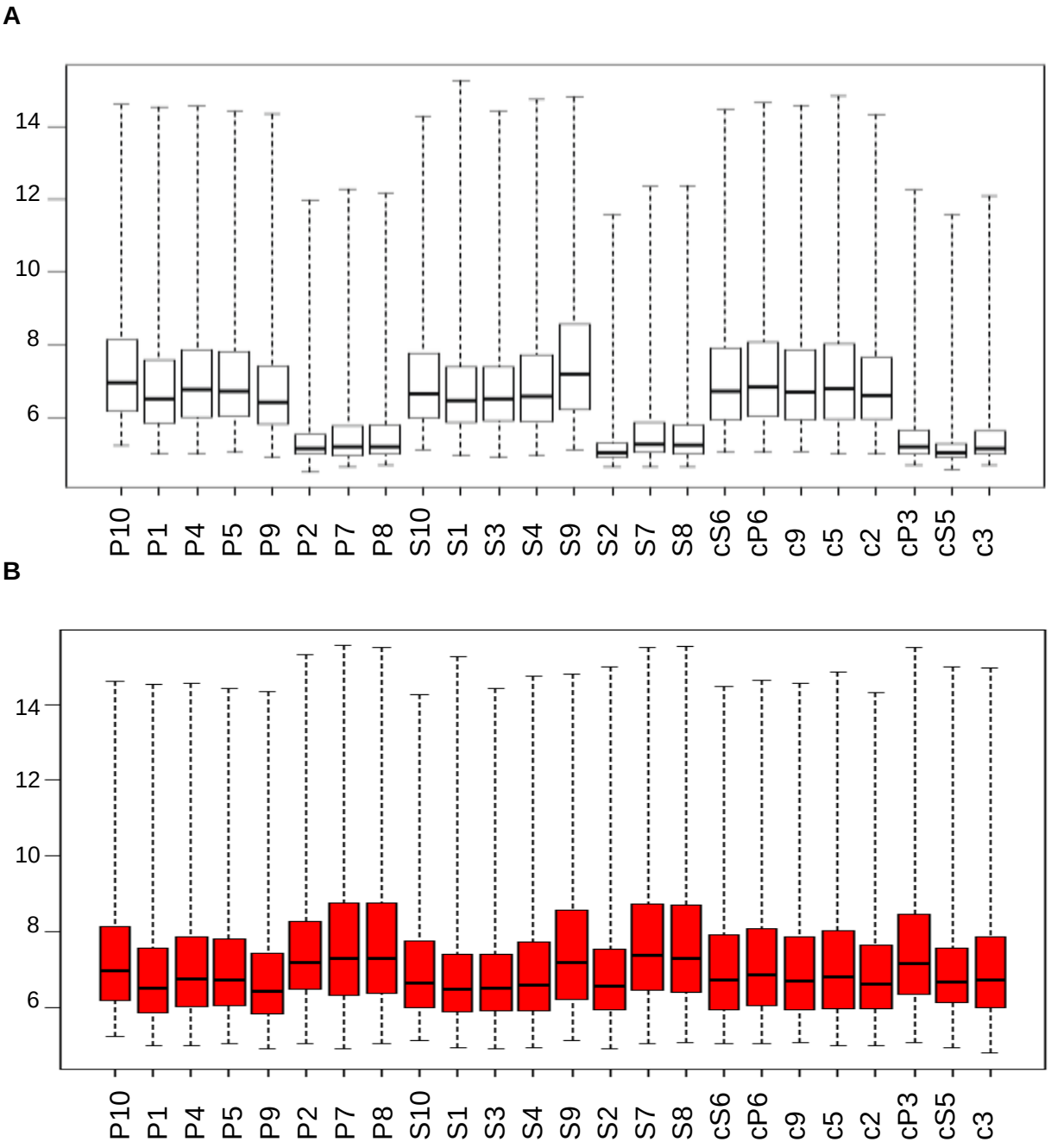


Figure 1. Raw intensity boxplots for each array. In the first analysis the intensity of the arrays corresponding to the first sample set was quite low compared to the rest of the chips (**A**). After repeating the staining of the first sample set the signal intensities were more homogeneous among all the arrays (**B**).

Concerning the third sample set, we observed a very low amount of aRNA following the amplification step of the microarray protocol. After some tests it was found that the problem was the eluent used during the experiments. By changing the eluent stock the aRNA could be rescued in high concentration. Once we solved these technical issues, further microarray quality control (MAQC) was performed to assess the quality of each array. Relative log expression (RLE) and normalized unscaled standard error (NUSE) plots were examined in order to avoid procedural failures and the presence of degraded RNA samples. On the RLE plot, arrays with lower quality will have boxes that are centred higher and/or have a larger spread than the other good quality arrays from the same experiment. As shown in **Figure 2A**, we found that the array corresponding to the control sample cS5 was centred higher and had a larger spread compared to the rest of the arrays. In line with RLE plot, also the NUSE plot indicated a low quality for this sample. NUSE represents normalized standard error (SE) estimates from the probe level model (PLM) fit. The SE estimates are normalized such that for each probe set, the median standard error across all arrays is equal to 1. Typically, boxes centred above 1.1 represent arrays that have quality problems. Indeed, sample cS5 was centred above 1.1 and had a much larger spread compared to the rest of the samples (**Figure 2B**).

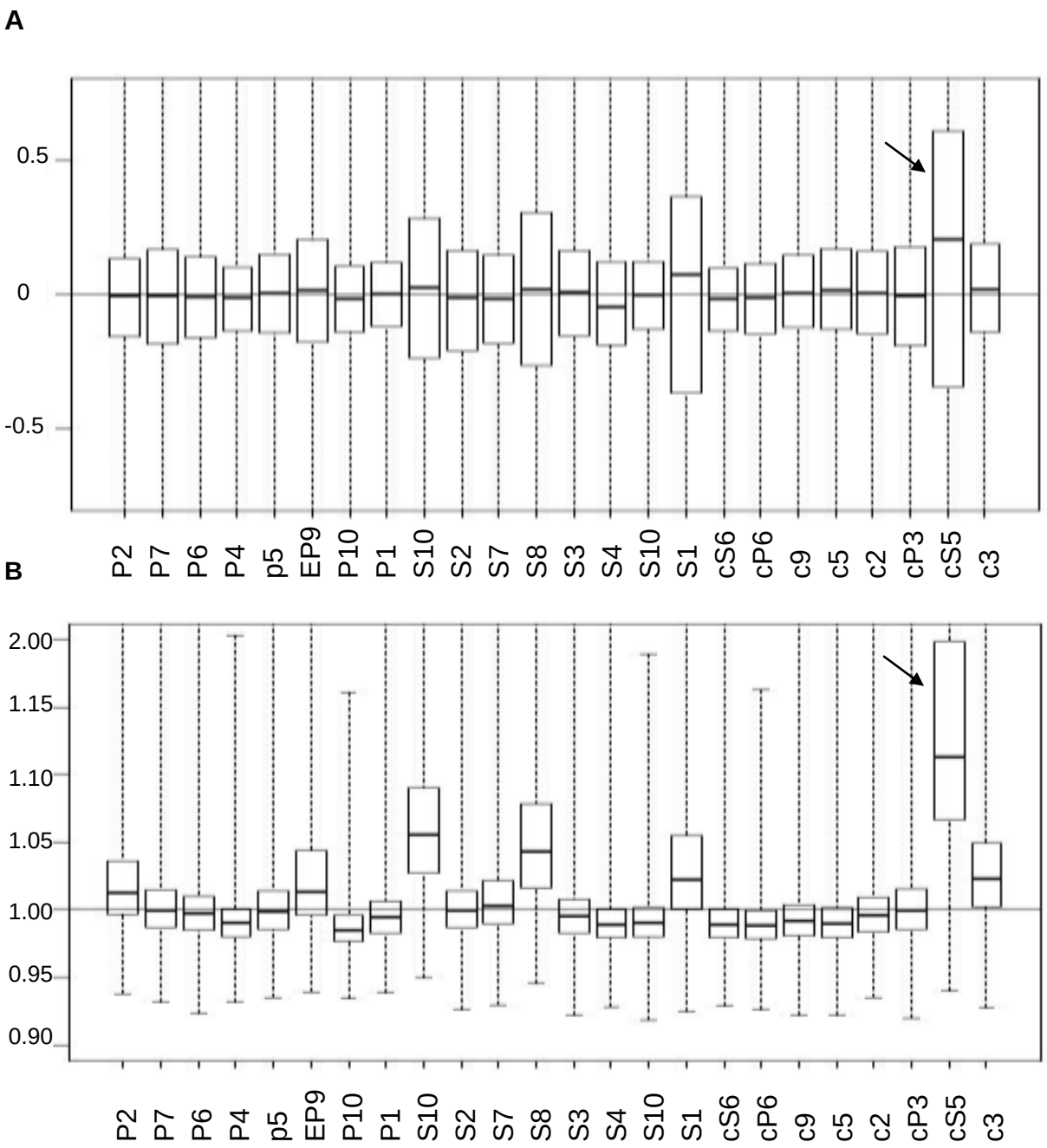


Figure 2. Post hybridization quality assessment. Relative log expression (RLE) (**A**) and normalized unscaled standard error (NUSE) (**B**) plots are used to check for technical problems and to spot outlier samples after GCRMA normalization. One outlier was easily identified by post hybridization quality assessment (black arrow in panel A and B, control sample cS5).

Due to these quality problems, we decided to exclude the control sample cS5. However, in order to see in which degrees the results could be affected by the removal of such sample, we carried out two preliminary statistical analyses, one including and one excluding the sample. We could find 141 differentially expressed probes in the first analysis (all infected samples *vs* all control samples) and 144 in the second analysis (all infected samples *vs* all control samples excluding sample cS5). 86 genes were in common between the two analyses. To be the most conservative as possible, for the final selection of DEGs to be validated by RT-qPCR, in addition to the *p* value and the fold change, we took into account also this preliminary comparison (thus favouring those genes which remained significant after the removal of sample cS5). Finally, we could improve our data by substituting sample P9, which displayed the lowest RIN value (2.2 in Table 1), with a new high quality RNA batch (named as sample EP9) isolated from the same animal (RIN value for sample EP9 was equal to 9.2). Summarizing, after removal of one outlier control sample (cS5) and substitution of one low-quality preclinical sample (P9->EP9), the final microarray statistical analyses were performed using 23 samples: 8 preclinical (P1, P2, P4, P5, P7, P8, EP9 and P10), 8 clinical (S1, S2, S3, S4, S7, S8, S9 and S10), and 7 control samples (c2, c3, cP3, c5, cP6, cS6 and c9). The final statistical analyses, showed in detail in the next paragraph, were four: infected (H- and L-type) *versus* control (I*vs*Ctrl), preclinical versus control (P*vs*Ctrl), clinical versus control (C*vs*Ctrl) and clinical versus preclinical (C*vs*P) analysis.

3.2 Identification of differentially expressed genes (DEGs) in the blood of atypical BSE-infected cattle by microarray analysis.

Statistical analysis was performed on microarray results using the oneChannelGUI Bioconductor package (Sanges, Cordero et al. 2007). The raw microarray data were deposited in the Gene Expression Omnibus (GEO) repository and assigned the accession number GSE69048 (see section: **Availability of supporting data**).

Since the goal of this project was to identify a common pattern of DEGs in atypical BSE infection, we defined the 4 H- and the 4 L- type inoculated-cattle as one single group of 8 animals named as atypical infected cattle. This approach allowed us to increase the sample size and obtain more reliable results. Indeed, in a preliminary analysis comparing the 4 H-type and the 4 L-type infected animals we found a limited number of DEGs: only 15 had a *p* value lower than 0.01 and only 16 showed a fold change higher than 3 (see **Table 4**).

Table 4. List of DEGs found in H*vs*L statistical comparison.

Probe ID	Gene symbol	Gene name	*P* value	*Fold Change*
Bt.16070.3.A1_at	LOC786352	apolipoprotein L, 1-like	4.36E-06	-7.064
Bt.8382.2.S1_at	RHOB	ras homolog gene family, member B	6.07E-05	2.285
Bt.28461.2.S1_a_at	INSR	insulin receptor	7.58E-05	-6.156
Bt.7220.1.S1_at	BLA-DQB	MHC class II antigen	1.82E-04	-112.648
Bt.9173.1.A1_at	NBEAL2	neurobeachin-like 2	2.57E-04	2.410
Bt.215.1.S1_at	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 5	4.45E-04	-170.841
Bt.20925.1.S1_at	BOLA-DQB	major histocompatibility complex, class II, DQ beta	6.49E-04	-62.998
Bt.28461.1.S1_a_at	INSR	insulin receptor	8.66E-04	-3.242
Bt.9605.1.S1_at	MS4A8B	membrane-spanning 4-domains,	1.35E-03	2.461

		subfamily A, member 8B		
Bt.23226.1.S1_at	ACADSB	acyl-CoA dehydrogenase, short/branched chain	1.45E-03	-2.300
Bt.27760.1.S1_at	BoLA /// BOLA-A	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A	1.57E-03	-4.832
Bt.22734.1.A1_at	ENPP4	ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative)	2.03E-03	3.447
Bt.16070.2.S1_at	LOC786352	apolipoprotein L, 1-like	2.76E-03	-3.006
Bt.29692.1.S1_at	TRD@	T-cell receptor delta chain	3.42E-03	-6.658
Bt.6147.1.S1_a_at	METTL12	methyltransferase like 12	4.53E-03	-2.034
Bt.22785.1.S1_at	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	6.56E-03	2.175
Bt.27261.2.S1_at	LOC100847574	multidrug resistance-associated protein 4-like	0.010996	-4.342
Bt.1658.1.S1_at	DUSP1	dual specificity phosphatase 1	1.30E-02	2.072
Bt.24441.1.S1_at	LOC100336589	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6-like	1.36E-02	3.580
Bt.11525.1.S1_a_at	RBPMS	RNA binding protein with multiple splicing	1.64E-02	2.145
Bt.5230.1.S1_at	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.64E-02	-2.674
Bt.4762.1.S1_at	BOLA-NC1	non-classical MHC class I antigen	1.65E-02	-2.403
Bt.1736.1.A1_at	SOCS1	suppressor of cytokine signaling 1	1.67E-02	2.327
Bt.29403.1.S1_at	TFF2	trefoil factor 2	1.69E-02	-5.802
Bt.155.1.S1_at	IL8	interleukin 8	1.70E-02	2.201
Bt.4609.1.S1_at	LOC100847474	uncharacterized LOC100847474	1.76E-02	-2.893
Bt.24154.1.A1_at	TFDP2	transcription factor Dp-2 (E2F dimerization partner 2)	1.78E-02	2.316
Bt.18566.1.A1_at	LOC539805	uncharacterized LOC539805	1.84E-02	-2.014
Bt.21546.1.S1_at	LOC516579	probable phospholipid-transporting ATPase IIA-like	1.93E-02	2.524
Bt.27854.1.S1_at	NFIL3	nuclear factor, interleukin 3 regulated	1.98E-02	2.064
Bt.5847.1.S1_at	GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	2.01E-02	-4.420
Bt.27973.1.S1_at	SLCO3A1	solute carrier organic anion transporter family, member 3A1	2.09E-02	2.151
Bt.29715.1.S1_at	PTPRC	protein tyrosine phosphatase, receptor type, C	2.15E-02	-2.045
Bt.22143.1.A1_at	SYT4	synaptotagmin IV	2.18E-02	2.088
Bt.19937.2.S1_at	LOC532189	carboxypeptidase D-like	2.25E-02	2.280

