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REVIEW



Human prion disease: molecular pathogenesis, and possible therapeutic targets and strategies

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ABSTRACT

Introduction: Human prion diseases are heterogeneous, and often rapidly progressive, transmissible neurodegenerative disorders associated with misfolded prion protein (PrP) aggregation and self-propagation. Despite their rarity, prion diseases comprise a broad spectrum of phenotypic variants determined at the molecular level by different conformers of misfolded PrP and host genotype variability. Moreover, they uniquely occur in idiopathic, genetically determined, and acquired forms with distinct etiologies.

Area covered: This review provides an up-to-date overview of potential therapeutic targets in prion diseases and the main results obtained in cell and animal models and human trials. The open issues and challenges associated with developing effective therapies and informative clinical trials are also discussed.

Expert opinion: Currently tested therapeutic strategies target the cellular PrP to prevent the formation of misfolded PrP or to favor its elimination. Among them, passive immunization and gene therapy with antisense oligonucleotides against prion protein mRNA are the most promising. However, the disease's rarity, heterogeneity, and rapid progression profoundly frustrate the successful undertaking of well-powered therapeutic trials and patient identification in the asymptomatic or early stage before the development of significant brain damage. Thus, the most promising therapeutic goal to date is preventing or delaying prion conversion in carriers of pathogenic mutations by lowering prion protein expression.

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1. Introduction

Prion diseases are a group of typically rapidly progressive and transmissible neurodegenerative disorders affecting humans and other mammalian species. The pathogenic hallmark is the deposition of pathological conformers of the prion protein (PrP), mainly in the central and less frequently in the peripheral nervous system and organs such as spleen, tonsils, appendix, etc. In humans, prion diseases are rare, with an annual mortality rate of about 2 cases per 1 million population [1].

Current terminology and classification of human prion diseases use historical eponyms (Creutzfeldt – Jakob disease, CJD, and Gerstmann – Sträussler – Scheinker disease, GSS) and clinicopathological or protein chemistry descriptions (Fatal Familial Insomnia, FFI, and Variably Protease-Sensitive Prionopathy, VPSPr). These terms are combined with the designation of the putative etiology (i.e. sporadic, genetic, or acquired). Finally, a terminology mainly referring to molecular features is used to classify CJD, the most common human prion disease, in several distinct subtypes (see below).

Based on pathological features, two main groups of human prion disease are distinguished. The first, the most prevalent, including CJD and most FFI cases, is characterized by widespread spongiform change involving multiple brain areas. The

second comprises the PrP-amyloidoses, including GSS and other rare genetic prion diseases characterized by parenchymal and vessel-related cerebral amyloid angiopathy (CAA), with or without systemic PrP deposits [2]. Notably, this significant difference in the distinctive histopathology of the two groups (spongiform change vs. amyloid plaques) strongly correlates with the physicochemical properties of abnormal PrP (PrP^{Sc}) aggregates. In spongiform encephalopathy, proteinase K (PK) treatment of brain homogenates generates N-terminally truncated PrP^{Sc} fragments retaining the glycosylphosphatidylinositol (GPI) anchor and the glycosylation sites referred to as PrP^{27–30}. In contrast, in the PrP-amyloidoses, PK-resistant PrP^{Sc} comprises unglycosylated anchorless fragments ragged at both N- and C- termini [2]. As an exception to this general rule, a subgroup of rare prion disorders, including VPSPr and some GSS cases linked to the *PRPN* P102L-129M haplotype are characterized by the coexistence of both PrP^{Sc} fragments correlating with a mixed phenotype that includes both spongiform change and PrP^{Sc} amyloid deposits.

Sporadic prion disease, which accounts for more than 80% of all human cases, is thought to originate spontaneously from unknown stochastic cellular events leading to the conversion of the cellular prion protein (PrP^C) into PrP^{Sc}. The genetic form

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Article highlights

- Human prion diseases are rare, often rapidly progressive, transmissible neurodegenerative disorders caused by prion protein misfolding, characterized by broad clinical, histopathological, and molecular heterogeneity.
- No pharmacologic treatment has shown a positive effect on survival in patients with prion disease, but novel promising approaches, including immunotherapy and genetic modulation, are now available.
- A first observational trial using an anti-prion protein antibody (PRN100) documented a possible brain clearance of the abnormal prion protein in one Creutzfeldt-Jakob disease-affected brain and no adverse events in six individuals with clinical disease.
- Antisense oligonucleotides effectively reduced the levels of physiologic cellular prion protein in animal models and currently represent the most promising approach for inherited prion disease, mainly to prevent phenocconversion in *PRNP* mutation carriers.
- The rarity of disease limits the enrollment of large patient cohorts, impairing the ability to successfully undertake statistically meaningful trials.
- Future clinical trials should consider the molecular heterogeneity of the disease related to different prion strains, validate novel biomarkers of phenocconversion in at-risk individuals, and further standardize clinical and/or biological outcomes.

is causally linked to autosomal-dominant pathogenic mutations in the prion protein gene (*PRNP*) and contributes to about 10–15% of cases. Finally, the acquired form originates either from the accidental human-to-human transmission through surgical/medical procedures as in iatrogenic CJD (iCJD), blood transfusions as in variant CJD (vCJD), and ritual cannibalism as in kuru, or from the ingestion of bovine spongiform encephalopathy (BSE)-derived prions as zoonosis (vCJD).

At the molecular level, two types of PrP^{27–30} with distinct physicochemical properties (i.e. type 1 and type 2), in combination with the *PRNP* genotype at the polymorphic codon 129 (coding for methionine/M or valine/V), largely determine the CJD phenotype, allowing a histo-molecular disease classification [3,4]. Six molecular groups, MM1, MV1, VV1, MM2, MV2, and VV2, correlating with the clinical and neuropathological phenotypic heterogeneity of sporadic CJD (sCJD), were initially defined. However, given that two of these molecular combinations were found to be associated with two distinct clinicopathological subtypes rather than one, ‘histopathological’ hallmarks were also introduced. Accordingly, the MM2 group includes a ‘cortical’ (MM2C) and a ‘thalamic’ variant (MM2T), and the MV2 group comprises a prevalent subtype with cerebellar kuru-type amyloid plaques (MV2K subtype) and a rare variant phenotypically indistinguishable from the MM2C group (MV2C subtype). Interestingly, the classification also applies to the prevalent genetic forms of CJD (gCJD), where the histotypes mainly depend on the codon 129 polymorphism in the mutated allele/PrP^{Sc} type 1 or 2 combinations rather than on the mutation per se [5]. The classification of human prion diseases, including the CJD subtypes, is summarized in Table 1.

