Rationale for early therapeutic intervention in genetic prion disease

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Abstract

Prion disease is a fatal, incurable neurodegenerative disease caused by a conformational change in the prion protein (PrP). Reducing brain PrP expression is a well-supported therapeutic strategy, and may be achievable in humans using intrathecally delivered antisense oligonucleotides (ASOs). Preclinical evidence indicates that some therapies may be more effective, or may only be effective, before symptom onset. Predictive genetic testing provides an opportunity to intervene early and preserve full quality of life in healthy individuals with prion protein gene (*PRNP*) mutations. Here, we report four advances that simultaneously strengthen the rationale for clinical trials in these pre-symptomatic individuals, and provide key data to enable such trials.

First, by comparing allele frequencies in prion disease cases and population controls, we quantify *PRNP* variant penetrance and determine which individuals are at high lifetime risk — a potential patient population for trials. We also characterize healthy humans with heterozygous *PRNP* loss-of-function variants, supporting the safety of PrP-lowering strategies.

Second, we characterize age of onset in genetic prion disease, and show that randomization to a disease endpoint is infeasible. This motivates development of biomarkers as surrogate endpoints, and our natural history dataset could aid in long-term confirmation of clinical benefit.

Third, we develop a method for quantifying cerebrospinal fluid (CSF) PrP by mass spectrometry. CSF PrP decreases in active prion disease, potentially confounding any pharmacodynamic

readout for a PrP-lowering drug in symptomatic patients, thus further motivating pre-symptomatic trials. Accordance between mass spectrometry and immunoassay results builds confidence in CSF PrP as an analyte, supporting its use as a biomarker.

Fourth, we assess the efficacy of PrP-lowering ASOs against established brain prion infection in mice. We observe efficacy even at timepoints with detectable neuropathology and near the onset of obvious symptoms, encouraging further development of this modality. We also find that efficacy is maximized when treatment is initiated earlier, emphasizing the need to establish a clinical pathway for pre-symptomatic trials.

Our findings help to establish both the need for, and feasibility of, early therapeutic intervention in genetic prion disease.

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Acknowledgments

2,661 days

The worst day of my life was October 8, 2011. On that day, I learned that my soulmate, Sonia Vallabh, was at risk for inheriting the dominant genetic prion disease mutation that had taken her mother's life. The next day, I looked up dominant inheritance on Wikipedia. I hadn't studied biology since high school. Sonia was scarcely better informed than I was.

We finally learned, on December 9, 2011, through predictive genetic testing, that Sonia had indeed inherited the mutation. That was day 0, the day things started to get better. To both of our immense surprise, we began re-training as scientists and made it our life's work to develop a drug to prevent Sonia's disease.

Seven and a half years on, we will receive our PhDs in Biological and Biomedical Sciences. We are now scientists, and we live the dream of working every day to develop a drug to prevent Sonia's disease. The journey to here has been hard. Very hard, harder than the me of 2010 knew it was possible for anything to be. Not necessarily in the ways we expected. Our impatience is our greatest asset, yet biology always, always demands patience. Our awareness of the big picture — getting a safe and effective drug into humans — is our guiding star, yet biology insists on being details, a giant fractal ocean of individually unsatisfying small pictures. There is relief in focus, total immersion in one problem and forgetfulness that other problems exist, yet biology has its own immovable pace, and so demands distraction, as it is only through parallel progress on many fronts that a problem can be compressed down to — hopefully — one lifetime. Only occasionally, when we tell our whole story from beginning to end, we can catch a glimpse of a reconciliation between the days — 2,661 of them and counting, none of which has

yet concluded with a tidy and satisfying nugget of scientific progress — and the years, over which somehow, despite the days, we have climbed partway up a once-unscalable mountain. In the process, we have been called upon, again and again, to find within ourselves sources of patience, positivity, and sanity that we didn't know we had. A reward for this has been getting to see the person who I already loved so deeply for the incredible whole of what she is truly capable of.

In these 2,661 days, as our dissertations describe, we have revised our understanding of the genetics of prion disease, have launched a patient registry and a clinical research study, have developed a biomarker for use in clinical trials and engaged with regulators about a pathway to prevention, and have catapulted a rational targeted therapy into preclinical development. We can now imagine, not just in an imaginary way, but in a concrete, this is what most of the details could look like kind of way, what it may mean to prevent genetic prion disease.

This kind of progress does not happen in isolation. We are immensely, *immensely* grateful to have received support and mentorship of every imaginable kind, from every unexpected direction. What makes this support generous beyond belief is that we have never yet managed to be normal people making a normal ask. Every success has been thanks to people who took a chance on us, hearing our story and our passion and deciding to give us opportunities that simply unheard-of, or, at a minimum, for which we were, on paper, still underqualified.

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Many others deserve mention, and my efforts to make this list complete only make the inevitable omissions the more offensive, so apologies in advance for that.

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Chapter 1. Introduction

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Overview

Prion disease is a fatal and incurable neurodegenerative disease caused by conversion of the prion protein (PrP) into an autocatalytically self-replicating conformer called a prion. In humans, prion disease may be sporadic, genetic, or acquired, and may present in various subtypes known historically by names including Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Sträussler-Scheinker disease (GSS). Regardless, the molecular mechanism of the disease is singular, and relies absolutely on expression of PrP as the substrate for prion formation. There exist strong proofs of concept that reducing PrP expression should be both safe and effective as a therapeutic strategy in prion disease. To date, clinical trials have recruited symptomatic patients for testing of repurposed drugs without compelling preclinical evidence for efficacy. The rapid progression and complex differential diagnosis pose challenges for trials in this population, and preclinical proofs of concept suggest that therapeutics effective at delaying or preventing prion disease prophylactically may not be effective after disease onset. Lessons from preclinical drug discovery efforts indicate that an effective drug will need to distribute broadly across the brain, be effective independent of prion strain, and be tested clinically at a disease timepoint when it may reasonably be expected to be effective. In this thesis, we provide rationale, tools, and data to support testing of targeted, PrP-lowering therapeutics in pre-symptomatic individuals at risk for genetic prion disease, where there exists an opportunity to intervene early and preserve full quality of life.

Molecular mechanism of prion disease

Prion disease is a fatal neurodegenerative disease caused by misfolding of the prion protein (PrP), which is encoded by the gene *PRNP* in humans[1] (Figure 1.1A-B). Prion disease naturally afflicts a wide range of mammalian species in addition to humans, including sheep and goats[2], cattle[3], mink[4], deer and elk[5], and camels[6]. Prion disease is unique in that it can arise — in humans and in other animals[7–9] — by three different etiologies: sporadic (apparently due to spontaneous protein misfolding events), genetic (due to gain-of-function coding mutations in the *PRNP* gene), and acquired (due to exposure to exogenous prion infectivity). Prion disease is otherwise known by several historical names, including Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Sträussler-Scheinker disease (GSS) in humans. Regardless of species, etiology, and historical name, however, all cases of prion disease arise from a single molecular pathway: conversion of normal, properly folded cellular prion protein (PrP^{C}, Figure 1.1C) into a self-templating conformer known as the scrapie prion protein (PrP^{Sc}, or simply a prion, Figure 1.1D).

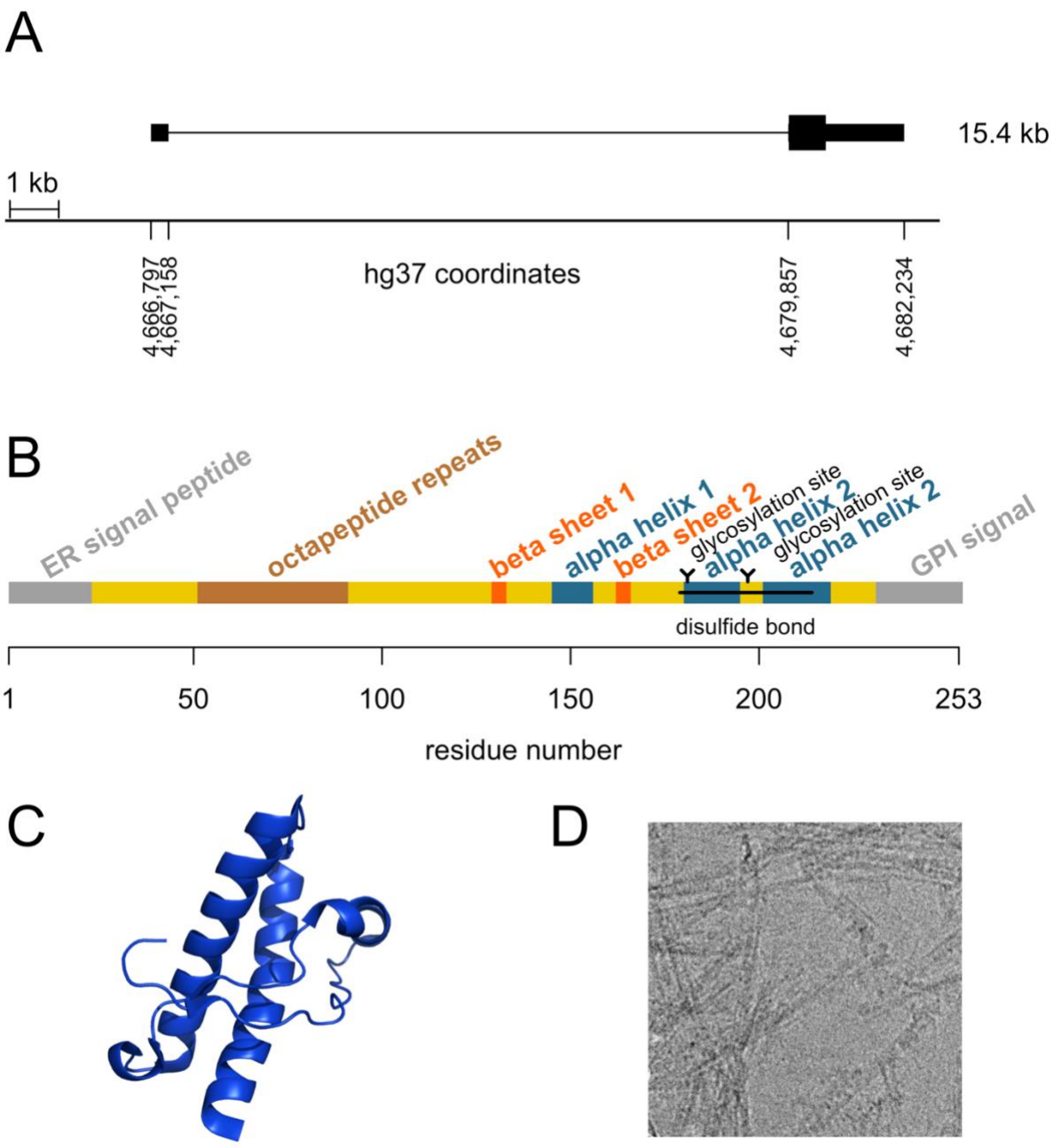


Figure 1.1* | *Prion protein gene, domains, and structure. A) PRNP *in humans is located on the short arm of chromosome 20 and consists of two exons, the first of which contains only 5'UTR sequence; the entire protein-coding region exists within exon 2.* ***B)*** *The open reading frame consists of 253 codons, which yields a 208-residue protein after post-translational modification.* ***C)*** *Structure of the properly folded globular C-terminal domain of human PrP, PDB #2W9E from Antonyuk et al*[10]*.* ***D)*** *Cryo-electron microscopy image of prion fibrils reproduced from Vázquez-Fernández et al*[11]*.*

PrP is a cell surface glycosylphosphatidylinositol (GPI)-anchored protein encoded by a simple gene structure (Figure 1.1A). In humans, it is encoded by 253 codons, with an ER signal peptide and GPI signal removed to yield a 208-residue mature protein (HuPrP23-230) with two variably N-linked glycosylation sites (N181 and N197) and one disulfide bond (C179-C214). The structure of bacterially expressed recombinant PrP has been solved by nuclear magnetic resonance (NMR) and X-ray crystallography[12,13]. The C-terminus contains three alpha helices and two beta sheets while the N-terminus is intrinsically unstructured. The structure of PrP^{Sc} has been accessible only at low resolution owing to its intrinsically aggregated nature[14]. Prions are known to come in different "strains" associated with distinct biochemical, symptomatic, and neuropathological properties[15]. Ample indirect evidence indicates that strains are encoded in distinct conformations of PrP^{Sc}, although how exactly those conformations differ remains unclear[16–18].

The native function of PrP is not clear. In the peripheral nervous system, it appears to undergo a native proteolytic event to release an N-terminal peptide with a signaling function related to myelin maintenance[19,20]. Although tens of knockout phenotypes have been asserted in the literature[21], only a mild, age-dependent demyelinating peripheral neuropathy is well-established[19,22,23], and no native function has yet been clarified in the central nervous system. Regardless, prion disease is caused by a gain of function, whereas loss of function appears well tolerated (see below).

Prion disease is naturally transmissible among many mammalian species. It is commonly modeled in mice by intracerebral (i.c.) inoculation of brain homogenate from terminally prion-sick mice into naïve wild-type mice[24]. This method produces fatal neurological symptoms after a highly predictable silent incubation period.

Prion disease in humans

Prion disease in humans is rare, but to avoid confusion as to just how rare, it is important to disambiguate the different metrics used. In terms of *incidence* (new cases presenting per year), prion disease affects 1-2 people per million population per year[25–27]. Countries that surveil prion disease more intensely, as measured by the number of referrals, observe statistics closer to 2 per million[26]. Dividing this number by the all-cause death rate (~1 per 100 annually[28]) yields a *lifetime risk* of ~1 in 5,000. In other words, prion disease is responsible for 1 in 5,000 deaths. This figure is supported by death certificate analyses[29].

