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Chapter

TSE Monitoring in Wildlife Epidemiology, Transmission, Diagnosis, Genetics and Control

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Abstract

Among the transmissible spongiform encephalopathies (TSEs), chronic wasting disease (CWD) in cervids is now the rising concern within Europe. CWD will be outlined in this chapter gathering its epidemiology, transmission, diagnosis, genetics, and control. Prion diseases are fatal neurodegenerative diseases characterized by the accumulation of an abnormal isoform of the prion protein (PrP^{c}), usually designated by PrP^{sc} or prion. CWD is a prion disease of natural transmission affecting cervids detected mainly in North America. The first European case was detected in Norway, in 2016, in a wild reindeer; until April 2018, a total of 23 cases were described. The definite diagnosis is *postmortem*, performed in target areas of the brain and lymph nodes. Samples are first screened using a rapid test and, if positive, confirmed by immunohistochemistry and Western immunoblotting. It is not possible to establish a culling plan based on the genotype, once affected animals appear with all genotypes. However, some polymorphisms seem to result in longer incubation periods or confer a reduced risk. The control is not easy in captive cervids and even more in the wildlife; some recommendations have been proposed in order to understand the danger and impact of CWD on animal and public health.

Keywords: prion, cervids, chronic wasting disease, *PRNP* gene, pathology

1. Introduction

The study and monitoring of wildlife diseases are key points for establishing conservational policies for wild fauna.

Wildlife are often in double risk from disease due to the high number of infectious diseases of wildlife origin that affects humans and (or) domestic animals and

also the opposite, humans and domestic animals' diseases that could affect the wildlife. Likewise, due to habitat changes, the introduction of pathogens from domestic animals as well as other actions with long-term adverse effects for the conservation of species needs to have supervising.

As in other diseases, some wildlife animal species could be at risk for transmissible spongiform encephalopathies (TSEs), acting as a potential prion reservoir, threatening the livestock and public health.

TSEs are fatal neurodegenerative diseases characterized by the accumulation of an abnormal isoform, partially resistant to the enzymatic digestion, of the cellular prion protein (PrP^{c}), usually designated by PrP^{sc} or prion. Due to its conformational arrangement, it is very resistant to common inactivation procedures used on conventional infectious agents. As PrP^{c} is host-encoded by the *PRNP* gene, polymorphisms in this gene can act upon the susceptibility or the resistance to TSEs.

The most common and well-known diseases of this group are scrapie in small ruminants, bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD) in humans, and chronic wasting disease (CWD) in cervids.

Scrapie and CWD are recognized as natural transmitted TSEs, so wildlife can be naturally affected by these two TSEs.

Since scrapie was identified in mouflon sheep (*Ovis musimon*) [1], wild species of sheep and goats, like Iberian wild goat (*Capra pyrenaica*), and Pyrenean chamois (*Rupicapra P. pyrenaica*) can also be infected by scrapie as well as by BSE. Furthermore, according to some studies, European wild ruminants have a *PRNP* genetic background that is compatible with TSE susceptibility [2, 3].

Albeit limited, some countries, namely, Estonia, Finland, Spain, and Cyprus, reported negative results on samples tested for TSE in other wild animal species—mink (*Mustela lutreola*), fox (genus *Vulpes*), raccoon dog (*Nyctereutes procyonoides*), bison (*Bison bison*), and Cyprus mouflon (*Ovis gmelini ophion*) [4].

Considering that CWD is a TSE affecting several cervid species, a very contagious disease with an efficient horizontal transmission, appearing to be enzootic and to be expanding both geographically and in prevalence [5], all the sections in this chapter are focused in order to better characterize the epidemiology, transmission, pathogenesis, diagnosis, genetics, and control of this disease.

2. Molecular basis of transmissible spongiform encephalopathies (TSEs)

2.1 Biology of the etiologic agent

Initially, TSEs were thought to be caused by "slow viruses" (reviewed in [6]). However, as the agent causing scrapie was not deactivated by both chemical and physical procedures, which modify or destroy nucleic acids, it was suggested that this infectious agent was not harboring nucleic acids. Thus, in 1967 Griffith proposed a model in which the scrapie agent could be a protein, but it was Prusiner in 1982, after confirming that procedures used to modify or destroy proteins deactivated the scrapie agent, who published that the etiologic agents of TSEs were proteinaceous infectious particles, called prions [6, 7].

According to this protein-only hypothesis, TSEs are caused by the conversion of the physiological cellular prion protein (PrP^{c}) into a pathogenic misfolded isoform (designated PrP^{sc}) that is able to propagate by recruiting and transforming more PrP^{c}, by an increase in β-sheet structure and a propensity to aggregate into oligomers (reviewed [6, 8]). Moreover, this conformational change confers to PrP^{sc} a greater insolubility in nonionic detergents, high resistance to heat and chemical sterilization, and partial resistance to protease digestion—the truncated

PrP 27–30 kDa (reviewed in [8–10]). Until now, this latter feature has been used for diagnostic purposes, being PrP^{sc} a diagnostic marker for these diseases (see Section 6 Diagnosis of CWD, in this chapter).

PrP^{c} is host-encoded by the *PRNP* gene (see Section 2.3. Prion protein gene, in this chapter) and is normally present in the cell surface in different organs and tissues of mammals and other vertebrates but with high expression levels in the central and peripheral nervous systems. It is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein of 33–35 kDa with a C-terminal globular domain, an N-terminal flexible tail, a single disulfide bond, and an α-helix content. The N-terminal tail includes two charged clusters, the octarepeat region and a hydrophobic domain. At the C-terminus, in the globular domain upstream of the sialylated GPI anchor, there are two N-glycosylation sites [11]. Thus, PrP^{c} has two putative sites of glycosylation, and three glycoforms of PrP can be described: di-, mono-, and non-glycosilated PrP. The relative proportions of these glycoforms and the size of the unglycosylated PrP^{sc} fragment are dependent on the strain of prion (reviewed in [12]).

Regarding the physiological function of the PrP^{c}, it has not been clarified yet; nevertheless, there are several proposed roles; ones are supported by compatible results of different experiments (neuronal excitability; glutamate receptor function, neurite outgrowth; neuroprotection; copper, zinc, iron, and lactate metabolism; and peripheral myelin maintenance), while others are yielding inconsistent results (synaptic transmission and plasticity, memory formation, stabilization of sleep and circadian rhythm, calcium homeostasis, and toxicity elicited by oligomeric species) [11].

While the structure of PrP^{c} is well studied and identified, the structure of PrP^{sc}, the mechanism by which PrP^{c} converts into PrP^{sc} in a posttranslational process, and the molecular mechanisms behind prion strains are still not known, despite all the experimental attempts [8–10].

PrP^{sc}, as a physical template, compels PrP^{c}, with the same primary but different secondary, tertiary, and quaternary structures, to adopt the PrP^{sc} conformation, probably on a complete unfolding of PrP^{c}, followed by refolding. To thoroughly comprehend this molecular process, it is essential to know the architecture of PrP^{sc}. As extensively reviewed by Requena and Wille [9, 10], distinct molecular models have been proposed for PrP^{sc} (27–30 kDa): (1) a four-stranded β-sheet plus two C-terminal α-helices [13, 14]; (2) an antiparallel, intertwined β-helix structure [15]; (3) a parallel β-helix [16]; (4) a fold modeled on the human TATA-box-binding protein containing a five-stranded β-sheet, with the C-terminal α-helices [17]; (5) a "spiral model" with three α-helices of the original structure and four β-strands [18]; (6) a unit consisting of a left-handed four-rung parallel β-helical fold for the N-terminal part and α-helical state for the C-terminal portion, assembled as a trimer [19]; (7) a PrP primary structure based onto the β-helical template and the C-terminal α-helices [20]; (8) a β-helical architecture with a threefold domain in the trimeric unit and α-helical structure for the C-terminal portion [21]; (9) a two-rung β-helix with the C-terminal α-helices [22]; (10) a parallel in-register intermolecular β-sheet (PIRIBS) architecture [23, 24]; and (11) a parallel β-helix extended to the C-terminal portion of the PrP molecule [25].

Nevertheless, none of these models have explained all the experimental results. Recently, methodologies like cryo-electron microscopy and X-ray fiber diffraction have pointed out a four-rung β-solenoid as the basic structural element structure of PrP^{sc} [26], though the available structural information is still limited to determine the PrP^{sc} architecture in atomic details (reviewed in [10]).

To explain strain diversity of prions, the prion-only hypothesis considers that, in the absence of a nucleic acid, the variety of PrP^{sc} conformers and its mixture

may result in different prion strains. Each prion strain presents a specific disease phenotype (incubation times, clinical signs, histopathological lesions, PrP^{sc} deposition patterns in the brain, and PrP^{sc} biochemical characteristics) which is exactly preserved upon serial passage within the same host genotype. Nevertheless, the molecular mechanisms by which a range of PrP^{sc} conformers would be produced and selected have not been yet established (reviewed in [8]).

In relation to chronic wasting disease (CWD) prion strains, two prevalent CWD prion strains (CWD1 and CWD2) were identified in North America based on transmission in transgenic cervid (TgCerPrP) mice of several CWD isolates from different cervid species and geographic areas. CWD1 and CWD2 showed distinct incubation time, clinical signs, and neuropathologic profile but with indistinguishable electrophoretic migration patterns of di-, mono-, and non-glycosyl forms of PrP^{sc} [27], reviewed in [28]. These bioassay results showed that elk may be infected with either CWD1 or CWD2 strains, while in deer CWD1/CWD2 strain mixture can be present ([27], reviewed in [28]) (**Figure 1**).

Regarding the CWD prion strain(s) responsible for the outbreaks of the disease in new geographic areas—South Korea, Norway, and Finland—the available data is still limited. The strain characterization of the Korean CWD cases in elk in 2001 and 2004 suggested a single strain responsible for the outbreaks imported from Canada [29], without identifying if it was CWD1, CWD2, or both. The biochemical analysis and immunohistochemistry (IHC) distribution of PrP^{sc} from Norway reindeer revealed a pattern indistinguishable from North America isolates [30]. Remarkably, in CWD-affected moose in Norway, a different phenotype was observed in both PrP^{sc} distribution and biochemical features, suggesting a presence of a different type of CWD prion strain in moose from this country (designated Nor-16CWD) [31] (**Figure 1**).