1.1. Molecular pathogenesis and potential therapeutic targets

The primary mechanism governing prion disease pathogenesis in all disease forms is the misfolding of physiologically

expressed PrP^C into the abnormal isoform PrP^{Sc} that is partially and variably resistant to protease digestion and insoluble in detergents [6]. Prion protein misfolding prompts PrP^{Sc} accumulation in highly ordered aggregates that mediate neurotoxicity [7].

PrP^C expression is the absolute requirement for prion formation and replication since mice devoid of PrP^C do not develop the disease after experimental injection of prion-infected tissue homogenates [8]. Human PrP^C is encoded by the *PRNP* located in the short arm of chromosome 20 and in mature form includes 208 amino acids (PrP23–231). After synthesis as a 253 amino acid precursor, N- and C-terminal signal peptides are removed, and chaperones guide the nascent PrP^C into the endoplasmic reticulum (ER) and Golgi apparatus to fold correctly and undergo post-translational modifications, including the variable attachment of glycans at residues Asn181 and Asn197, and the addition of a GPI anchor to the C-terminus. Following these steps, the PrP^C fold in absence of Cu²⁺ comprises an intrinsically disordered N-terminal domain and a structured, predominantly α -helical C-terminal domain [9].

There is incomplete knowledge about the key events triggering PrP^{Sc} formation. Pathogenic *PRNP* mutations and/or external factors such as oxidative stress, age, and inflammatory insults may all play a role [10]. Failure of cellular quality control mechanisms involving molecular chaperones, such as heat shock proteins, that normally favor the elimination of misfolded species by refolding or degradation through the ubiquitin-proteasome quality control (UPS) [11,12], may also promote the stabilization of soluble oligomers, leading to the formation of higher-ordered structures [10].

There is growing convergence on seeded nucleation being the mechanism underlying prion aggregation. During this process, PrP^{Sc} oligomers incorporate and convert PrP^C monomers, growing in size and eventually generating protofibrils and fibrils [13]. Then, fibril fragmentation creates new nucleation sites that amplify the reaction by incorporating new PrP^C monomers [7]. Using animal-derived prions (i.e. 263K and anchorless RML), recent cryo-EM studies demonstrated the assembly of PrP^{Sc} fibrils with parallel in-register intermolecular β sheets, in which each misfolded PrP^{Sc} monomer represents one rung of the fibril core and templating surface for incoming monomers at fibril ends, where prion growth occurs [14].

In physiologic conditions, PrP^C is localized primarily on the cell surface, where it is GPI-anchored in the lipid rafts of the membrane. Evidence suggests that this site is critically involved in converting PrP^C into PrP^{Sc} [15]. As part of this process, PrP^C could act as a toxicity-inducing receptor or trap and concentrate PrP^{Sc} molecules on the cell surface that induce toxic effects by compromising the integrity of cell membranes [16]. Additionally, given the attachment to the membrane through the GPI moiety, during the conversion phase, PrP^C twists along one side of the fibril while binding to membranes, causing its distortion and promoting cellular damage [14]. Besides the cell surface, PrP^C conversion can also occur shortly after internalization during an endocytic process. In such conditions, the internalized PrP^{Sc} aggregates can foster the seeded conversion by blocking the UPS system and activating the unfolded protein response (UPR) signaling pathways related to ER stress [17]. As a result, the impairment of

Table 1. Summary of human prion disorders, their prevalence and main histo-molecular features.

Aetiology	Disease	Prevalence	Phenotype	Prevalent misfolded PrP ^s
Idiopathic		85%		
	Sporadic Creutzfeldt-Jakob disease <u>Subtypes:</u> - MM/MV1 (typical form) - VV2 - MV2K - MM/MV2C - MM2T - VV1	>95% - 65% - 15% - 10% - rare - rare - rare	Spongiform encephalopathy	PrP ²⁷⁻³⁰
	Variably protease sensitive proteinopathy	rare	Mixed features (spongiform change +/- PrP amyloidosis)	PrP-amyloid + PrP ²⁷⁻³⁰
Genetic		10-15%		
	Genetic Creutzfeldt-Jakob disease <u>Subtypes:</u> - M1 - V2 - V1 - M2C - Mi-E200K Not classifiable, associated to specific <i>PRNP</i> haplotypes*	5-10% - >80% - rare - rare - rare - rare - rare	Spongiform encephalopathy	PrP ²⁷⁻³⁰
	Fatal familial insomnia (or subtype M2T)	rare	Spongiform encephalopathy	PrP ²⁷⁻³⁰
	Gerstmann-Sträussler-Scheinker disease	rare	PrP amyloidosis	PrP-amyloid
	PrP-cerebral amyloid angiopathy	rare	PrP amyloidosis	PrP-amyloid
	Specific <i>PRNP</i> haplotypes**	rare	Variable phenotype(s), often with mixed features	PrP-amyloid + PrP ²⁷⁻³⁰
Acquired		<1%		
	Iatrogenic Creutzfeldt-Jakob disease	rare	Spongiform encephalopathy	PrP ²⁷⁻³⁰
	Variant Creutzfeldt-Jakob disease	rare	Spongiform encephalopathy	PrP ²⁷⁻³⁰
	Kuru	extinct	Spongiform encephalopathy	PrP ²⁷⁻³⁰

Note: ^sIn prion disorders, misfolded PrP accumulates either as N-terminally truncated PK-resistant form of PrP^{Sc} with an unglycosylated core fragment of 19–21 kDa (PrP²⁷⁻³⁰), or as N- and C-terminally truncated PK-resistant form of 6–11 kDa (PrP amyloid).

*T183A-129 M, 5/6 octapeptide repeat insertions-129 V.

**P102L-129 M, P105S-129 V, P105T-129 V and 7 octapeptide repeat insertions-129 V/M.

protein synthesis leads to synaptic dysfunction and neuronal loss, which may also depend on the activation of the apoptotic cascade stimulated by the saturation of the UPS system [18] (Figure 1). Additionally, protracted UPR response leads to deacylation and degradation of PIKfyve, reducing phosphoinositide diphosphate PI(3,5)P₂ levels. This, in turn, alters endosome maturation, resulting in enlarged endolysosomes that eventually become intracellular vacuoles reminiscent of prion-induced spongiosis [19].

All evidence above indicates that PrP^C and PrP^{Sc} are both potential therapeutic candidates for prion disease because of their pivotal role in disease pathogenesis. Strategies to deplete the PrP^C substrate or prevent recruitment and contact between PrP^C and PrP^{Sc} should hinder the conversion mechanism. Additionally, an intervention targeting cell-surface PrP^C should prevent its binding with the neurotoxic

pathologic aggregates [7]. Given the current modeling of prion strain selection [20], removing PrP^C as substrate, compared to targeting PrP^{Sc}, has the potential additional advantage of avoiding the risk of selecting a resistant PrP^{Sc} conformer generating a novel dominant strain.