Approximately 85% of human prion disease cases are classified as sporadic, a term given a special meaning in prion disease: not caused by any known genetic or environmental factor. The occurrence of sporadic prion disease is geographically and temporally random[30], and is suspected to originate from spontaneous, stochastic protein misfolding events[1]. ~15% of cases are considered genetic[31,32], meaning these individuals harbor rare variants in *PRNP*, which include highly penetrant (>90% lifetime risk) variants as well as some moderate risk and occasional benign variants (see Chapter 2). Prion disease may also be acquired[30,33] (<1% of cases in recent years[27]), with known routes of transmission in humans including ingestion of prions through contaminated beef (vCJD)[34] or ritualistic cannibalism (kuru)[35] and medical procedures involving contaminated human tissues or instruments, including cadaveric hormone infusions[36,37], transplants[38,39], neurosurgery[40], and blood transfusion[41].

The progression of prion disease is in most cases exceptionally rapid. Median survival for sporadic prion disease is 5 months from first symptom to death[42]. Most genetic prion disease is rapid as well, with duration <1 year, although ~25% of genetic cases harbor mutations classified as slowly progressive, with duration ranging from a few years to a few decades[42,43] (see Chapter

3). Age of onset is highly variable, peaking in one's 60s for sporadic prion disease[42] and in one's 50s or earlier for many forms of genetic prion disease[43–45]; it is not predicted by any known factor[43] (see Chapter 3).

Several criteria are used clinically in the diagnosis of prion disease[46,47], including clinical symptoms, neuroimaging and electrophysiology, and cerebrospinal fluid markers. Clinical symptoms used as diagnostic criteria include progressive dementia, myoclonus, visual or cerebellar disturbance, pyramidal or extrapyramidal dysfunction, or akinetic mutism[47]. These more informative symptoms are associated with fairly advanced disease; the first presenting symptoms can be exceptionally diverse and non-specific[48,49]. Electroencephalography (EEG)[50] and magnetic resonance imaging (MRI)[51,52] are often used. Cerebrospinal fluid markers include 14-3-3 protein[50,53], total tau[54,55], and real-time quaking-induced conversion (RT-QuIC)[56–59]. RT-QuIC, an *in vitro* assay for the presence of prion "seeds" in CSF capable of triggering fibrillization of recombinant PrP[56,57], is particularly sensitive and specific[60–63]. None of these tests performs particularly well in genetic prion disease[44,45,61,64–68]. In genetic subtypes, *PRNP* sequencing remains the only highly informative test, but may not always be pursued because many cases, even with highly penetrant variants, lack a positive family history[44,45]. Even in the most common subtype of prion disease, sporadic Creutzfeltdt-Jakob disease, differential diagnosis from other rapidly progressive dementias remains challenging[69], and prion disease diagnosis is only considered "definite" following autopsy or brain biopsy[46]. Retrospective studies of autopsy-confirmed cases indicate that most individuals were not diagnosed with prion disease until approximately two-thirds of the way through their disease course (time from first symptom to death)[69,70], and diagnostic tests for prion disease were not even ordered until a median of three months after the patient's first symptom[70]. Neurologists are often advised to prioritize treatable conditions in the differential diagnosis[71], suggesting that a first effective treatment for prion disease might be a key factor in enabling future earlier diagnosis.

In contrast to slowly progressive neurodegenerative disorders, such as Huntington disease and Alzheimer disease, where biomarkers of neuropathology can be detected decades in advance of symptoms[72–74], there is no known pathological prodrome in prion disease. Individuals with pathogenic *PRNP* mutations appear to be completely normal until the sudden onset of frank disease. Longitudinal MRI and ^{18}F fluorodeoxyglucose positron emission topography (FDG-PET) studies have reported changes in brain imaging features only ~1 year prior to symptom onset, and even then, these changes were so subtle as to be noticed only in hindsight[75–80]. Analysis of neuronal damage markers neurofilament light chain (NfL) and total tau (t-tau) in CSF and serum has been described in only one pre-symptomatic patient, with no clear evidence for elevation above the level seen in controls[64]. In slowly progressive prion disease associated with the P102L mutation, neurophysiological markers such as warm and cold threshold and H-reflex may provide early indications of phenotypic conversion, but do not pre-date symptom onset[81]. Prodromal markers could yet emerge with further study of pre-symptomatic individuals, but it is also possible that the accumulation of prions and of attendant neuropathology occur on a very rapid time scale, in keeping with the rapid progression after disease onset.

Predictive genetic testing for *PRNP* mutations is available, and should provide an opportunity for early therapeutic intervention to preserve full quality of life. Laying the groundwork to make good on this opportunity will be the major theme of this dissertation. At present, it appears that only approximately one quarter of those at risk pursue predictive testing[82], perhaps because people at risk are often counseled that the lack of an available treatment makes the results medically inactionable. In fact, genetic testing can be medically actionable for some individuals, as it facilitates the use of *in vitro* fertilization with pre-implantation genetic diagnosis to avoid transmitting mutant alleles to the next generation[83]. Moreover, the development of preventive

therapies may well depend upon research participation by individuals at risk, highlighting the need for increased engagement with this community.

PrP as the therapeutic target in prion disease

Decades of research in prion disease, with converging lines of evidence from diverse scientific approaches, have established conclusively that PrP is the cause of prion disease.

Biochemical purification approaches first identified PrP as the constituent protein comprising infectious prions extracted from brain tissue[84–86]. Conversely, prion infectivity can be created *de novo* using bacterially expressed recombinant PrP[87–89]. Different conformations of misfolded PrP are also now understood to encode the "strain" properties of prions[16–18]. There is evidence that lipid and/or polyanion co-factors bound by PrP may play a role in determining prion strain and in achieving high infectious titer in synthetic prion preparations[87,90–92], but no non-PrP proteins or nucleic acids have been identified as components of prions.

Reverse genetics studies (engineering genotype and then examining phenotype) in animals subsequently confirmed the centrality of PrP to prion disease. Knockout mice revealed that PrP expression is required for prion disease, prion propagation, and prion neurotoxicity[93–96]. Transgenics established that PrP amino acid sequence is the apparently sole determinant of the "species barrier" for prion transmission[97–99] and that PrP gene dosage controls the pace of prion replication and disease progression[100,101].

Forward genetics studies (ascertaining on a phenotype and then working to find the causal genotype) in animals and humans have likewise agreed that PrP is the cause of prion disease. Prion protein genotype was first linked to prion incubation time in mice[102,103] and then to

Mendelian forms of prion disease in humans[104,105]. All multiplex families with prion disease have since been traced to protein-coding variants in *PRNP*[106]. Conversely, certain common missense variants in *PRNP* have been identified as protective against prion disease in humans[107–109] and in sheep[110–113]. Genome-wide association studies have confirmed the association of *PRNP* genotype to prion disease risk[114,115].

The overwhelming evidence implicating PrP as the cause of prion disease suggests that any disease-modifying therapeutic for prion disease will need to, whether directly or indirectly, affect PrP. While other proteins have been nominated as potential drug targets in prion disease, none yet has any of the above lines of evidence in its favor. These non-PrP targets are supported by, at most, small changes in prion disease incubation time observed upon knockout or pharmacological inhibition in a mouse[116–119]. There has been some success in phenotypic screening for antiprion compounds (see below), suggesting that other targets, or at least, other targetable mechanisms, may exist which indirectly affect PrP. To date, however, no such mechanisms have been elucidated, so for a target-oriented drug discovery approach, PrP remains the only target.

Therapeutic hypotheses in prion disease

Excellent proofs of principle support the hypothesis that lowering PrP would be therapeutically effective against prion disease. PrP knockout confers total resistance to prion disease in mice[94–96], and PrP knockout brain tissue is exempt from prion disease-associated neuropathological sequelae even adjacent to prion-infected tissue grafted from a PrP-expressing mouse[93]. The relationship between PrP expression and the pace of prion propagation and disease progression is gene dosage-dependent across a wide range of expression levels (Figure 1.2). Likewise, the expression level of spontaneous disease-causing PrP transgenes in mice is

inversely correlated with age of onset[120,121]. Finally, conditional knockout or reduction of PrP expression is strongly protective, even after prion infection is well underway[122–124].

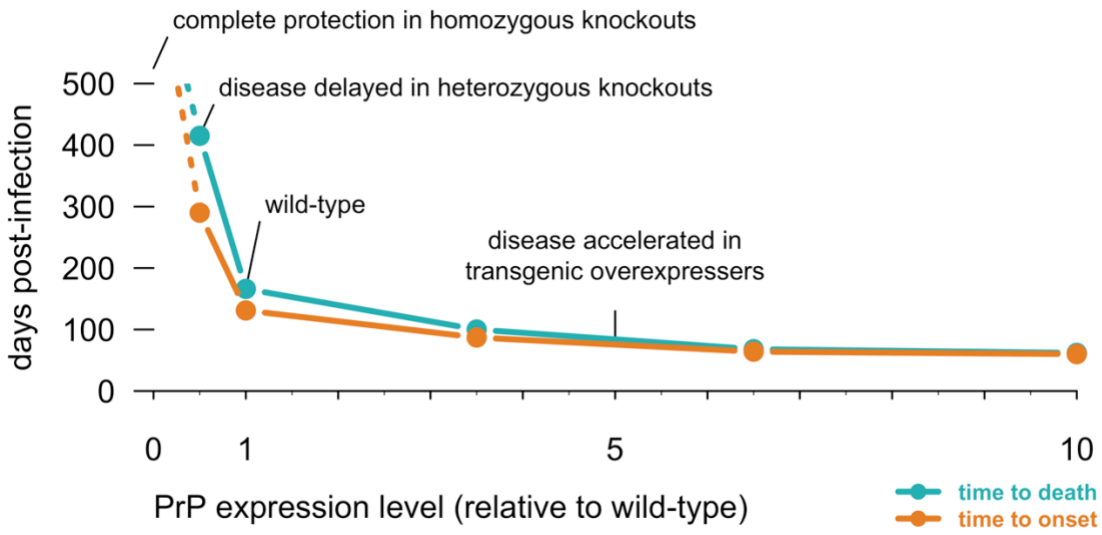


Figure 1.2 | The pace of prion disease is PrP gene dosage-dependent. *Time to disease onset and death in mice intracerebrally inoculated with the RML strain of prions. Adapted from Fischer et al*[101]*.*

Meanwhile, available evidence strongly supports the safety of reducing PrP expression as a therapeutic strategy. PrP knockout mice appear grossly normal[23,96,125], as do knockout cows[126] and both engineered[127] and naturally occurring[128] knockout goats. As with any well-studied gene, many knockout phenotypes have been claimed in the scientific literature, but most lack strong evidence and some have been refuted[21,129]. After 25 years of study, the only robustly demonstrated phenotype is a mild, age-dependent peripheral neuropathy, not present in the CNS nor in heterozygous knockout mice[19], apparently due to the deficiency of an N-terminal PrP fragment with a role as a signaling peptide that promotes myelin maintenance by receptor Adgrg6[20]. Healthy humans with one loss-of-function allele of *PRNP* have also been identified[32,130] (see Chapter 2). For broader context, it is worth noting that both essential and non-essential genes can make highly successful drug targets, and even a lethal knockout phenotype, or a total lack of loss-of-function alleles in humans, should not rule out a gene as a

potential drug target[130]. That said, the fact that *PRNP* is required for disease while also having such a mild knockout phenotype and no evidence for natural selection against loss-of-function in humans[130] is particularly encouraging.

A second strategy is to stabilize PrP so that it cannot misfold. From first principles, this should be effective, because conversion from PrP^{C} to PrP^{Sc} involves a profound conformational change[131–133]. Accordingly, "stapling" of PrP by introduction of non-native disulfide bonds antagonizes prion formation[134], and monoclonal antibodies to PrP^{C} clear prion infection in cultured cells[135,136], and antagonize peripheral prion infection in animals[137], possibly by locking PrP in its native conformation. These data come from cell culture and peripheral prion infection models, however, and the lack of any direct proofs of principle in the CNS makes this approach somewhat less well-supported than the concept of PrP lowering. Certain porphyrin compounds have also been reported to bind PrP and to have antiprion effects in cell culture and animals[138–141], however, these data are difficult to interpret because the binding event is not specific to PrP[142] and there is not yet evidence for a monovalent binding mode.

A third approach is to antagonize the accumulation of misfolded PrP. The proof of principle here comes from the *in vivo* efficacy of small molecules identified in phenotypic screening. Using mouse N2a neuroblastoma cells that propagate the RML strain of prions, together with protease digestion and immunodetection of protease-resistant PrP, it is possible to screen for compounds that reduce prion accumulation[143]. At least four series of small molecules identified as being active in such assays[144–147] have since gone on to exhibit unambiguous *in vivo* efficacy[144,148–150] (see below). The mechanisms of action are not known for any of these compounds, however[151], and all have proven ineffective against human prion strains[149,149,150,152], so it is unclear whether or not they actually provide a proof of principle that the same approach could work for human prions.

Overall, while therapeutic approaches to stabilize PrP or to antagonize its misfolding through indirect mechanisms remain worth pursuing, the strongest proof of principle exists for lowering PrP expression, and this is an area of research that should be prioritized.

Clinical trials in prion disease

A variety of approved drugs have been tested clinically in prion disease patients, usually in just a handful of cases on an off-label compassionate use basis[153]. Drugs that have been tested in a randomized trial or in a larger compassionate use case series are listed in Table 1.1.