Novel cervid prion strains have been experimentally generated by adaptation of prions from other species, for instance, scrapie (SSBP1, an American classical scrapie isolate) [28] and BSE [32], demonstrating that cervid species can also be susceptible to other prions. Moreover, CWD prions easily adapt to new species (see Section 4), including sheep, cattle, and squirrel monkeys. Thus, there is a putative risk of development of novel CWD-related prion disease in livestock by grazing in CWD-contaminated pasture (**Figure 1**). Lastly, the ability of the CWD prions to cross the human species barrier has to be further evaluated, but amino acid residues (residues 165–175) in the β2–α2 loop sequence of human PrP^{c} can constitute a species barrier to its conversion by CWD prions [33], reviewed in [28].

2.2 Prions and the deviations in the central dogma of molecular biology

According to the central dogma of molecular biology, first published in 1958 and revisited later [34], heritable information is stored in DNA, expressed as RNA, and translated into protein. Nevertheless, this paradigm has been updated by many aspects of the regulation of gene expression, namely, by the identification and characterization of alternative splicing, alternative promoters, alternative polyadenylation events, and the increasing number of noncoding RNAs (ncRNAs) with critical importance in the regulation of messenger RNA (mRNA) [35] and the discovery of "prions": prion proteins can adopt multiple conformations, at least one of which has the capacity to self-template [36, 37] (**Figure 2**).

2.3 Prion protein gene (PRNP)

The astounding improvement in genetic tools and bioinformatic programs/algorithms and the incredible amount of data deposited freely in the main scientific

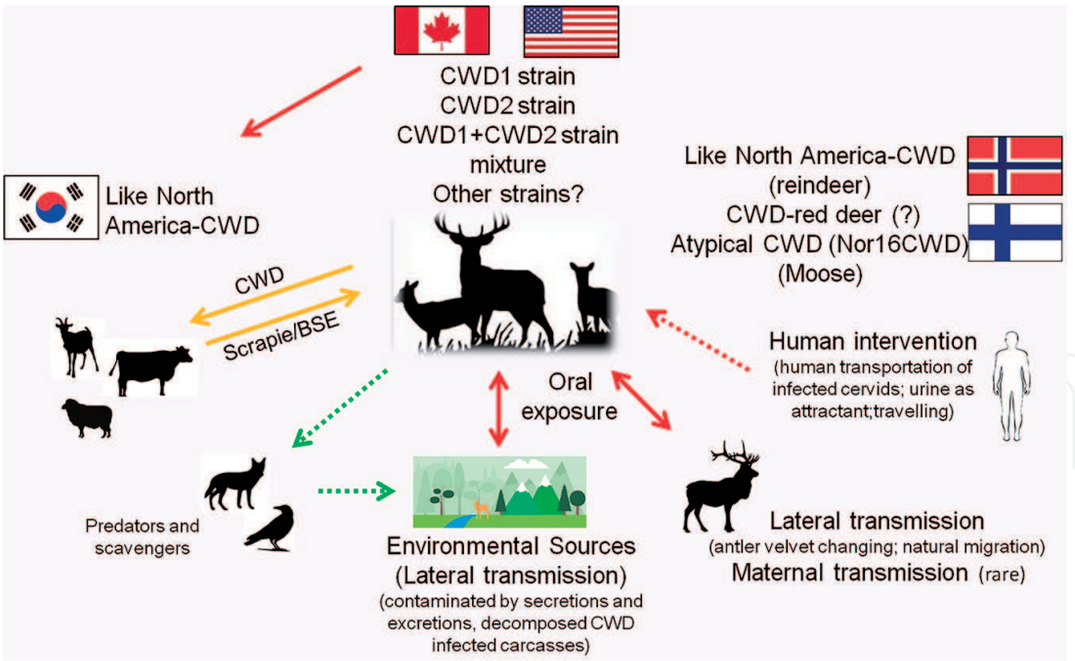


Figure 1.
Summary of CWD prions and transmission. Like North America-CWD-isolates with PrPres biochemical and PrPres distribution similar to that described in North America CWD cases. ➔ natural transmission; ➔ putative transmission; ➔ experimental transmission; ➔ potential spread of CWD prions or decrease of PrPres environmental reservoir at the carcass site due to scavenging process, (silhouettes and pictures from freepik.com and img.linkfrog.com).

databases allow us to use comparative genomics in an effective manner. **Figure 3** contains the representation of the *PRNP* gene in *Homo sapiens*, used as a reference and establishing a comparison with other animal species according to the Ensembl database. This gene is constituted by two exons, although a single exon is responsible for the open reading frame (ORF) and the remaining sequence contains untranslated and regulatory regions. Some animal species have the same distribution, while others contain a single exon (e.g., *Canis lupus familiaris*, *Felis catus*, and *Ovis aries*), three exons (*Cervus elaphus* and *Odocoileus virginianus* from *Cervidae* family), or four exons (like *Bos taurus),* according to Ensembl database. Nevertheless, a high level of conservation at the coding sequence and corresponding protein sequence is maintained (as confirmed in **Figure 4**).

The fundamental event in the pathogenesis of TSE is not a primary structure modification but the conversion of the normal cellular prion protein (PrP^{c}) into the misfolded pathogenic isoform (PrP^{sc}) [38]. In fact, PrP^{c} and PrP^{sc} share the same amino acid sequence, but differences in the secondary structure originate the tertiary and quaternary structures that dictate the PrP^{sc} with new physicochemical properties, namely, insolubility in nondenaturing detergents and partial resistance to proteolysis. It is therefore important to identify the specific codons and amino acids with relevant importance in the protein structure dynamics.

Rongyan and collaborators [39] compared the *PRNP* gene sequences among 83 species and reinforced a remarkable degree of conservation among the mammalian sequences. In order to confirm this statement, a DNA, RNA, and protein comparison of *PRNP* among humans, bovine, ovine, caprine, and deer was performed. According to this comparison, human *PRNP* is less similar compared to the others, which is understandable since they are phylogenetically more distant species. However, the protein comparison showed a high similarity between all these species (above 90%), indicating a high conservation of PrP.

In order to add supplementary information especially regarding wild species, the PrP protein sequences from 13 different species were compared and are presented in

Figure 4. The high level of conservation suggests the preservation of some important functional characteristics of PrP through evolution.

According to NCBI-SNP database (accessed in December 2018), 3683 variations in the human *PRNP* gene were presented. Some of these variations are located in the coding sequence and originate modifications in the protein. Once PrP is highly conserved, the variations already described in humans can be used to predict variations in other species. In order to simplify this process, all the missense and nonsense mutations associated with prion diseases described in humans are presented in **Figure 2**.

Variations in PrP sequences exist between species and also between individuals of the same species. It was already demonstrated that this fact can influence the susceptibility to prion infection and ultimately to disease. Chronic wasting disease appear to have a higher potential of transmissibility than other forms of prion disease, and it has been confirmed that some genetic variations are associated with lower rates of infection and slower progression of clinical manifestations [40]. A total of 17 polymorphic sites have been reported in the PrP in *Cervidae* species.

Historically, O'Rourke and collaborators [41] were the first authors that reported that *PRNP* gene from *Cervus elaphus* was polymorphic at codon 132 encoding methionine (M) or leucine (L). This codon is equivalent to codon 129 in

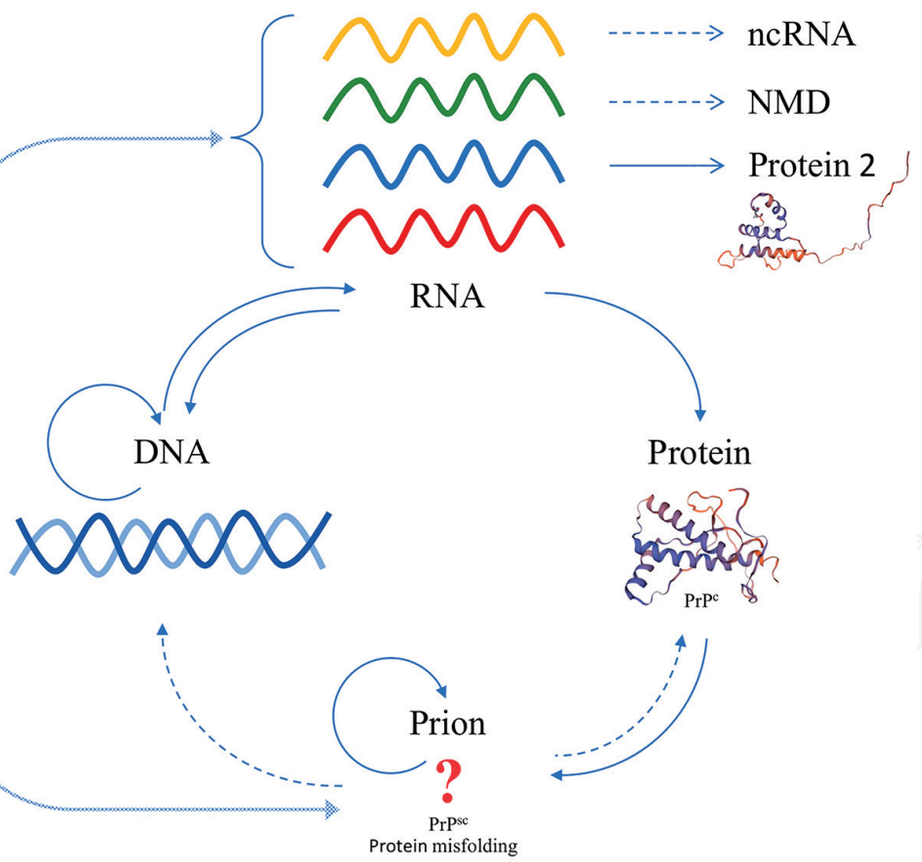


Figure 2.
Updated vision of the classical central dogma of the molecular biology. A DNA sequence can originate multiple RNAs (by using different promoters, by alternative polyadenylation and alternative splicing events). Some of these RNAs can be degraded by nonsense mediated decay (NMD), normally if they contain a premature termination codon (PTC). Other RNAs are not translated but still have a possible regulatory function (noncoding RNAs, ncRNA) and supplementary RNAs are translated originating a different protein, with similar or unrelated function comparing to the canonical protein. The prion postulation assumes that an abnormal protein conformation (PrPsc) propagates itself via an autocatalytic mode by recruiting the normal cellular isoform (PrPc) as a substrate and acting as the disease transmissible agent. This misfolding can be reversible and prion proteins have the ability to interact with nucleic acids (DNA and RNA) and other polyanions (as lipids).