Another critical point in the pathogenesis of prion disease is the ability of protein aggregates to propagate within the central nervous system (CNS), spreading from cell to cell and between different brain areas [21]. Different mechanisms may explain how PrP^{Sc} aggregates enter and spread between cells, including exosomes [22], nanotubes [23], and receptor-mediated internalization [21]. All these mechanisms are potential targets of therapeutic interventions aiming to slow the diffusion of the disease to different brain regions. In this regard, it is noteworthy that the susceptibility of cellular populations and brain regions to prion strains is variable and

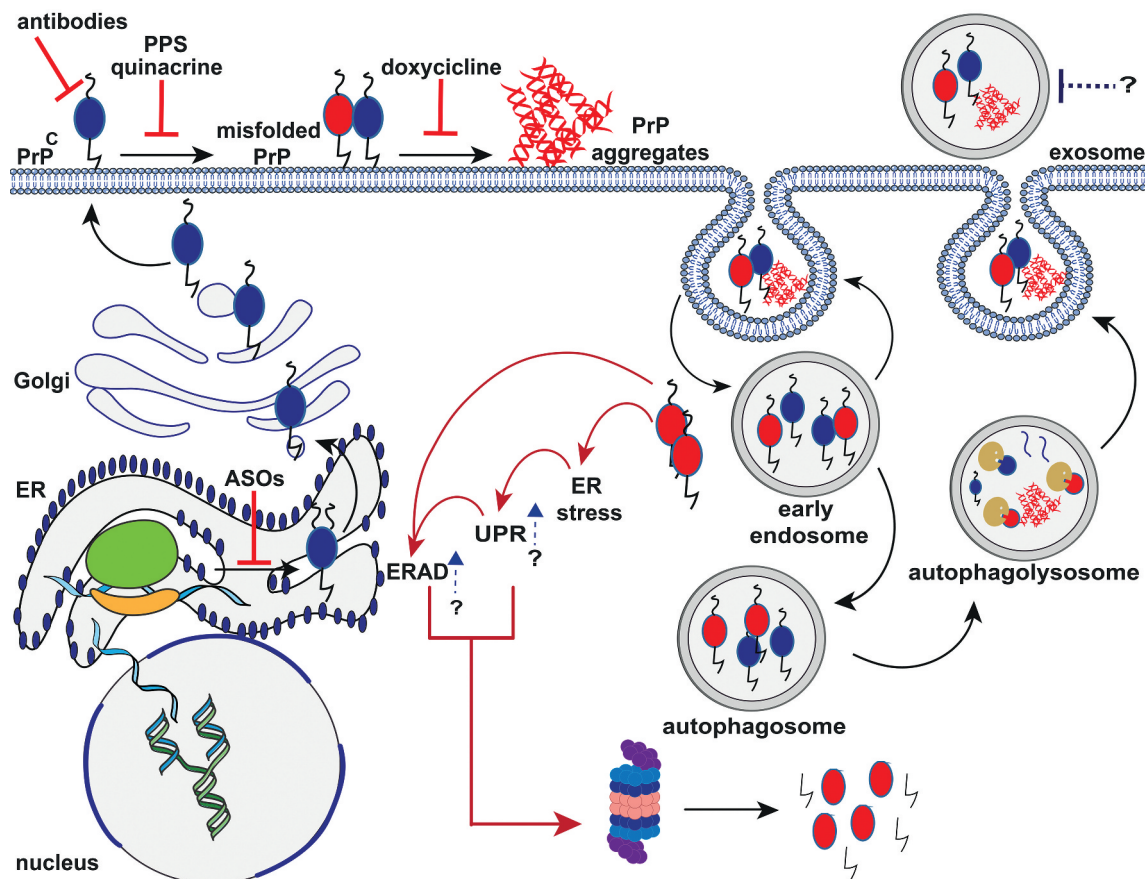


Figure 1. Schematic representation of the putative mechanisms of PrP aggregate formation and clearance and mechanism of action of different anti-prion compounds. After synthesis, the nascent PrP^C enters the lumen of the ER, where the N-terminal signal peptide is removed. Then, the protein moves to the Golgi apparatus to undergo post-translational modifications. Once fully folded, PrP^C moves along the secretory pathway to the outer leaflet of the plasma membrane, where it anchors via the C-terminal GPI moiety to lipid rafts. Both cell surface and endosomal pathways are putative sites of PrP^C misfolding and oligomerization. Endocytosed PrP^C and misfolded PrP are rapidly recycled into the plasma membrane or removed through macroautophagy following the extracellular release of exosomes. Internalized aggregates promote the ER stress resulting in the activation of UPR and ERAD response, which, in turn, induce the degradation of misfolded PrP by the UPS system. Red solid lines indicate potential therapeutic intervention points, including ASOs targeting PrP mRNA, prion clearance (antibody therapy), prion replication (PPS and Quinacrine), and oligomers stabilization (Doxycycline). Blue arrows and dotted lines represent new potential sites of intervention (UPR and ERAD response enhancement; exosome blockade). Black solid arrows indicate molecular steps (endocytosis of PrP^C; conversion of PrP^C to PrP^{Sc}; macroautophagy pathways). Red solid arrows indicate the cascade triggered by the accumulation of PrP aggregates.

List of abbreviations: ASOs, antisense oligonucleotides; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation; GPI, glycosylphosphatidylinositol; PPS, pentosan polysulfate; PrP, prion protein; PrP^C, cellular PrP; PrP^{Sc}, PrP scrapie; UPR, unfolded protein response; UPS, ubiquitin-proteasome quality control.

leads to distinguishable disorders with unique phenotypic features [24]. At the cellular level, there is an involvement of both neurons and glial cells. Evidence from transcriptomic studies indicated that microglial and astrocytic activation occurs since the early phase of the disease, and changes in these cell groups precede neuronal damage. Microglial cells are involved in either neuroprotection by clearing PrP^{Sc} or neurodegeneration by prompting the release of inflammatory cytokines that induce neuronal apoptosis [25]. Therefore, modulating the expression of cell-specific cofactors related to neuroinflammatory pathways, such as receptors and chaperones, could be an additional therapeutic strategy [26].

1.2. History of treatment failures

In the last 30 years, scientists tested several chemical compounds for their potential anti-prion effect in cell cultures

and animal models of prion disease. However, only a few eventually reached a trial in patients affected by human prion disease. Moreover, most of these studies included a limited number of individuals and were non-comparable in their experimental design since they comprised case reports, observational and case-control studies, as well as randomized placebo-controlled clinical trials (Table 2).