Bt.7145.1.S1_at	GZMB /// LOC100125946	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// uncharacterized LOC100125946	2.50E-02	-2.850
Bt.16350.2.A1_s_at	GBP5	guanylate binding protein 5	2.51E-02	-2.468
Bt.8724.1.S1_at	LOC100299281	quinone oxidoreductase-like protein 2-like	2.68E-02	2.035
Bt.2749.1.S1_at	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.73E-02	2.833
Bt.25269.1.S1_at	GCA	grancalcin, EF-hand calcium binding protein	2.78E-02	2.879
Bt.21724.2.S1_a_at	FDFT1	farnesyl-diphosphate farnesyltransferase 1	2.99E-02	-2.370
Bt.13628.1.S1_at	TGM3	transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)	3.02E-02	2.126
Bt.23399.1.S1_at	PKM	pyruvate kinase, muscle	3.04E-02	2.058
Bt.19308.1.S1_at	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	3.07E-02	-2.451
Bt.28027.1.S1_at	LOC785752 /// LOC789653	uncharacterized LOC785752 /// TRD@ protein-like	3.15E-02	-4.060
Bt.49.1.S1_at	CD40LG	CD40 ligand	3.16E-02	-2.364
Bt.21225.1.S1_at	EYA3	eyes absent homolog 3 (Drosophila)	3.51E-02	2.183
Bt.21986.1.A1_a_at	USP7	ubiquitin specific peptidase 7 (herpes virus-associated)	3.86E-02	-2.315
Bt.27261.1.S1_at	LOC100337108	uncharacterized LOC100337108	3.98E-02	-10.885
Bt.28732.1.S1_at	TRD@	T-cell receptor delta chain	4.04E-02	-2.147
Bt.5075.1.S1_at	SPINT2	serine peptidase inhibitor, Kunitz type, 2	4.30E-02	-2.093
Bt.1847.1.S1_at	LOC789748	Sialic acid-binding Ig-like lectin 14-like	4.59E-02	2.047
Bt.29747.1.S1_at	LOC100851578	uncharacterized LOC100851578	4.77E-02	-2.484

The final microarray analysis consisted in 4 statistical comparisons: infected (preclinical and clinical) versus control (I*vs*Ctrl), preclinical versus control (P*vs*Ctrl), clinical versus control (C*vs*Ctrl) and clinical versus preclinical (C*vs*P). Statistical comparison between the infected animals and the control group (I*vs*Ctrl) revealed a total of 101 differentially regulated probe sets (*p* value lower than 0.05 and changes in expression higher than 2-

fold) as shown in **Table 5**. Gene annotation revealed that some of these probe sets encoded for the same gene and that 93 of the identified DEGs had a known function.

Table 5. Differentially expressed genes found in infected animals versus control group (I*vs*Ctrl) by microarray analysis[a].

Probe ID	Gene symbol	Gene name	*P* value	Fold Change
Bt.6653.2.A1_at	ALKBH4	alkB, alkylation repair homolog 4 (E. coli)	1.35E-07	-2.317
Bt.8586.1.S1_at	LOC512150	myeloid-associated differentiation marker-like	4.17E-05	4.192
Bt.21996.1.S1_at	IGHE	Immunoglobulin heavy constant epsilon	4.77E-05	-5.104
Bt.16101.1.S1_s_at	GNLY /// LOC100300483	granulysin /// antimicrobial peptide NK-lysin-like	5.70E-05	-4.177
Bt.28383.1.S1_at	GNLY	granulysin	8.98E-05	-4.834
Bt.9265.2.S1_at	BATF	basic leucine zipper transcription factor, ATF-like	1.12E-04	-2.571
Bt.14153.1.S1_at	NEB	nebulin	1.45E-04	-4.896
Bt.16101.1.S1_at	GNLY	granulysin	2.12E-04	-4.810
Bt.12986.1.S1_at	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	2.35E-04	-2.722
Bt.9265.1.A1_at	BATF	basic leucine zipper transcription factor, ATF-like	2.36E-04	-2.553
Bt.23123.1.S1_at	BHLHE40	basic helix-loop-helix family, member e40	5.75E-04	-2.369
Bt.26326.1.A1_at	MTBP	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104kDa	6.23E-04	2.785
Bt.22526.1.S1_at	HSPB8	heat shock 22kDa protein 8	8.58E-04	-2.393
Bt.16916.3.S1_at	KLF11	Kruppel-like factor 11	9.29E-04	4.456
Bt.24923.2.S1_a_at	SEL1L3	sel-1 suppressor of lin-12-like 3 (C. elegans)	1.06E-03	-3.342
Bt.8804.1.S1_at	NELL2	NEL-like 2 (chicken)	1.17E-03	-2.350
Bt.28654.1.S1_at	LOC100850906 /// USP42	ubiquitin carboxyl-terminal hydrolase 42-like /// ubiquitin specific peptidase 42	1.32E-03	3.550
Bt.18321.1.A1_at	GNB4	guanine nucleotide binding protein (G protein), beta polypeptide 4	1.34E-03	8.268
Bt.24630.2.S1_at	41527	septin 10	1.37E-03	-3.330
Bt.21975.1.S1_at	PRF1	perforin 1 (pore forming protein)	1.55E-03	-2.006
Bt.20330.1.S1_at	PRSS23	protease, serine, 23	1.70E-03	-3.423
Bt.24929.1.A1_at	RG9MTD3	RNA (guanine-9-) methyltransferase domain	1.90E-03	2.421

		containing 3		
Bt.6968.1.S1_at	IPCEF1	Interaction protein for cytohesin exchange factors 1	1.96E-03	2.425
Bt.9675.1.S1_at	LOC100847724	extracellular peptidase inhibitor-like	2.12E-03	-3.528
Bt.21979.1.S1_at	CXCR6	chemokine (C-X-C motif) receptor 6	2.16E-03	-2.209
Bt.9504.1.A1_at	CCL4	chemokine (C-C motif) ligand 4	2.85E-03	-2.650
Bt.28040.1.S1_at	LOC781494	myeloid-associated differentiation marker-like	3.00E-03	-3.596
Bt.23505.1.S1_at	PDK4	pyruvate dehydrogenase kinase, isozyme 4	3.18E-03	3.186
Bt.24236.1.S1_at	DLC1	deleted in liver cancer 1	3.56E-03	2.297
Bt.26636.1.S1_at	NKG7	natural killer cell group 7 sequence	3.81E-03	-2.169
Bt.6147.1.S1_a_at	METTL12	methyltransferase like 12	4.46E-03	-2.151
Bt.49.1.S1_at	CD40LG	CD40 ligand	4.52E-03	-3.237
Bt.26259.1.A1_at	ZNF462	zinc finger protein 462	4.81E-03	3.969
Bt.15915.1.S1_at	LRRC70	leucine rich repeat containing 70	5.10E-03	2.154
Bt.17280.1.S1_at	PLEKHH1	pleckstrin homology domain-containing family H member 1-like	5.12E-03	2.357
Bt.29009.1.A1_at	RYR3	ryanodine receptor 3	5.25E-03	-5.464
Bt.2129.1.S1_at	LOC100850064	versican core protein-like	5.94E-03	2.002
Bt.22415.2.A1_at	LOC512863	sialic acid-binding Ig-like lectin 14-like	6.31E-03	2.231
Bt.19308.1.S1_at	BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	6.60E-03	3.175
Bt.16861.1.A1_at	LOC515128	major facilitator superfamily domain-containing protein 4-like	6.68E-03	2.319
Bt.22301.1.S1_at	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4	6.82E-03	-2.307
Bt.15705.1.S1_at	DSTN	destrin (actin depolymerizing factor)	6.91E-03	2.216
Bt.28637.1.S1_at	LOC100848843	myeloid-associated differentiation marker-like	7.71E-03	2.740
Bt.24983.1.A1_at	DYNC2H1	dynein, cytoplasmic 2, heavy chain 1	8.14E-03	-2.085
Bt.24543.1.A1_at	KCTD1	potassium channel tetramerisation domain containing 1	1.02E-02	2.673
Bt.16916.1.S1_at	KLF11	Kruppel-like factor 11	1.02E-02	2.110
Bt.11259.1.S1_at	IFI27	putative ISG12(a) protein	1.09E-02	3.109
Bt.17081.2.S1_at	LMO2	LIM domain only 2 (rhombotin-like 1)	1.16E-02	-2.375
Bt.22214.1.S1_at	CD180	CD180 molecule	1.16E-02	-2.044
Bt.7145.1.S1_at	GZMB /// LOC100125946	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// uncharacterized LOC100125946	1.50E-02	-3.156
Bt.20540.1.S1_at	CD79B	CD79b molecule, immunoglobulin-associated beta	1.51E-02	-3.586

Bt.20257.2.S1_at	BCNT2	Bucentaur-2	1.59E-02	-2.289
Bt.155.1.S1_at	IL8	interleukin 8	1.60E-02	2.250
Bt.24112.1.A1_at	CXHXorf57	chromosome X open reading frame, human CXorf57	1.60E-02	-2.760
Bt.18253.1.A1_at	KIAA1324L	KIAA1324-like ortholog	1.61E-02	2.360
Bt.27043.2.S1_at	FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	1.62E-02	-2.063
Bt.13469.1.S1_at	SDSL	serine dehydratase-like	1.63E-02	-2.506
Bt.9262.1.A1_at	SPIB	Spi-B transcription factor (Spi-1/PU.1 related)	1.67E-02	-3.493
Bt.9974.1.S1_at	CCL3	chemokine (C-C motif) ligand 3	1.71E-02	-2.110
Bt.19014.1.A1_at	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	1.74E-02	4.152
Bt.2899.1.S2_at	FOS	FBJ murine osteosarcoma viral oncogene homolog	1.77E-02	2.487
Bt.6438.1.A1_at	TGFB2	transforming growth factor, beta 2	1.91E-02	3.437
Bt.3352.1.S1_at	ASIP	agouti signaling protein	1.97E-02	-2.093
Bt.9163.2.S1_at	P2RY10	purinergic receptor P2Y, G-protein coupled, 10	1.97E-02	-2.047
Bt.27760.1.S1_at	BoLA /// BOLA-A	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A	2.07E-02	-3.409
Bt.3805.1.S1_at	BOLA-N /// JSP.1 /// LOC100125916	MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016	2.13E-02	3.897
Bt.17019.1.A1_at	FOXP1	Forkhead box P1	2.14E-02	2.003
Bt.20640.1.A1_at	C22H3orf64	chromosome 22 open reading frame, human C3orf64	2.18E-02	2.039
Bt.411.1.S1_at	NRG1	neuregulin 1	2.19E-02	2.758
Bt.11847.1.A1_at	XIST	X (inactive)-specific transcript	2.30E-02	-21.298
Bt.24923.1.S1_at	SEL1L3	sel-1 suppressor of lin-12-like 3 (C. elegans)	2.36E-02	-3.251
Bt.13367.1.A1_at	XIST	X (inactive)-specific transcript	2.42E-02	-34.673
Bt.29820.1.S1_s_at	BOLA	MHC class I heavy chain	2.49E-02	-6.083
Bt.23911.1.A1_at	XIST	X (inactive)-specific transcript	2.52E-02	-29.733
Bt.22139.1.S1_at	COBLL1	COBL-like 1	2.64E-02	-2.766
Bt.22854.1.S1_at	CA2	carbonic anhydrase II	2.68E-02	-2.336
Bt.13330.1.S1_at	PDK4	pyruvate dehydrogenase kinase, isozyme 4	2.82E-02	2.400
Bt.16070.2.S1_at	LOC786352	apolipoprotein L, 1-like	2.89E-02	-2.314
Bt.17473.2.S1_at	RPE	ribulose-5-phosphate-3-epimerase	3.04E-02	2.209
Bt.16966.1.S1_at	CXCL10	chemokine (C-X-C motif) ligand 10	3.05E-02	-3.066

Bt.23172.1.S1_at	BAX	BCL2-associated X protein	3.07E-02	-2.288
Bt.4609.1.S1_at	LOC100847474	uncharacterized LOC100847474	3.18E-02	-2.619
Bt.20687.1.A1_at	C5H12orf35	chromosome 5 open reading frame, human C12orf35	3.24E-02	2.091
Bt.3651.2.A1_at	C15H11orf96	chromosome 15 open reading frame, human C11orf96	3.52E-02	2.093
Bt.5408.1.A1_at	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	3.52E-02	2.097
Bt.7015.1.S1_at	EED	embryonic ectoderm development	3.65E-02	-2.089
Bt.14010.1.S1_at	PTGR1	prostaglandin reductase 1	3.67E-02	-2.194
Bt.2899.1.S1_at	FOS	FBJ murine osteosarcoma viral oncogene homolog	3.78E-02	2.466
Bt.1075.1.A1_at	ZNF24	zinc finger protein 24	3.83E-02	2.708
Bt.20494.1.S1_at	RYBP	RING1 and YY1 binding protein	3.86E-02	2.158
Bt.27261.2.S1_at	LOC100847574	multidrug resistance-associated protein 4-like	4.03E-02	3.296
Bt.23094.1.A1_at	AKR1C4 /// LOC506594	aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4) /// prostaglandin F synthase 1-like	4.11E-02	-2.137
Bt.20540.3.A1_at	CD79B	CD79b molecule, immunoglobulin-associated beta	4.23E-02	-2.063
Bt.24372.1.S1_at	ZRSR2Y	zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2	4.27E-02	3.787
Bt.7122.1.S1_at	HELZ	helicase with zinc finger	4.36E-02	2.564
Bt.213.1.S1_at	CD163L1	CD163 molecule-like 1	4.73E-02	2.500
Bt.20216.2.S1_at	RPIA	ribose 5-phosphate isomerase A	4.83E-02	-2.068
Bt.27759.2.S1_at	IDO1	indoleamine 2,3-dioxygenase 1	4.85E-02	-3.729
Bt.29815.1.S1_x_at	BOLA	MHC class I heavy chain	4.85E-02	-3.122
Bt.15705.1.S2_at	DSTN	destrin (actin depolymerizing factor)	4.86E-02	2.055
Bt.16933.2.A1_at	LOC100298891	aTP-binding cassette, sub-family C (CFTR/MRP), member 4-like	4.91E-02	2.748

Enrichment analysis revealed the involvement of many pathways and functional categories, including chemotaxis, inflammatory response and antigen presentation via MHC, which may be involved in peripheral prion pathogenesis. The most relevant functional groups are reported in **Table 6.**

Table 6. Functional classification of differentially expressed genes in blood of infected cattle versus control group[a].