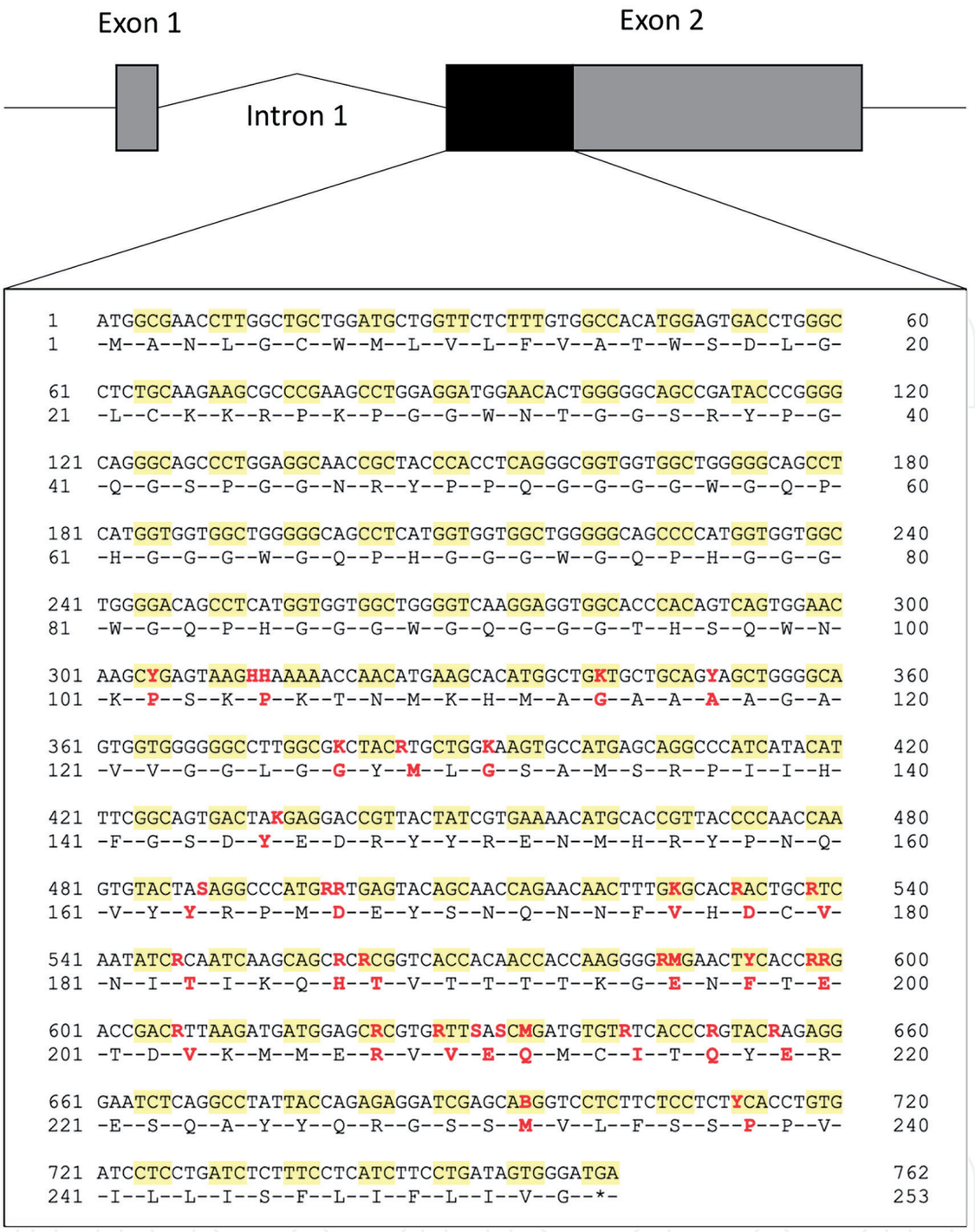


Figure 3.
Organization of the Homo sapiens PRNP gene. From the two exons, only exon 2 is codifying for the PrP protein (black square). The coding sequence is presented below with the top lines showing the nucleotide sequence and the bottom lines showing the amino acid sequence. Missense and nonsense mutations in PrP associated with human prion diseases are marked in red. Information retrieved from Ensembl and NCBI databases were used to create this scheme.

the human protein encoding either M or valine (V) [42]. According to Collinge and colleagues [43] and Palmer and collaborators [44], this variation has an important impact on human prion disease presentation. In the same direction, regarding CWD, O'Rourke and collaborators [45, 46] indicated that the L132 allele protected against this disease in *Cervidae*. Although all *PRNP* genotypes can be affected with CWD, there are some polymorphisms that appear to result in longer incubation periods in some species. The polymorphisms Q95H and G96S are related to the reduction of the risk of infection [47]. S96S or G96S and G95H

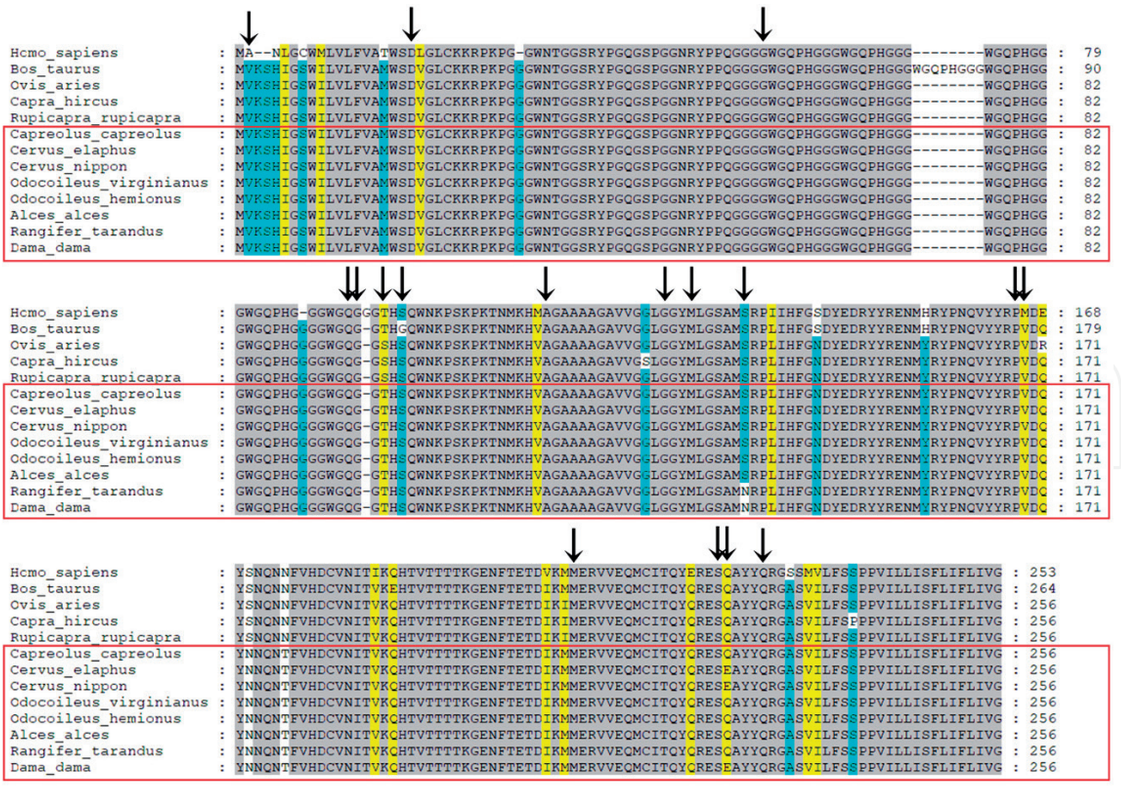


Figure 4.
Alignment of PrP protein sequences among 13 different species. T-coffee was the multiple alignment tool used from EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/tcoffee/) and Genedoc version 2.7.000 was the multiple sequence alignment editor. The accession numbers of each species considering a short name (by order of presentation in the alignment) are: Hs_ENSP00000368752.4; Bt_ENSBTAP00000043233.2; Oa_ENSOARP00000004991.1; Ch_ NP_001301176; Rr_UniProt_spQ5XVM4; Cc_AY639096; Ce_UniProt_spP67987; Cn_UniProt_trQ6DN38; Ov_UniProt_trQ7JIQ1; Oh_UniProt_trQ6VS46; Aa_UniProt_trQ693S2; Rtg_UniProt_trQ3Y673; Dd_UniProt_trQ7YSF3. Besides human (the reference), the four following species belong to Bovidae family and the last eight species belong to Cervidae family (highlighted with a rectangle frame). The arrows locate the seventeen polymorphic amino acids described in Cervidae.

seem to produce a reduced susceptibility, with longer survivor period [48], being underrepresented in CWD-affected populations [45]. Regarding S225F polymorphism there is a differential susceptibility to experimental oral exposure and incubation periods [45, 49].

Table 1 presents the information concerning the amino acidic variations reported until 2018, with the references and functional implications in the susceptibility to CWD.

3. Epidemiology of chronic wasting disease (CWD)

3.1 Natural history and geographical distribution of CWD

Descriptions of CWD date back to 1967, in which a body-wasting syndrome associated with behavioral changes was described in Colorado in a closed herd of captive mule deer. However, only a few years later, in 1980, the pathologists Elizabeth Williams and Stewart Young observed histological lesions that made it possible to identify the disease as transmissible spongiform encephalopathy, in deer from wildlife research facilities in Colorado and Wyoming. These lesions included neuropil spongiform transformation, intracytoplasmic vacuoles in neuronal perikaryons, and significant astrocytic hyperplasia and hypertrophy [60].

In 1991, amyloid plaques reactive to antibodies produced against scrapie were identified in the cerebral gray and white matter and in molecular, pyramidal, and granular layers of the cerebellum of infected captive mule deer (*Odocoileus hemionus hemionus*) [61]. In the same year, a similar study identified scrapie amyloid-immunoreactive plaques in Rocky Mountain elk (*Cervus elaphus nelsoni*) and hybrids of mule deer and white-tailed deer (WTD) (*Odocoileus virginianus*) [62].

After its first identification, CWD was detected in a mule deer from Wyoming, and, until 1980, they had already identified 53 cases in mule deer (*Odocoileus hemionus hemionus*) and 1 case in a black-tailed deer (*Odocoileus hemionus columbianus*) from wildlife facilities in Colorado and Wyoming [60].

The disease continued to be detected in new cervid species as Rocky Mountain elk (*Cervus elaphus nelsoni*) in Colorado and Wyoming wildlife facilities [63]. Subsequently, the disease was identified in free-ranging animals: mule deer and elk in the same states [64] and white-tailed deer in Nebraska and South Dakota in 2001 [65, 66].

The limited area of southern Wyoming, Northern Colorado, and Western Nebraska has been considered the endemic area. This area spreads rapidly, and new epidemic outbreaks were identified in other states including areas not contiguous geographically to endemic areas [66].