Pursuing the 'slow virus' hypothesis of CJD etiology, initial attempts to treat CJD involved the antiviral Amantadine, a drug initially developed against the influenza virus. In anecdotal CJD case reports, the drug positively affected survival time [27], or clinical symptoms [28,29]. However, other single-case observations did not confirm the positive effect [30,31]. In a study by Terzano and colleagues, Amantadine treatment did not increase survival in 4 CJD patients compared with 5 CJD individuals undergoing only palliative/supportive care [32]. As the only distinguishing feature between groups, subjects

Table 2. Summary of anti-prion molecules tested by clinical studies on human prion diseases.

Molecule	Class	Route	Type of study	Patients/controls	Dose	Primary outcome	Secondary outcome	Country	Ref.
Amantadine	Antiviral	Oral	Case-control	4 (cases): CJD 5 (controls): CJD	300–800 mg/day	No extended survival	Slight improvement of vigilance	Italy	[32]
Flupirtine	Aminopyridine compounds	Oral	Case-control	4 (cases): CJD 4 (controls): CJD	300 mg/day	No extended survival	-	Italy	[33]
			RCT double blind	13 (treatment): 12 sCJD, 1 gCJD (D178N) 15 (placebo): 14 sCJD, 1 gCJD (E200K)	100–400 mg/day	No extended survival	Less cognitive decline	Germany	[40]
Pentosan polysulphate	Anticoagulant	Intraventricular	Observational	11 (cases): 6 sCJD, 2 gCJD (V180I), 1 GSS (P102L), 2 iCJD 7 (cases): 3 vCJD, 2 iCJD, 2 GSS (P102L)	1–120 µg/kg/day	Possibly extended	Slow progression	Japan	[44]
Quinacrine	Antiprotozoal	Oral	Case-control	32 (cases): 30 sCJD, 2 vCJD 125 (controls)*: all sCJD	1000 mg, then 100 × 3 mg/day	Possibly extended survival	No differences at neuropathology	UK	[43]
			Open-label patient-preference trial	32 (treatment): 8 sCJD, 1 iCJD, 6 vCJD, 17 IPD 69 (no treatment): 36 sCJD, 1 iCJD, 10 vCJD, 22 IPD	300 mg/day	No extended survival	Transient improvement on functional scales in 10% of cases	France	[53]
			RCT double blind, stratified	23 (treatment): sCJD 28 (placebo): sCJD	300 mg/day	No extended survival	Less decline on functional scales#	USA	[55]
Doxycycline	Antibiotic	Oral	RCT double blind	62 (treatment): 60 sCJD, 2 gCJD (E200K, V210I) 59 (placebo): 57 sCJD, 2 gCJD (E200K, V210I)	100 mg/day	No extended survival	No differences in loss of autonomous feeding, sphincter control and reaching akinetic mutism	France/Italy	[60]
			RCT double blind	7 (treatment): sCJD 6 (placebo): sCJD	100 mg/day	No extended survival	No improvement of life quality	Germany	[61]
			Observational	55 (treated): sCJD 33 (untreated): sCJD		Extended survival	Effect on survival 129 MM > VW	Germany	[61]
PRN100	Anti-PrP ^C antibody	Intravenous	Observational	6 (cases): 5 sCJD, 1 iCJD Age- and codon 129 matched controls*	80–120 mg/kg/2 weeks	Extended survival	Reduced brain PrP accumulation at neuropathology in 1 case (iCJD)	UK	[85]

Note: The Table includes studies with at least 5 participants. *Historical controls; #modified Rankin and Clinical Dementia Rating. List of abbreviations: RCT, randomized controlled trial; CJD, Creutzfeldt-Jakob disease; sCJD, sporadic CJD; iCJD, iatrogenic CJD; gCJD, genetic CJD; vCJD, variant CJD; IPD, inherited prion disease.

treated with Amantadine showed a transient improvement in wakefulness [32]. A subsequent study confirmed the lack of a positive effect in 8 CJD patients [33]. Additionally, approximately 35 other CJD patients were treated unsuccessfully with Amantadine [34]. Similarly, antiviral drugs such as Acyclovir and Interferon failed to show a positive clinical effect in CJD case reports [35–37].

Flupirtine, a pyridine derivative with analgesic and anti-apoptotic properties [38], reduced cell death in neuronal cells treated in vitro with the neurotoxic PrP(106–126) fragment [39]. The drug was then evaluated in a double-blind prospective clinical trial including 28 sCJD patients (13 treatment arm, 15 placebo arm). The treatment demonstrated some beneficial effects on cognition but failed to prolong survival [40].

In vitro and animal studies demonstrated that pentosan polysulphate (PPS), a polyanion compound, reduced PrP^{Sc} formation [41,42]. Intra-cerebroventricular administration of PPS in transgenic mice intracerebrally infected with different prion strains (263K, RML, Fukuoka-1) prolonged incubation time. Consistently, the drug reduced abnormal PrP deposition, the extent of neuropathologic change (atrophy, neuronal loss), and the associated infectivity [42]. Two different observational studies in the United Kingdom (UK) and Japan evaluated PSS in 18 individuals affected by prion disorders, including 6 sCJD (all from the Japanese study), 4 iCJD (2 from each study), 3 GSS-P102L (2 from the UK, one from Japan) and 3 vCJD patients (all from the British study) [43,44]. Both studies reported a likely extended survival in treated patients. Moreover, the treatment decreased the PrP^{Sc}/total PrP and the oligomeric PrP/total PrP ratios in some CJD cases in one study [45]. However, PPS did not significantly affect neurological functions, and brain post-mortem pathology [43,44].

One of the most studied anti-prion agents is Quinacrine, an antiprotozoal drug used in the past as an antimalarial. The drug targets the conversion of PrP^C into PrP^{Sc} [46]. In early 2000s, initial evidence demonstrated its efficacy in inhibiting PrP^{Sc} accumulation in scrapie-infected neuroblastoma cells [47,48]. In subsequent animal studies, Quinacrine had mixed results. Some reported no effect on incubation time and disease duration [49,50], while others showed an extension of both incubation time and survival after prion injection [51]. A first study involving 4 CJD patients documented minimal and transient improvement of arousal and integrative functions [52]. In the three following independent studies with case-control [53], observational [54], and randomized trial designs [55], which included 95 individuals undergoing active Quinacrine treatment, there were no significant clinical effects. Of note, the study populations were significantly different among studies: one included sCJD patients ($n=23$) [55], another both sCJD ($n=30$) and vCJD ($n=2$) cases [53], whereas the last included patients of various etiologies (sporadic, iatrogenic, inherited, and vCJD) [54]. Some authors attributed the inefficacy of Quinacrine *in vivo* to the selection of drug-resistant prions after continuous treatment exposure [56].