Table 1.1 | Drugs tested clinically against prion disease.

drug	location	*N*	design	endpoint	ref
flupirtine	Germany	28	double-blind	ADAS-Cog	[154]
pentosan polysulfate	U.K.	7	open label	survival	[155]
pentosan polysulfate	Japan	11	open label	survival	[156]
quinacrine	France	32	open label	survival	[157]
quinacrine	U.K.	40	open label	survival	[158]
quinacrine	U.S.	51	double-blind	survival	[159]
doxycycline	France, Italy	121	double-blind	survival	[160]
doxycycline	Germany	55	open label	survival	[161]

All of these studies recruited symptomatic patients, mostly with sporadic prion disease, and followed them to a survival, cognitive, or functional endpoint. While these trials establish the feasibility of this approach, they also illustrate the serious challenges posed by the rarity, rapid progression, and difficult differential diagnosis of prion disease. In the U.S. quinacrine trial, for example, out of 425 patients referred over nearly four years, only 51 sporadic CJD patients were successfully randomized, and 19 (37%) died before the two-month follow-up visit[159]. At the time of enrollment, subjects were typically unable to function independently, with a mean Barthel index score of 60-65. In the French/Italian doxycycline trial, at the time of randomization, patients had already been sick for an average of 5 months, and approximately half had already entered a state of akinetic mutism and/or become incontinent or unable to eat[160]. It is important

to consider whether a therapeutic could plausibly still be effective at such a disease stage, and whether an increase in survival time at this level of impairment would constitute a benefit to patients or their families.

An important lesson from these clinical trials is that open label studies may give the false appearance of increased symptomatic survival time when treated patients are compared to historical controls. For both doxycycline and quinacrine, some open-label studies had suggested a substantial increase in survival time[159,161], yet subsequent randomized trials demonstrated no difference between drug and placebo[159,160]. A possible explanation is that next of kin are more likely to seek experimental treatment for patients who still retain relatively more cognitive and executive function, meaning individuals who are either progressing more slowly, were diagnosed earlier in disease, or both. More profoundly debilitated patients, who are progressing rapidly and/or were diagnosed later, are more likely to be moved into hospice immediately upon diagnosis, and less likely to enroll in clinical trials, yet still count among the historical controls to whom open-label trial patients are compared. This observation affects interpretation of the literature. For example, even though several prion disease patients who received pentosan polysulfate have had exceptionally long survival times from disease onset to death[162], one should not conclude from these data that the treatment was effective.

Of the drugs listed in Table 1.1, none had convincing evidence of *in vivo* efficacy to support advancement to human trials. Flupirtine was nominated for trials based solely on studies in cell culture models of Batten disease[163], amyloid beta toxicity[164], and toxicity of a PrP peptide[165]. Quinacrine was known to antagonize PrP^{Sc} accumulation in cell culture[166,167], but had already been shown ineffective in prion-infected mice[168]. One study reported efficacy in three cohorts of intracerebrally prion-infected mice treated with doxycycline[169], but the reported effect sizes were small (<11% increase in survival time) and all three reported *P* values were marginal (0.023 –

0.063) — both causes for skepticism[170,171] — and the findings were not replicated with a related compound[172]. Only pentosan polysulfate had clear evidence for efficacy in prion-infected mice[173], but it was only effective early in the incubation period, well before the onset of symptoms, whereas the humans treated with pentosan polysulfate were often profoundly debilitated by the time of treatment[155]. Based on this lack of preclinical justification, it is unsurprising that none of these trials observed a survival benefit.

Lessons from preclinical development of prion therapeutics

In addition to the handful of therapeutic candidates tested in the clinic (see above), over one hundred candidate therapeutic agents have been tested in various *in vitro*, cell culture, or animal systems intended to model prion disease. These have been reviewed exhaustively elsewhere[174,175]. While many were reported as positive results, the vast majority of these agents have never shown any convincing evidence for benefit in any credible model system. On the other hand, several agents do bring about clear and reproducible benefit in prion-infected animals, yet none have advanced to an approved drug. By examining the reasons why these candidates failed, it is possible to draw several lessons about the properties that a successful therapeutic must possess. These lessons are broadly summarized below.

1. Efficacy against peripheral prion infection does not imply efficacy against prions in the brain.

Because prions are transmissible agents, prion disease can be modeled in wild-type mice by inoculating infectious material, usually brain homogenate from terminally prion-sick animals, into naïve animals[24]. Studies most commonly use intracerebral inoculation, meaning freehand injection into the animal's brain, but some animal studies use various peripheral routes of

exposure, such as intraperitoneal or oral infection with prions. In multiple instances, therapies that were effective against peripherally acquired prion infection in mice were ineffective against intracerebral infection. For example, monoclonal antibodies to PrP delivered peripherally against a peripheral prion infection could completely prevent neuroinvasion and disease, yet were ineffective once prions had entered the brain[137]. Certain metallated porphyrins have likewise delayed the neuroinvasion of peripherally acquired prions, but have shown lesser or no efficacy against intracerebral inoculation[139,140].

The inefficacy of these treatments against brain-based infections may simply be due to pharmacokinetics. The size of antibodies and the size and charge of porphyrins make them unlikely to cross the blood-brain barrier in significant amounts, and meanwhile brain PrP expression is higher than peripheral PrP expression[176,177]. Together, these factors may make the stoichiometry of drug and target unfavorable. However, it is also possible that some fundamental biological difference in prion replication in the CNS versus the periphery contributes to the failure of these therapeutic approaches. Direct infusion of monoclonal antibodies into the mouse brain failed to produce convincing benefit, with nominally positive results not significant after multiple testing correction[178]. No proof-of-concept experiments (such as using transgenic mice to constitutively express anti-PrP antibodies in the brain) have yet addressed the question of whether antibodies to PrP would be effective against brain-based infections if the pharmacokinetic barrier could be overcome.

In recent history, some hundreds of humans have died of prion disease acquired through peripheral infection routes including ritualistic cannibalism (kuru), consumption of infected beef products (variant CJD) or iatrogenic exposure[33]. It is possible that peripherally infected mice do in fact accurately recapitulate the neuroinvasion process in such humans, and that these models would be a suitable way to test therapeutics for this population. However, these humans

were not aware of their infected status prior to the onset of symptoms, so it is not clear that there would have been any opportunity to intervene with a therapeutic prior to neuroinvasion if such a therapeutic had existed. Moreover, thanks to preventive measures put in place in response to these epidemics, acquired prion disease is now vanishingly rare[30]. Today, there are effectively only two patient populations with prion disease: those diagnosed after symptom onset (including sporadic and some genetic cases), and those with advance notice from predictive genetic testing (some genetic cases). In sporadic and genetic prion disease, prion infectivity is concentrated chiefly in the brain, with lower infectivity found variably in tissues proximate to the brain[179,180]. This suggests that the disease likely originates in the brain, leaving no opportunity to intercept disease in the periphery. Thus, for any prion therapeutic to be useful in humans, it will need to be effective against prion infection in the brain.

2. A therapeutic agent will need to reach most or all of the brain in order to be effective.

Prion disease is a disease of the whole brain. Although certain brain regions may be disproportionately affected in certain subtypes of the disease[181,182], misfolded PrP is found throughout the brain[183] and all neurological functions are impaired. Thus, in contrast to, for example, Parkinson's disease, where some therapies have been targeted specifically to the substantia nigra[184], there is no single brain region to which a prion disease drug could be targeted to meaningfully impact disease.

Some therapeutic approaches have been based upon sound therapeutic hypotheses but have suffered from poor distribution to or throughout the brain. For example, PrP lowering is an extremely strong therapeutic hypothesis, and RNA interference (RNAi) can be an effective means of lowering a target protein in cell culture or in certain human tissues, but there currently exists no method for broad delivery of RNAi agents to the brain. Several studies have examined

RNAi against the PrP gene as a therapy for prion disease, but without significant success. Two studies used peripherally administered lipid nanoparticle formulations that did not achieve sufficient brain uptake[185,186], and two studies used intraparenchymal injections of viral vectored RNAi, without significant distribution beyond the focal injection site[187,188]. Any survival benefit was marginal.

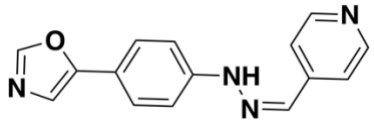
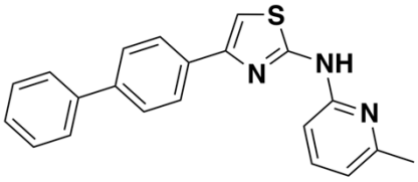
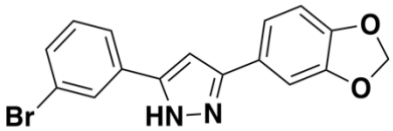
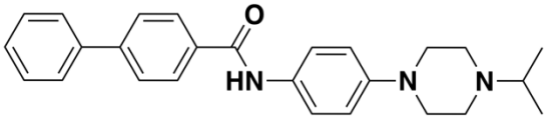
Brain distribution limitations might also have contributed to the failure of pentosan polysulfate as a prion therapeutic. Unlike RNAi or monoclonal antibodies, pentosan polysulfate showed a clear therapeutic benefit in intracerebrally infected animals, more than doubling survival time when administered early in prion infection[173]. As noted above, pentosan polysulfate was only effective in mice well before symptomatic stage of disease[173], whereas the individuals enrolled in human trials of the drug were symptomatic and often already profoundly debilitated[155]. This difference in the timepoint of treatment initiation in mice versus humans is sufficient to explain why there is no clear evidence for a therapeutic benefit of pentosan polysulfate in humans with prion disease[162]. However, it is also worth noting that pentosan polysulfate is a polar, high molecular weight substance for which no pharmacokinetic studies have examined distribution across brain parenchyma. Postmortem findings in prion disease patients treated with the drug have reported conflicting evidence as to how broadly, if at all, prion neuropathology was impacted across the brain[162,189,190]. It is possible that pentosan polysulfate diffuses only a limited distance into the parenchyma, sufficient to impact global neuropathology in a 0.5g mouse brain but insufficient for a >1kg human brain.

3. Treatments may be effective against some prion strains but not others.

Prions come in different “strains” associated with different neuropathologic and phenotypic outcomes[15]. While the structure of the prion has never been solved to atomic resolution for any

prion strain[14], an abundance of indirect evidence indicates that strain is encoded in the conformation of the misfolded protein[16–18]. Although prion strains have been a topic of intellectual fascination for decades, their significance for therapeutics has been appreciated only more recently. *In vivo* studies of cpd-b, the first small molecule to prove effective at extending survival in intracerebrally infected mice, demonstrated that efficacy was not necessarily equal among all prion strains tested[148]. This issue has since proven to be a roadblock for all otherwise promising small molecules that have shown efficacy in mice, none of which have proven effective against any human prion strain (Table 1.2).

Table 1.2 | Antiprion small molecules effective against mouse but not human prions.

name	structure	effective against mouse prions (RML)?	effective against human prions (MM1 sCJD)?
cpd-b		yes[148,191]	no[191]
IND24		yes[149]	no[149]
anle138b		yes[144]	no[152]
arylamide 1		yes[150]	no[150]

The molecular mechanism of action is not known for any of these small molecules[151], making it difficult to draw inferences about why their efficacy is strain-specific. Experiments with IND24 demonstrate that the problem is related to prion conformation, not to the primary structure (amino acid sequence) of PrP. Those experiments used transgenic mice (Tg1014) expressing a

mouse/human chimeric PrP molecule, which are capable of being infected with either mouse or human prions[192]. In that mouse model, IND24 was effective against mouse RML but not human MM1 sCJD prions, despite the amino acid sequence of PrP expressed in the mouse brain being the same[149].

In addition to the fact that prion strains exhibit differential drug sensitivity at baseline, there is also the problem that strains are apparently capable of developing drug resistance. Cell culture and animal studies have provided evidence that prions can evolve through conformational change[193,194], such that prion strains can develop resistance to chronic drug treatment[149,195–197]. Experiments with IND24 showed that some, though not all, prion strains can develop drug resistance to the compound *in vivo*, limiting its therapeutic efficacy[149,198]. When brain homogenate from mice infected with RML prions and treated with IND24 was inoculated into naïve mice, which were then treated with IND24 or vehicle, IND24 proved ineffective, indicating that a resistant strain had emerged[149]. Accordingly, IND24 appears more effective under an intermittent dosing regime than under a continuous dosing regime[152]. While some investigators have pointed to drug resistance as a major roadblock to antiprion therapeutics[149], IND24 is remarkably effective against mouse prions despite the resistance phenomenon, and perhaps could have advanced to the clinic if not for its lack of any efficacy against human prions and the timepoint dependence of its efficacy (see below).

4. Therapeutic efficacy depends upon the timepoint when treatment is initiated.

For all compounds that have shown convincing evidence of efficacy in prion-infected mice, a universal finding has been that the increase in survival time depends upon the timepoint in disease when the treatment is initiated. To compare results between different mouse models, it is useful to normalize both variables and compare the relative survival time (1X = no benefit, 2X

= doubling of survival time) to the relative timepoint (percentage of the disease course that passed prior to treatment initiation, with 0% = treatment initiated on the day of prion infection, and 100% = treatment initiated on the day of endpoint — spontaneous death or euthanasia of the animal). Results for the four compounds with the greatest number of timepoints tested are shown in Figure 1.3.