The establishment of disease surveillance and detection programs in both wild and captive cervids contributes to the knowledge of the geographical distribution of the disease [67]. However, the lack of well-defined limits of the affected areas, the low incidence, and the insufficient sampling could lead to underestimated prevalence rates [66].

a.a Position	Consensus a.a	Variation	Reference
2	V	M	[50]
20	D	G	[51, 52]
59	G	S	[3, 52]
95	Q	H	[40, 52, 53]
96	G	S	[52, 53]
98	T	A	[3]
100	S	N	[54]
116	A	G	[52]
129	G	S	[55]
132	M	L	[45, 56, 57]
138	S	N	[55]
168	P	S	[3]
169	V	M	[55]
209	M	I	[50]
225	S	F	[58]
226	Q	E/K	[3, 52, 58, 59]
230	Q	L	[52]

The position considers the Cervidae prion protein. Legend: amino acid codes—A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine.

Table 1.
Amino acid variations and susceptibility to CWD.

At present, the disease has been identified in captive and free-ranging cervids in 24 states of the United States of America (USA) [68]. Considering only free-ranging cervids, CWD was identified in 270 counties in 24 states, according to March 2019 data, namely, Arkansas, Colorado, Illinois, Iowa, Kansas, Maryland, Michigan, Minnesota, Mississippi, Missouri, Montana, Nebraska, New Mexico, New York, North Dakota, Pennsylvania, South Dakota, Tennessee, Texas, Utah, Virginia, West Virginia, Wisconsin, and Wyoming [69, 70].

In Canada, the first case was detected in 1996 in Saskatchewan, in a captive elk [71]. The second case was diagnosed in 1998 in a farmed elk and the third case, in 2001, in a captive 24-month-old Rocky Mountain elk. In this herd, no other cases had been detected; however, an animal which died 2 years earlier without postmorten examination had been introduced previously from an infected herd [72]. In 2002, the first case was identified in farmed white-tailed deer [71]. Nevertheless, considering a retrospective study done in Toronto Zoo cervids that died between 1973 and 2003, CWD exists in Canada since the 1980s. CWD infection was detected in seven mule deer and one black-tailed deer [73].

According to the Canadian Food Inspection Agency, data about cervid herds infected with CWD are reported since 1996 [69]. In 2001, CWD was identified in 21 herds, the highest number between 1996 and 2018. Until last year, the disease was circumscribed to two provinces: Alberta and Saskatchewan [72]. A screening for CWD in caribou (*Rangifer tarandus)* in northern Quebec revealed no positive animals [74]. However, data from 2018 reported the disease in Quebec in a red deer herd [69, 70, 75].

Recent data, from 2018 official reports, describe CWD in captive elks (in the province of Alberta and Saskatchewan), white-tailed deer (in Saskatchewan), and red deer (in Quebec and Saskatchewan) [69, 75]. CWD was also identified in free-ranging cervid populations in Canadian Alberta and Saskatchewan provinces [69, 71, 75].

The first case outside North America was detected in 2002, in South Korea, associated with a 7-year-old elk imported from Canada [76].

CWD was first recognized in Europe in March 2016 in a free-ranging female reindeer (*Rangifer tarandus*) in the Nordfjella mountain range of Southern Norway [30]. Since then, Norway further intensified its monitoring by sampling with the aid of hunters and governmental culling. A total of 41,125 cervids had been tested leading to the detection of CWD in 19 wild reindeers (*Rangifer tarandus*), 3 wild moose (*Alces alces alces*), and 1 red deer (*Cervus elaphus*) [77, 78]. In Finland, one CWD-affected moose was detected in 2500 cervids tested [78].

3.2 Host range of CWD

CWD has been identified in several members of the family Cervidae: white-tailed deer (*Odocoileus virginianus*) [79], mule deer (*Odocoileus hemionus*) [60], Rocky Mountain elk (*Cervus elaphus nelsoni*) [80], moose (*Alces alces shirasi*) [81], reindeer (*Rangifer tarandus*) [30], moose or Eurasian/European elk (*Alces alces alces*), and red deer (*Cervus elaphus*) [77].

There are other animals that can experimentally be infected, both cervid and noncervid species. The experimental host range includes squirrel monkey [82, 83], several species of voles [84, 85], white-footed mice [29], Syrian golden hamsters [86], ferrets [87], raccoons [88], cats [89], pigs [90], and sheep [91]. Other cervids are susceptible to experimental CWD infection: fallow deer (*Dama dama*) [92] and muntjac deer (*Muntiacus reevesi*) [93]. However, CWD was not yet detected naturally in these species.

3.3 Prevalence and dissemination of CWD

The prevalence of the CWD is quite different in wild or captive cervids. In free-ranging cervids, the disease spreads slowly, often with a stable prevalence and low diffusion rate [94]. However, CWD prevalence in wild cervids and geographical areas with animals infected are increasing every year. Prevalence could reach 31% in hunting deer from Wyoming (USA) [95] and exceed 10% in elk from Rocky Mountain National Park [96]. In Europe, the prevalence in cervids in Norway is 0.05% (23 in 41,125 cervids) and 0.04% in Finland (1 in 2500 cervids), which is very low, but it is expected to increase, as it is a very contagious form of TSE, endemic, and difficult to eradicate [78].

In captive cervids the prevalence is higher and may reach 79% or more of affected animals on the herd. In young animals, this number could be even higher [66, 97]. The annual incidence may be higher than 50% in white-tailed deer and mule deer [95].

The diffusion of CWD, despite the many studies existent, is not well understood. The disease spreads slowly, in free-ranging cervids, probably following river corridors or associated with the movements of males, as proposed for white-tailed deer (*Odocoileus virginianus*) [98]. However, there are no definite conclusions about the origin of the disease in wild cervids. CWD foci in these animals could be extensions of the initial endemic focus, secondary foci, or they could correspond to the spontaneous development of different foci of the disease [94]. In captive animals, CWD spread probably followed slightly different pathways. The occurrence of the disease and its irregular propagation with their appearance in two geographically distant endemic regions must be associated with the commercialization of infected animals between the different herds. Occasionally the disease was detected simultaneously in herds and wild animals in the same region, previously or later, in wild animals suggesting a spillover of the infection from captive to free-ranging animals [94].

4. Transmission

Concerns about transmission between species of TSEs, and particularly transmission of animal TSEs to humans, have existed since the infectious nature of these diseases was demonstrated, but there was no evidence of zoonotic transmission until the link between BSE and a new human TSE (variant CJD or vCJD) was established in the late 1990s. Evidence for the transmission of BSE from cattle to humans came from a diversity of sources, combining careful surveillance and epidemiological studies, often involving the development of new techniques and animal models. The demonstration of the zoonotic transmission of BSE also renewed concerns about the public health risks of other TSEs leading to enhanced surveillance and efforts to control scrapie and CWD [99].

Efficient control measures were applied for BSE and scrapie in cattle, sheep, and goats. However, CWD prions present a challenging risk, affecting both captive and wild cervids [100].

The experimental transmission of BSE in cervids and the bioassay in transgenic mice strongly support the claim that CWD agents are not related to classical BSE. However, the possible relationships between CWD and other TSE agents circulating in animal populations (transmissible mink encephalopathy (TME), scrapie) are still uncertain [99].

TSEs are horizontally transmitted by oral ingestion (kuru, BSE, vCJD, TME, feline spongiform encephalopathy (FSE), CWD), by environmental contact with

infectious prions (scrapie, CWD), by blood transfusion (vCJD, CWD, scrapie), or by iatrogenic exposure (vCJD, sCJD) [93]. Horizontal transmission of CWD also includes skin, oral, and nasal routes [101].

CWD prions can be discarded in urine, feces, saliva, blood, and velvet antler (clinical and preclinical stage of the infection) and can be found in skeletal muscle, fat, viscera, and the central nervous system [102, 103]. For this, there is a high risk of exposure to CWD prions, not only for cervids and other wild animals but also for hunters who may contact directly with infected animals and people consuming venison [104] (**Figure 1**).

Although still unclear, natural CWD transmission between animals has occurred. Environmental contamination and infectivity can persist in pastures for 2 years after the death of animals affected with CWD, as shown by studies performed in grazing presumably highly contaminated [49].

Both horizontal and environmental transmissions efficiently disseminate CWD. Decomposing carcasses and the placenta also contribute for this transmission as the prion agent is environmentally very stable persisting for many years where TSE-infected animals stood [94]. Other putative sources for CWD spread are predators/raptors and scavengers as prions remained infectious after passage through the digestive system of American crows [105, 106] and coyotes [107] which were orally infected with scrapie and CWD, respectively (**Figure 1**).

High infectivity and rapid transmission between cervids result in a high prevalence and can exceed 90% in captive animals [108] leading to the increased CWD exposure of humans and other animals, mainly by the consumption of prion-infected animal products or grazing on prion-contaminated pastures, respectively. So, studies concerning the zoonotic risk of CWD and transmission to other animal species are very important with a high interest for public and animal health. The effect of the species barrier (lower transmission efficiency compared to that within the donor species) is very present while studying the interspecies transmission of CWD prions. Investigations identified a key influence of amino acid sequence differences in the prion protein between species as well as a strong effect of the prion strain type [109, 110]. For prions, strain type is characterized by a set of properties that define infection and pathogenesis not explained by differences in amino acid sequence [111]. Both factors influence species interaction, dose, route of inoculation, and age of the host [109, 110]. Furthermore, prion agents can adapt to a new host species, only becoming virulent after repeated passage [112].

For the study of CWD interspecies transmission, experimental inoculations were conducted mainly by intracerebral (IC) inoculation, resulting in a successful transmission to ferrets (*Mustela putorius furo*) [113] but unsuccessful to Syrian golden hamsters (*Mesocricetus auratus*) even after multiple attempts [64]. The ferret-adapted CWD was, however, and after several passages, transmitted to hamsters, demonstrating that interspecies transmission can be altered by the adaptation to a new host [113]. The IC inoculation of mule deer CWD to goats presented an incubation period of about 6 years, a longer period when compared to what happens with scrapie [64, 114].

Transmission experiments of CWD prions from white-tailed deer, mule deer, and elk to transgenic mice expressing deer, elk, sheep, cattle, or human PrP^c suggest that the transmission barrier for CWD prions among different species of the cervid family is low, whereas the transmission barrier for CWD prions to sheep, cattle, and humans is high ([115–119], cited by [114]). Although still unclear, species barrier is thought to be controlled, at least in part, by the differences in the primary PrP sequences of donor and recipient animals [120–122] that affect the structure and folding of each prion protein ([123] cited by [114]). Studies of amino acid differences have demonstrated that there is a high level of conservation

of the prion protein between species (90%) being the energy barrier increased for conversion, when compared to others, with some major differences in the amino acid sequence [33].

Although the hypothesis that CWD is transmitted to humans is not supported (reviewed by [100]), results from in vitro experiments suggest that the species barrier is not absolute.