Pathway	Probe name	Gene symbol	Gene name	*P* value	Fold Enrichment
Autoimmune thyroid disease	BT.21975.1.S1_AT	PRF1	perforin 1 (pore forming protein)	1.01E-04	19.14728682
	BT.29815.1.S1_X_AT	BOLA	MHC class I heavy chain		
	BT.29820.1.S1_S_AT	BOLA	MHC class I heavy chain		
	BT.3805.1.S1_AT	BOLA-N /// JSP.1 /// LOC100125916	MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016		
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
	BT.7145.1.S1_AT	GZMB /// LOC100125946	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) /// uncharacterized LOC100125946		
Extracellular region	BT.155.1.S1_AT	IL8	interleukin 8	2.22E-04	3.036995516
	BT.16070.2.S1_AT	LOC786352	apolipoprotein L, 1-like		
	BT.16966.1.S1_AT	CXCL10	chemokine (C-X-C motif) ligand 10		
	BT.20330.1.S1_AT	PRSS23	protease, serine, 23		
	BT.20640.1.A1_AT	C22H3orf64	chromosome 22 open reading frame, human C3orf64		
	BT.2129.1.S1_AT	LOC100850064	versican core protein-like		
	BT.213.1.S1_A	CD163L1	CD163 molecule-like		

	T		1		
	BT.29009.1.A1_AT	RYR3	ryanodine receptor 3		
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
	BT.6438.1.A1_AT	TGFB2	transforming growth factor, beta 2		
	BT.8804.1.S1_AT	NELL2	NEL-like 2 (chicken)		
	BT.9504.1.A1_AT	CCL4	chemokine (C-C motif) ligand 4		
	BT.9974.1.S1_AT	CCL3	chemokine (C-C motif) ligand 3		
	BT.3352.1.S1_AT	ASIP	agouti signaling protein		
Immune response	BT.155.1.S1_AT	IL8	interleukin 8	4.91E-04	5.451442755
	BT.16966.1.S1_AT	CXCL10	chemokine (C-X-C motif) ligand 10		
	BT.27760.1.S1_AT	BoLA /// BOLA-A	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A		
	BT.29815.1.S1_X_AT	BOLA	MHC class I heavy chain		
	BT.29820.1.S1_S_AT	BOLA	MHC class I heavy chain		
	BT.3805.1.S1_AT	BOLA-N /// JSP.1 /// LOC100125916	MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016		
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
	BT.9504.1.A1_AT	CCL4	chemokine (C-C motif) ligand 4		
	BT.9974.1.S1_AT	CCL3	chemokine (C-C motif) ligand 3		
Chemokine	BT.155.1.S1_A	IL8	interleukin 8	5.42E-04	24.18039

activity	T				216
	BT.16966.1.S1_AT	CXCL10	chemokine (C-X-C motif) ligand 10		
	BT.9504.1.A1_AT	CCL4	chemokine (C-C motif) ligand 4		
	BT.9974.1.S1_AT	CCL3	chemokine (C-C motif) ligand 3		
Locomotory behavior	BT.155.1.S1_AT	IL8	interleukin 8	0.001387806	9.982954545
	BT.16966.1.S1_AT	CXCL10	chemokine (C-X-C motif) ligand 10		
	BT.5408.1.A1_AT	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)		
	BT.9504.1.A1_AT	CCL4	chemokine (C-C motif) ligand 4		
	BT.9974.1.S1_AT	CCL3	chemokine (C-C motif) ligand 3		
Inflammatory response	BT.155.1.S1_AT	IL8	interleukin 8	0.001604508	9.598994755
	BT.16966.1.S1_AT	CXCL10	chemokine (C-X-C motif) ligand 10		
	BT.49.1.S1_AT	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)		
	BT.9504.1.A1_AT	CCL4	chemokine (C-C motif) ligand 4		
	BT.9974.1.S1_AT	CCL3	chemokine (C-C motif) ligand 3		
Antigen processing and presentation via MHC class I	BT.27760.1.S1_AT	BoLA /// BOLA-A	major histocompatibility complex, class I, A /// major histocompatibility complex, class I, A	0.002359977	39.93181818
	BT.29820.1.S1_S_AT	BOLA	MHC class I heavy chain		

	BT.3805.1.S1_AT	BOLA-N /// JSP.1 /// LOC100125916	MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016		
	BT.29815.1.S1_X_AT	BOLA	MHC class I heavy chain		
B cell proliferation	BT.23172.1.S1_AT	BAX	BCL2-associated X protein	0.033775277898711 7	57.045454545454 5
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
Cell adhesion molecules (CAMs)	BT.2129.1.S1_AT	LOC100850064	versican core protein-like	0.038771205	5.105943152
	BT.29820.1.S1_S_AT	BOLA	MHC class I heavy chain		
	BT.3805.1.S1_AT	BOLA-N /// JSP.1 /// LOC100125916	MHC class I antigen /// MHC Class I JSP.1 /// uncharacterized protein 100125016		
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
	BT.29815.1.S1_X_AT	BOLA	MHC class I heavy chain		
Regulation of secretion	BT.27043.2.S1_AT	FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	0.048207557	8.319128788
	BT.49.1.S1_AT	CD40LG	CD40 ligand		
	BT.29009.1.A1_AT	RYR3	ryanodine receptor 3		

[a] The gene enrichment analysis was performed using DAVID bioinformatics tool 6.7 (NIAID/NIH, USA). Only genes with a known GO and belonging to the most relevant functional categories are reported in the list.

To evaluate to what extent gene expression alterations in blood were related to the preclinical or clinical stage of the disease, two distinct statistical analyses were performed comparing each group with the control one: clinical *versus* control group (C*vs*Ctrl) and preclinical *versus* control group (P*vs*Ctrl). In the clinical stage, a total of 207 probe sets showed significant alteration in gene expression levels compared to the control group. Among these, 87 were up-regulated while 120 had a reduction in expression. Interestingly, a pronounced alteration in the gene expression profile was also found in the preclinical stage, with a total number of 113 differentially expressed probe sets (55 genes were up-regulated while 58 were down-regulated). Two heat maps representing the differentially expressed probe sets in preclinical and clinical cattle are shown in **Figure 3**. The complete probe set lists with the relative *p* values and fold changes can be found in **S1** and **S2 Tables** attached to this thesis.

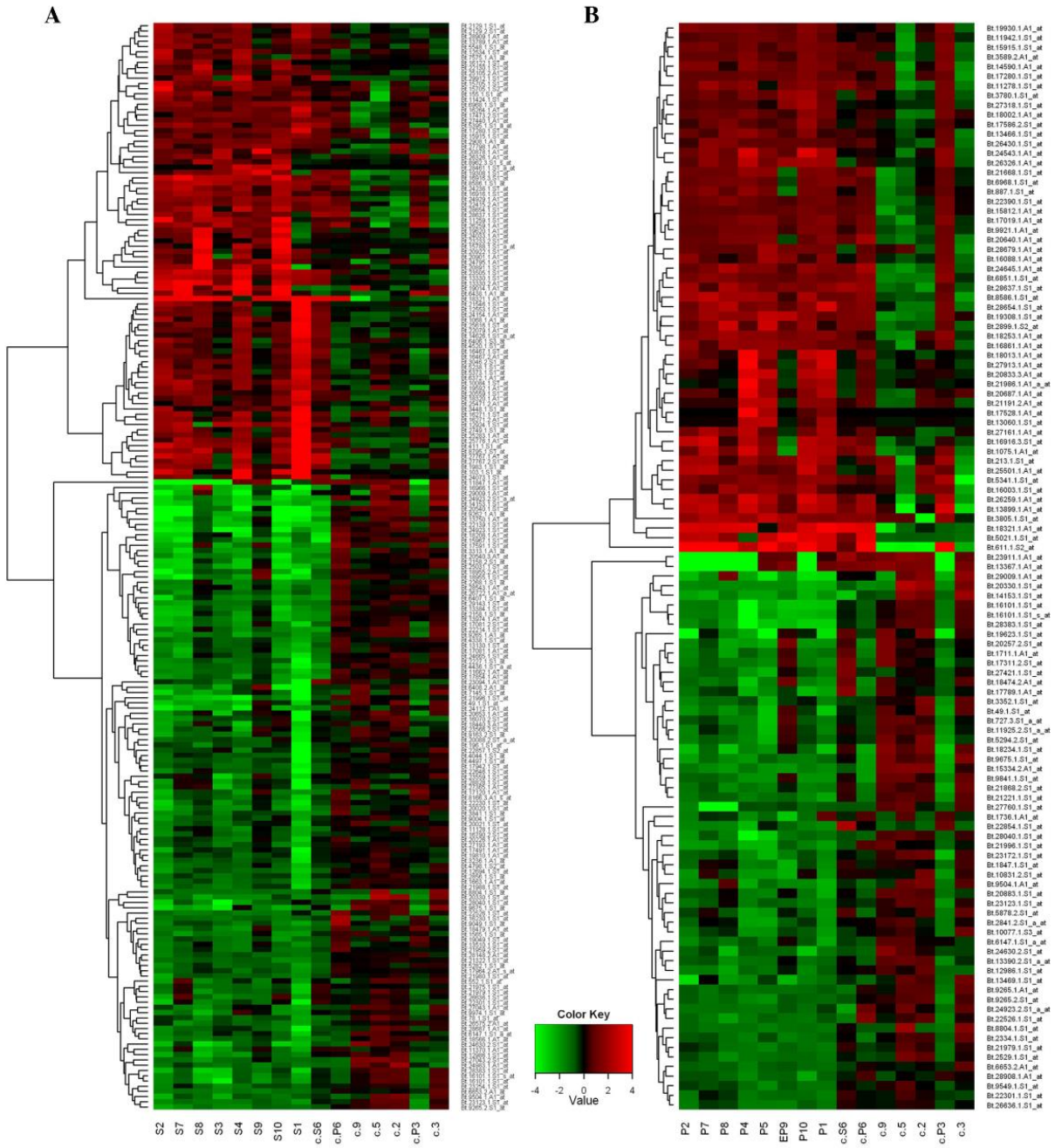
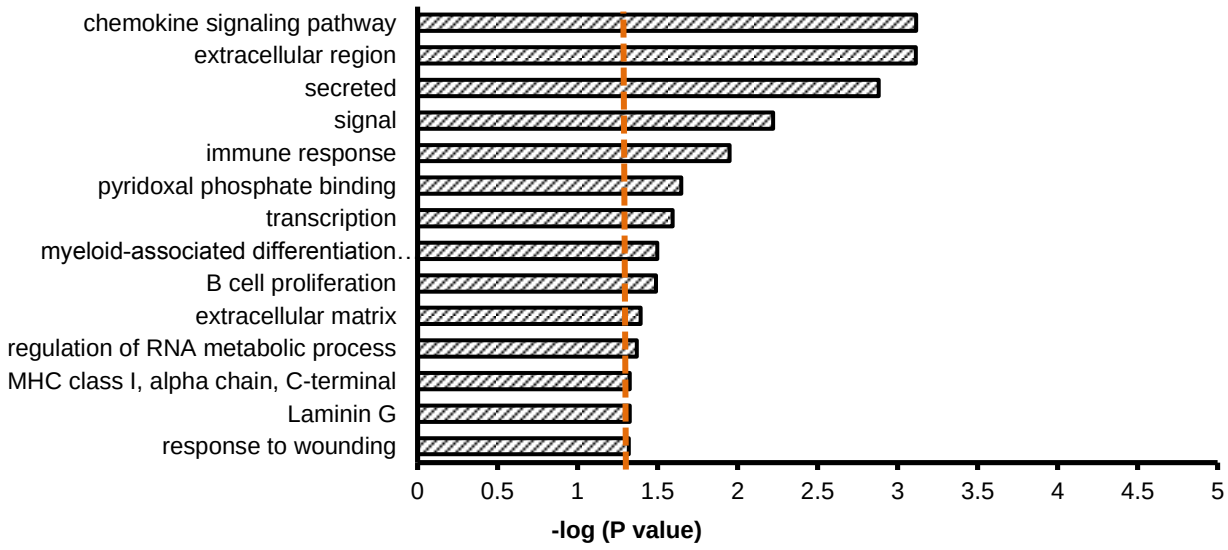


Figure 3. **Heat maps representing the DEGs found in clinical and preclinical cattle with atypical BSE**. Two heat maps were generated using the *heatmap.2* function from the *gplots* library in *R* statistical environment. DEGs were hierarchically clustered with complete linkage using the Euclidean metric. The heat maps represent the most significant DEGs (*p* value ≤.0.05 and fold change ≥ 2) in clinical (A) and preclinical (B) animals compared to the control group. Animals are reported in the x-axis while the differentially expressed probes are in the y-axis.

A gene enrichment analysis was performed to identify the most enriched GO terms in the clinical and preclinical groups (**Figure 4**). The preclinical stage was characterized by enrichment in gene clusters related to chemokine signaling pathway, extracellular region, secreted protein, immune response, pyridoxal phosphate binding, transcription, myeloid-associated differentiation marker, B cell proliferation, extracellular matrix, RNA metabolic process, MHC class I, Laminin G and response to wounding (**Figure 4A**). DEGs specific of the clinical group were clustered in functional categories related to cytokine-cytokine receptor interaction, regulation of leukocyte activation, inflammatory response, autoimmune thyroid disease, chemokine activity, B cell proliferation and differentiation, regulation of apoptosis, kinase inhibitor activity, and membrane raft (**Figure 4B**).