After the Quinacrine trial failures, Doxycycline, a tetracycline-class antibiotic commonly used to treat bacteria and parasite infections, received rising scientific attention. Doxycycline acts as an anti-prion drug by binding PrP^{Sc} conformers, hindering their assembly into amyloid fibrils, and sensitizing their proteolytic degradation [57,58]. In scrapie-infected (263K) transgenic mice, Doxycycline was effective in prolonging the incubation time independently from the route of administration [59]. In humans, a randomized controlled trial conducted in France and Italy evaluated the use of Doxycycline in 121 individuals affected by sCJD, iCJD, vCJD, and gCJD (62 included in the treatment arm, and 59 in the placebo group) [60]. The trial was interrupted ahead of schedule after an interim analysis revealed no superiority of Doxycycline compared to the placebo. In 2018, a combined analysis including a group of 12 patients (7 under treatment and 5 controls) undergoing a randomized, double-blind controlled trial and a separate group of 88 sCJD cases (55 treated with Doxycycline and 33 controls) revealed a slightly statistically significant prolongation of survival [61]. This effect was more pronounced in subjects carrying the genotype MM at *PRNP* codon 129 [61]. A clinical trial with Doxycycline (DOXIFF) involving asymptomatic individuals that bear the *PRNP* D178N-129M haplotype linked to FFI is ongoing in Italy [62]. The first results are expected by the end of 2023. Mutation carriers will receive doxycycline 100 mg/day (200 mg/day since May 2019) over a 10 year-period [63]. The trial represents the first attempt to assess the feasibility of preventive treatment in asymptomatic individuals with a high risk of developing prion disease.

1.3. Promising treatments

1.3.1. Immunotherapy and the development of anti-PrP antibodies

Immunotherapy represents a promising approach for the treatment of a variety of neurological disorders, including neurodegenerative diseases. Accordingly, in the last decade, enormous efforts have been made to develop immunotherapies for Alzheimer's (AD) and Parkinson's (PD) related proteinopathies [64,65].

In the prion field, the phenomenon of self-tolerance, determined by the widespread PrP^C expression in tissues and organs, hindered the application of active immunotherapy (i.e. the production of anti-PrP antibodies by the host following vaccination) [66]. Nonetheless, a wide range of small molecules, including truncated or modified recombinant PrP peptides, PrP dimers, and heterologous PrP peptides, have been tested in vitro for their ability to elicit an anti-PrP^C immunity [67], with contrasting results [68–70]. Even if some studies documented a delayed symptom onset in vaccinated mice, the overall effect of active immunization was marginal.

Given the difficulty of developing vaccination strategies breaking the self-tolerance efficiently, researchers pursued passive immunization (i.e. administering a pre-made antibody able to block or modulate a specific target). However, the development of antibodies that selectively bind PrP^{Sc} proved

challenging. Therefore, most researchers focused on targeting PrP^C. Indeed, PrP^{Sc} requires PrP^C for its propagation [71], and limiting the conversion of PrP^C into PrP^{Sc} prevents neurotoxicity [72].

In the first study of this kind, conducted in the mid-80s, a polyclonal rabbit PrP antiserum raised against PrP²⁷⁻³⁰ reduced prion infectivity *in vitro* [73,74]. Later, the development of several monoclonal antibodies by immunization of homozygous PrP^C knock-out mice [8] allowed the targeting of various epitopes and the search for the most favorable efficacy-side effect profile. Transgenic expression of the Mu heavy chain of anti-PrP^C antibody 6H4 provided the first evidence of neuroprotection against prions *in vivo* [75]. The analysis of the efficacy-side effect profile of the anti-prion antibodies in prion-infected cellular models and, to a lesser extent, in prion-infected mice showed a significant association with the target epitope [76]. Antibodies targeting the globular domain of PrP^C (POM1, D18, ICSM18) reduced PrP^{Sc} formation [75,77], but also induced intracellular toxicity [78,79], likely by disrupting the intramolecular docking between N- and C-terminal domains of PrP^C [80]. Specifically, a recent study revealed that the formation of an intramolecular R208-H140 hydrogen bond ('H-latch') that alters the flexibility of the α 2- α 3 and β 2- α 2 loops of PrP^C mediates the neurotoxic effect of these antibodies [81]. Accordingly, abolishing the H-latch formation confers resistance to POM1 toxicity [81].

In contrast, antibodies raised against the flexible tail of PrP^C, including those targeting the octapeptide repeat region, did not show significant toxicity. Yet, most of these antibodies conferred neuroprotection against prions [66,79]. As a significant exception, 4H11, an anti-PrP^C antibody targeting the octapeptide repeat region, caused neuronal and glial toxicity associated with behavioral deficits in BSE-infected mice with no therapeutic effects [82]. Moreover, the safety profile of the ICSM18 antibody, targeting the PrP^C α 1 region in the globular domain, thus expected to be neurotoxic, has been the object of intense debate. The antibody effectively reduced PrP^{Sc} levels and prion infectivity in a mouse model [83]. However, while the intracerebral stereotactic injection of 2 μ g of ICSM18 was well tolerated [84], a dose escalation up to 6 μ g showed a dose-dependent neurotoxic effect with an estimated 3.1 μ g for the upper limit of the ICSM18 intracerebrally injected safe dose [79]. These data illustrate that anti-prion antibodies' efficacy and toxicity profiles are complex and likely depend on multiple intrinsic (e.g. targeted epitope) and extrinsic (e.g. route of administration, dosage) factors.

Nonetheless, the impressive therapeutic effect of ICSM18 in mice infected by intraperitoneal injection of RML prions with a survival time >500 days with no clinical sign of disease (N.B. when the administration started after 7 or 30 days after injection) [83], prompted the first human trial using the humanized version of ICSM-18, PRN100. The study included six patients (5 sCJD and one iCJD) who received PRN100 through repeated intravenous administrations [85]. PRN100 was not effective in modifying the clinical trajectory of the disease. Still, in 1 out of 2 cases undergoing postmortem brain examination (i.e. the only iCJD case), neuropathologic examination suggested that treatment could clear disease-associated PrP. Remarkably, no treatment-related clinical side effects were reported, and no evidence of subclinical neurotoxicity was noted at the

neuropathologic examination, although PRN100 reached therapeutic levels only in 4 out of 6 individuals. Considering the small number of patients included, these are encouraging results and highlight the feasibility of antibody-based clinical trials for CJD. However, it should be noted that in mice, ICSM18 was ineffective in blocking prion disease when the administration started after clinical onset [84]: this could at least partially explain the preliminary observations in CJD patients. Given these findings, the late administration during the symptomatic phase should also be considered a possible cause of the negative outcome of previous trials with other drugs (e.g. Doxycycline, Quinacrine, etc.).