No correlation between CWD prion exposure and human prion disease was demonstrated by epidemiological studies, and humanized transgenic mice did not demonstrate transmission. CWD transmission to macaques has been unsuccessful to date, although squirrel monkeys seem to be susceptible to CWD prions [100].

The negative transmission results reported in seven studies support the conclusion that the transmission barrier associated with the interaction of human PrP and CWD prions is fairly strong and is stronger than the species barrier between human PrP and the BSE prion ([124] cited by [100]).

It is hypothesized that, as a result of an extensive human exposure, a CWD prion strain able to be transmitted to humans can emerge, even with no current evidence that it can cause disease [125]. So there is a risk of exposure for those who directly contact with cervids or contaminated environment or who consume venison [104]. However, variation in CWD prion strains, human genetic heterogeneity, other factors contributing to individual variation, and some evidence that intraspecies and interspecies passage of CWD prions may increase the risk that CWD prions adapt to have a lower species barrier for transmission to humans, complicating this area of research [112].

Wild-type inbred strains of mice (e.g., RIII, C57BL, and VM) were important for the identification and characterization of different types of scrapie prions [126–131], but CWD transmission to wild-type mice presented only few results [29]. In order to optimize time and resources as well as the results obtained, when performing studies of scrapie prions, transgenic (Tg) mice for ovine or cervid prion protein gene were developed [115, 117, 118, 132–134].

According to several studies, cervidized mice have been established as appropriate mouse bioassay models for the study of CWD. CWD prions were transmitted efficiently to TgElk mice with no evidence of transmission to ovinized mice—Tg338 (transgenic mouse line expressing the ovine VRQ prion protein) [119].

There are currently no known cases of interspecies transmission of CWD prions to sheep or goats through natural exposure, and transmission resulting from direct inoculation has been relatively inefficient. Nevertheless, the potential for natural exposure of sheep and goats to CWD prions is presumably rising in parallel with the increasing incidence and geographic range of CWD [119]. In this study transmission of classical scrapie and CWD prions from their native hosts to transgenic mouse lines expressing the ovine (Tg338) or cervid PrP was assessed. Inoculation of transgenic mice with scrapie prions from small ruminants or CWD prions from WTD (white-tailed deer) resulted in distinct transmission patterns. Scrapie prions transmitted efficiently to Tg338 but not to TgElk, whereas CWD prions from a natural host efficiently transmitted to TgElk but not Tg338. Unlike these distinct transmission patterns, efficient transmission of CWD prions following primary passage in sheep was observed in both Tg338 and TgElk mice. Experimentally transmitted CWD in small ruminants has been difficult to distinguish from natural cases of classical scrapie [119].

Peripheral lymphoid tissues may be a target for accumulation in cross-species transmission as observed with Tg338 mice intracerebrally inoculated with elk CWD prions with high rates in the spleen but low doses in the brain [110]. Transmission of CWD from mule deer or elk to sheep has been achieved following intracerebral inoculation [91, 135]. Yet, inoculation with brain homogenate from WTD with

CWD did not result in brain accumulation of PrP^{sc} during primary passage in ovinized mice (Tg338). One factor that may have contributed to the lack of transmission could be varying levels of PrP^{sc} and PrP^{CWD} between brain homogenates from animals with scrapie and CWD, respectively [119].

Non-Tg mice have been described as resistant to CWD infection [115], while the VM/Dk inbred strain of mice infected with wapiti CWD prions reported limited infection [29], suggesting the existence of some variable species barriers to the transmission of CWD [77].

Nalls et al. [93] have demonstrated mother-to-offspring transmission and disease progression to a viable cohort of offspring that were born to CWD-infected dams.

Several researchers noted that dissimilarity between PrP sequences alone does not accurately predict the transmissibility between species and other factors are under investigation. One that was used in recent studies was the conformational selection model, which proposes that compatibility between the three-dimensional molecular shapes of PrP^{sc} and PrP^{c} is more important than the sequences of the protein backbones [136].

5. Pathogenesis of CWD: the interaction of prions and immune system

The unique features of prions as infectious agents are also blatant when considering its interaction with the host's immune system. As stated previously, PrP^{c} is involved in important neurologic functions; however, the depletion of neuronal PrP^{c} in experimental models, such as mice, cows, and goats, did not result in altered phenotypes [11]. This suggests the existence of redundant mechanisms that compensate the absence of PrP^{c}, albeit its ubiquitous cellular expression. Pertaining the immune system, PrP^{c} is expressed in lymphocytes, granulocytes, and stromal cells. This abundant expression may explain why PrP^{sc} does not elicit specific immune responses despite its accumulation in high levels within the host's secondary lymphoid tissues (SLT) in the initial phases of the infection. In particular, gut-associated lymphoid tissues (GALT), such as the Peyer's patches in the small intestine, seem to be pivotal for the infection and disease process. As in most natural occurring prion diseases, CWD of cervids is acquired peripherally, mainly after oral exposure, after which prions replicate in GALT. The importance of GALT in the pathogenesis of prion diseases is well documented by several studies that revealed impaired neuroinvasion in the absence of Peyer's patches [137–140].

5.1 *M* cells

Within GALT, studies suggest that prions depend on host *M* cells to enter the organism, as they seem to be involved in the initial transfer of the prion agent across the gut epithelium. These are specialized epithelial cells widely spread among the follicle-associated epithelium, which are involved in the transcytosis of particulate antigens and microorganisms from the gut lumen into the GALT [141]. *M* cells are essential to the gut immune system being fundamental to antigen recognition and presentation of pathogens or commensal microflora. As such, factors that increase *M*-cell density in the gut epithelium (coinfections or miscellaneous inflammatory conditions) may also increase susceptibility to orally acquired prion infections. A study by Wyckoff et al. [142] mentions the binding of prions to certain types of soil particles that may result in an increased uptake of prions from the gut lumen, but it is not yet clear if this uptake is mediated by *M* cells. Host infection by

M-cell-independent mechanisms cannot be disregarded, namely, those involving directly enterocytes from the follicle-associated epithelium [143] or other *M*-cell-independent pathways [144].

5.2 Follicular dendritic cells (FDC) and B and T lymphocytes

Several studies show that in prion-infected hosts, high accumulations of disease-specific PrP are detectable within the B-cell follicles of the SLT [145–149]. After internalization, evidence suggests that prions accumulate and replicate in the follicular dendritic cells (FDC) that lie in the B-cell follicles and germinal centers of SLT [150–156]. FDC possess multiple slender and long dendritic processes, differentiate from ubiquitous perivascular precursor cells (pericytes), and are a distinct lineage from bone marrow-derived conventional dendritic cells (DC) [157, 158]. FDC extend throughout the B-cell follicle being able to trap and retain large amounts of native antigens upon their surfaces in the form of immune complexes composed by antigen–antibody and/or opsonizing complement components. Apparently, prions can be bound by opsonizing complement components [152–155], internalized, and replicated within FDC, as it was demonstrated that they accumulate upon PrP^c-expressing FDC in the SLT of experimentally infected mice, sheep with natural scrapie, cervids with CWD, and patients with vCJD [145–149]. Subsequently, immunohistochemistry studies identified FDC as the first sites of prion conversion and replication in experimentally infected mice, in which increased prion labeling was observed on the plasma membranes of FDC in the Peyer's patch germinal centers [143]. Furthermore, evidence suggests that FDC maturation and regression cycle are impaired by disease-associated prion protein, thus affecting immune function [159]. Despite this, some prion strains can establish infection within SLT regardless of FDC, as it was demonstrated by experimental studies in mice, where infection occurred associated to the high endothelial venules in the lymph nodes [160].

The role of B and T cells in prion disease pathogenesis remains uncertain. Experimental studies showed that mice lacking mature B and T cells were not susceptible to peripheral infection with prions [145, 161–163], despite prion disease pathogenesis being unaffected in T-deficient mice [162, 164, 165]; however, it seems that prions are not capable of replicating within B and T cells [166, 167]. These cells appear to be intermediary actors in prion diseases, by providing physical and humoral support to other cells, thus contributing to the homeostatic state of the immune system. However, B cells, in particular, seem to play a more active role during prion infection. The ability of secreting tumor necrosis factor-α (TNF-α) and lymphotoxins (LT) turns B cells pivotal in keeping FDC in their differentiated state, since in the absence of stimulation from these cytokines, FDC rapidly dedifferentiate [168]. In Peyer's patches, B cells migrate to the mesenteric lymph nodes and then return to the circulation [169]. It is this constant rotation between Peyer's patches and SLT that may help B cells to disseminate prions acquired from FDC as they both migrate together through the germinal centers [170, 171]. Additionally, the accumulation of prions in the spleen and subsequent neuroinvasion are significantly decreased in the specific absence of B cells [162]. It is also noteworthy that prions were detected in B cells from the blood of sheep with scrapie [172] and deer with CWD [173].

As it was already mentioned, FDC in the Peyer's patches are major sites of early prion replication in orally infected animals, both experimentally [137–140, 174] and in the natural forms of the disease. The study of natural scrapie cases in sheep showed that GALT of the upper gastrointestinal tract exhibited early accumulation

of prions when compared to the large intestinal GALT, such as the cecal patches [175–178]. The rectoanal mucosa-associated lymphoid tissues (RAMALT), which also accumulate prion in naturally occurring cases of scrapie in sheep and goats, as well as in CWD in cervids, also displayed reduced incidence of prion accumulation at the earlier stages of disease [96, 179–184]. Taken together, these studies point out to the fact that prions first replicate within the GALT of the upper gastrointestinal tract and then spread to the local lymph nodes and to other SLT, such as the spleen. Additionally, it was observed that FDC in the spleen expressing PrP^{c} were able to display high levels of prion replication that was impeded after PrP^{c} expression blockage in these cells [185].