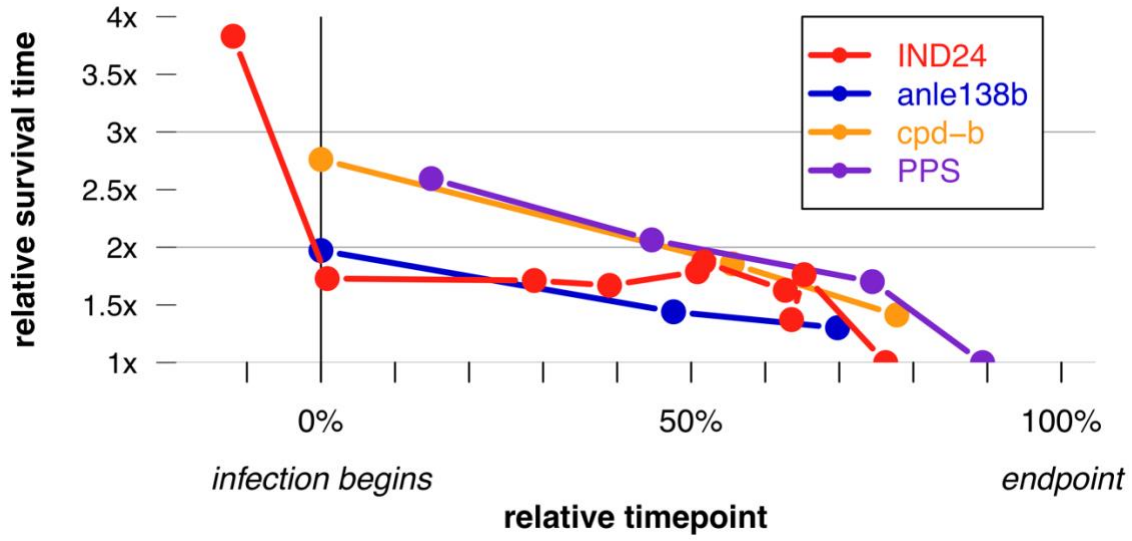


Figure 1.3 | The efficacy of antiprion treatments depends upon the timepoint when treatment is initiated. *PPS = pentosan polysulfate. Adapted from previously published data*[144,148,152,173].

Each of these compounds was capable of doubling or tripling survival time if given early enough, but none showed efficacy after the onset of symptoms. The result for IND24 is the most profound: it nearly quadrupled survival time when given prophylactically, two weeks prior to prion infection, but less than doubled survival time when given after prion infection began, and became ineffective by 90 dpi, even though symptoms did not begin until 118 dpi[152].

It is not clear that any treatment has ever shown clear evidence of efficacy after symptom onset in a prion-infected mouse. The compound anle138b showed some benefit at 120 dpi and this was reported as a symptomatic timepoint, but without clear evidence for the presence of symptoms[144]. Polythiophenes infused directly into the brain displayed a clear survival benefit

early in disease, but had only a marginal benefit when given at the first moment when infected mice began to decline in average rotarod performance[199]. To some extent, the definition of symptom onset in prion-infected mice is fuzzy, and when mice are monitored very closely, subtle behavioral changes can sometimes be detected before frank clinical signs[124,200]. Thus, it is possible that some of the compounds reviewed here were in fact effective at a time when the very earliest symptoms had begun. Regardless, these timepoints in mice should not be assumed to correspond to a timepoint when symptomatic humans could realistically be identified and treated. As noted above, the majority of a patient's disease course in prion disease is spent searching for a diagnosis, and patients are often profoundly debilitated by the time prion disease is even suspected.

Although preclinical studies suggest that effective prophylactic treatment may be easier to achieve than efficacy at the symptomatic stage, it is important to remember that preventive treatment of prion disease in humans is not a solved problem. All of the agents described above that proved effective in wild-type mice have now failed. IND24, cpd-b, anle138b, and arylamide 1 proved ineffective against human prions[149,150,152,191] and have never been advanced to trials. Pentosan polysulfate failed in symptomatic patients[155,156], and while this in itself does not rule out efficacy in pre-symptomatic population, the delivery via intraventricular catheter proved wrought with complications[155] and would be untenable in healthy individuals. As noted above, people pursuing predictive genetic testing for *PRNP* mutations today are still counseled that the results are not medically actionable. Thus, the search for a therapeutic agent that is effective at a pre-symptomatic stage in mice, and that also has the ability to translate to humans, must continue.

Challenges and strategy for developing a prion disease drug

Building off of the lessons listed above, it is possible to arrive at constraints on the properties that a successful prion disease drug must have.

First, a drug must be capable of treating a prion infection in the brain, and this means either crossing the blood-brain barrier or being capable of achieving broad brain distribution upon delivery directly into the CNS. At present, small molecules could meet this criterion, although optimization for ability to cross the blood-brain barrier is certainly non-trivial[201]. Antisense oligonucleotides (ASOs) delivered by bolus intrathecal injection also achieve broad brain distribution in primates[202,203]. Monoclonal antibodies so far do not appear to achieve sufficient brain distribution to treat prion disease[137,178]. Despite ongoing efforts to develop gene therapy options, at present no viral vectors appear to be ready to treat a whole-brain disease[204].

Second, a drug must have a mechanism of action that is effective against human prion strains, and indeed, ideally against all prion strains. Based on present knowledge, strategies aimed at PrP prior to its misfolding, or anywhere further upstream (DNA, RNA) are likely to meet this criterion, because PrP is the universal substrate for prion formation. It is not yet clear whether any strategies aimed downstream — say, at misfolded PrP or at neurotoxicity — could achieve strain-independent efficacy.

Third, a drug should be suitable for clinical testing a disease stage or timepoint when it can be reasonably expected to be effective. Consider statins, which can prevent heart attacks by lowering cholesterol years in advance, but which are ineffective at treating a heart attack once it is in progress. This example illustrates that some drugs may be effective only in prevention, and not in treatment, of a disease or condition. The fact that all antiprion therapeutics discovered to

date exhibit timepoint dependence suggests that success in developing a drug for prion disease could well depend upon the ability to test a drug in pre-symptomatic individuals. Such treatment should be initiated as early as possible, ideally before the disease process has even begun on the molecular level. Not all potential mechanisms of action are suited to this trial population: for example, a drug targeting misfolded PrP or neurotoxicity might have no target and no pharmacological effect at all in this population, leaving no obvious route forward for clinical evaluation of the drug.

The ability to conduct trials in the pre-symptomatic population will depend upon several factors. It will be necessary to identify individuals at high risk years in advance, which will depend upon uptake of predictive genetic testing as well as the ability to estimate penetrance and age of onset in the patient population. It will be necessary to identify an endpoint — a measurable clinical trial outcome — that could merit approval of a drug for this population. This in turn constrains the set of possible mechanisms of action a drug could have, because as explained above, not all potential therapeutic mechanisms are relevant before the disease process begins.

Summary of this dissertation

In an accompanying work[205], we describe preclinical proof-of-concept experiments as well as a biomarker and regulatory pathway to support the development of PrP-lowering antisense oligonucleotides (ASOs) to delay the onset of genetic prion disease. This therapeutic strategy appears to meet the requirements described above. PrP-lowering ASOs are effective in prophylactic treatment of prion infection in mice and are not prion strain-specific. The PrP-lowering mechanism of action permits measurement of a pharmacodynamic biomarker in pre-symptomatic individuals, CSF PrP, which we are working with regulators to evaluate as a

surrogate biomarker for the U.S. Food and Drug Administration Accelerated Approval program[205].

In this dissertation, we present four studies which both deepen the rationale and help to establish the feasibility of pre-symptomatic trials of ASOs or of any other PrP-lowering agent.

In order to identify pre-symptomatic individuals who could receive a preventive drug, it is essential to determine which *PRNP* mutations actually cause prion disease. While many *PRNP* mutations have been reported as disease-causing — 70 as of this writing — most lack clear evidence for pathogenicity. Moreover, genome-wide analyses show that DNA mutations reported to cause genetic disease in humans are, in the aggregate, far more common in the human population than are genetic diseases[206], suggesting that many such variants must either be falsely associated to a disease, or confer only a low lifetime risk of developing the disease. In Chapter 2 (published as Minikel et al, 2016[32]), we demonstrate that mutation penetrance in a rare disease such as prion disease can be quantified by comparing allele frequencies in disease case series with population controls. By comparing 16,025 prion disease cases with 60,706 individuals from the Exome Aggregation Consortium and 531,575 individuals from the 23andMe research database, we identify reportedly pathogenic *PRNP* variants that span a spectrum from benign to highly penetrant. This finding has already affected clinical practice, leading to revision of prognoses for individuals who harbor, or are at risk for, low- or no-risk variants[207,208], and affecting the way that novel *PRNP* variants are interpreted[209]. We also identify heterozygous loss-of-function mutations in *PRNP* in healthy humans and show that *PRNP* has not been depleted of such variants by natural selection, a finding we have since extended[130]. This supports the safety of therapeutic lowering of PrP.

Because preventive drugs might not be effective after symptom onset, development of a drug intended for use in pre-symptomatic individuals requires a viable pathway for its clinical evaluation in pre-symptomatic individuals. It is generally expected that trials for new drugs should demonstrate clinical benefit — a change in how patients feel, function, or survive — but regulators may consider alternatives when the parameters of the disease make conventional trials difficult or impossible[210]. In Chapter 3 (pre-print posted as Minikel et al, 2018[43]), we assess the feasibility of randomized prevention trials that follow pre-symptomatic *PRNP* mutation carriers to an endpoint of disease onset. We show that genetic prion disease age of onset is highly variable and is not predicted by any known factor. Power calculations show that randomized trials following pre-symptomatic individuals to onset would have enrollment requirements likely exceeding the number of eligible individuals who have ever been identified. This strengthens the argument for use of a surrogate biomarker endpoint in clinical evaluation of drugs in pre-symptomatic *PRNP* mutation carriers. In addition, by aggregating the world's largest dataset on age of onset in genetic prion disease, we provide a dataset that should prove useful in determining when to begin treatment, and could figure in the design of a long-term, post-approval surveillance mechanism for ultimately confirming clinical benefit by determining whether age of onset has been delayed.

In an accompanying work[205], we show that measurement of CSF PrP using an enzyme-linked immunosorbent assay (ELISA) kit is technically reproducible and that CSF PrP has the right properties to serve as a pharmacodynamic biomarker — it is brain- rather than blood-derived, and it has good short-term, within-subject, test-retest reliability. However, this and previous studies relying on ELISA have also found that CSF PrP decreases in the symptomatic phase of prion disease, suggesting that this pharmacodynamic biomarker might only be informative in pre-symptomatic individuals. Because PrP misfolding and proteolytic cleavage — both of which occur in disease — could render CSF PrP invisible to ELISA, we developed an orthogonal

method to quantify CSF PrP. In Chapter 4, we describe PrP multiple reaction monitoring (MRM), a targeted mass spectrometry assay which can be used to precisely quantify multiple tryptic peptides of PrP, corresponding to distinct protein domains, in CSF as well as brain tissue from humans and preclinical species of interest. We find that all PrP peptides are uniformly decreased in the CSF of symptomatic prion disease patients, confirming the findings from ELISA studies. This suggests that dose-finding studies for a PrP-lowering therapeutic may need to be conducted in pre-symptomatic individuals. By using an orthogonal method to measure the same analyte, we also validate other reported findings from ELISA studies, and our findings support the interpretability of CSF PrP generally.

While PrP-lowering ASOs are effective prophylactically against prion infection in mice[205], we also sought to determine whether they are effective after the disease process is already underway in the brain. In Chapter 5, we report preclinical studies of PrP-lowering ASOs in mice with established CNS prion infections. We identify ASOs that are effective at extending survival in mice after neuropathology is already detectable and close to the time of symptom onset. This supports further development of PrP-lowering ASOs. We also determine that ASOs are more effective when treatment is initiated earlier in the disease process. Thus, it is important to establish a clinical pathway for drug evaluation in pre-symptomatic individuals that does not depend upon the outcome of trials in symptomatic patients.

In summary, the work reported in this dissertation underscores the need for biomarker-based pre-symptomatic trials in genetic prion disease, and also lays groundwork that will be critical to making such trials a reality.

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Chapter 2. Quantifying penetrance in prion disease

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Attributions:

I conceived and designed the study together with Sonia Vallabh and Daniel G. MacArthur. I analyzed the data, generated the figures, and wrote the manuscript. I reviewed literature and IGV screenshots together with Sonia Vallabh. Kaitlin Samocha contributed the constraint analysis. Other authors participated in data collection and interpretation, and manuscript revision.

Abstract

More than 100,000 genetic variants are reported to cause Mendelian disease in humans, but the penetrance — the probability that a carrier of the purported disease-causing genotype will indeed develop the disease — is generally unknown. Here we assess the impact of variants in the prion protein gene (*PRNP*) on the risk of prion disease by analyzing 16,025 prion disease cases, 60,706 population control exomes, and 531,575 individuals genotyped by 23andMe, Inc. We show that missense variants in *PRNP* previously reported to be pathogenic are at least 30X more common in the population than expected based on genetic prion disease prevalence. While some of this excess can be attributed to benign variants falsely assigned as pathogenic, other variants have genuine effects on disease susceptibility but confer lifetime risks ranging from <0.1% to ~100%. We also show that truncating variants in *PRNP* have position-dependent effects, with true loss-of-function alleles found in healthy older individuals, supporting the safety of therapeutic suppression of prion protein expression.