1.3.2. Therapeutic gene modulation

Several lines of evidence suggest that lowering the PrP^C levels could represent an effective disease-modifying therapy. Indeed, the conversion of PrP^C into PrP^{Sc} is the critical molecular event for disease development and propagation after infection, as demonstrated by conditional and constitutive knock-out models [72,86,87].

Gene therapy in prion disease may be especially suitable for patients or presymptomatic individuals carrying heterozygous pathogenic *PRNP* mutations with variable penetrance associated with gCJD, FFI, GSS, and PrP-CAA with or without systemic amyloidosis phenotypes, which is supported by the successful development of genetic modulation for other genetic neurodegenerative disorders [88,89]. Although no trials adopting therapeutic gene modulation for prion disease are ongoing, a wealth of evidence has been collected to answer the following preliminary questions: 1) can humans tolerate PrP^C lowering without relevant side effects? 2) What is the best modality to lower PrP^C expression?

Abolishing PrP^C expression in mice, goats, and cattle did not induce CNS pathology or a clinical phenotype indicating abnormalities of CNS functions [90-92] but showed an effect on peripheral myelin maintenance [93,94]. The reduction of PrP^C was linked to a late onset peripheral neuropathy, demyelinating type, in homozygous knock-out mice (*Prnp*^{-/-}) but not in heterozygous (*Prnp*^{+/-}) [93]. In humans, research has focused on loss-of-function genetic variants. Interestingly, the finding in apparently healthy mid-age adults of heterozygous loss of function truncating mutation localized in the N-terminal region (before codon 131) indicates that a 50% reduction of PrP^C expression might be well-tolerated [95].

Additionally, inhibitory drugs could successfully target loss-of-function variants involving essential genes [96], indicating that lowering PrP^C expression represents a potential therapeutic approach even if *PRNP* loss-of-function has harmful effects.

Remarkably, although the minimal effective PrP^C lowering necessary to accomplish clinical outcomes remains elusive, *in vitro* findings of dose-dependent efficacy were recently replicated *in vivo*. Indeed, a study reported that a transient pharmacologic 21% PrP^C knockdown (i.e. treatment administered twice at -14 and 76 days post-inoculation during the presymptomatic phase) is sufficient to extend survival in RML prion-infected mice [97]. This finding aligns with evidence that a 50% reduction of PrP^C expression in *Prnp*^{+/-} mice are beneficial against prion infection.

Currently, there is no univocal therapeutic method to lower the PrP^C levels. The approaches investigated include RNA interference [98], the use of small molecules identified on the surface of human glioblastoma (T98G) and neuroblastoma (IMR32) cells [99], adeno-associated virus vector type 2 encoding a short hairpin RNA targeting Prnp mRNA (AAV2-PrP-shRNA) [100], and antisense oligonucleotides (ASOs) targeting PrP RNA [101]. In recent years, different ASOs have been designed and extensively studied [97,102,103]. Researchers found that intracerebroventricular administration of PrP-lowering ASOs extends survival by 61–98% in RML prion-infected mice [102]. Treatment efficacy was strictly dependent on the ability of specific ASOs to lower PrP^C expression (i.e. aptameric interaction between ASOs and PrP was ineffective) as evaluated by CSF PrP concentrations. Even a single administered dose close to the clinical disease onset, or even after the appearance of neuropathologic change, showed a benefit [102]. A subsequent study reported the efficacy of ASOs against four additional strains at different time points, even during the symptomatic phase of the disease [97]. However, the administration of some ASOs induced a subacute decline requiring euthanasia in a subgroup of mice with established prion neuropathology [97,101,102]. The finding is apparently uncoupled from PrP-lowering since it was also observed with ASOs not targeting PrP RNA [103]. Before evaluating the translation of ASO-based therapy in human trials, the mechanisms underlying this phenomenon must be clarified and prevented.

1.4. Open issues, challenges, and possible solutions

1.4.1. Low disease incidence and the design of well-powered clinical trials

An annual incidence of approximately 2 cases per million people makes prion disease an exceedingly rare condition [95,104]. Besides, the prevalence differs according to the etiology and phenotype. Finally, prion disease is a phenotypically heterogeneous disorder, including several disease subtypes. All these features prevent the inclusion of sizable homogeneous patient cohorts in clinical trials [76]. To date, all therapeutic trials, mainly designed as single-center studies and including small patient cohorts (<50 individuals), have focused on symptomatic individuals diagnosed with prion disease. In such a context, an early and accurate diagnosis would be pivotal since diagnostic delay could be primarily responsible for the failure of anti-prion therapies. Indeed, several studies demonstrated that the effectiveness of therapies increases with early treatment. However, despite the significant advances in the development and validation of diagnostic tests, the diagnosis of prion disease is still reached with significant delay attended by severe brain damage. To overcome this limit, identifying groups at increased risk of developing the disease and eventually close to the clinical onset would be an option for future trials. However, no specific factors are known to predict with a high level of certainty the age of disease onset [105,106], although etiology and genetic host factors are known to play a role in the age distribution of clinical onset [3,5,107], and lifetime disease risk [95]. The recent development of gene modulation therapeutic strategies has raised interest in asymptomatic *PRNP* mutation carriers. A recent international collaborative study including 1094 individuals with highly penetrant *PRNP* mutations (i.e. E200K, D178N, P102L) found a broad and not predictable variability in age at onset [106]. Based

on the age-dependent hazards, it has been estimated that randomized preventive trials would require hundreds (or even thousands) of at-risk individuals to be statistically powered for an endpoint of clinical onset [106]. Given the exceedingly high number of required individuals for a feasible preventive trial, post-marketing trials possibly using historical controls appear to be the best option [106].

1.4.2. Heterogeneity of disease (strains, subtypes, and mixed phenotypes)

Prion disease includes a highly heterogeneous spectrum of phenotypes determined at the molecular level by different PrP^{Sc} conformers and host genotype variability. Focusing on sCJD, the most common prion disorder, the current classification recognizes six clinicopathological subtypes (MM1/MV1, VV2, MV2K, MM2C, MM2T, VV1) [3,24]. Five of them behaved as prion strains after transmission to syngeneic hosts, producing a distinctive phenotype [108]. Evidence suggests that the same human prion strains are also responsible for genetic and iatrogenic CJD forms [2]. Interestingly, the combination of two or more subtypes co-occurs within the same brain in about one-third of sCJD cases [4]. However, most demonstrate a predominant subtype since the non-dominant one usually shows a focal (regional) distribution [109].