A



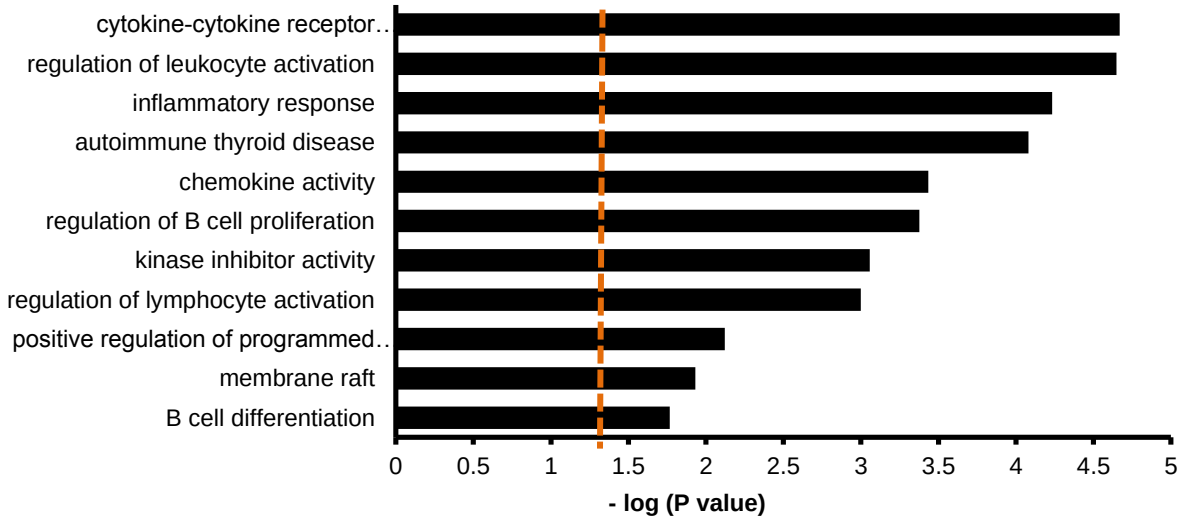


Figure 4 Gene enrichment analysis of DEGs specific of the clinical and preclinical stage of the disease. The most relevant GO terms (y axis) associated to preclinical (**A**) and clinical (**B**) phase are listed according to decreasing statistical significance from top to bottom. The threshold for statistical significance is marked by the orange lines. The enrichment analysis was performed using DAVID bioinformatics tool 6.7 (NIAID/NIH, USA).

When comparing the differentially regulated probe sets identified in the preclinical and clinical groups, it was found that 35 differentially expressed probe sets (corresponding to 32 DEGs) were common between the two stages of disease (**Figure 5**), leaving 172 DEGs specific to clinical and 78 genes specific to preclinical animals. Remarkably, all of the 32 common DEGs displayed a very similar pattern of expression in the clinical and preclinical groups, as shown in **Figure 5B.** These genes are listed in bold in **S1** and **S2 table** attached to this thesis.

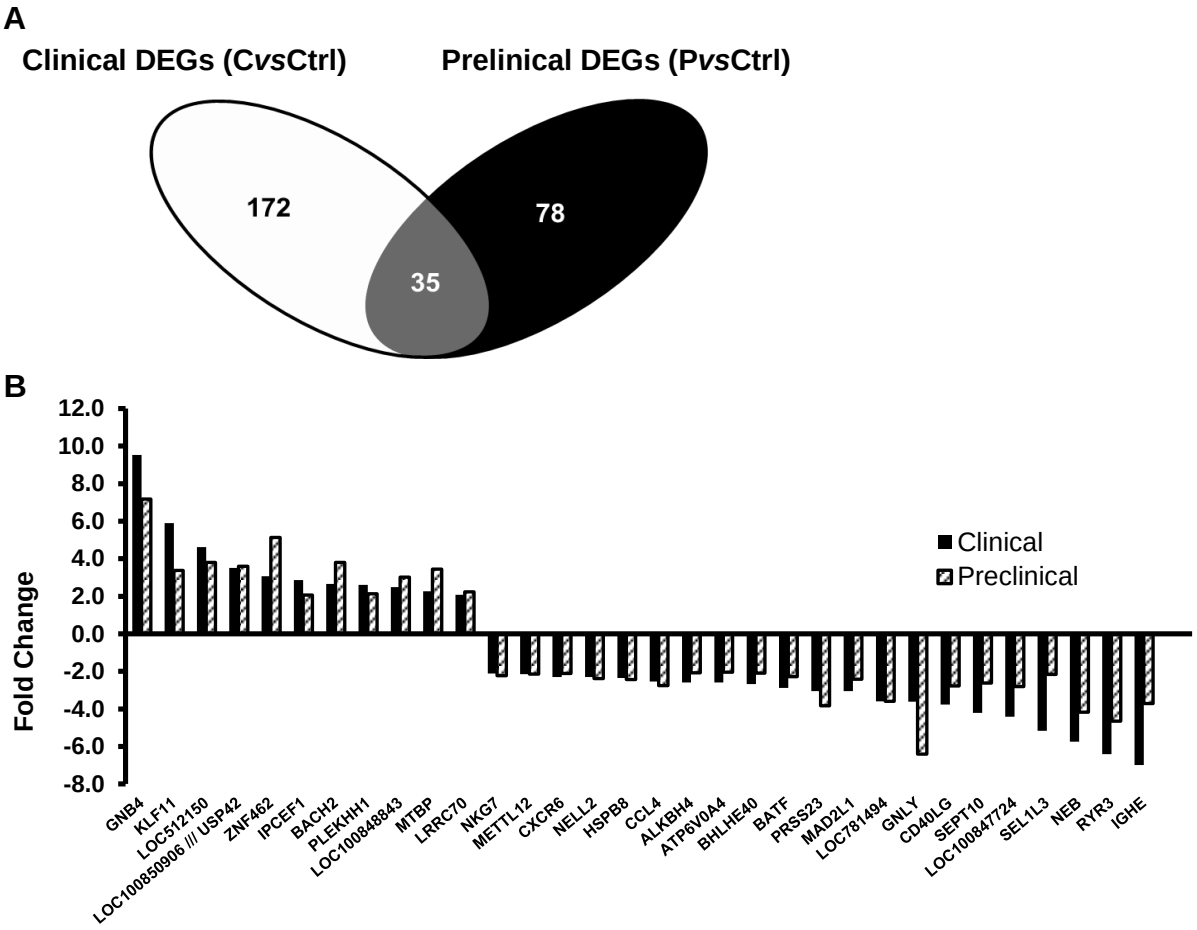


Figure 5. Identification of common DEGs in blood of preclinical and clinical atypical BSE-infected cattle. Venn diagram showing the number of differentially expressed probe sets in blood of clinical and preclinical cattle. The intersection in grey represents 35 differentially expressed probe sets corresponding to 32 differentially expressed genes (DEGs) that are in common between the two stages of the disease (**A**). Expression pattern of the common 32 DEGs. The histograms represent the fold change relative to the control group. P*vs*Ctrl= preclinical versus control, C*vs*Ctrl=clinical versus control (**B**).

To further dissect gene expression alterations during the progression of the disease, we performed a statistical analysis to identify specific changes between the clinical and preclinical stages (C*vs*P). Indeed, we found 235 DEGs, which were significantly enriched in pathways related to immune response (regulation of B cell proliferation, leucocyte activation, ISG15-protein conjugation and chemokine signaling were among

the most significant). The list of the most relevant enriched probe sets can be found in **S3 Table** attached to this thesis.

We used a Venn diagram to compare the DEGs found in P*vs*Ctrl, C*vs*Ctrl and C*vs*P analyses that were previously performed and then we examined the expression levels of common DEGs (**Figure 6**). Venn diagram revealed the presence of one DEG in common between P*vs*Ctrl, C*vs*Ctrl and C*vs*P comparisons, while 22 genes were differentially expressed in P*vs*Ctrl and C*vs*P but not in C*vs*Ctrl comparisons (**Figure 6A**). We found that these 22 DEGs had an opposite fold change sign in P*vs*Ctrl and C*vs*P, thus indicating an up/down-down/up pattern of expression (see **Figure 6B**, **6C** and **Table 7**). In particular, 9 out of 22 DEGs were up-regulated in the preclinical phase and then went back roughly to the expression level of the controls in the clinical stage (**Figure 6B**). The remaining 13 out of 22 DEGs were down-regulated in the preclinical phase and then went back almost to control levels in the clinical phase (**Figure 6C**). The only gene in common between the three comparisons, namely Sel-1 Suppressor Of Lin-12-Like 3 **(***SEL1L3*), was progressively down-regulated during the preclinical and the clinical phase of the infection, as shown in **Figure 6D**.

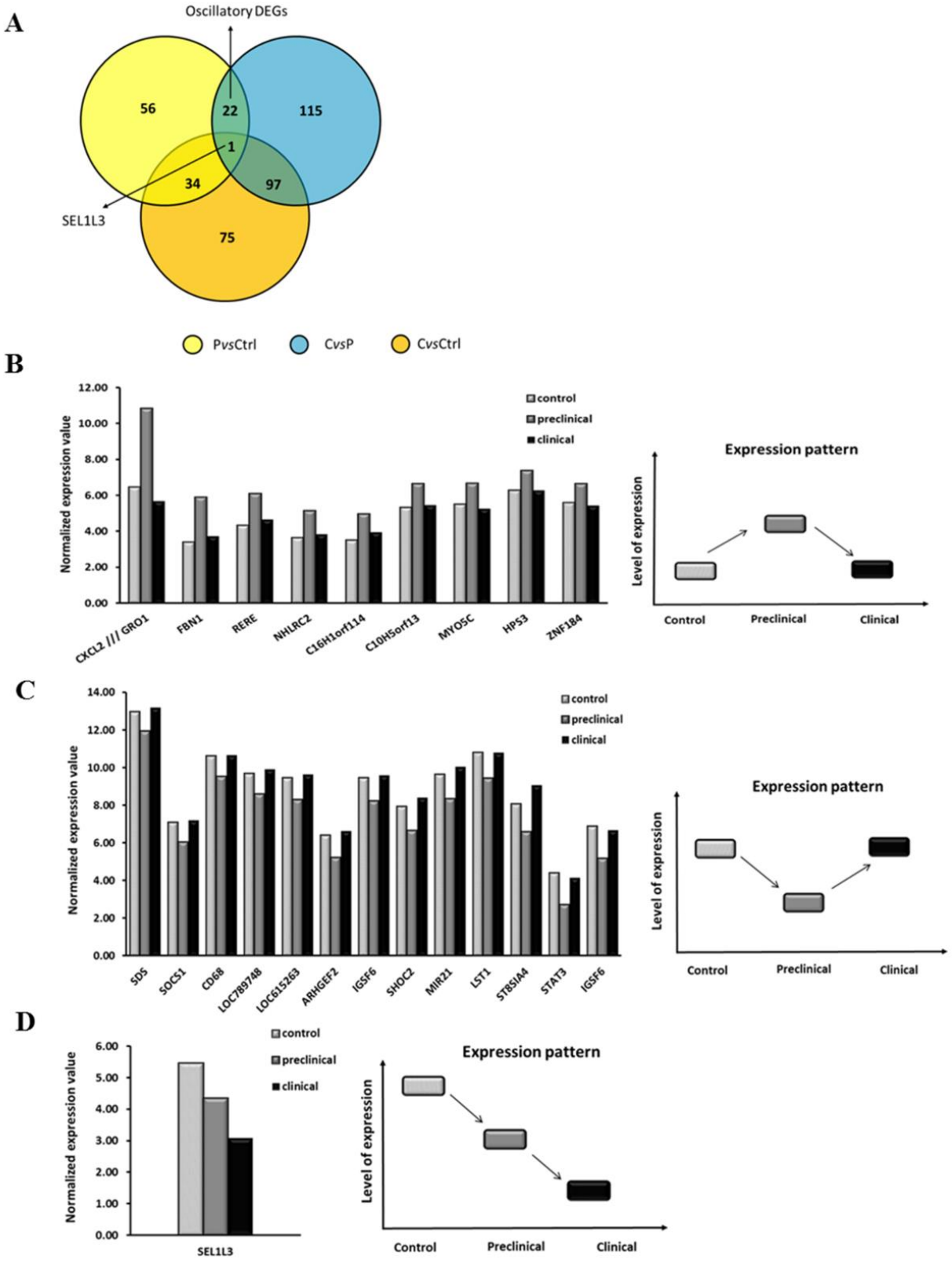

A
Oscillatory DEGs
56
22
115
1
34
97
75
SEL1L3
PvsCtrl
CvsP
CvsCtrl
B
Normalized expression value
control
preclinical
clinical
CXCL2 /// GRO1
FBN1
RERE
NHLRC2
C16H1orf114
C10Horf13
MYO5C
HPS3
ZNF184
Expression pattern
Level of expression
Control
Preclinical
Clinical
C
SD5
SOCS1
CD68
LOC789748
LOC615263
ARHGEF2
IGSF6
SHOC2
MIR21
LST1
ST8SIA4
STAT3
IGSF6
D
SEL1L3

Figure 6. DEGs in common between C*vs*P, P*vs*Ctrl and C*vs*Ctrl comparisons. (A) Venn diagram revealed the presence of 22 DEGs in common between C*vs*P and P*vs*Ctrl comparisons. One DEG (SEL1L3, Sel-1 Suppressor Of Lin-12-Like 3) was common in the three comparisons. (B-C) The normalized expression values of the 22 common DEGs for C*vs*P and P*vs*Ctrl comparisons are represented by the histograms. As indicated by the schematic figures on the right, these 22 DEGs showed an up/down-down/up pattern of expression: (B) 9/22 were upregulated in P*vs*Ctrl and then downregulated in C*vs*P comparisons, while (C) 13/22 were downregulated in P*vs*Ctrl and upregulated in C*vs*P comparison, respectively. (D) SEL1L3**,** the only gene found in common among the three comparisons (C*vs*P, C*vs*Ctrl and P*vs*Ctrl), showed a progressive downregulation during the preclinical and the clinical phase of the infection. P=preclinical, C=clinical and Ctrl=control, *vs*=*versus*. DEGs=differentially expressed genes.

Table 7. **List of DEGs characterized by an up-down/down-up pattern of expression.**[a]

Probe Name	Gene Symbol	Gene Name	Fold Change in P*vs*Ctrl	Fold Change in C*vs*P
Bt.611.1.S2_at	CXCL2	chemokine (C-X-C motif) ligand 2	20.968	-35.428
Bt.5021.1.S1_at	FBN1	fibrillin 1	5.661	-4.474
Bt.18013.1.A1_at	RERE	arginine-glutamic acid dipeptide (RE) repeats	3.399	-2.668
Bt.18234.1.S1_at	IGSF6	immunoglobulin superfamily, member 6	-3.289	2.812
Bt.15334.2.A1_at	STAT3	Signal transducer and activator of transcription 3	-3.238	2.679
Bt.20833.3.A1_at	NHLRC2	NHL repeat containing 2	2.825	-2.435
Bt.27161.1.A1_at	C16H1orf114	chromosome 16 open reading frame, human C1orf114	2.783	-2.035
Bt.17789.1.A1_at	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	-2.771	5.522
Bt.21221.1.S1_at	LST1	leukocyte specific transcript 1	-2.555	2.531
Bt.3780.1.S1_at	C10H5orf13	chromosome 10 open reading	2.481	-2.248

		frame, human C5orf13		
Bt.9841.1.S1_at	MIR21	microRNA mir-21	-2.452	3.249
Bt.18474.2.A1_at	SHOC2	soc-2 suppressor of clear homolog (C. elegans)	-2.406	3.303
Bt.21868.2.S1_at	IGSF6	immunoglobulin superfamily, member 6	-2.341	2.568
Bt.27421.1.S1_at	ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	-2.263	2.640
Bt.16088.1.A1_at	MYO5C	myosin VC	2.261	-2.646
Bt.1711.1.A1_at	LOC615263	uncharacterized LOC615263	-2.203	2.512
Bt.22390.1.S1_at	HPS3	Hermansky-Pudlak syndrome 3	2.147	-2.131
Bt.1847.1.S1_at	LOC789748	Sialic acid-binding Ig-like lectin 14-like	-2.125	2.459
Bt.2334.1.S1_at	CD68	CD68 molecule	-2.116	2.157
Bt.26430.1.S1_at	ZNF184	zinc finger protein 184	2.099	-2.319
Bt.1736.1.A1_at	SOCS1	suppressor of cytokine signaling 1	-2.061	2.221
Bt.5878.2.S1_at	SDS	serine dehydratase	-2.027	2.368

[a] For each gene the fold changes found in P*vs*Ctrl (preclinical versus control) and C*vs*P (clinical versus preclinical) comparisons are reported.