5.3 Mononuclear phagocytic cells

A heterogeneous population comprised of monocytes, conventional dendritic cells (DC), and macrophages seem to have also an important role in host infection. From a different lineage of the stromal-derived FDC, conventional DC [157, 158, 186], the antigen-presenting cells par excellence, are also involved in the transport of antigens both within Peyer's patches and toward the mesenteric lymph nodes [187–189]. Their strategic position allows them not only to sample their local environment for pathogens and their antigens, processing them, but also to capture and retain unprocessed (native) antigens [190, 191]. Processed or native antigens are then delivered to B and T cells as DC undergo maturation and migrate toward the local SLT to initiate an immune response [190–193]. Prions are wrapped by DC either by complement opsonization, namely, by C1q and C3 components [155, 194], or in a non-specifically manner, through fluid-phase micropinocytosis [144]. Specific chemokines have been reported to play important roles in prion infections by regulating FDC and DC migration within the SLT. CXCL13 chemokine is expressed by FDC and other stromal cells in the B-cell follicles of SLT and recruits CXCR5-expressing cells toward them [195, 196]. On the other hand, CXCL13-CXCR5 signaling mediates the migration of certain populations of DC toward the FDC-containing B-cell follicles [192, 193, 197], and studies have shown that the early accumulation of prions upon FDC in Peyer's patches was impaired and disease susceptibility reduced, in the absence of CXCR5 expression by DC [198]. The interplay between FDC and DC during prion infection was also patent by the finding that early replication of prions upon FDC in the local SLT was inhibited when conventional DC were transiently depleted at the time of exposure [199–202]. Apparently, after being transferred across the gut epithelium either directly by enterocytes or by *M* cells, prions may be then internalized by DC [143, 199] and propagated by them toward FDC in Peyer's patches [198]. The ability of DC to migrate into B-cell follicles [192, 193, 203] suggests that they may also propagate prions to and within SLT, namely, to the mesenteric lymph nodes.

Regardless of FDC and DC roles in the propagation of prions, there are other cell populations that appear to phagocytose and destroy them [204, 205]. In fact, it was observed that tingible body macrophages of the germinal centers display heavy PrP^{sc} accumulations within their endosomal compartments during prion disease [143, 159, 185]. It is speculated that macrophages may scavenge and degrade prions in an attempt to protect the host from infection, as it is suggested by studies that show that macrophage depletion resulted in an enhanced accumulation of PrP^{sc} within SLT [206, 207].

5.4 Neuroinvasion

After being acquired orally, prions undergo replication and accumulation upon FDC, reaching a threshold above which neuroinvasion occurs. Experiments

in which immunohistochemical tracing was performed revealed that prions then infect enteric nerves and spread along efferent fibers of both the sympathetic (e.g., splanchnic nerve) and parasympathetic (e.g., vagus nerve) nervous systems, spreading within them to the CNS, where they ultimately cause neurodegeneration [143, 208–212]. The initial transfer of prions from FDC to the peripheral nerves continues surrounded by knowledge gaps, but it appears that tunneling nanotubes (TNT) may play an important role at this stage of the disease. These are slender membrane-bound cylinders of cytoplasm that connect cells to enable cell-to-cell communication and the intercellular transfer of plasma membrane or cytoplasmic components [213]. Since mononuclear phagocytes and DC are in close proximity with enteric nerves [214–217] and in vitro studies showed that prions can be transferred between DC and neurons via endolysosomal vesicles within TNT [215–218], it is possible that the same transfer also occurs in vivo. Another hypothesis is that the transfer of prions may also occur in association with small endosomal-derived vesicles, termed exosomes [219], as it was demonstrated experimentally. Studies supporting this theory are based on the observation that DC infected with prions can release exosomes that in turn may infect neighboring cells [220]. In the CNS, prion aggregates accumulate to a point where they overcome the structures within it, starting from the synapses and then proceeding to axons, where axonal retraction is observed, and then to the cell body, in which atrophy and neuronal loss are lastly observed [221–224]. The abovementioned process is, however, slowly and progressive, depending on factors such as prion agent strains, the existence of concurrent infections, and hosts' age and immune status [144].

The onset of neurodegeneration is preceded by the activation of glial cells, microglia, and astrocytes [225, 226]. Microglial cells, in particular, by intervening in synaptic remodeling and in the removal of dead and dying cells, are important for the course of neurological changes during prion infection. Organotypic cerebellar culture devoid of microglia exhibited enhanced prion accumulation [227], whereas CNS prion disease pathogenesis was exacerbated in vivo in the absence of microglia [228]. Studies revealed that during prion infections, a cytokine response mediated by TGF-β and PGE2 develops within the CNS, stimulating microglia to adopt an anti-inflammatory status [223, 229, 230]. Evidence suggests that this anti-inflammatory ambiance may be beneficial for the host, as CNS prion infection in mice deficient in the anti-inflammatory cytokines interleukin (IL)-4, IL-10, and IL-13 displays an aggravated disease pathogenesis [231, 232]. Other studies supported this hypothesis by the finding that blocking colony-stimulating factor (CSF) receptor-1 resulted in the expression of selective anti-inflammatory microglial markers, slowing the development of neuropathology and extending survival times [233]. Activated microglia scavenge and clear prions and prion-affected cells, but neural degeneration seems to occur when prion accumulation is associated to an inflammatory response in the microglia [234]. Together, these data point out to the existence of both anti-inflammatory and pro-inflammatory responses by microglial cells during CNS prion infections [226, 234] contributing to the symptoms of this disease.

6. Diagnosis of CWD

Classification of CWD as TSE required histopathological examination of brains from diseased animals [60]. These subsequent analyses [80] were limited to microscopic evaluation of the CNS to detect neuropathological features typical of, but not necessarily exclusive to, prion diseases including neuronal vacuolation, attendant spongiform degeneration of the neuropil, reactive astrocytic gliosis, and florid amyloid plaques.

6.1 Clinical signs and macroscopic lesions

The main clinical features of progressive CWD disease in adults is weight loss and behavioral changes that typically span weeks or months. Besides weight loss, diseased animals may show a wide range of behavior changes for a long time that include somnolence, lassitude, repetitive walking, polydipsia, and polyuria. Gradually, the affected cervids may exhibit ataxia, mainly in the hind limbs, head tremors, drooped ears, and fixed gaze, and may have difficulty in swallowing. In the end stage, they may show signs of sialorrhea and teeth grinding [235, 236]. The clinical signs may be subtle requiring that the animal keeper/observer is familiar with and has some knowledge of the normal behavior of the species. As a result, the disease is most commonly detected in emaciated cervids hunted, killed, or injured by road accidents or found dead [235].

At necropsy, severe emaciation, poor hair coat condition, megaesophagus, froth or watery rumen contents (often containing sand), abomasal or omasal ulcers, serous atrophy of bone marrow and pericardial fat, enlarged adrenal glands, muscle atrophy [237], and aspiration pneumonia are common findings. As the clinical signs and the macroscopic findings at necropsy are not pathognomonic [49], other diseases must be ruled out, namely, listeriosis, meningoencephalitis, brain abscesses, starvation, nutritional deficiencies or severe parasitism, bluetongue disease, epizootic hemorrhagic disease, meningeal worms (*Parelaphostrongylus tenuis*), and locoweed intoxication [49, 235, 238].

TSEs present very specific diagnostic challenges because of their strain variation, their very long incubation period, and the lack of pathognomonic clinical signs. Moreover, the disease pathogenesis can range both among and within species due to the influence of strain, host genotype, or a combination of the two [239].

6.2 Rapid screening testing

The initial diagnosis of TSE in all species was based on passive surveillance (e.g., clinical presentation) and assessment via conventional histopathology and subsequently detection of PrP^{sc} by IHC, both time-consuming, technically demanding, and expensive for large surveillance plans [77]. For that reason, the development of commercial rapid immunologically based screening tests (RT) for the detection of PrP^{sc} in tissue homogenates allowed a large-scale active surveillance program for TSE in cattle and small ruminants.

These were originally developed for the detection of BSE in the cattle brain, and extensive formal test evaluation of a wide range of these RT was undertaken at Europe level [240, 241]. Mainly, there are two types of these RT kits allowing the purification, concentration, and detection of PrP^{sc} from samples of tissues obtained from infected animals. Both assays consisted in an immuno-enzymatic technique: one is a sandwich format using two antibodies for the detection of the antigen PrP^{sc} after proteinase K digestion (e.g., TeSeE BioRad®), and the other uses a PrP^{sc}-specific ligand immobilized on the surface of the antigen-capture plate (e.g., HerdCheck IDEXX®). Both are suitable for the surveillance of CWD as diagnostic methods for the detection of the disease [77].

The limited experience in CWD so far in Europe has not allowed us to know the best tissue to be proposed as the most sensitive for surveillance in all circumstances. Sampling only the brain stem could reduce the diagnostic sensitivity for strain-host combinations that are characterized by early lymphoid PrP^{sc} accumulation. Consequently, lymphoid tissues (preferably the tonsils, retropharyngeal lymph nodes, and lymphoid tissues of rectal mucosa) and the brain stem at the level of the obex should be tested to maximize the diagnostic sensitivity in any surveillance program [77].

6.3 Confirmatory testing

Like in other recognized animal TSEs, confirmation of an initial CWD "screening positive" (or "suspect") sample can be undertaken by histopathological examination and immunodetection methods either by IHC or Western blot (WB). The former enables PrP^{sc} accumulations to be assessed regarding the types of deposition and anatomical distribution (including cellular) location, while the latter gives some classification data based on the molecular mass and glycosylation profiles of the PrP^{sc} [77].

Histopathologic lesions in the encephalon are similar to those described for ruminant TSEs: perikaryon neuronal vacuoles, microcavitation of the gray matter, astrogliosis, neuronal degeneration and loss, and PrP positively labeled prion deposits and plaques [242]. Duration of clinical disease does not significantly affect the distribution or severity of lesions, and inflammatory cell response is not apparent, unless associated with intercurrent disease [49]. In clinically affected cervids, examination of well-fixed medulla oblongata at the level of the obex is considered sufficient for diagnosis of CWD [80], and sections at this level were used for CWD surveillance before availability of immunohistochemistry [49].

Neuropathology varies slightly between deer and elk: elk have more severe lesions in the thalamus and in some white matter areas. Congo red birefringent and PAS-positive amyloid plaques have been seen in the deer brain but not in elk [80]. The cerebral cortex and basal ganglia of the elk with CWD show minimal fine spongiform degeneration and astrogliosis with focal distribution. The spongiform degeneration with astrogliosis is more prominent in the thalamus where it forms clusters of coarse vacuoles. Fine spongiosis, often in small clusters, is present in the molecular layer of the cerebellum, in dorsal nuclei of the pons, and in the substantia gelatinosa of the spinal cord. Occasional large neurons in various nuclei of the pons show a vacuole [243]. Amyloid plaques are relatively common and can be detected on hematoxylin and eosin (HE)-stained brain sections, most prominently and with decreasing frequency, in white-tailed deer, mule deer, and elk [49]. Neuronal loss and astrogliosis are minimal except for the molecular layer of the cerebellum, which shows rarefaction of granule cells with no indication of apoptosis [243].

The PrP^{sc} immunostaining is consistently present in the cerebral cortex, basal ganglia, and thalamus. In the cerebellum the immunostaining is present in both molecular and granule cell layers as well as in the dentate nucleus. In the pons it is widespread over gray structures, whereas in the spinal cord, it is generally confined to the dorsal part of the dorsal horns [243]. Patterns of PrP^{sc} deposition in CWD-affected cervid brains include perineuronal and perivascular accumulation, extracellular plaques and granular deposits, and subependymal and subpial deposition [49] (**Figure 5**).