Introduction

The study of pedigrees with Mendelian disease has been tremendously successful in identifying variants that contribute to severe inherited disorders[1–3]. Causal variant discovery is enabled by selective ascertainment of affected individuals, and especially of multiplex families. Although efficient from a gene discovery perspective, the resulting ascertainment bias confounds efforts to accurately estimate the penetrance of disease-causing variants, with profound implications for genetic counseling[4–7]. The development of large-scale genotyping and sequencing methods has recently made it tractable to perform unbiased assessments of penetrance in population controls. In several instances, such studies have suggested that previously reported Mendelian variants, as a class, are substantially less penetrant than had been believed[8–11]. To date, however, all of these studies have been limited to relatively prevalent (>0.1%) diseases, and point estimates of the penetrance of individual variants have been limited to large copy number variations[8,11].

Here we demonstrate the use of large-scale population data to infer the penetrance of variants in rare, dominant, monogenic disease, using the example of prion diseases. These invariably fatal neurodegenerative disorders are caused by misfolding of the prion protein (PrP, the product of *PRNP*)[12] and have an annual incidence of 1 to 2 cases per 1 million population[13]. A small, albeit infamous, minority of cases (<1% in recent years[14,15]) are acquired through dietary or iatrogenic routes. The majority (~85%) of cases are defined as sporadic, occurring in individuals with two wild-type *PRNP* alleles and no known environmental exposures. Finally, ~15% of cases occur in individuals with rare, typically heterozygous, coding variants in *PRNP*, including missense variants, truncating variants, and octapeptide repeat insertions or deletions (Supplemental Table S2.1). Centralized ascertainment of cases by national surveillance centers

(Methods) makes prion disease a good test case for using reference datasets to assess the penetrance of these variants.

PRNP was conclusively established as a dominant disease gene due to clear Mendelian segregation of a few variants with disease[16–18]. Yet ascertainment bias[19], low rates of predictive genetic testing[20], and frequent lack of family history[21,22] confound attempts to estimate penetrance by survival analysis[19,23–26]. Meanwhile, the existence of non-genetic etiologies leaves doubt as to whether novel variants are causal or coincidental.

A fully penetrant disease genotype should be no more common in the population than the disease that it causes. This observation allows us to leverage two large population control datasets to re-evaluate the penetrance of reported disease variants in *PRNP*. The recently reported Exome Aggregation Consortium (ExAC) dataset[27] contains variant calls on 60,706 people ascertained for various common diseases, without any ascertainment on neurodegenerative disease. 23andMe's database contains genotypes on 531,575 customers of its direct-to-consumer genotyping service who have opted in to participate in research, pruned to remove related individuals (first cousins or closer; Methods), preventing enrichment due to large families with prion disease.

Results

We began by asking whether reportedly pathogenic variants are as rare as expected in these population control datasets. The proportion of people alive in the population today who harbor completely penetrant variants causal for prion disease can be approximated by the product of three numbers: the annual incidence of prion disease, the proportion of cases with such a genetic variant, and the life expectancy of individuals harboring these variants. Based on upper

bounds of these numbers (Figure 2.1A), and assuming ascertainment is neutral with respect to neurodegenerative disease, we would expect no more than ~1.7 such individuals in the 60,706 exomes in the ExAC dataset[27], and ~15 such individuals among the ~530,000 genotyped 23andMe customers who opted to participate in research.

Through reviews[28–30] and PubMed searches, we identified 63 rare genetic variants reported to cause prion disease (Supplemental Table S2.2). We reviewed ExAC read-level evidence for every rare (<0.1% allele frequency) variant call in *PRNP* (Materials and Methods; Supplemental Table S2.3-4) and found that 52 individuals in ExAC harbor reportedly pathogenic missense variants (Figure 2.1B), at least a 30-fold excess over expectation if all such variants were fully penetrant. Similarly, in the 23andMe database we observed a total of 141 alleles of 16 reportedly pathogenic variants genotyped on their platform (Supplemental Table S2.5).

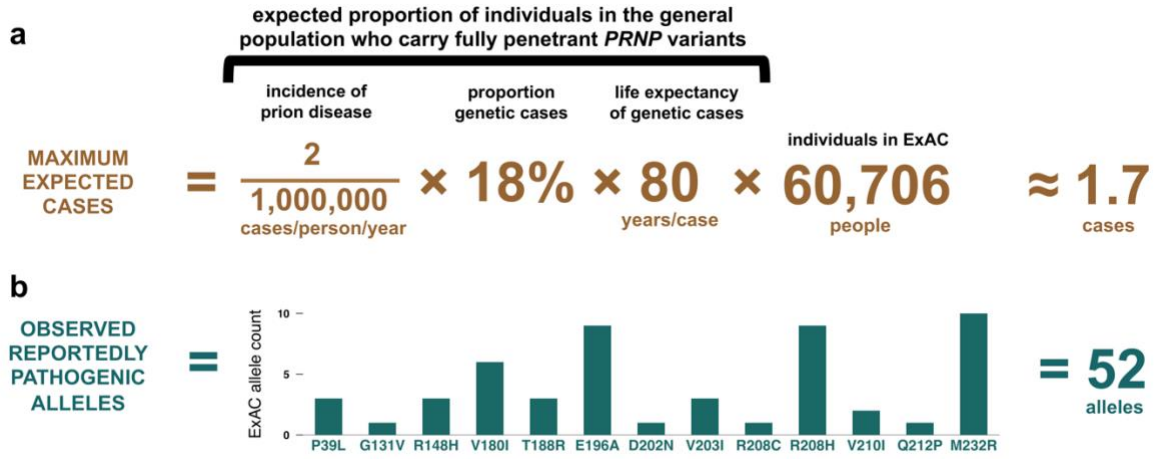

Figure 2.1 | Reportedly pathogenic PRNP variants are >30 times more common in controls than expected based on disease incidence. *Reported prion disease incidence varies with the intensity of surveillance efforts [13], with an apparent upper bound of ~2 cases per million population per year (Materials and Methods). In our surveillance cohorts, 65% of cases underwent PRNP open reading frame sequencing, with 12% of all cases, or 18% of sequenced cases, possessing a rare variant (Supplemental Table S2.1), consistent with an oft-cited estimate that 15% of cases of Creutzfeldt-Jakob disease are familial [31]. Genetic prion diseases typically strike in midlife, with mean age of onset for different variants ranging from 28 to 77 [22,32] (Supplemental Table S2.10); we accepted 80, a typical human life expectancy, as an upper bound for mean age of onset, and to be additionally conservative, we assumed that all individuals in ExAC and 23andMe were below any age of onset, even though both contain elderly individuals [33] (Supplemental Figure S2.1). Thus, no more than ~29 people per million in the general population should harbor high-penetrance prion disease-causing variants. Therefore at most ~1.7 people in ExAC (**A**) and ~15 people in 23andMe would be expected to harbor such variants. In fact, reportedly pathogenic variants are seen in 52 ExAC individuals (**B**) and on 141 alleles in the 23andMe database.*

Individuals with reportedly pathogenic *PRNP* variants did not cluster within any one cohort within ExAC (Supplemental Table S2.6), arguing against enrichment due to comorbidity with a common disease ascertained for exome sequencing. ExAC does include populations, such as South Asians, in which prion disease is not closely surveilled and we cannot rule out a higher incidence than that reported in developed countries, yet the individuals with reportedly pathogenic variants in either ExAC or 23andMe were of diverse inferred ancestry (Supplemental Table S2.7-9). These individuals' ages were consistent with the overall ExAC age distribution (Supplemental Figure S2.1), rather than being enriched below some age of disease onset. ExAC genotypes at the prion disease modifier polymorphism M129V[34] were consistent with population allele frequencies (Supplemental Table S2.7), rather than enriched for the lower-risk

heterozygous genotype. Certain *PRNP* variants are associated with highly atypical phenotypes[35,36], which are mistakable for other dementias and may not be well ascertained by current surveillance efforts. Most of the variants found in our population control cohorts, however, have been reported in individuals with a classic, sporadic Creutzfeldt-Jakob disease phenotype[22,28,30,37–39], arguing that the discrepancy between observed and expected allele counts does not result primarily from an underappreciated prevalence of atypical prion disease.

Having observed a large excess of reportedly pathogenic variants over expectation in two datasets, and having excluded the most obvious confounders, we hypothesized that the unexpectedly high frequency of these variants in controls might arise from benign and/or low-risk variants.

We investigated which variants were responsible for the observed excess (Figure 2.2). Variants with the strongest prior evidence of pathogenicity are absent from ExAC and cumulatively account for ≤5 alleles in 23andMe, consistent with the known rarity of genetic prion disease. Much of the excess allele frequency in population controls is due, instead, to variants with very weak prior evidence of pathogenicity (Figure 2.2 and Supplementary Discussion). For four variants observed in controls (V180I, R208H, V210I, and M232R), pathogenicity is controversial [40,41] or reduced penetrance has been suggested [42,43], but quantitative estimates of penetrance have never been produced, and the variants remain categorized as causes of genetic Creutzfeldt-Jakob disease[21,22]. Although we cannot prove that any one of the variants we observe in population controls is completely neutral, the list of reported pathogenic variants likely includes false positives. Indeed, the observation that 0.4% (236 / 60,706) of ExAC individuals harbor a rare (<0.1%) missense variant (Supplemental Table S2.4) suggests that ~4 of every 1000 sporadic prion disease cases will, by chance, harbor such a variant, which in

many cases will be interpreted and reported as causal given the long-standing classification of *PRNP* as a Mendelian disease gene.

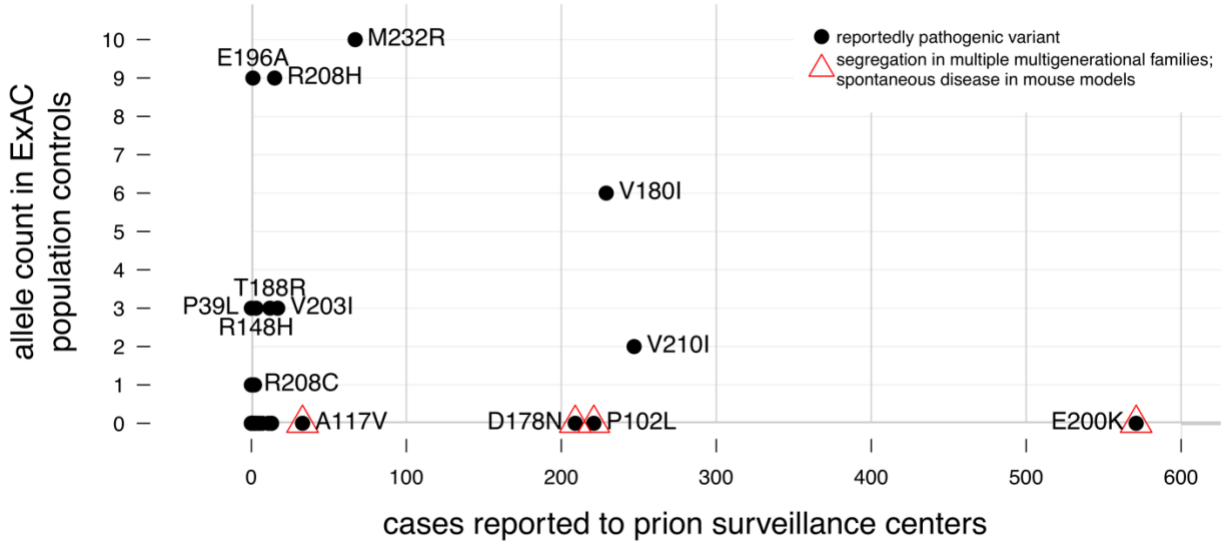

Figure 2.2 | Reportedly pathogenic PRNP variants include Mendelian, benign, and intermediate variants. *Prior evidence of pathogenicity is extremely strong for four missense variants - P102L, A117V, D178N and E200K - each of which has been observed to segregate with disease in multiple multigenerational families [16–18,44–48] and to cause spontaneous disease in mouse models [49–54]. These account for >50% of genetic prion disease cases (Supplemental Table S2.1), yet are absent from ExAC (Supplemental Table S2.3), and collectively appear on ≤5 alleles in 23andMe's cohort (Supplemental Table S2.5), indicating allele frequencies sufficiently low to be consistent with the prevalence of genetic prion disease (Figure 2.1). Conversely, the variants most common in controls and rare in cases had categorically weak prior evidence for pathogenicity. R208C (8 alleles in 23andMe) and P39L were observed in patients presenting clinically with other dementias, with prion disease suggested as an alternative diagnosis solely on the basis of finding a novel PRNP variant [55,56]. E196A was originally reported in a single patient, with a sporadic Creutzfeldt-Jakob disease phenotype and no family history [37], and appeared in only 2 of 790 Chinese prion disease patients in a recent case series [57], consistent with the ~0.1% allele frequency among Chinese individuals in ExAC (Tables S5 and S8). At least three variants (M232R, V180I, and V210I) occupy a space inconsistent with either neutrality or with complete penetrance (see main text and Figure 2.3). R148H, T188R, V203I, R208H and additional variants are discussed in Supplementary Discussion.*

At least three variants, however (V180I, V210I, and M232R) fail to cluster with either the likely benign or likely Mendelian variants (Figure 2.2). Because each of these three appears primarily in one population in both cases and controls (Tables S1, S5, S7), we compared allele frequencies in matched population groups. Each has an allele frequency in controls that is too

high for a fully penetrant, dominant prion disease-causing variant, and yet far lower than the corresponding allele frequency in cases (Figure 2.3).