3.3 Selection of the target and reference genes for the RT-qPCR validation analysis

To confirm the array results using an independent and more sensitive technique, we performed RT-qPCR analyses based on SYBR® green assay for a subset of DEGs. The selection of the genes to be validated was made on the basis of their *p* value, fold change, and possession of an interesting function as indicated by the enrichment analysis or according to the literature**.** Moreover, because we performed several statistical analyses of the microarray data (I*vs*Ctrl, P*vs*Ctrl, C*vs*Ctrl and C*vs*Ctrl), we chose genes that appeared as differentially expressed with the highest frequency in different resulting datasets. The list of target genes selected for the validation is reported in **Table 8.** Genomic DNA amplification (RT- control), primer dimer amplification (NTC control) and yield of signal intensities (Cq values) were carefully examined in order to select the best primer pairs for all the genes examined in this study. Also, primer specificity was assesed by agarose gel electrophoresis of the RT-qPCR amplicons.

Table 8. Candidate target genes analyzed by RT-qPCR[a]

Gene	Primer sequence		Amplicon length (bp)	Accession number
CD40L	F:	ACA ACC TCT GTT CTC CAG TG	82	NM_174624.2
	R:	GTC GTT TCC CGT TTT CGA GG		
XIST	F:	GTG GCA AGG ACC AGA ATG GA	112	NR_001464.2
	R:	TCC GAC CCC AGT ATT AGC CA		
GNLY	F:	AGC CCG ATG AGA ATA CCG TT	120	NM_001075143.1
	R:	CGA TGT CCT CAG CGA TGG TA		

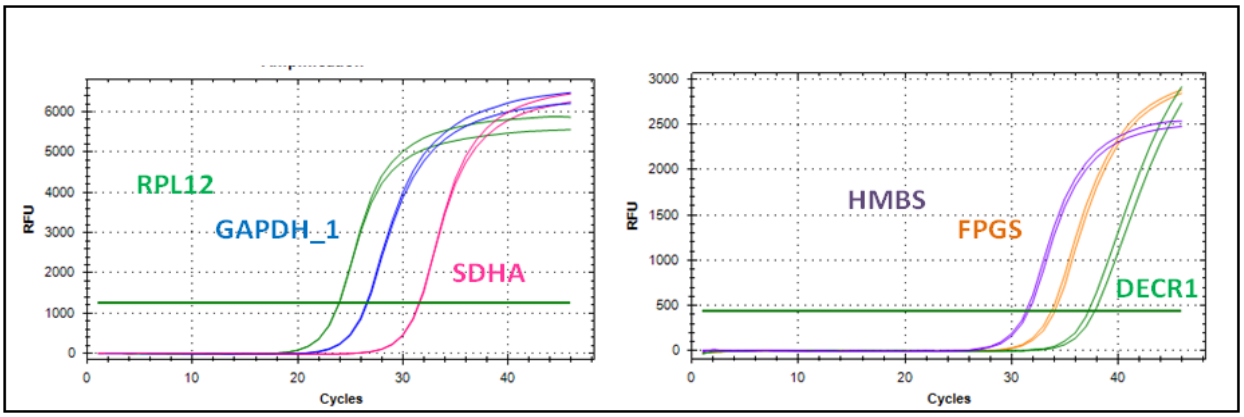
PDK4	F:	TGG TGT TCC CCT GAG AGT CA	109	NM_001101883.1
	R:	GTA ACC AAA ACC AGC CAG CG		
HBA2	F:	ACA AGG GCA ATG TCA AGG CC	124	NM_001077422.3
	R:	TCG AAG TGG GGG AAG TAG GT		
GNB4	F:	AGA TCG TGC AGG TGT TCT TG	96	NM_001099033.1
	R:	CTG TCC CAA GAC CCT GTT G		
IDO1	F:	ATT GGT GGA GTC CCT GCA GAC	150	NM_001101866.2
	R:	CTG CAG GGT AGC ATT GCC TT		
BOLA	F:	CTC GTA GTA GCT GTG GTG GC	96	NM_001038518.1
	R:	ACT GTC ACT GCT TGC AGC C		
SEL1L3	F:	TGA AGG AGT GGTTTC GCC TG	79	NM_001206556.2
	R:	TTC AAA TCC TGC CCA GTG CT		

[a] Primers (F, forward; R, reverse) used for gene amplification, amplicon size, and GenBank® accession numbers for the bovine cDNA sequences used for primer design. All primers were designed according to the genome sequence of *Bos taurus.*

For the reference genes selection, we outlined a list of possible candidates based on blood transcriptomics literature and on previous experiments carried out in our laboratory (Gubern, Hurtado et al. 2009, Stamova, Apperson et al. 2009, Ledderose, Heyn et al. 2011, Barbisin, Vanni et al. 2014). We designed primer pairs corresponding to six of these genes: DECR1 (2,4-dienoyl CoA reductase 1, mitochondrial), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), HMBS (hydroxymethyl-bilane synthase), FPGS (folylpolyglutamate synthase), RPL12 (ribosomal protein L12) and SDHA (Succinate dehydrogenase complex, subunit A). Based on the level of expression assessed by a preliminary RT-qPCR analysis, we first selected two genes from the above mentioned list. RPL12 and GAPDH were the most robust reference genes (see

Figure 7A). We tested these genes on additional samples and noticed that in some case the RT- negative control for GAPDH was amplified with a Cq value (threshold cycle) too close to that of the RT+ samples. Therefore, we designed two new primer pairs for GAPDH that worked better in our conditions (**Figure 7B**)

A



B

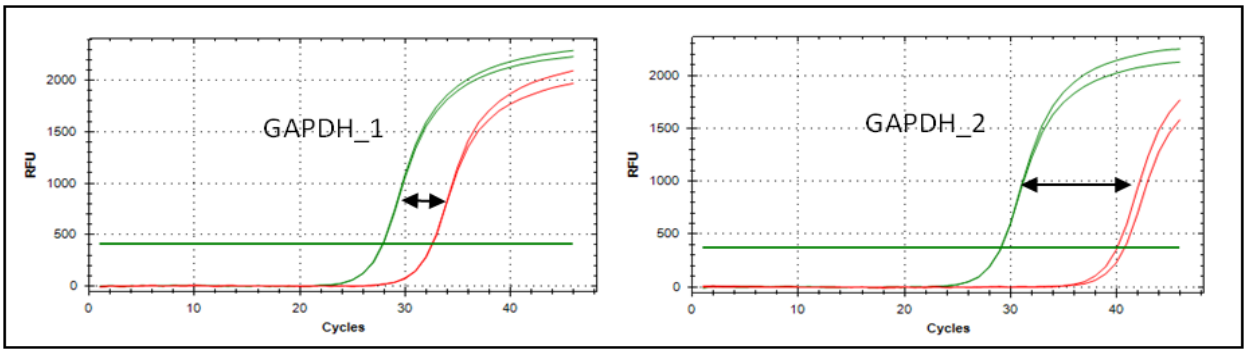


Figure 7. Reference genes selection and GAPDH primer optimization. The expression level of 6 candidate reference genes (RPL12, GAPDH, SDHA, HMBS, FPGS and DECR1) was assessed by RT-qPCR. RPL12 and GAPDH were selected as they displayed the lowest Cq values (**A**). Primer pair for GAPDH (GAPDH_1) was optimized to reduce the detection of genomic DNA. GAPDH_ is the new optimized primer pair that we used for subsequen analyses. In red are shown the amplification curves of the RT- control and in green those relative to the RT+ sample (**B**).

To verify if our sample population was characterized by an uniform expression of the selected reference genes, we performed an RT-qPCR analysis measuring the Cq value for GAPDH and RPL12 across all the samples. As shown in **Figure 8**, the expression trend of GAPDH and RPL12 was quite similar. The stability of the two genes was comparable between preclinical, clinical and control population, with Cq ranges quite similar among the three groups. In all the samples GAPDH was less expressed compared to RPL12, with the exception of sample S1. The variability of the reference genes, measured as standard deviation (SD) of the Cq values, was slightly higher for GAPDH (SD=1.19) compared to RPL12 (SD=0.98).

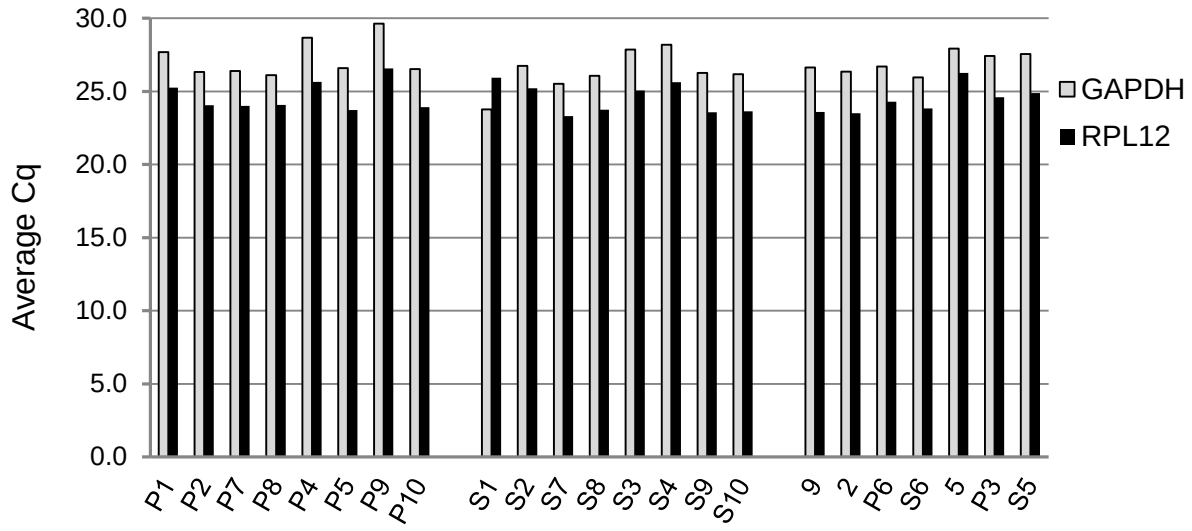
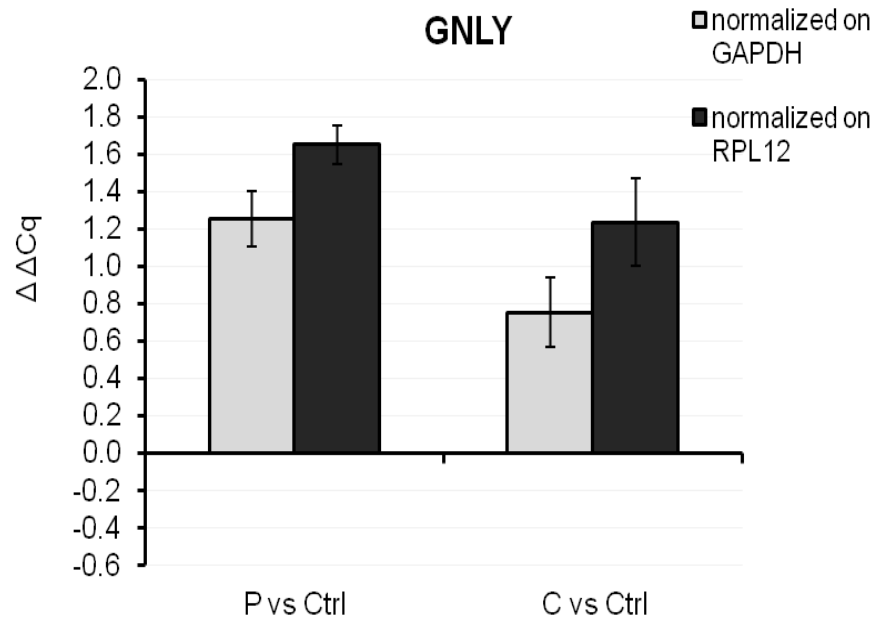


Figure 8. Variability of C_q values among preclinical, clinical and control samples.

At this point we performed a preliminary RT-qPCR analysis on a subgroup of samples (4 preclinical, 4 clinical and 4 control) to verify that the results obtained normalizing with RPL12 and GAPDH were comparable. As target genes to test in this preliminary analysis, we selected GNLY and GNB4. Concerning GNLY, we found that the ΔΔCq values were quite similar using GAPDH and RPL12 as reference. However, for GNB4

the results changed more consistently depending on the reference gene used for the normalization (**Figure 9**).

A



B

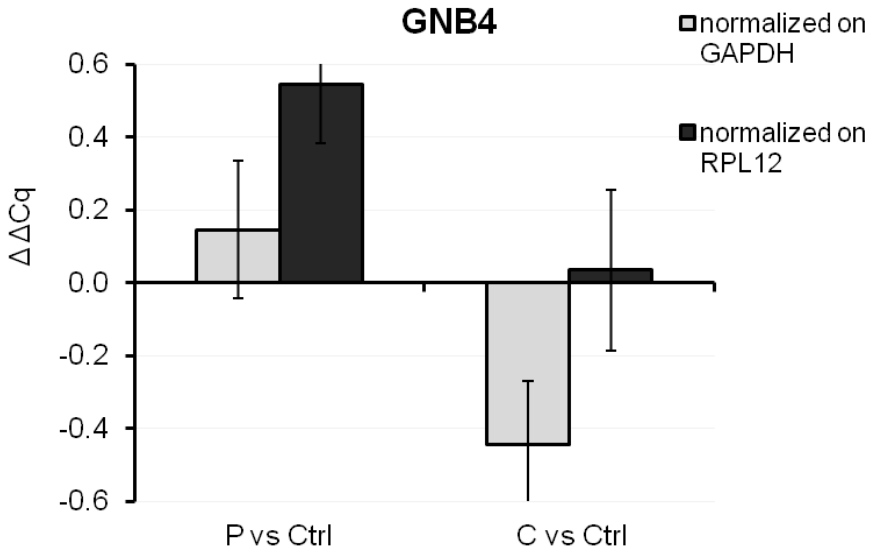


Figure 9. GNLY (A) and GNB4 (B) ΔΔCq values in preclinical and clinical samples using GAPDH or RPL12 as reference genes.