Recently, a different neuropathologic phenotype, characterized mainly by intraneuronal deposition of PrP^{sc} and few immunostaining at the dorsal motor of the vagus nerve, was observed in the putative atypical CWD detected in Norwegian moose [31].

Deposition of PrP^{sc} occurs widely in lymphoid tissues (**Figure 5**) during CWD incubation in the absence of histologic lesions in these tissues, resembling classical scrapie in small ruminants [149, 244]. Therefore, lymphoid tissues are very useful for diagnostic purposes and surveillance. Nevertheless, in the referred atypical CWD, no PrP^{sc} was detected in lymphoid tissue [31], similar to that described in heterozygote ARR sheep affected with classical scrapie as well as atypical scrapie. In those cases, lymphoid tissues infectivity should be further studied like it was in atypical scrapie, demonstrating that infectivity can accumulate in lymphoid tissues even with no detectable PrP^{sc} [245].

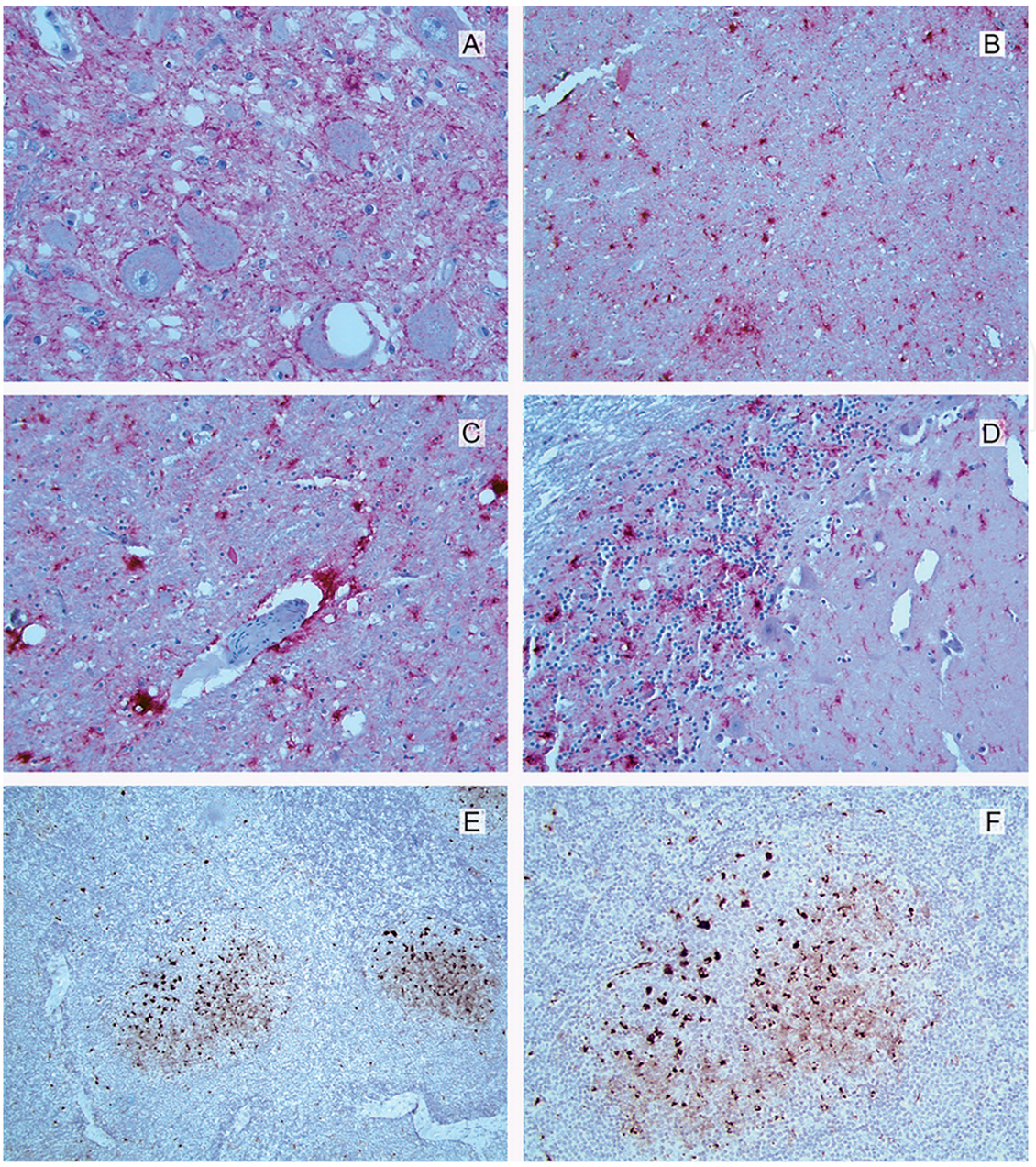


Figure 5.
PrPsc immunohistochemistry in CWD affected deer. (A) Perineuronal and granular deposits in neuropil at dorsal vagal nucleus (brainstem), X400; (B) Extracelullar plaque-like deposits (cerebral cortex), X200; (C) perivascular type (brainstem), X200; (D) distribution in both molecular and granular layers of the cerebellum, X200; (E) and (F) presence of PrPsc in several follicules in a lymph node, X100 and X200, respectivelly. (A–D) sections from CWD control samples kindly provided by professor Stefanie Czub (Canadian food inspection agency, National Center for animal diseases) in 2003; (E-F) sections from the CWD proficiency testing 2008 organized by the European reference laboratory for TSEs (APHA, Weybridge). (A-D) specific PrPsc signal was visualized with F99/97.6.1 monoclonal antibody (raised against bovine residues 220–225; VMRD Inc., Pullman, WA; 1:1250 dilution) StreptABC-alkaline phosphatase, new Fuchsin system (DAKO); (E and F) IHC with 2G11 monoclonal antibody (raised against ovine PrP peptide sequence 146-R154-R171–182; Institute Pourquier;1:200 dilution). All tissue sections were counterstained with Mayer's hematoxylin.

If histopathological examination cannot be carried out due to poor state of the sample and/or IHC results which are not conclusive, then Western immunoblotting is the remaining confirmation method available. This technique relies on detergent extraction followed by treatment with proteinase K to digest any PrP^{c} and detect only PrP^{sc} by a specific antibody presenting bands that correspond to proteins within a range of molecular mass from 17 kDa (unglycosylated PrP^{sc}) to 27 kD (diglycosylated PrP^{sc}) [238].

WB and IHC allow a comparison of labelling patterns with antibodies that recognize different epitopes of the protein and help to clarify specific proteinase K cleavage inherent of strains type. This approach forms the basis of the discriminatory testing of small ruminant isolates, to differentiate between isolates that can be classified as scrapie, and those that are considered BSE-like by looking at the lower molecular weight for the unglycosylated protein band and the ratio of the mono- and diglycosylated fragments, for example, between classical, atypical/Nor98, and CH1641 scrapie in sheep and goats and BSE and scrapie [246, 247].

The Norwegian atypical CWD (Nor-16CWD) also presented an unusual PrP^{sc} electrophoretic pattern distinguishable from previous CWD cases and from known ruminant prion diseases in Europe, with the possible exception of sheep CH1641. Transmission studies in several rodent models are ongoing to clarify if this different phenotype could reflect the presence of a new cervid prion strain in moose from Norway [31].

6.4 New diagnostic methods

Regarding diagnosis in vivo, more sensitive diagnostic methods are desirable. Using in vitro conversion such as protein misfolding cycling amplification (PMCA) or real-time quaking-induced conversion assay (RT-QuIC), CWD prions are detectable already at a preclinical stage in specimen that can be obtained antemortem by noninvasive methods, such as blood, urine, feces, or saliva.

During PMCA, the normal form of PrP (PrP^{c}) is converted into protease K-resistant PrP (PrP^{sc}) using small amounts of infectious PrP^{sc}. Continued recruitment and conversion of PrP^{c} by PrP^{sc} are accomplished by sonication in a process analogous to amplification of DNA by the polymerase chain reaction (PCR). This amplification process enhances detection sensitivity by several orders of magnitude as compared to WB and has been used to confirm the presence of CWD prions in muscle [248] and feces [249].

In the assay referred to as real-time quaking-induced conversion (RT-QuIC) [250], prion seeds are thought to induce recombinant PrP to adopt a β-sheet structure. Thioflavin T, added to the reaction, is incorporated into the growing amyloid causing an altered spectrofluorimetric emission pattern, which is monitored over time.

CWD still represents a challenge in TSE research for which transmission and dissemination remain unchecked. The tools of diagnostic available for identifying infected animals have steadily progressed over time from clinical and pathological descriptions to antibody–antigen-dependent immunoassays and more recently qualitative and quantitative prion amplification techniques. These tools have provided a deep understanding of disease pathogenesis and transmission and allowed animal health technicians to monitor the expanding geographical presence of CWD [251].

7. Surveillance and control of CWD

7.1 Surveillance

Of further concern is the fact that CWD is the only transmissible spongiform encephalopathy known to be expanding both geographically and in prevalence [5].

After the confirmation in 2016 of two cases of CWD in a wild reindeer (April) and a wild moose (May) in Norway (the first CWD cases in Europe), this country intensified its surveillance program for CWD in cervids and detected a number

of other cases of CWD in reindeers and in moose. In the meantime, the European Commission (EC) requested the European Food Safety Agency (EFSA) to recommend surveillance activities and, if necessary, additional animal health risk-based measures to prevent the introduction of the disease and the spread into/within the European Union (EU), specifically Estonia, Finland, Iceland, Latvia, Lithuania, Norway, Poland, and Sweden [252]. Based on EFSA opinion, the EC places in force the Commission Regulation (EU) 2017/1972, amending Annexes I and III of Regulation (EC) No. 999/2001 as regards a surveillance program for chronic wasting disease in cervids in Estonia, Finland, Latvia, Lithuania, Poland, and Sweden and repealing Commission Decision 2007/182/EC (which provided requirements for a survey on CWD in cervids which was carried out from 2007 to 2010). According to this new regulation, the member states (MSs), which have a wild and/or farmed and/or semidomesticated population of moose band/or reindeer (aforementioned MSs), shall carry out a 3-year monitoring program for CWD from 1 January 2018 to 31 December 2020.