Because we lack genome-wide SNP data on cases we are unable to directly correct for population stratification, which thus may contribute to the observed differences in allele frequencies. Geographic clusters of genetic prion disease have been recognized for decades[26,31,58]. For example, nearly half of Italian prion disease cases with the V210I variant are concentrated within two regions of Italy[59], so any non-uniform geographic sampling in cases versus controls would add some uncertainty to our penetrance estimates.

Nonetheless, the magnitude of the enrichment of certain variants in cases over controls in our datasets makes substructure an implausible explanation for the entire difference. In order for V210I to be neutral and yet appear with an allele frequency of 8.1% in Italian cases despite an apparent allele frequency of 0.02% in Italian controls, it would need to be fixed in a subpopulation comprising 8% of Italy's populace. Under this scenario, this subpopulation would need to be virtually unsampled in any of our control cohorts, and V210I cases would contain many homozygotes. In reality, no cases have been reported homozygous for this variant. Conversely, if V210I were fully penetrant, family history would be positive in most cases, and the variant's appearance on 13 alleles in 23andMe (Supplemental Table S2.5) would indicate that this variant alone accounts for three times the known prevalence of genetic prion disease (Figure 2.1A). Finally, if the low family history rate were due to many *de novo* mutations, then V210I cases would be more uniformly distributed across populations (Supplemental Table S2.1). Similar arguments rule out V180I being either benign or Mendelian. M232R, though clearly not Mendelian, could still be benign as it exhibits only 4- to 6-fold enrichment in cases, an amount that might conceivably be explained by Japanese population substructure alone. However, because even common variants in *PRNP* affect prion disease risk with odds ratios of

3 or greater[60–62], it is not implausible that M232R has a similar effect size, and our data suggest this a more likely scenario than it being neutral.

Satisfied that these three variants are likely neither benign nor Mendelian, we estimated lifetime risk in heterozygotes (Methods). The ~2 in 1 million annual incidence of prion disease translates into a baseline lifetime risk of ~1 in 5,000 in the general population (Methods). Because prion diseases are so rare, even the massive enrichment of heterozygotes in cases (Figure 2.3), implying odds ratios on the order of 10 to 1,000, corresponds to only low penetrance, with lifetime risk for M232R, V180I and V210I estimated near 0.1%, 1%, and 10%, respectively. Although our estimates are imperfect due to population stratification, they accord well with family history rates (Figure 2.3) and explain the unique space that these variants occupy in the plot of case versus control allele count (Figure 2.2). These data indicate that *PRNP* missense variants occupy a risk continuum rather than a dichotomy of causal versus benign.

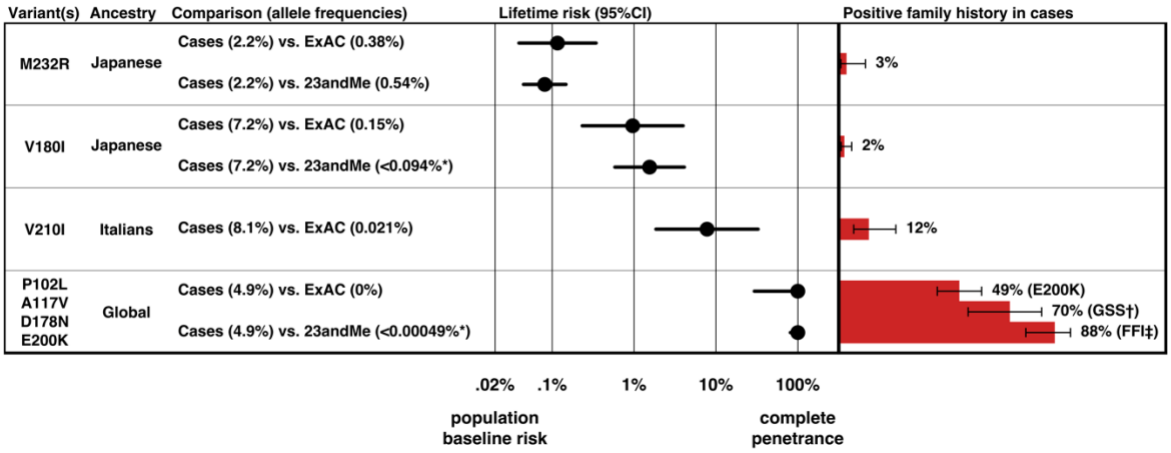

Figure 2.3 | Certain variants confer intermediate amounts of lifetime risk. *M232R, V180I, and V210I show varying degrees of enrichment in cases over controls, indicating a weak to moderate increase in risk. Best estimates of lifetime risk in heterozygotes (Materials and Methods) range from ~0.08% for M232R to ~7.8% for V210I, and correlate with the proportion of patients with a positive family history. Allele frequencies for P102L, A117V, D178N and E200K are consistent with up to 100% penetrance, with confidence intervals including all reported estimates of E200K penetrance based on survival analysis, which range from ~60% to ~90%[19,23–26]. Rates of family history of neurodegenerative disease in Japanese cases are from (Supplemental Table S2.10) and in European populations are from Kovacs et al[21], with Wilson binomial 95% confidence intervals shown. *Based on allele counts rounded for privacy (Materials and Methods). †GSS, Gerstmann Straussler Scheinker disease associated with variants P102L, A117V and G131V. ‡FFI: fatal familial insomnia associated with a D178N cis 129M haplotype.*

We asked whether the same was true of protein-truncating variants. *PRNP* possesses only one protein-coding exon, so premature stop codons are expected to result in truncated polypeptides rather than in nonsense-mediated decay. Prion diseases are known to arise from a gain of function, as neurodegeneration is not seen in mice, cows, or goats lacking PrP[63–66], and the rate of prion disease progression is tightly correlated with PrP expression level[67]. Yet heterozygous C-terminal (residue ≥145) truncating variants are known to cause prion disease, sometimes with peripheral amyloidosis[35]. Some of these patients also experience sensorimotor neuropathy phenotypically similar to that present in homozygous, but not heterozygous, PrP knockout mice[68], but attributed to amyloid infiltration of peripheral nerves, rather than loss of PrP function[35].

We identified, for the first time, heterozygous N-terminal (residue ≤131) truncating variants in four ExAC individuals and were able to obtain Sanger validation (Supplemental Figure S2.1) and limited phenotype data (Supplemental Table S2.11) for three. These individuals are free of overt neurological disease at ages 79, 73, and 52, and report no personal or family history of neurodegeneration nor of peripheral neuropathy. Therefore, the pathogenicity of protein-truncating variants appears to be dictated by position within PrP's amino acid sequence (Figure 2.4). Observing three *PRNP* nonsense variants in ExAC is consistent with the expected number (~3.9) once we adjust our model[69] to exclude codons ≥145, where truncations cause a dominant gain-of-function disease. Thus, we see no evidence that *PRNP* is constrained against truncation in its N terminus. This, combined with the lack of any obvious phenotype in individuals with N-terminal truncating variants, suggests that heterozygous loss of PrP function is tolerated.

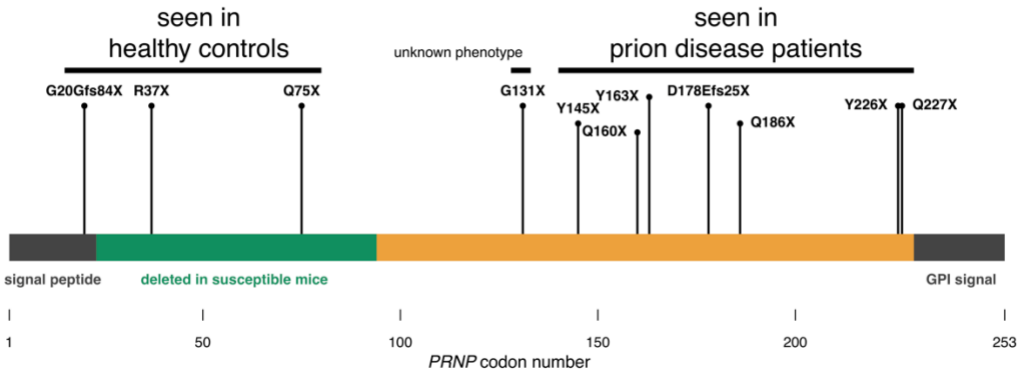

Figure 2.4 | Effects of truncating variants in the human prion protein are position-dependent. *Truncating variants reported in prion disease cases in the literature (Supplemental Table S2.2) and in our cohorts (Supplemental Table S2.1) cluster exclusively in the C-terminal region (residue ≥145), while truncating variants in ExAC are more N-terminal (residue ≤131). The ortholog of each residue from 23-94 is deleted in at least one prion-susceptible transgenic mouse line[70]. C-terminal truncations abolish PrP's glycosylphosphatidylinositol anchor but leave most of the protein intact, a combination that mediates gain of function through mislocalization, causing this normally cell-surface-anchored protein to be secreted. Consistent with this model of pathogenicity, mice expressing full-length secreted PrP develop fatal and transmissible prion disease[71,72]. By contrast, the N-terminal truncating variants that we observe retain only residues dispensable for prion propagation, and are likely to cause a total loss of protein function.*

Discussion

Over 100,000 genetic variants have been reported to cause Mendelian disease in humans[73,74]. Many such reports do not meet current standards for assertions of pathogenicity[75,76], and if all such reports were believed, the cumulative frequency of these variants in the population would imply that most people have a genetic disease[27]. It is generally unclear how much of the excess burden of purported disease variants in the population is due to benign variants falsely associated, and how much is due to variants with genuine association but incomplete penetrance.

Here we leverage newly available large genomic reference datasets to re-evaluate reported disease associations in a dominant disease gene, *PRNP*. We identify some missense variants as likely benign while showing that others span a spectrum from <0.1% to ~100% penetrance. Our analyses provide quantitative estimates of lifetime risk for hundreds of asymptomatic individuals who have inherited incompletely penetrant *PRNP* variants.

Available datasets are only now approaching the size and quality required for such analyses, resulting in limitations for our study. The confidence intervals on our lifetime risk estimates span more than an order of magnitude, and our inability to perfectly control for population stratification injects additional uncertainty. We have been unable to reclassify those *PRNP* variants that are very rare both in cases and in controls (Supplementary Discussion). We have avoided analysis of large insertions that are poorly called with short sequencing reads, though we note that existing literature on these insertions is consistent with a spectrum of penetrance similar to that which we observe for missense variants[28,77]. Penetrance estimation in Mendelian disease will be improved by the collection of larger case series, particularly with genome-wide SNP data to

allow more accurate population matching. This, coupled with continued large-scale population control sequencing and genotyping efforts, should reveal whether the dramatic variation in penetrance that we observe here is a more general feature of dominant disease genes.

Because PrP is required for prion pathogenesis and reduction in gene dosage slows disease progression[67,78–80], several groups have sought to therapeutically reduce PrP expression using RNA interference[81–83], antisense oligonucleotides[84], or small molecules[85,86]. Our discovery of heterozygous loss-of-function variants in three healthy older humans provides the first human genetic data regarding the effects of a 50% reduction in gene dosage for *PRNP*. Both the number of individuals and the depth of available phenotype data are limited, and lifelong heterozygous inactivation of a gene is an imperfect model of the effects of pharmacological depletion of the gene product. With those limitations, our data provide preliminary evidence that a reduction in *PRNP* dosage, if achievable in patients, is likely to be tolerated. Increasingly large control sequencing datasets will soon enable testing whether the same is true of other genes currently being targeted in substrate reduction therapeutic approaches for other protein-folding disorders.

Together, our findings highlight the value of large reference datasets of human genetic variation for informing both genetic counseling and therapeutic strategy.

Methods

Prion disease case series

Prion disease is considered a notifiable diagnosis in most developed countries, with mandatory reporting of all suspect cases to a centralized surveillance center. Surveillance was carried out broadly according to established guidelines[87,88], with specifics as described previously for

Australia[89], France[90], Germany[91–93], Italy[94], Japan[22], and the Netherlands[95]. Sanger sequencing of the *PRNP* open reading frame was performed as described [96]. We included only prion disease cases classified as definite (autopsy-confirmed) or probable according to published guidelines[88]. Criteria for genetic testing vary between countries and over the years of data collection, with testing offered only on indication of family history in some times and places, and testing of all suspect cases with tissue available in other instances. Summary statistics on the total number and proportion of cases sequenced are presented in Supplemental Table S2.1.