3.4 Optimization of the RT-qPCR normalization method.

Since a considerable grade of variability of the results was found using GAPDH or RPL12 as control gene, we decided to introduce a third one (ACTB, beta actin) and to adopt a normalization factor based on geometric averaging of the three selected reference genes. In this way we could improve the accuracy of the analysis and avoid erroneous interpretation due to the variability associated to the use of a single reference gene per time (Vandesompele, De Preter et al. 2002). Moreover, we included in our study two inter-run calibrators to reduce plate to plate variability. The stability of the selected reference genes was determined by calculating their geNorm M value (M) and the coefficient of variation (CV) on the normalized relative quantities (**Figure 10)** (Hellemans, Mortier et al. 2007). M and CV values were then compared against empirically determined maximum thresholds for acceptable stability (~ 1 and ~ 0.5 for M and CV values, respectively) (Hellemans, Mortier et al. 2007). The statistical analysis and the fold change calculation for the final analyses were carried out using qBasePlus 1.1 software (Hellemans, Mortier et al. 2007).

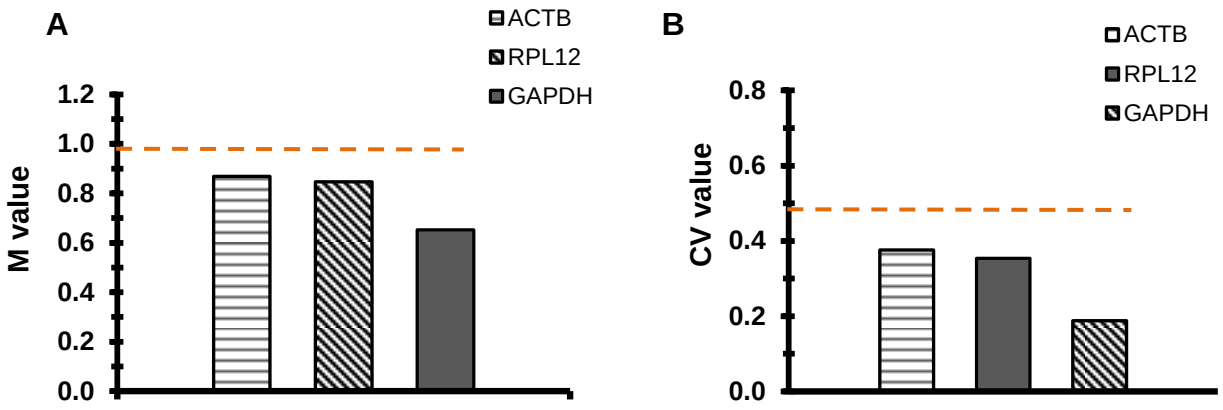


Figure 10. Reference gene expression stability. Stability of the selected reference genes was determined by calculating their geNorm M value (M) (**A**) and their coefficient of variation (CV) (**B**) on the normalized relative quantities (CNRQ). The dashed green lines in panel A and B indicate the maximum acceptable threshold for M and CV values, respectively. These thresholds have been empirically determined by previous experiments performed by Hellemans and colleagues (Hellemans, Mortier et al. 2007)

3.5 Setting of the minimal RNA starting amount for RT-qPCR amplification.

Since we had a limited quantity of RNA for each sample, before proceeding with subsequent analyses we had to set the minimal RNA template amount for the RT-qPCR analysis. Based on previous protocols used in our laboratory, we decided to test two different RNA concentrations: 1.25 ng/µL and 0.625 ng/µL, corresponding to a dilution of 1:10 and 1:20 of the initial RNA, respectively. For this preliminary experiment we used 1 µL of RNA solution and two primer pairs which amplified a reference gene (RPL12) and a target gene (GNB4). Forward and reverse primers were used at a concentration of 400 nM each. The results showed that these two different RNA amount

resulted in around 1 Cq difference between the two amplification curves, as expected (**Figure 11**). Amplification of the target gene GNB4 resulted in a Cq equal to 30.96 and 32.13 using 1.25 and 0.625 ng as starting RNA amount, respectively. Such Cq values were quite high if we consider that 30-35 Cq is the generally accepted maximum threshold for a reliable quantification. For this reason, we decided to use the higher RNA starting amount (1.25 ng) for all the subsequent RT-qPCR analyses.

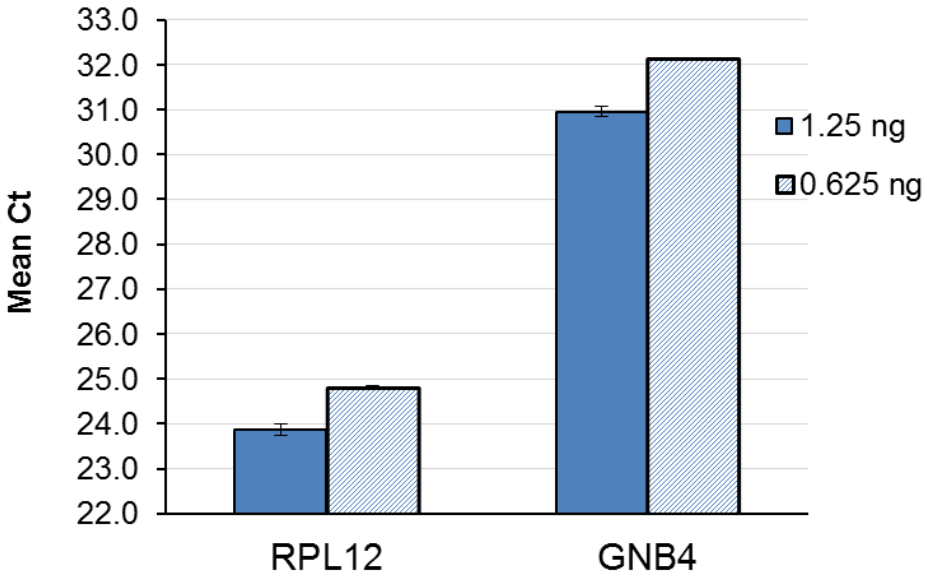


Figure 11. Mean Cq values for RPL12 and GNB4 using two different RNA starting amounts.

3.6 RT-qPCR final results.

We performed RT-qPCR analysis for all the genes reported in **Table 8**. We were able to confirm the microarray results for six out of nine genes selected (*XIST, CD40L*, *GNLY*, *PDK4*, *HBA2* and *SEL1L3*), which are represented in **Figure 12** and **Table 9**: *CD40L*, *XIST*, *SEL1L3* and *GNLY* downregulation was confirmed in both preclinical and clinical groups, while *HBA2* was significantly down-regulated only in preclinical animals. Significant *PDK4* upregulation was found in the clinical stage, but not in the preclinical one. These results were in line with the microarray data (**Table 9**).

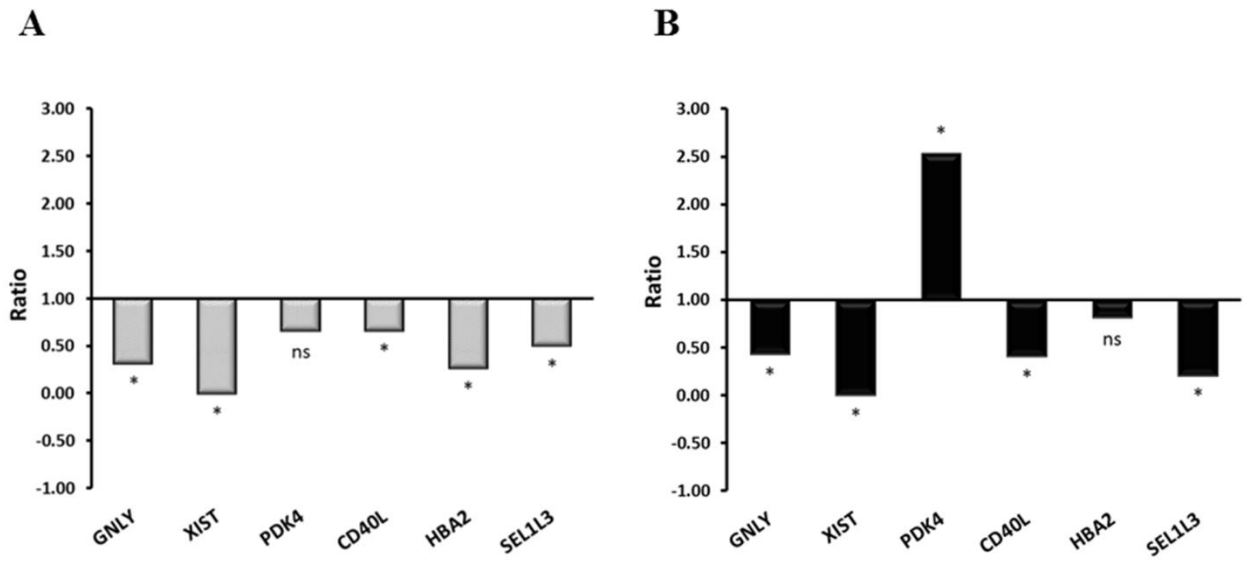


Figure 12. Validation of microarray data by RT-qPCR. Differential expression of selected genes in blood from preclinical (A) and clinical (B) atypical BSE-infected cattle. Ganulysin (GNLY), X-inactive specific transcript (XIST), pyruvate dehydrogenase kinase 4 (PDK4), CD40 ligand (CD40L), haemoglobin, alpha 2 (HBA2) and Sel-1 Suppressor Of Lin-12-Like 3 protein (SEL1L3). Gene expression (ratio) values are represented as relative to RNA levels in control animals. Ns = not significant; **P* value ≤ 0.05.

Table 5. Differential expression of selected genes quantified by microarray and RT-qPCR analysis [a].

Gene name	Gene symbol	Microarray fold change		RT-qPCR ratio/FC	
		preclinical	clinical	preclinical	clinical
Granulysin	GNLY	-6.318	-3.698	0.322/-3.106	0.433/-2.309
X-inactive specific transcript	XIST	-33.240	-22.612	0.003/-333.333	0.004/-250.000
Pyruvate dehydrogenase kinase 4	PDK4	1.752 (ns)	5.793	0.665/ -1.503 (ns)	2.536
CD40 ligand	CD40L	-2.786	-3.761	0.667/-1.499	0.408/-2.450
Haemoglobin, alpha 2	HBA2	-3.866 (ns)	-2.259 (ns)	0.269/-3.717	0.816/-1.225 (ns)
Suppressor Of Lin-12-Like Protein 3	SEL1L3	-2.16	-5.17	0.504/-1.984	0.213/-4.695

[a] For an easier interpretation, the differential expression of the downregulated genes measured by RT-qPCR is reported both as original ratio and as fold change calculated as -1/ratio. Ns=not significant. FC=fold change.

4 Discussion

Whilst the major neuropathological features of TSEs are well documented, the host response to prion infection and the molecular events that characterize the peripheral prion pathology are largely unknown. Genomic approaches are a potential tool to understand the molecular bases of complex mechanisms such as prion disorders. Further, they are powerful tools for the identification of target genes that could be used as disease biomarkers. Indeed, one of the major challenges in the field of neurodegenerative diseases is the finding of a preclinical diagnostic test that would allow the patient to begin therapy before irreversible neuronal loss has occurred. An early treatment could increase the possibility to slow disease progression and perhaps even reverse it. In prion disorders, such as vCJD or BSE, the development of a preclinical test is particularly desirable due to their transmissible nature, even at early stages of the infection.

In the present study, gene expression changes occurring in the blood of atypical BSE-infected cattle were identified with the aim to understand the peripheral mechanisms of prion infection and to detect some candidate genes that could be further investigated as biomarkers of the disease. Since our aim was to find a common pattern in H- and L-type infection, we pooled these two groups. Indeed, in a preliminary analysis we compared the 4 H-type and the 4 L-type infected animals and found a limited number of DEGs. Therefore, we concluded that, at least in terms of number of DEGs, these two groups did not display large differences. This observation is in agreement with a previous work of Konold and colleagues, which carried out a behavioral study on the same animals that we used in our analysis (Konold, Bone et al. 2012). In their study it was also found

a large similarity between the 4 H-type and the 4 L-type infected groups in terms of behavioral and clinical signs. Also, another study by Priemer and colleagues highlighted a similarity between H and L type BSE (Priemer, Balkema-Buschmann et al. 2013). Indeed they found comparable PrP^{Sc} anatomical distribution for atypical strains, with only slight differences in the overall intensities between L- and H-type. Taking into account all these considerations, we decided to focus our attention on finding a common gene pattern among all the atypical BSE-infected cattle and therefore we pooled the two groups. Further studies in larger animal cohorts would be required to investigate in detail the strain-specific gene expression changes occurring during the progression of the disease.

During the last decade, several high-throughput genomic profiling studies have been performed on murine, ruminant and primate models. The majority of these studies focused on the gene expression changes occurring in the CNS in course of prion infection. However, despite being informative of the central disease mechanisms, direct analyses of the brain or spinal cord are not suitable for rapid diagnosis of infected animals and patients. To our knowledge, we present here the first microarray study of whole blood from BSE-infected cattle. Whole blood is an accessible source of transcriptional information and offers many advantages compared to blood fractioning, such as minimization of sample handling artifacts, reduction of sample variability due to fractionation and a shorter protocol. One of the major caveats in using peripheral blood is that its cellular components may change dramatically during infections or inflammation. The animals used in the present study did not show any apparent side pathology, they were monitored daily by the husbandry staff and their blood was

examined for serum aspartate aminotransferase (AST), creatine kinase (CK) and manganese (Konold, Bone et al. 2012). Nonetheless, possible interference due to hidden pathologies or to inter-individual variations in hematocrit and white blood cell count may affect interpretation of expression data and should be considered as an important variable in future studies. In light of this, the present results should be read as a first attempt to explore whole blood transcriptomics in course of a prion infection.