The 3-year CWD monitoring program shall cover animals over 12 months of age, estimated on the basis of dentition, obvious signs of maturity, or any other reliable information, of the following cervid species:

- Eurasian tundra reindeer (*Rangifer tarandus tarandus*)
- Finnish forest reindeer (*Rangifer tarandus fennicus*)
- Moose (*Alces alces*)
- Roe deer (*Capreolus capreolus*)
- White-tailed deer (*Odocoileus virginianus*)
- Red deer (*Cervus elaphus*)

According to some authors [252, 253], in order to avoid unbiased prevalence or incidence estimates and to obtain a representative set of animals, a random sampling is always recommended to design monitoring program. This classical approach may be possible to achieve for farmed cervids, but, for wildlife population, random sampling may not be possible to design as sampling frames are not available [252]. However, when disease detection is the first objective, a sampling targeting high-risk animals may be more efficient. That, in fact, was the case of the Norwegian monitoring program that detected the two CWD cases through sampling of "suspect" cervids [252].

Since the aims of the proposed surveillance system were to detect disease in countries where CWD has not yet been detected and to estimate prevalence in areas where disease has been detected, the 3-year surveillance system includes a two-stage sampling program (Commission Regulation (EU) 2017/1972):

- At the first stage, a random sampling is applied for wild/semidomesticated cervids [primary sampling (PSU)] corresponding to geographical areas containing cervid populations, whereas for farmed cervids they will correspond to farms.
- At the second stage, a convenience sampling is applied targeting high-risk animals of the selected species within PSU (**Figure 6**).

To date, according to Commission Regulation (EU) 2017/1972, member states shall carry out additional monitoring for TSEs in cervids based on a risk assessment which may take into account the detection of a TSE in cervids in the same or

neighboring regions. Efforts to prevent the introduction of CWD should consider whether prior surveillance data are sufficient to assure that the disease has not already spread into the area of interest.

Based on the main provisions in the TSE regulation currently applicable to CWD, TSE-positive cases in cervids must be notified to the commission and the member states (Article 11 of Regulation (EC) No. 999/2001), and all parts of the body of a cervid positive for TSE must be sent to disposal as category 1 materials in accordance with the Animal By-Product Regulation 10 (Article 13.1. (a) of Regulation (EC) No. 999/2001).

7.2 TSE control

As it was previously referred (see Section 4), CWD is a very contagious form of TSE. Therefore, control in captive cervids is not easy and even more in the wildlife. According to Uehlinger and colleagues [254], the combination of direct and environmental transmission of CWD prions; the persistence of environmental infectious prion for at least for 2.5 years [255]; the absence of a rapid, practical, and reliable antemortem field tests for detecting preclinical CWD; and the nonavailability of licensed vaccines nor therapies together with the impossibility of quarantine suspicions populations in wild cervids make the control of this disease in wild populations very difficult.

The management of CWD in affected populations is a proven difficult task, based on population reduction in the case of wild deer or in quarantine and depopulation in commercially reared animals.

Measures to control the spread of the disease will depend on whether we are dealing with commercially exploited herds, confined to demarcated areas, or with wild populations of deer. In the first case, and similar to other infectious diseases and other livestock, programs to detect and eliminate positive animals may be used [94].

This task is not easy, however, as a shown by a 2018 study conducted on a private farm in Colorado where the use of disease control management through rectal biopsy diagnosis was not able to prevent the increase in prevalence suggesting the need to refine management criteria or antemortem diagnostic methods [5].

Thus, the science available to inform effective CWD management and control strategies remains relatively incomplete as it was concluded by different studies [254, 256] during their review on CWD control in North America. Nevertheless, it may be stated that approaches for CWD management generally fall into three categories in order to prevent the introduction of the disease and its spread [252]:

a. Prevention of entry in free regions (where CWD is assumed absent or is not believed to occur)

b. Containment procedures of a CWD focus to avoid disease spread

c. Control/suppression in an affected herd or population

Regarding preventive and containment strategies, it was referred that these measures tend to focus on regulations (e.g., bans on movements of live animals, carcasses, or specified risk materials) and efforts to prevent the introduction of CWD should consider whether prior surveillance data are sufficient to assure that the disease has not already spread into the area of interest [252].

Haley and collaborators [94] suggest that outer mechanisms of disease control could be useful like the development of simple antemortem test to use prior to animal movements among farms, the breeding for resistant genotypes, the installation of effective fences to prevent transmission between commercial

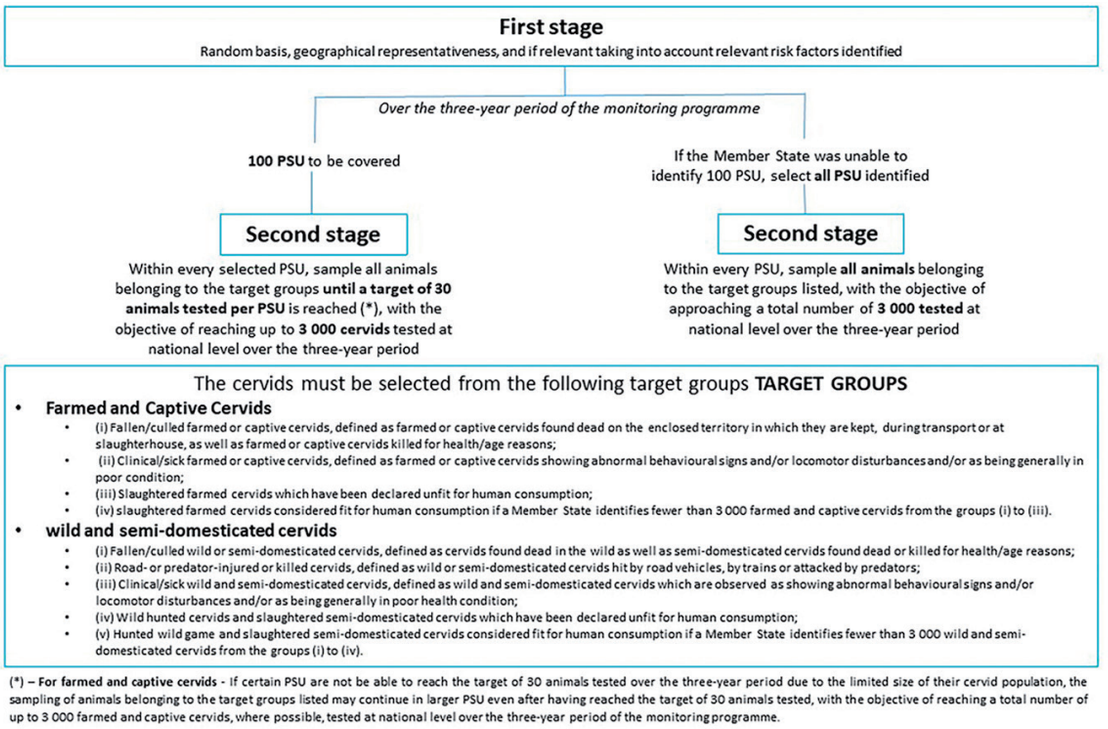


Figure 6.
Surveillance program for CWD in cervids. Schematic presentation of two-stage sampling approach of the 3-year surveillance programme for CWD in cervids in Estonia, Finland, Latvia, Lithuania, Poland and Sweden in cervids defined on commission regulation (EU) 2017/1972.

and wild populations, and equipment cleaning and quarantine prior to animal introduction.

Free-ranging deer population reduction is empirically effective in reducing a disease with direct contact transmission, but several studies have shown that in the case of CWD, the results are below expected [94, 257].

Capturing, testing, and recapturing positive free-ranging mule deer did not reduce prevalence in the female population [258].

Concerning the prevention of entry in free regions, according to Regulation (EC) No. 999/2001, in the EU, the feeding to cervids of proteins derived from animals is prohibited, with the exception of milk and milk products, eggs and egg products, hydrolyzed proteins from nonruminants or from ruminant hides and skins, and gelatine and collagen from nonruminants (Article 7 and Annex IV). Also, at import into the EU, an attestation is required for meat and meat products from wild and farmed cervids coming from the USA or Canada (Chapter F of Annex IX), confirming that the products:

- Exclude the offal and spinal cord
- Are derived from animals tested for CWD with negative results
- Are derived from animals which do not come from a herd (for farmed animals) or a region (for wild animals) where CWD has been confirmed or officially suspected

As a result of the detection of the first case in Norway, in Europe, in addition to the permanent measures as described in the aforementioned regulation, the Commission Implementing Decision (EU) 2016/1918 enforced temporary safeguard measures in relation to CWD (until December 31, 2017). According to this

decision, the movement of live cervids from Norway into the European Union was prohibited with several derogations permitting its movements under certain circumstances (e.g., movements of live cervids from Norway to Sweden or Finland for direct slaughter; movements of live reindeer for seasonal grazing from Norway to some listed areas in Sweden and Finland).

According to the European Food Safety Agency [252], the derogations that lay down in this decision present a risk of introduction of CWD into the EU, since it was concluded that the most likely pathway of introduction of CWD into the EU is the movement of live cervids, either by deliberate transportation or by the movement of wild animals across the border of Norway to Sweden or Finland. Additionally, these authors present other measures to reduce the probability of introduction of CWD into the EU, namely, the use of natural cervid urine lures and awareness campaigns targeting both local Norwegian hunters and hunters visiting Norway from (and returning to) other countries regarding the personal protective equipment (PPE), disinfection, the safe dressing of carcasses, and the appropriate disposal of carcass trimmings.

In general, according to several authors [252, 254, 256], other measures can be implemented in order to contain and/or to control/suppress CWD in a region or country where the disease is present. Some of those measures may include:

- Reducing environmental contamination (e.g., interdict the use of dead cervids to feed wildlife nor to use as baits for hunting, unless they have been tested negative, prior to use).

- Reduction of animal-to-animal contact (e.g., ban artificial feeding, lick blocks or other congregating management procedures).

- Decrease population densities by definition of strategic plan for hunting management policies (e.g. increasing harvest permits and/or hunting season) and culling practices.

Regarding the last measure (decrease population), Uehlinger and colleagues [254] alert that:

- To date, the evidence is unclear that increased nonselective culling pressure has a beneficial effect on the spread or prevalence of CWD.

- Culling of wildlife is often unpopular. For that reason, any control program that includes culling of animals must take into account the public acceptance and hunters' attitudes and behaviors.

Since dispersion of infected deer may not be limited by political boundaries, regional and interstate/interprovincial cooperation and collaboration will be an important part of any successful management strategy. Also, it is always important to evaluate the costs in order to understand the feasibility of the control strategy proposals (when developing any intervention program) [254].

This review gathers known and recent features of this progressive and fatal neurodegenerative disease affecting cervid species, contributing for chronic wasting disease awareness among scientific community and stakeholders. Conscious that research in this field has a long way to go to answer many of the questions that remain open, we believe that this review will contribute to the understanding of the occurrence of prion diseases in wildlife.

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