Exome sequencing and analysis

The ascertainment, sequencing, and joint calling of the ExAC dataset have been described previously[97]. We extracted all rare (<0.1%) coding variant calls in *PRNP* with genotype quality (GQ) ≥10, alternate allele depth (AD) ≥3 and alternate allele balance (AB) ≥20%. Read-level evidence was visualized using Integrative Genomics Viewer (IGV)[98] for manual review. Because most ExAC exomes were sequenced with 76bp reads and the *PRNP* octapeptide repeat region (codons 50-90 inclusive) is 123bp long, it was impossible to determine whether genotype calls in this region were correct, and they were not considered further. After review of IGV screenshots, 87% of genotype calls were judged to be correct and were included in Supplemental Table S2.3. Of the genotype calls judged to be correct, 99% had genotype quality (GQ) ≥95, 99% had allelic balance (AB) between 30% and 70%, and 97% had ≥10 reads supporting the alternate allele. All participants provided informed consent for exome sequencing and analysis. The Exome Aggregation Consortium's aggregation and release of exome data have been approved by the Partners Healthcare Institutional Research Board (2013P001339). ExAC data have been publicly released at http://exac.broadinstitute.org/ and IGV screenshots of the rare *PRNP* variants deemed to be genuine and included in this study are available at https://github.com/ericminikel/prnp_penetrance/tree/master/supplement/igv

23andMe research participants and genotyping

Participants were drawn from the customer base of 23andMe, Inc., a personal genetics company (accessed February 6, 2015). All participants provided informed consent under a protocol approved by an external AAHRPP-accredited IRB, Ethical & Independent Review Services (E&I Review). DNA extraction and genotyping were performed on saliva samples by National Genetics Institute (NGI), a CLIA-licensed clinical laboratory and a subsidiary of Laboratory Corporation of America. Samples were genotyped on one of four Illumina platforms (V1-V4) as described previously[99]. Of the *PRNP* SNPs considered, two (P105L and E200K) were genotyped on all four platforms while the other 14 were genotyped only on V3 and V4, resulting in differing numbers of total samples genotyped (Supplemental Table S2.5). Genotypes were called with Illumina GenomeStudio. A 98.5% call rate were required for all samples. As with all 23andMe research participants, individuals whose genotyping analyses failed to reach the desired call rate repeatedly were recontacted to provide additional samples. A maximal set of unrelated individuals was chosen based on segmental identity-by-descent (IBD) estimation[100]. Individuals were defined as related if they shared more than 700 cM IBD (approximately the minimal expected sharing between first cousins). Allele counts between 1 and 5 were rounded up to 5 to protect individual privacy (Supplemental Table S2.5). Rounding down to 1 instead would raise our estimates of penetrance for V180I to 7.7% (95%CI, 1.2% - 50%) and for P102L, A117V, D178N and E200K collectively to 100% (95%CI, 100% - 100%), but the confidence intervals would still overlap those based on ExAC allele frequencies, and the overall conclusions of our study would remain unchanged.

23andMe ancestry composition

Ancestral origins of chromosomal segments were assigned on a continental level (European, Latino, African, and East Asian) and a country level (Japanese) as described by Durand et al[101]. Briefly, after phasing genotypes using an out-of-sample implementation of the Beagle

algorithm[102], a string kernel support vector machine classifier assigns tentative ancestry labels to local genomic regions. Then an autoregressive pair hidden Markov model was used to simultaneously correct phasing errors and produce reconciled local ancestry estimates and confidence scores based on the initial assignment. Finally, isotonic regression models were used to recalibrate the confidence estimates.

Europeans and East Asians were defined as individuals with more than 97% of chromosomal segments predicted as being from the respective ancestries. Because African Americans and Latinos are highly admixed, no single threshold of genome-wide ancestry is sufficient to distinguish them. However, segment length distributions of European, African, and Native American ancestries are different between African Americans and Latinos, due to distinct admixture timing in the two ethnic groups. Thus, a logistic classifier based on segment length of European, African, and Native American ancestries was used to distinguish between African Americans and Latinos.

At the country level, individuals were classified as Japanese based on the fraction of the respective local ancestry using a threshold of 90% for classifying Japanese ancestry. This threshold is based on the average fraction of local ancestry in the reference population (23andMe research participants with all four grandparents from the reference country): 94% (5% SD, *N*=533) for Japanese. Using the same approach, we were unable to obtain a confident set of Italian individuals for analysis of V210I due to extensive admixture. 23andMe research participants with all four grandparents from Italy only have 66% (18% SD, *N*=2090) Italian ancestry, and only ~60 participants have >90% Italian ancestry.

ExAC ancestry inference

We computed ten principal components based on ~5,800 common SNPs as described[27,103]. A centroid in eigenvalue-weighted principal component space was generated for each HapMap population based on 1000 Genomes individuals in ExAC. The remaining individuals in ExAC were assigned to the HapMap population with the nearest centroid according to eigenvalue-weighted Euclidean distance. Ancestries of all individuals, including those with reportedly pathogenic variants, are summarized in (Tables S7, S8).

Prion disease incidence and baseline risk

The reported incidence of prion disease varies between countries and between years, with much of the variability explained by the intensity of surveillance, as measured by the number of cases referred to national surveillance centers[13]. Rates of ~1 case per million population per year have been reported, for instance in the U.S.[104] and in Japan[22], however, the countries with the most intense surveillance (greatest number of referrals per capita), such as France and Austria, observe incidence figures as high as 2 cases per million population per year[13]. Only in small countries where the statistics are dominated by a particular genetic prion disease founder mutation, such as Israel and Slovakia[23,26], has an incidence higher than 2 per million been consistently observed[105]. We therefore accepted 2 cases per million as an upper bound for the true incidence of prion disease. Assuming an all-causes death rate of ~10 per 1,000 annually[106], this incidence corresponds to prion disease accounting for ~0.02% of all deaths, which we accepted as the baseline disease risk in the general population.

Lifetime risk estimation

By Bayes' theorem, the probability of disease given a genotype (penetrance or lifetime risk, P(D|G)) is equal to the proportion of individuals with the disease who have the genotype (genotype frequency in cases, P(G|D)) times the prevalence of the disease (baseline lifetime

risk in the general population, P(D)), divided by the frequency of the genotype in the general population (here, population control allele frequency, P(G)). The use of this formula to estimate disease risk dates back at least to Cornfield's estimation of the probability of lung cancer in smokers[107], with later contributions by Woolf[108] and a synthesis by C.C. Li with application to genetics[109].

We used an allelic rather than genotypic model, such that lifetime risk in an individual with one allele is equal to case allele frequency (based on the number of prion disease cases that underwent *PRNP* sequencing) times baseline risk divided by population control allele frequency, $P(D|A) = P(A|D) \times P(D)/P(A)$. Note that we assume that our population control datasets include individuals who will later die of prion disease, thus enabling direct use of the ExAC and 23andMe allele frequencies as the denominator P(A). Following Kirov[11], we compute Wilson 95% confidence intervals on the binomial proportions P(A|D) and P(A), and calculate the upper bound of the 95% confidence interval for penetrance using the upper bound on case allele frequency and the lower bound on population control allele frequency, and vice versa for the lower bound on penetrance.

Source code availability

Data processing, analysis, and figure generation utilized custom scripts written in Python 2.7.6 and R 3.1.2. These scripts, along with vector graphics of all figures and tab-delimited text versions of all supplemental tables, are available online at

https://github.com/ericminikel/prnp_penetrance

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Chapter 3. Age of onset and preventive trial design in genetic prion disease

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Attributions:

I principally conceived and designed the study, with contributions from Sonia Vallabh, Margaret Orseth, and Simon Mead. I aggregated and analyzed the data, wrote the code, created the figures and tables, and wrote the manuscript. Sonia Vallabh contributed to data curation and figure design. Other authors participated in data collection, curation, and interpretation, and in revision of the manuscript.

Abstract

Regulatory agencies worldwide have adopted programs to facilitate drug development for diseases where the traditional approach of a randomized trial with a clinical endpoint is expected to be prohibitively lengthy or difficult. Here we provide quantitative evidence that this criterion is met for the prevention of genetic prion disease. We assemble age of onset or death data from *N*=1,094 individuals with high penetrance mutations in the prion protein gene (*PRNP*), generate survival and hazard curves, and estimate statistical power for clinical trials. We show that, due to dramatic and unexplained variability in age of onset, randomized preventive trials would require hundreds or thousands of at-risk individuals in order to be statistically powered for an endpoint of clinical onset, posing prohibitive cost and delay and likely exceeding the number of individuals available for such trials. Instead, the characterization of biomarkers suitable to serve as surrogate endpoints will be essential for the prevention of genetic prion disease. Biomarker-based trials may require post-marketing studies to confirm clinical benefit. Parameters such as longer trial duration, increased enrollment, and the use of historical controls in a post-marketing study could provide opportunities for subsequent determination of clinical benefit.

Introduction

Placebo-controlled, double-blind, randomized trials with a clinical endpoint — a measure of how patients feel or function — constitute the gold standard for demonstration of therapeutic efficacy and, where feasible, are strongly preferred for approval of new drugs. Regulators worldwide have recognized, however, that in some diseases the duration of such trials may unduly delay patient access to potentially life-saving drugs. Many agencies have therefore created programs to support drug development in this situation. For instance, the United States Food and Drug Administration (FDA) Accelerated Approval program[1] provides for conditional approval based on trials using surrogate endpoints, including biomarkers, with a requirement for post-marketing studies to confirm clinical benefit[2]. Honoring the specifics of each disease, FDA "consider[s] how to incorporate novel approaches into the review of surrogate endpoints… especially in instances where the low prevalence of a disease renders the existence or collection of other types of data unlikely or impractical"[3]. Here we present evidence that genetic prion disease meets this criterion.

Prion disease is a fatal and, at present, incurable neurodegenerative disease caused by the misfolding of the prion protein, PrP, encoded by the gene *PRNP*[4]. Most subtypes of prion disease are extremely rapid, leading from first symptom to death in several months[5]. Prion diseases are transmissible, but today few cases are known to be acquired by infection. ~85% of prion disease cases are termed "sporadic," meaning they arise spontaneously with no known environmental or genetic trigger, while ~15% of cases possess protein-altering rare variants in *PRNP*, a subset of which are highly penetrant[6]. Various genetic subtypes of prion disease include fatal familial insomnia, genetic Creutzfeldt-Jakob disease, and Gertsmann-Sträussler-Scheinker disease.

To date, all completed clinical trials in prion disease have recruited only symptomatic patients, mostly with sporadic prion disease, and have used cognitive, functional, or survival endpoints[7–14]. By the time of diagnosis many prion disease patients are in a state of advanced dementia, and even a therapy that halted the disease process entirely at this stage might only preserve the patient in a state with little or no quality of life[15]. Moreover, preclinical proofs of concept argue that a preventive, rather than therapeutic, approach is more likely to be effective. Multiple antiprion agents have been discovered that extend the survival time of prion-infected mice by 2-4X when administered long before symptoms, yet these have diminished effects at later timepoints, and no effect when administered after clinical onset[16–19]. These observations indicate a need to enable preventive trials in presymptomatic individuals at risk for genetic prion disease.

The ongoing preventive trial of crenezumab, an anti-amyloid β antibody, for *PSEN1* E280A early-onset Alzheimer's disease, follows a design where presymptomatic individuals are randomized to drug or placebo and followed for five years to a cognitive endpoint[20]. While this represents one model for preventive trials in neurodegeneration, we hypothesized that this approach might be challenging for genetic prion disease due to its variable age of onset[21,22], small presymptomatic patient population[23], and more limited financial incentives for pharmaceutical companies. To test this hypothesis, we set out to aggregate age of onset data in genetic prion disease, generate survival and hazard curves, and simulate statistical power for randomized pre-approval trials with a clinical endpoint. We also set out to investigate the feasibility of one potential alternative: post-marketing studies using historical controls to confirm clinical benefit, following Accelerated Approval on a surrogate biomarker endpoint.

Results

Age of onset in genetic prion disease

We reasoned that any preventive trial with a clinical endpoint in genetic prion disease would derive most of its statistical power from individuals with high penetrance *PRNP* variants. Some *PRNP* variants can be identified as highly penetrant by their extreme enrichment in cases over population controls, but many variants are too rare in both groups for meaningful comparison[6]. We therefore reviewed the literature on 69 reportedly pathogenic *PRNP* variants and identified 27 variants for which Mendelian segregation has been reported in at least one family with at least three affected individuals and/or for which a *de novo* mutation in a case has been identified (Supplemental Table S3.1), thus suggesting high penetrance.

We examined the frequency of these putative high penetrance variants in a recent case series[6]. The top three variants — E200K, P102L, and D178N — collectively explain 85% of high penetrance cases (Supplemental Figure S3.1). Each of these arises from a CpG transition (a C to T DNA change where the adjacent base is G), a type of variant which occurs by spontaneous mutation 10-100X more often than other mutation types[24,25], explaining the recurrence of these three mutations on multiple *PRNP* haplotypes in families worldwide[6,26,27]. Therefore, regardless of the population studied, these three variants are likely to account for a large fraction of genetic prion disease cases with high penetrance variants. For this reason, we focused our analysis primarily on individuals with these three variants. We aggregated age of onset and/or age of death data on *N*=1,001 individuals with the E200K, P102L, or D178N mutations from nine study centers worldwide (Table 3.1 and Supplemental Table S3.2), encompassing both direct clinical reports and family histories (see Methods), and including censored individuals. Statistics on *N*=93 individuals with the next four mutations most common in cases — 5-OPRI (insertion of five extra octapeptide repeats), 6-OPRI, P105L, and A117V — are included in Supplemental

Table S3.3. We used these data to compile life tables and computed the annual hazard — risk of onset in each year of life — for each mutation (Supplementary Life Tables).

We found wide variability in age of onset (Table 3.1), consistent with previous reports[21,22,28]. An implication of this variability is that high lifetime risk arises not from certain onset at a specific age, but from modest risk in any given year of life, accumulated over many decades of exposure. This poses a challenge for following presymptomatic individuals to onset in a preventive clinical trial, as it is difficult to ascertain a group of individuals for whom onset is imminent. For example, even at age 57, an E200K individual has only a 5% probability of disease onset occurring in any given year. This means that 20 person-years of follow-up for E200K individuals around this age would be expected to result in only one observed disease onset. Annual hazards do rise with age, but as they reach high levels, the number of surviving individuals also dwindles (Figure 3.1). For the three most common mutations, the annual hazard remains below 10% until after the majority of people have already died (Figure 3.1, Supplemental Figure S3.3, and Supplementary Life Tables). Similarly, the median number of years until onset, conditioned on an individual's current age, remains ≥5 years until after the median age of onset has passed (Supplementary Life Tables). The next four most common mutations have tighter age of onset distributions, and so reach higher annual hazards sooner (Supplemental Figure S3.3), but these mutations are also much rarer, accounting for only 10% of cases with a high penetrance variant[6].