A comparison of our results with other studies would be important to identify the most promising signature genes of the disease to be further tested in larger cohorts and in other animal models. Unfortunately, at present very little information is available on gene expression changes related to atypical BSE infections. In 2011 Panelli et al. (Panelli, Strozzi et al. 2011) investigated the gene expression changes in fractionated white blood cells from L-type orally-infected cattle. In this study 56 DEGs were identified using a custom array. Surprisingly, only a few of them were present in our list as well. Different infection routes, microarray platforms, statistical analysis stringency and *p* value cut-off may explain such discrepancy. Additionally, the white blood cells used in the study of Panelli *et al.* were isolated from 1 year post-infection animals, while in our study we used whole blood from preclinical and clinical infected cattle (at 6 months and 22-26 months post-infection, respectively). Finally, in addition to leukocytes, whole blood contains many cell populations such as immature dendritic cells, erythrocytes with residual transcription activity (Kabanova, Kleinbongard et al. 2009) and platelets, which may respond to prion infection by regulating different sets of genes.

The scavenger receptor CD163L1 (CD163-like molecule 1) was one of the genes found upregulated in both our and Panelli's study (we found significant changes in I*vs*Ctrl and

L*vs*Ctrl). CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily class B that is highly expressed on resident tissue macrophages and monocytes. Besides a function as innate immune sensor for bacteria and inducer of local inflammation (Fabriek, van Bruggen et al. 2009), CD163 play a role in erythropoiesis (Fabriek, Polfliet et al. 2007) and has been identified as an endocytic receptor for hemoglobin-haptoglobin (Hb-Hp) complexes (Kristiansen, Graversen et al. 2001). These findings are interesting especially in light of previous data reporting the differential expression of an erythroid differentiation-related factor (EDRF) that was suggested as a possible molecular marker for early stages of TSE infection (Miele, Manson et al. 2001). In this context, it is of note that a therapeutic strategy focused on hematopoietic cells was able to suppress the pathological effects of Sandhoff disease, a neurodegenerative pathology that, in a similar manner to TSEs, present inflammatory response involving microglial activation that leads to neuronal apoptosis (Wada, Tifft et al. 2000). The involvement of the hematopoietic system in prion infection is also supported by the HBA2 downregulation that we confirmed in the preclinical stage of prion infection by RT-qPCR analysis. Haemoglobins are iron-containing proteins that transport oxygen in the blood of most vertebrates. Beside blood, *HBA* and *HBB* are also expressed in mesencephalic dopaminergic neurons and glial cells (Biagioli, Pinto et al. 2009) and are down regulated in AD, PD and other neurodegenerative diseases (Kabanova, Kleinbongard et al. 2009). Haemoglobin gene expression alteration during preclinical scrapie was also found in the spleen and CNS of infected animals (Booth, Bowman et al. 2004, Kim, Snyder et al. 2008), as well as in the brains of nonhuman primates infected with BSE (Barbisin, Vanni et al. 2014). Further, it has been found that

both HbA and HbB protein distribution is altered in mitochondrial fractions from PD degenerating brain (Shephard, Greville-Heygate et al. 2014). Taken together, these findings strongly suggest a central role for haemoglobin in neurodegenerative processes.

An aspect to be taken into account when reading the present results is that additional negative control cattle, aged from 12 to 37 months and derived from a different herd compared to the Konold's study group (Konold, Bone et al. 2012) were introduced in the analyses. The addition of these controls was useful to balance the samples from infected animals and allowed a preliminary exploration of the differentially expressed transcripts. However, age-related and environmental variability may have affected to some degree the data and need to be further considered for their correct interpretation. Despite some limitations, since several statistical analyses were performed (including the C*vs*P comparison, in which all the animals derived from the same herd) a cross comparison of all them, as we did with the Venn diagram, is useful in order to define a set of genes for further validation. In light of this, we believe that our data constitutes an important starting point for future experiments.

A technical issue that we had to solve in the present study consisted in the fact that the RNA samples that we received were very low concentrated, even after reducing the final RNA elution volume. According to the standard one-cycle amplification and labeling protocol developed by Affymetrix (Santa Clara, CA), a minimal mRNA concentration is required to perform the RNA target preparation for the microarray experiments. According to this protocol, the recommended input RNA amount should be equal to 100 ng in a volume smaller than 3 µL. Many of our samples had a concentration lower than

this threshold, prompting us to concentrating them. It is known that such a procedure may affect the final integrity of the samples, especially when long time and/or high temperature conditions are set for the concentration. However, the protocol that we adopted was specifically designed to speed up the evaporation of solvents from very small samples and is commonly used in this field, especially when the 260/280 and 260/230 ratios are good, as for our samples; thus we did expect that the concentrate would adequately represent the initial mRNA products. Further, all our samples were processed in the same way, at room temperature and for a very short time (around 5 minutes). Their integrity was assessed by capillary electrophoresis and was found to be mostly unchanged than before concentration.

In the first statistical analysis we performed (I*vs*Ctrl), we found that among 101 DEGs, 93 had known functions and were involved in several biological processes and molecular pathways, such as autoimmune thyroiditis, chemokine and cytokine activity, regulation of the secretion pathway, immune system components and antigen processing presentation. Previous studies on CNS tissues from BSE-infected animals also showed the involvement of many of these pathways in prion pathogenesis. For example, a decrease in GNLY expression, which we could validate in both the preclinical and clinical group, was also found in the medulla oblongata from preclinical sheep affected by natural scrapie (Filali, Martin-Burriel et al. 2012). GNLY is a powerful antimicrobial protein contained within the granules of cytotoxic T lymphocyte and natural killer cells. Its downregulation could be interpreted as a mechanism exerted by prions to escape the immune system and could contribute to explain a lack of NK activation during the course of prion infection.

The similarity between brain and blood –omics is not surprising, since it has already been established in the literature that blood transcriptomics can identify genes that are relevant to the pathological processes occurring in the CNS (Tylee, Kawaguchi et al. 2013). Indeed, measuring disease-related gene expression in peripheral blood may be a useful proxy measure for gene expression in the CNS (Borovecki, Lovrecic et al. 2005, Tylee, Kawaguchi et al. 2013). Nonetheless, transcriptional changes occurring in blood do not always overlap with those found in the brain, a fact that does not necessarily detract from their suitability as indicators of CNS pathology. For example, an increase in CCL3 and CCL4 chemokine expression was observed in CJD infected mouse brains during preclinical stage of the disease (Lu, Baker et al. 2004), while in our study we found a downregulation for such genes (CCL3 downregulation was found in I*vs*Ctrl and C*vs*Ctrl comparison while CCl4 was found downregulated in C*vs*Ctrl). Interestingly, it has been shown that these chemokines are involved in the migration of bone marrow-derived mesenchymal stem cells to brain lesions caused by prions (Song, Honmou et al. 2011). Thus, we could hypothesize that a reduction of their expression in the periphery paralleled by an increase in the CNS could be relevant in this migration phenomenon. As for CCL3 and CCL4, we found downregulation for CCL5, a chemokine involved in the activation of NK cells. The lack of reported NK cell response to prion pathogenesis suggests that the reduction of CCL5 expression, together with the already mentioned GNLY downregulation, may be the consequence of a prion-induced mechanism to evade NKs.

An interesting DEG found by microarray analysis in I*vs*Ctrl was IDO1 (indoleamine 2,3-dioxygenase 1). IDO1 is a heme-containing protein that acts as a rate-limiting enzyme

of tryptophan catabolism through kynurenine pathway. Metabolites in the kynurenine pathway, generated by tryptophan degradation, are thought to play important roles in neurodegenerative disorders, including Alzheimer's and Huntington's diseases. Notably, it has been shown that kynurenine 3-monooxygenase inhibition in the blood ameliorates neurodegeneration (Zwilling, Huang et al. 2011). Microarray analysis performed in the present study revealed a downregulation for IDO,1 but RT-qPCR analysis did not confirm this result.

To characterize the gene expression profile in the preclinical and clinical stages, we performed the P*vs*Ctrl and the C*vs*Ctrl statistical comparisons. We found that 113 probe sets were differentially regulated in the preclinical stage of the disease, while 207 probe sets had an altered expression in the clinical phase. Importantly, the present results indicated that, at least in blood, a consistent gene expression alteration is present from the early stages of the disease. This finding is in agreement with microarray analysis carried out by Tang *et al.*, which revealed the highest degree of differential gene regulation in brains of cBSE-infected cattle at 21 months post infection, which is prior to the detection of infectivity (Tang, Xiang et al. 2009). Also, Tortosa and colleagues found a significant number of DEGs at early stages of the disease in the CNS from cBSE-infected transgenic mice (Tortosa, Castells et al. 2011).

Venn diagram revealed that 35 DEGs were in common between the clinical and preclinical groups and, remarkably, they had a very similar pattern of expression in both stages of the disease. The fact that these genes are altered in both subgroups of infected animals should be read as an internal comparison which may help in the identification of the most promising candidate genes for further validation experiments.

Based on GO enrichment analysis, we found that immunity and inflammation processes were strongly involved during the progression of the disease stages. Expression of many chemokines was altered in both the preclinical and clinical groups. Besides the aforementioned chemokines of the C-C family, we also found a downregulation of CXCL10 (I*vs*Ctrl and C*vs*Ctrl analyses) CXCL3 (P*vs*Ctrl analysis), CXCR6 (I*vs*Ctrl, P*vs*Ctrl and C*vs*Ctrl analyses) and CXCR5 (C*vs*Ctrl and S*vs*P analyses). CXCL3 controls the migration and adhesion of monocytes and neutrophils, while CXCR6 is found primarily on activated T cells, NKT cells, bone marrow, plasma cells and smooth muscle cells. At present, there are no reports in literature indicating any specific role for these two chemokines in prion pathophysiology. We can only hypothesize that their downregulation could be part of the immune escape strategy exerted by prions in the periphery. CXCL10 is known to be a potent chemoattractant for Th1 T cells, NK cells, and monocytes/macrophages. Also, it is known as a potent angiostatic factor (Angiolillo, Sgadari et al. 1995). Therefore, its downregulation could be read both as an immune escape mechanism and as strategy for prion propagation within the organism. Of note, expression of CXCR5 has been found to be involved in the regulation of prion neuroinvasion. Indeed, CXCR5 ablation juxtaposes FDCs to major splenic nerves, and accelerates the transfer of intraperitoneally administered prions into the spinal cord (Prinz, Heikenwalder et al. 2003). In light of this evidence, the downregulation of CXCR5 also supports the hypothesis of an active process exerted by prions to evade and hijack the immune system.

Interestingly, we found that antigen processing and presentation via MHC (major histocompatibility complex) molecules and the autoimmune thyroiditis pathway were

significantly altered in atypical BSE-challenged animals. The majority of MHC class I molecule coding-genes were down-regulated in infected cattle (three out of four probes) and, also, MHC class II molecule coding transcripts were found to be down-regulated during the progression of the clinical signs (four out of four probes were down-regulated in the C*vs*P comparison). In line with the trend found by the microarray analysis, the RT-qPCR validation experiments indicated a downregulation for MHC class I heavy chain (*BOLA*), even though the results failed to reach the statistical significance (data not shown). The involvement of MHC transcripts in prion pathogenesis is supported by another microarray study published by Khaniya and colleagues (Khaniya, Almeida et al. 2009).In this study it was found a reduction in the expression of two genes related to MHC II and one gene involved in MHC class I presentation in Peyer's Patches of BSE-infected cattle. The authors hypothesized that, since MHC class I molecules are associated with the development of T cytotoxic cells, by limiting this process the PrP^{Sc}-expressing cells, like macrophages or peripheral blood mononuclear cells (PBMCs), may escape immune surveillance.

Interestingly, gene enrichment of the autoimmune thyroiditis pathway was found in the clinical stage of the disease. It is well established in literature that Hashimoto's encephalitis, together with the associated thyroiditis, is a differential diagnosis for CJD, since the two pathologies share a very similar clinical symptomatology (Seipelt, Zerr et al. 1999). As hypothesized previously by Prusiner and colleagues, the clinical similarities between CJD and Hashimoto's thyroiditis raise the possibility that protein misprocessing may underlie both neurodegenerative and autoimmune diseases (Prusiner 2001).

A fourth statistical analysis was performed to identify any specific changes between the clinical and the preclinical stages of disease (C*vs*P). We found that the last phases of the disease were accompanied by the overactivation of several genes involved in the immune defense response. In particular, the shift from the preclinical towards the clinical stage was characterized by the upregulation of genes involved in B cell proliferation and the ISG15 (IFN-induced 15-kDa protein) conjugation system. ISG15 is a ubiquitin-like molecule that is tightly regulated by specific innate immunity signaling pathways (Blomstrom, Fahey et al. 1986). Interestingly, it has been shown in the literature that this protein is over-activated in the spinal cord of amyotrophic lateral sclerosis mice models (Wang, Yang et al. 2011) and it has been indicated as a general marker for both acute and chronic neuronal injuries (Wang, Kaul et al. 2012). Also, some ficolins and PTAFR (platelet-activating factor receptor) were found upregulated in C*vs*Ctrl analysis. Ficolins are a group of oligomeric lectins involved in complement activation in the absence of antibody binding. Notably, it has been shown that complement activation facilitates early prion pathogenesis (Klein, Kaeser et al. 2001). On the same line, upregulation of PTAFR could be linked to the spread of prions, since activated platelets have been shown to release PrP^C on exosomes (Robertson, Booth et al. 2006).

To further analyze the data, we compared the list of DEGs found in P*vs*Ctrl, C*vs*P and C*vs*Ctrl and found 22 genes with an up/down-down/up pattern of expression, being differentially expressed in the preclinical stage and then going back roughly to the control level in the clinical stage. These genes, in addition to the aforementioned 32-gene pattern, may be the preferential candidates to be further investigated as diagnostic

biomarkers and for monitoring the progression of the disease. Interestingly, some of the up/down-down/up DEGs are involved in regulation of transcription, thus suggesting that the gene expression during atypical BSE infection is tightly regulated. Venn diagram revealed that one gene, *SEL1L3*, was down-regulated in all the comparisons (P*vs*Ctrl, C*vs*Ctrl, C*vs*P). SEL1L3 codes for a transmembrane protein whose function is unknown. Interestingly, an important paralog of *SEL1L3*, *SEL1L*, is involved in the retrotranslocation of misfolded proteins from the lumen of the endoplasmic reticulum to the cytosol, where they are degraded by the proteasome in an ubiquitin-dependent manner (Gardner, Swarbrick et al. 2000). Therefore, we could hypothesize that its down-regulation in prion-infected animals would lead to a reduced degradation of PrP^{Sc}, thus supporting the progression of the disease. We validated this gene by RT-qPCR, confirming its down-regulation in both the preclinical and clinical stages of the disease. Further investigation on the function of *SEL1L3L* would be of great interest since this gene may play an important role in prion disease and maybe other neurodegenerative illnesses.