Identifying these subtypes accurately *in vivo* has multiple implications. Firstly, they are biologically distinct disorders that accumulate structurally different isoforms of PrP^{Sc}, and consequently, treatments selectively targeting PrP^{Sc} (e.g. anti-PrP^{Sc} antibodies) should account for this conformational heterogeneity. Secondly, sCJD subtypes have different clinical trajectories, including a remarkable variability of disease duration that largely depends on PrP^{Sc} regional distribution, speed of replication and spreading, and neurotoxicity of aggregates. Similar considerations can be extended to other rarer prion disease (i.e. VPSPr, GSS) that are also characterized by significant clinicopathological heterogeneity. Therefore, future clinical trials should consider phenotypic diversity for patient selection and outcome assessments.

1.4.3. Identification of the presymptomatic phase, prediction of clinical conversion, and early diagnosis

Two possible recruitment scenarios are predictable for patient enrollment into future clinical trials: the inclusion of symptomatic or presymptomatic (at-risk) individuals.

Given the rapid clinical progression of prion disorders, a requisite for clinical trials including symptomatic individuals is the possibility of reaching an early and accurate diagnosis. In this regard, the development and the introduction in clinical practice of ultrasensitive seeding assays, such as the real-time quaking-induced conversion (RT-QuIC) assay and the protein misfolded amplification assay (PMCA), represented a breakthrough for the diagnostic work-up of these disorders [110]. RT-QuIC is particularly useful for diagnosing sCJD and PMCA of vCJD, while genetic testing remains the gold standard for inherited forms. A recent meta-analysis including cases with definite, probable, and possible CJD reported a pooled 94% accuracy (91% sensitivity and 97% specificity) of prion RT-QuIC [111]. However, the meta-

analysis has the limitation of not considering factors such as the CJD subtype, the reaction protocol adopted, and the tissue/biofluid analyzed that substantially influence RT-QuIC sensitivity [112]. Recent studies involving surveillance centers from Europe and the US demonstrated that the introduction of RT-QuIC in the diagnostic criteria for sCJD significantly improved diagnostic accuracy [113–115], even at the time of the first neurological evaluation [116].

In two different studies, cerebrospinal fluid (CSF) RT-QuIC has demonstrated effective detection of prion seeding activity in a few presymptomatic E200K carriers (3 out of 28) and a single carrier of the P102L mutation (out of 23 tested) [117,118]. By contrast, there was no seeding activity in 6-OPRI, A117V, and D178N carriers. Interestingly, two of the three E200K carriers remained asymptomatic after two and three years of follow-up, revealing that CSF RT-QuIC could show prion seeding activity long before conversion to the symptomatic stage. Together, these findings confirmed the effectiveness of CSF RT-QuIC in gCJD [114,119–125], and in a lesser extent in GSS, and provided further insights for its potential uses in trials for presymptomatic individuals. In summary, the assay showed an overall low and variable sensitivity during the presymptomatic stage, being *PRNP* mutations at least in part responsible for such variability. Secondly, CSF RT-QuIC potentially detects prion seeding activity in the presymptomatic phase but within a large time frame before conversion to the symptomatic stage.

Other fluid biomarkers, including total-tau, neurofilament light chain (NfL), glial fibrillar acid protein (GFAP), and ubiquitin C-terminal hydrolase L1, have also been investigated in both CSF and plasma for their ability to predict the conversion from the presymptomatic to the symptomatic stage [113,114]. Among them, plasma NfL levels increase abruptly close to clinical conversion, especially in genetic prion disorders showing a fast disease progression (gCJD-E200K and FFI). In contrast, a more progressive (linear) elevation of both NfL and GFAP levels starting more than two years before phenocconversion has been observed in P102L-associated disease [118]. The finding of increased levels of NfL in prion-inoculated *Prnp* wild-type mice as early as 60 days post-inoculation also supports its use since the presymptomatic phase [97].

Evidence also supports the use of neuroimaging (i.e. brain magnetic resonance imaging, MRI, cerebral positron emission tomography with 18F-fluorodeoxyglucose) [126–128], or neuropsychologic testing [129] as proximity markers heralding the conversion to the symptomatic stage. However, they could be more helpful in identifying the early symptomatic stage rather than predicting conversion.

1.4.4. Definition of standardized outcomes

Defining and accurately measuring the outcomes is critical to any clinical trial. Identifying reliable and standardized evaluation strategies is mandatory to determine the efficacy of a specific treatment and promote reliability and comparison between trials. To date, the use of different outcome measures between studies, except for survival, has significantly prevented comparing drug compounds for CJD treatment in historical trials.

As a general rule, given the heterogeneity of prion disease, composite outcomes should be preferred to a single measure.

Acknowledging the disease's lethality, all trials investigated the survival time as the primary outcome. In this regard, researchers should be aware that the sCJD subtype influences disease duration (e.g. the natural disease duration is three months in sCJD MM1 vs. 18 months in sCJD MV2K) [3]. Therefore, survival assessment must be coupled with the subtype (neuropathological) definition. Additionally, trials should consider factors that may influence survival, such as enteral feeding [130]. In this regard, to consider the time from the clinical onset to akinetic mutism could be an alternative option. Although less standardized and relevant for individuals' prognoses, the severity of neuropathologic change compared to that described in historical cohorts could be an additional measure of treatment efficacy. Similarly, postmortem brain tissues can be analyzed for the amount of PrP^{Sc} accumulation by either neuropathology or western blot.

Quantifying disease-associated molecules in biofluid could help clinicians monitor treatment *in vivo*. CSF PrP concentration is stable in presymptomatic *PRNP* mutation carriers [117], and therefore represents a candidate biomarker for PrP lowering therapies, such as ASOs. Drug trials in other neurodegenerative diseases, such as Tofersen in amyotrophic lateral sclerosis [131], evaluated plasma NfL as a secondary endpoint to assess treatment efficacy. Similarly, monitoring NfL levels in plasma could be helpful in trials involving CJD patients since they are associated with survival [132,133].

Putaminal diffusion tensor MRI has also been proposed as a biomarker of disease severity. The decrease in putamen radial diffusivity predicted clinical worsening as defined by the Medical Research Council (MRC) scale [134]. However, compared with blood biomarkers, performing serial brain MRI has limitations related to availability in rural hospitals, costs, administration in poorly cooperative patients, and greater complexity in analyzing the results that could prevent its application in large patient cohorts.