As shown in the results, the preliminary RT-qPCR analyses were carried out using two reference genes as internal controls. However, we had problems in interpreting the results since in some case the gene expression changes were quite different depending on which gene was used for the normalization. Probably, when the variation in gene expression is very subtle, different fold change values are obtained across different reference genes. On the contrary, large gene expression changes are more robust and therefore can be detected in a comparable way by different reference genes. To overcome this issue, we decided to introduce a third reference gene and to shift from

the classic delta-delta-Ct method to multiple reference genes. In this way we could obtain more reliable data and also we had the possibility to evaluate the stability of the reference genes by calculating their M and CV value.

In this last part of the Discussion, we will briefly describe the functions and the possible roles in prion pathology of the genes confirmed by RT-qPCR analysis. Besides SEL1L3, GNLY and HBA2, which were already mentioned in previous sections of the Discussion, another three genes were validated: CD40L, XIST and PDK4.

CD40L was found to be downregulated in both the preclinical and clinical stage, in agreement with microarray results. Also, the microarray analysis highlighted a downregulation of CD40, the CD40L receptor. CD40–CD40L interactions mediate a broad variety of immune and inflammatory responses and have been implicated in the pathogenesis of Alzheimer's disease (AD) (Tan, Town et al. 1999, Calingasan, Erdely et al. 2002). Mice deficient for CD40 or CD40L have impaired immunoglobulin class switching, lack germinal center formation and are deficient for germinal center B cells (GCBs). There are conflicting reports regarding the role of CD40 in prion diseases. A rapid disease development was observed in scrapie-infected mice deficient for CD40L, thus suggesting a neuroprotective function of intact CD40-CD40L interactions (Burwinkel, Schwarz et al. 2004). In line with this, $CD40L^{-/-}$ mice inoculated with ME7 prions via skin scarification developed disease significantly earlier than did wild-type mice(Mohan, Bruce et al. 2005). In contrast, results obtained from $CD40L^{-/-}$ mice inoculated via an intraperitoneal route with the Rocky Mountain Laboratory (RML) scrapie strain detected no alteration of prion pathogenesis (Heikenwalder, Federau et

al. 2007). Although the importance of *CD40L* in prion disease progression has not yet been clarified, its downregulation in blood during both preclinical and clinical stages of atypical BSE-infection suggests that prion infection has an impact on the host immune system response and that immune tolerance may be an active process induced by prions.

A marked downregulation in *XIST* expression was found in our infected animals. *XIST* is a gene located on X chromosomes which codes for a long non-coding RNA (LncRNA) involved in X-chromosome dosage control (Penny, Kay et al. 1996, Nguyen and Disteche 2006). Since XIST mRNA has the same sequence as XIST gDNA, it was impossible to design a primer pair that could discriminate between the two. Indeed, RT-control for XIST presented quite low Cq values (meaning high expression) compared to the other genes that we validated. However, since the genomic contamination was uniform among the samples, we still considered the results reliable. LncRNAs are emerging as useful biomarkers for neurodegenerative diseases such as AD (Johnson 2012) and other disease processes (Sun, Wang et al. 2013), and they can be easily detected in blood and urine from patients. In addition, we cannot exclude the possibility that the alteration in *XIST* expression may have some role in gender-dependent response to prion infection (Loeuillet, Boelle et al. 2010).

RT-qPCR experiments confirmed the upregulation of *PDK4* in clinically affected animals. *PDK4* encodes for a mitochondrial protein involved in glucose metabolism through the inhibition of pyruvate dehydrogenase complex, which leads to a reduction in pyruvate conversion to acetyl-CoA (Harris, Bowker-Kinley et al. 2002). In the literature, a key role has been suggested for acetyl-CoA fueling for the survival of cholinergic

neurons in the course of neurodegenerative diseases (Szutowicz, Bielarczyk et al. 2013). *PDK4* overactivation can lead to a switch from glucose catabolism to fatty acid utilization (Zhang, Hulver et al. 2014), thus increasing the production of ketone bodies. Notably, it has been shown in the literature that these molecules are able to cross the blood brain barrier. We could speculate that in prion infection (or at least in atypical BSE infection) the concentration of ketone bodies would rise in blood, as a consequence of PDK4 upregulation, and act in the brain as neuroprotective molecules (Reger, Henderson et al. 2004, Henderson 2008). This would be an attempt by the organism to prevent the neurodegeneration induced by prions.

5. Conclusions

The present study has led to the identification of several gene expression changes in whole blood from atypical BSE infected cattle prior to and after the manifestation of the pathology. 35 DEGs were found in common between the preclinical and the clinical stage, with all of them displaying a similar expression pattern during the progression of the disease. Large differences in blood transcripts were also found in the comparison of preclinical and clinical stage, thus suggesting a role of the peripheral immune system in the prion-pathogenic mechanisms observed at late stages in the CNS. The comparison of all microarray analyses performed in this study led to the identification of a core of 22 DEGs that displayed an up/down-down/up pattern of expression. Such genes could be good candidate markers to be tested in larger animal cohorts and in blood from human patients. By comparing our results with other studies on various animal prion diseases,

we observed that some of the most significantly altered DEGs we found in blood were found differentially expressed also in brain tissue from BSE-infected cattle; this observation indicates that whole blood transcriptomics may serve as a proxy measure for monitoring the changes occurring in the CNS. Overall, our study confirmed the differential expression of 6 genes (*XIST*, *CD40L*, *GNLY*, *PDK4*, *HBA2* and *SEL1L3*), which may play several roles in atypical BSE pathogenesis and, possibly, in other prion infections. Indeed, they are involved in multiple pathways such as immune response, inflammation, and glucose catabolism, all of which have already been mentioned in other studies related to several prion pathogeneses. Even though further studies are required to investigate the specific involvement of all the identified genes in prion diseases, our data support the idea of a relationship of complicity and tolerance between prion and the host immune system. As concluding remark, our study underlined the importance of utilizing whole blood, without any additional manipulation, as a source tissue for the development of a preclinical diagnostic test.

6 Materials and Methods

All procedures involving animals were approved by the Home Office of the UK government according to the Animal (Scientific Procedures) Act 1986 and in conformity with the institutional guidelines of the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Turin, Italy (IZSPLV), that were in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circular No.8, G.U., 14 July 1994) and international regulations (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987). All the experimental protocols proposed were reviewed and approved by the IZSPLV Animal Care and Use Committee (IACUC).

6.1 Blood samples

Blood samples from 8 BSE-infected cattle (4 with H-type and 4 with L-type BSE) and 2 non-infected controls were provided by the Biological Archive Group at the Animal and Plant Health Agency (AHVLA), United Kingdom. All procedures involving animals were approved by the Home Office of the UK government according to the Animal (Scientific Procedures) Act 1986. The calves were born by crossing *Aberdeen angus* with females imported from Denmark (Danish Holstein, Danish milking red). The inoculation details have been reported previously (Konold, Bone et al. 2012). Briefly, experimental cattle were intracerebrally inoculated with 1 ml of a 10% brain homogenate of either L-type or H-type BSE at 10-11 months of age (Konold, Bone et al. 2012). All infected cattle used in this study were females. The negative controls were age and sex-matched with the infected group. For each animal, the blood sampling was performed at 2 different time points after inoculation, corresponding to the preclinical (6 months post-infection) and

the clinical (from 22 to 26 months post-infection) stage of the disease. In this way we obtained 16 samples, 8 in the preclinical and 8 in the clinical stage. The estimated clinical onset after infection was based on the presence of changes in behavior, unexpected startle responses, and difficulty in rising (Konold, Bone et al. 2012). Neurological examination and behavioral observations were conducted routinely until the culling of the animals. TSE infection was confirmed by post-mortem immunohistochemistry on brain sections of the animals (Konold, Bone et al. 2012). Detailed information on the husbandry procedures and the pathological signs have been described in a previous study published by Konold et al. (Konold, Bone et al. 2012). Finally, blood samples from 6 sex-matched *Aberdeen angus* from a different herd were added to the study and used as additional negative controls to obtain a sample size comparable to the one of the infected animals (8 samples).

6.3 RNA isolation

500 µL of fresh blood were stabilized in 1.3 mL RNA*later*® Solution and immediately frozen at -20 °C. Samples were sent in dry ice to IZSTO (Turin, Italy), where the RNA was isolated according to the RiboPure™-Blood Kit manufacturer's instructions (Ambion®). DNase I treatment (Ambion®) was included in the RNA extraction protocol to reduce DNA contamination. Purified RNA was initially eluted in 100 µL elution solution and the final concentration, as well as the absence of protein, was determined using a Thermo Scientific™ NanoDrop 2000 spectrophotometer. Since the RNA concentration was too low to proceed with the subsequent analysis, newly isolated RNA samples were eluted in 50 µL elution solution. Again the concentration was low, therefore the RNA samples were concentrated using a Labconco CentriVap concentrator. Sample were let

evaporate for 5 minutes at room temperature. The new concentration was assessed using a Thermo Scientific™ NanoDrop 2000 spectrophotometer and the integrity of the RNA was determined by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, USA).

6.4 Microarray hybridization, statistical analysis and data mining

24 RNA samples were used for the microarray analysis: 8 preclinical (P1, P2, P4, P5, P7, P8, EP9 and P10), 8 clinical (S1, S2, S3, S4, S7, S8, S9 and S10), and 8 control (c2, c3, cP3, c5, cS5, cP6, cS6 and c9) samples. 120 ng of each total RNA were used as template for the synthesis of biotin-labeled cRNA according to the standard one-cycle amplification and labeling protocol developed by Affymetrix (Santa Clara, CA). cRNA was then fragmented and hybridized on GeneChip® Bovine Genome Array, which contains over 24128 probe sets. The microarrays were washed, stained (Affymetrix fluidic station 450 DX) and scanned (Affymetrix scanner 3000 7G). Cell intensity values from the raw array data were computed using the Affymetrix GeneChip® Operating Software (GCOS). Microarray quality control and statistical analysis were performed in the software system *R* using the Bioconductor package OneChannelGUI (Gautier, Cope et al. 2004, Sanges, Cordero et al. 2007, Simmons, Spiropoulos et al. 2011). The LIMMA algorithm was used to compute a linear model fit (Smyth 2004). Data filtering and normalization was carried out using GC-Robust multi-array analysis (GCRMA) from imported Affymetrix data (.CEL) files. After the assessment and inspection of microarray quality controls (RNA degradation plot, RLE and NUSE plots) we identified one low

quality control sample (cS5) and excluded it from the analysis (see **Figure 2**). In the final analysis the low-quality RNA sample P9 was substituted by the high-quality RNA sample EP9, which was purified from blood collected from the same animal.

Gene probes with a *p* value ≤0.05 and fold-change ≥2 were considered to be differentially expressed. Differentially expressed probe sets were functionally classified using David Bioinformatics tool (Huang da, Sherman et al. 2009, Huang da, Sherman et al. 2009) on the Affymetrix bovine background. Heat maps were generated using the heatmap.2 function from the gplots library in the *R* statistical environment (Team 2013, Gregory R. Warnes 2014). Probe set data were hierarchically clustered with complete linkage using the Euclidean metric.

6.5 RT-qPCR

To confirm the microarray results, we performed RT-qPCR using SYBR® green assay (Bio-Rad Laboratories, Inc.) for a selected number of target genes. The RT-qPCR analysis was performed on 22 samples (from 7 control, 8 preclinical and 7 clinical animals). For each sample, 250 ng of total RNA were used as template for the cDNA synthesis with the SuperScript® III Reverse Transcriptase and the Oligo $(dT)_{20}$ Primer (Life Technologies). PCR primers were designed using the online tool Primer-BLAST (Ye, Coulouris et al. 2012) provided by NCBI. Whenever possible, the primer pairs were designed in order to span an exon-exon junction, thus preventing the amplification of genomic DNA. The primer sequences were as follows: for GNLY 5'-AGC CCG ATG AGA ATA CCG TT and 5'- CGA TGT CCT CAG CGA TGG TA; for CD40L 5'-ACA ACC TCT GTT CTC CAG TG and 5'-GCT GTT TCC CGT TTT CGA GG; for PDK4 5'-TGG

TGT TCC CCT GAG AGT CA and 5'-GTA ACC AAA ACC AGC CAG CG; for HBA2 5'-ACA AGG GCA ATG TCA AGG CC and 5'- TCG AAG TGG GGG AAG TAG GT; for XIST 5'-GTG GCA AGG ACC AGA ATG GA and 5'-TCC GAC CCC AGT ATT AGC CA; for GNB4 5'-AGA TCG TGC AGG TGT TCT TG and 5'-CTG TCC CAA GAC CCT GTT G; for IDO1 5'-ATT GGT GGA GTC CCT GCA GAC and 5'-CTG CAG GGT AGC ATT GCC TT; for BOLA 5'CTC GTA GTA GCT GTG GTG GC and 5'ACT GTC ACT GCT TGC AGC C; for SEL1L3 5'-TGA AGG AGT GGTTTC GCC TG and 5' TTC AAA TCC TGC CCA GTG CT; for RPL12 5'-AGG GTC TGA GGA TTA CAG TGA AA and 5'-GAT CAG GGC AGA AGC AGA AGG; for ACTB 5'-GGA CTT CGA GCA GGA GAT GG and 5'-TTC CAT GCC CAG GAA GGA AG; for GAPDH 5'-AGG TCG GAG TGA ACG GAT TC and 5'-ATG GCG ACG ATG TCC ACT TT. The RT-qPCR reactions were carried out by denaturing at 95 °C for 15s, annealing at 60 °C for 1 min and extension at 55 °C for 1 min for 45 cycles. Melt curve analysis and gel electrophoresis of amplification products were performed for each primer pair to confirm the production of a single PCR amplicon. The amplification was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). All the RT-qPCR reactions were run in triplicate and included the following controls: no template (NTC) and minus-reverse transcriptase (RT-) negative controls. The normalization accuracy was improved by geometric averaging of multiple reference genes (Vandesompele, De Preter et al. 2002) and using two inter-run calibrators to reduce inter-run variation. We decided to use a normalization factor based on three reference genes (*GAPDH*, *RPL12* and *ACTB*) since it has been shown in the literature that this is the minimal number required for a reliable normalization (Lyahyai, Serrano et al. 2010). Stability of the selected reference genes

was determined by calculating their geNorm M value (M) and the coefficient of variation (CV) on the normalized relative quantities (Hellemans, Mortier et al. 2007). M and CV values were then compared against empirically determined thresholds for acceptable stability (~ 1 and ~ 0.5 for M and CV values respectively) (Hellemans, Mortier et al. 2007) (see **Figure 10**). The statistical analysis and the fold change calculation were carried out using qBasePlus 1.1 software (Hellemans, Mortier et al. 2007).

Availability of supporting data

The data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) repository: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=mzgxuaagddwftcz&acc=GSE69048. The DEGs were analyzed for their functions, pathways and networks using DAVID Bioinformatic Tool 6.7 (NIAID/NIH, USA).

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