Finally, standardized scales and questionnaires are broadly used to evaluate trial outcomes. In the past, studies included a variety of scores on cognitive and functional tests, such as the Barthel index, the Mini-Mental State Examination, the modified Rankin score, and so on, that are tools commonly used in neurologic clinical practice but are not specific for the assessment of CJD patients. In 2013, Thompson and colleagues developed and validated the MRC prion disease rating scale, a particular outcome measure for prion disease therapeutic trials [135]. The scale has been drawn in English and currently needs translation and validation in non-English speaking countries. A further criticism of the MRC prion disease rating scale is that it measures functional impairment without focusing on the 'type' of deficits contributing to it. To overcome this limit, the same research group recently developed two novel scales, the sCJD Motor and Cognitive scales, allowing them to quantify how these functional domains are selectively impaired [136].

2. Conclusion

Human prion diseases are a heterogeneous group of disorders related to PrP misfolding, representing the prototype of

neurodegenerative diseases caused by protein misfolding, amyloidogenic aggregation, and transcellular propagation by seeding-induced conversion. Currently, without effective pharmacotherapies, PrP^C represents the main potential target of therapeutic interventions to slow PrP^C conversion into PrP^{Sc} and the diffusion of the disease to different brain regions. The strategies to target PrP^C include passive immunization with anti-PrP antibodies and lowering PrP^C expression by ASOs administration. Both approaches have shown promising results in animal models by prolonging survival. In a recent clinical trial involving six symptomatic CJD patients, an anti-PrP antibody was well-tolerated and showed no toxicity signs. The treatment was ineffective in modifying the disease's clinical trajectory, although neuropathologic examination suggested a possible positive effect on PrP^{Sc} clearing from the brain in one case. The main therapeutic challenge for most patients with prion disease is initiating treatment as soon as possible after the onset of symptoms in sporadic human prion disease before developing severe, irreversible brain damage. Thus, the most promising realistic scenario currently involves presymptomatic individuals carrying heterozygous pathogenic *PRNP* mutations in whom proximity biomarkers identify the disease onset in the pre-clinical stage.

3. Expert opinion

The study of prion diseases has provided fundamental insight into the pathogenesis of neurodegenerative disorders. The rapid propagation, the development of infectivity in most cases, and the prominent neurotoxicity of misfolded PrP have made the prion models more informative than those involving other proteinopathies. However, when it comes to therapy, many of these peculiar features of prion diseases represent challenges rather than advantages. Therefore, it is likely that the development of effective treatments for these disorders will follow the success obtained with other more prevalent and slowly progressive conditions such as AD and PD.

Given their pivotal and isolated role in disease pathogenesis, PrP^C and PrP^{Sc} represent the most logical and promising currently identified potential therapeutic target candidates for prion disease. The gene *STX6*, a risk variant recently identified in a genome-wide association study of sCJD [137], is the only possible exception. Indeed, the results of a study showing a modest positive effect on survival in experimentally infected mice with knockout of *Stx6* expression seem to support further exploration of *STX6* as a potential therapeutic target for prion disease and, possibly, other neurodegenerative disorders [138].

In the last 30 years, scientists tested several chemical compounds for their possible anti-prion effect in cell cultures and animal models of prion disease. However, only a few reached a trial in patients affected by prion disease, and all of them eventually failed to demonstrate a significant clinical effect. As for other neurodegenerative disorders, current strategies to deplete the PrP^C substrate or prevent recruitment and contact between PrP^C and PrP^{Sc} include immunization and gene therapy with ASOs against PrP mRNA. Regarding immunotherapy, developing vaccination

strategies that break the self-tolerance efficiently or antibodies that selectively bind PrP^{Sc} proved challenging. Following the demonstration of the efficacy of anti-PrP^C antibodies in reducing PrP^{Sc} levels in prion-infected cellular and mice models and extensive efforts to understand the structural basis of the neurotoxicity shown by some of them, anti-PrP^C antibodies have been recently administered to humans for the first time.

Several lines of evidence suggest that lowering PrP^C expression levels through the administration of ASOs targeting PrP mRNA could also represent an effective disease-modifying therapy. Gene therapy to lower PrP^C expression may be especially suitable for patients or presymptomatic individuals carrying heterozygous pathogenic *PRNP* mutations with variable penetrance. The results of preliminary studies in genetically determined neurodegenerative disorders, such as amyotrophic lateral sclerosis, spinal muscular atrophy and Huntington's disease, are promising for developing further ASOs, or other PrP-lowering therapies and the eventual translation to trials treating human prion disease.

Unfortunately, the low incidence of disease and the scarcity of factors predicting the clinical onset limit the design of randomized controlled (pre-approval) trials for both sporadic and genetic prion disorders. The inclusion of highly penetrant *PRNP* mutation carriers in post-marketing studies is, to date, the most promising approach to reduce the sample size and develop well-powered clinical trials in the field. Nonetheless, multicentric and long-lasting recruitments would be necessary to reach the required sample size. Monitoring blood markers of neuroaxonal damage (NfL), astroglial activation (GFAP), and CSF prion seeds by RT-QuIC are feasible and promising strategies to identify the conversion to the symptomatic stage in presymptomatic *PRNP* mutation carriers.

Future clinical trials dealing with the sporadic disease must also face the challenge of disease heterogeneity for patient selection and outcome assessments. Along this line, models are needed to predict disease duration and subtype *in vivo* based on demographic, laboratory, genetic, and clinical findings [139,140]. The application of these models will help physicians decide on patient eligibility and stratification. Future clinical trials should also include, besides survival time, composite outcomes accounting for disease progression and severity through functional (clinical) and biological (laboratory) measures. Clinical assessment must consist of standardized evaluation scales specifically developed for CJD, at least for the prevalent subtypes. At present, blood NfL is the most promising candidate biomarker for monitoring drug effects and disease progression.

List of abbreviations

CJD	Creutzfeldt-Jakob disease
PrP	prion protein
sCJD	sporadic Creutzfeldt-Jakob disease
GSS	Gerstmann – Sträussler – Scheinker disease
FFI	Fatal Familial Insomnia
VPSPr	Variably Protease-Sensitive Prionopathy
CAA	cerebral amyloid angiopathy
PK	proteinase K
BSE	bovine spongiform encephalopathy
GPI	glycophosphatidylinositol

PrP ^C	cellular prion protein
iCJD	iatrogenic CJD
vCJD	variant CJD
ER	endoplasmic reticulum
UPS	ubiquitin-proteasome quality control
UPR	unfolded protein response
CNS	central nervous system
PPS	pentosan polysulphate
AD	Alzheimer's disease
PD	Parkinson's disease
ASOs	antisense oligonucleotides
RT-QuIC	real-time quaking-induced conversion
PMCA	protein misfolding cyclic amplification
CSF	Cerebrospinal fluid
NfL	neurofilament light chain
GFAP	glial fibrillar acid protein
MRI	magnetic imaging
MRC	Medical Research Council

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