Table 3.1 | Variability in age of onset in genetic prion disease. *Censored data include individuals who were either alive and well at last follow-up or had died of an unrelated cause, and whose genetic status was known either through predictive testing or due to obligate carrier status. For D178N and E200K, because the majority of individuals have disease duration ≤1 year (Supplemental Figure S3.2), age of death is used where age of onset is unavailable. For P102L, which more often has a longer disease duration, only age of onset is used. IQR, interquartile range. Range indicates highest and lowest observed age of onset, except where * indicates that the longest survival is a censored data point.*

	without censored data		survival curve including censored data		
mutation	mean ± sd	N	median (IQR)	range	N
P102L	53.7 ± 10.6	193	56 (47 - 60)	22 - 75	206
D178N	51.3 ± 11.8	256	53 (46 - 60)	12 - 89*	289
E200K	61.3 ± 10.0	456	62 (55 - 68)	31 - 92	506

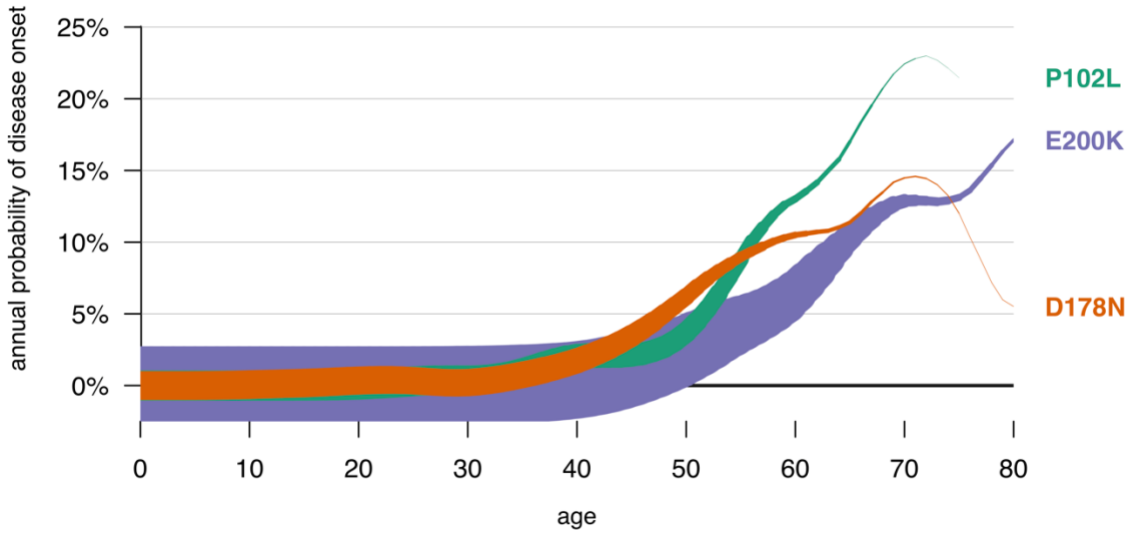

Figure 3.1 | Hazards and survival for the most common* PRNP *mutations. *The hazard, or probability of disease onset, in each year of life (y axis) is plotted against age (x axis) with curve thickness representing the number of individuals still living at each age, which is the product of age-dependent survival and mutation prevalence. Supplementary mutations, and conventional survival curves and hazard plots, are included in Supplemental Figure S3.3.*

Power for randomized pre-approval trials with a clinical endpoint

We set out to calculate how many individuals would need to enroll in order to power prevention trials with an endpoint of disease onset, using the calculated age-dependent hazards for each mutation. While younger individuals or those with a mutation of modest penetrance might seek to enroll in trials or take a preventive drug, they would not contribute much statistical power to

an endpoint of clinical onset. We therefore chose to base our power calculations on individuals with the three most common high penetrance mutations between age 40 and 80.

We estimated how many individuals in this age range have high penetrance *PRNP* mutations. It is estimated based on disease prevalence that 1-2 people per 100,000 in the general population harbor high penetrance *PRNP* mutations (ref. [6] and Supplemental Discussion), but at present, many remain unaware of their risk due to underdiagnosis[29] of affected family members, and few choose predictive testing[23] as the results are currently considered medically unactionable. The number of positive predictive genetic test results that have been provided in the U.S. is *N*=221 (Supplemental Discussion), and based on the estimated proportion of high penetrance variants[6] (75%), and the estimated proportion of positive test result recipients[23] over age 40 (36%) we estimate there are currently ~60 people in the U.S. who are age 40 or older and hold a positive predictive test for a highly penetrant variant.

We used published formulae[30] (see Methods) to calculate statistical power for a log-rank survival test in randomized clinical trials (Table 3.2). Across the three mutations and weighted by their prevalence among cases (Supplemental Figure S3.1) and number of surviving individuals at each age (Figure 3.1), the average annual probability of onset for individuals aged 40 to 80 is 4.6%. We used the 4.6% figure as a baseline hazard, and made the following assumptions: pre-symptomatic individuals are randomized half to drug and half to placebo and followed for 5 years with an endpoint of clinical onset; events in the first year are ignored as a "run-in" period to ensure sufficient drug exposure among individuals analyzed; the withdrawal rate is 15.2% annually (the median value from eight prevention trials reviewed, Supplemental Table S3.4); and the trial is designed for 80% power at the *P*=0.05 threshold. We then performed power calculations for such a trial as a function of the hazard ratio — the ratio of annual risk of onset in drug-treated individuals to that in placebo-treated individuals. For context,

we also determined the effect size, in median years of healthy life added, to which each hazard ratio corresponds (Table 3.2). The calculations are sensitive to which mutations are included, the "run-in" period, the number of years of follow-up, and the assumed withdrawal rate, but we explored a range of different assumptions and none support a different overall interpretation of the data (Supplemental Table S3.5 and Discussion). In particular, the assumption of a 15.2% annual withdrawal rate means that only 44% of original participants remain after 5 years, but even reducing the withdrawal rate to zero only lowers the numbers of participants required by one-third (Supplemental Table S3.5). Because FDA has cautioned against rare disease trial designs that assume a large effect size[31], we focus below on the moderate hazard ratio of 0.5, which would correspond to seven years of life added for treated individuals.

Table 3.2 | Preventive trial requirements under survival test power calculation. *For example, a hazard ratio of 0.5 means that placebo-treated individuals have a 4.6% annual probability of onset, while drug-treated individuals have only a 2.3% annual probability of onset. If a population of individuals were treated from an early age with such a drug, the median age of onset would be postponed by 7 years. To have 80% power at* P=*.05 to detect the effect of such a drug, 65 individuals would need to become symptomatic during the trial — given the 0.5 hazard ratio, about two-thirds of these would occur in the placebo group and one-third would occur in the drug group. Observing this number of disease onsets would require randomizing 813 people for 5 years (data from the first year would be ignored, and the remaining four years of data would be analyzed). *For a hazard ratio of 0.1, most individuals never become sick, thus, the increase in median age of onset (the age where 50% of people have had onset) is undefined.*

hazard ratio	years of life added	onsets required	participants required
0.1	undefined*	6	101
0.2	21	12	189
0.3	14	22	311
0.4	10	37	498
0.5	**7**	**65**	**813**
0.6	5	120	1,406
0.7	4	247	2,724
0.8	2	631	6,602
0.9	1	2,828	28,204

The above power calculations simplistically assume a uniform baseline hazard across all participants, regardless of age and *PRNP* mutation. We also used a simulation to account for the full shape of the hazard curve and diversity of genetic mutations, but the simulated power results were similar to those in Table 3.2 (see Supplemental Table S3.6). Stratification by *PRNP*

mutation did not improve power in our simulations (Supplemental Discussion), perhaps because age of onset distributions (Table 3.1) are wide and overlapping, such that *PRNP* mutation explains only a minority of the overall variance in age of onset (adjusted R^2 = 0.15, linear regression, $P < 1e-32$).

Statistical power might be improved by stratifying clinical trial analysis by relevant additional variables, but there are currently no variables that help to explain age of onset (Supplemental Discussion, Supplemental Table S3.7, and Supplemental Figure S3.4-5). For instance, we found no sex effect, and no evidence that parent and child age of onset are correlated after controlling for *PRNP* mutation and for child's year of birth, a variable that captures some effects of ascertainment bias[32] (Supplemental Table S3.7). A common genetic variant, *PRNP* M129V, is known to affect the clinical and pathological presentation of many forms of prion disease[33] as well as the risk of sporadic and acquired prion disease[34]. This variant has previously been reported to affect age of onset in some forms of genetic prion disease but not others[21,32,35,36]. We found no evidence that codon 129 affects age of onset for P102L or E200K individuals (Supplemental Table S3.7 and Supplemental Figure S3.4). For D178N, our data are suggestive that a 129VV genotype may predispose to earlier onset than MM or MV genotypes (Supplemental Figure S3.4 and Supplementary Discussion), but in the overall dataset, codon 129 failed to explain additional variance in age of onset (Supplementary Discussion).

Based on this analysis, at present it is not possible to adequately power a randomized pre-approval prevention trial with an endpoint of clinical onset in genetic prion disease. For example, for a drug that reduces annual risk by half (hazard ratio of 0.5), powering such a trial would require 813 participants age 40 or older, and even for a drug that reduces annual risk by ten-fold (hazard ratio of 0.1), 101 participants would be required (Table 3.2), versus the ~60 currently estimated to exist in the U.S. Key assumptions underpinning this analysis may change with

time: new stratifying variables could help to predict age of onset, or a first drug for prion disease could improve diagnosis and recruitment (see Discussion). However, the insight that randomized pre-approval prevention trials with a clinical endpoint may not be feasible today has implications for drug development efforts likely to reach the clinic while current assumptions hold. For this reason, we next turned our attention to the possibility that a preventive drug might be developed through the Accelerated Approval pathway using a surrogate biomarker endpoint.

Power for post-marketing studies

We asked whether, if Accelerated Approval were achieved, the required post-marketing studies to confirm clinical benefit could be adequately powered by following drug-treated individuals to clinical onset and comparing their survival to that of historical controls. Such a trial design could increase power but also introduce bias; we considered each issue in turn.

We identified several factors that are likely to decrease the number of participants required to power such a study compared to its randomized pre-approval equivalent: all, rather than half, of individuals are drug-treated; the number of historical controls can be large; a longer trial duration could be considered because the trial would overlap, rather than reduce, the drug's effective market exclusivity period (Supplemental Discussion); and a post-marketing surveillance program might allow newly drug-treated individuals to enter the program on a rolling basis, replacing any who withdraw. The effects of these assumptions (Table 3.3) are collectively to reduce the number of individuals required to demonstrate efficacy of a drug with hazard ratio of 0.5 from 813 to 37. At the same time, the number of individuals available for a trial might increase, because: an approved drug should have broader geographic reach than a pre-approval trial; a treatment might improve awareness and diagnosis of the disease; and a treatment might stimulate more individuals to pursue predictive genetic testing. For instance, of people at 50/50 risk for a *PRNP* mutation, currently only 23% pursue predictive testing,

compared to 60% (2.6X higher) for *BRCA1* or *BRCA2* mutations[37], which are considered medically actionable[38]. Thus, a post-marketing study could be adequately powered with available numbers of individuals for a hazard ratio of 0.5 (Table 3.3) and, over a range of assumptions, would bring power requirements into closer alignment with the number of available individuals (Supplemental Figure S3.6 and Supplemental Discussion).

Table 3.3 | Comparison of power calculations for pre-approval and post-marketing studies. *In each case the calculation is for a hazard ratio of 0.5, the N indicated is for 80% power at the P=0.05 threshold, and all assumptions other than those indicated in the table are the same as for Table 3.2. The number of individuals required for post-marketing studies is determined by simulation (Supplementary Discussion).*

scenario	*N* required	explanation
Randomized pre-approval — 5-year follow-up	813	See Table 3.2
Post-marketing with historical controls — 5-year follow-up	229	Increased power because all, rather than half, of individuals are treated, and *N*=1,000 historical controls are used for comparison.
Post-marketing with historical controls — 15-year follow-up	125	Increased power because longer trial duration may be financially tenable for post-marketing studies. Power is still limited, however, by withdrawal rate, which means that few participants remain at the end of 15 years.
Post-marketing with historical controls — 15-year follow-up, no withdrawal	37	Increased power because the withdrawal rate is set to zero, simulating a scenario where individuals who go on drug can continuously enter the surveillance program, and the cohort being monitored can maintain its size over time.

While we conducted tests to ensure that our power simulation was not itself biased (Supplementary Discussion), a post-marketing study could still be biased in real life, if the historical controls used do not accurately estimate the true hazard rates facing the trial participants[39]. There are no environmental, demographic, or non-*PRNP* genetic factors known to affect prion disease risk or age of onset, although these might nonetheless exist[40,41]. Perhaps of greater concern is that most of our historical controls were collected retrospectively — individuals are only ascertained if they become sick — and may overestimate the hazard rates for individuals followed prospectively[32]. To assess this possible source of bias, we compared the

survival of the limited number of individuals followed prospectively in our dataset (*N*=24 individuals, with a cumulative 145 person-years of follow-up), conditioned on their ages at first ascertainment, to those of individuals with no prospective follow-up. We did not observe a significant difference in hazard (*P*=0.59, Cox proportional hazards test) between these two groups, although this could be due to a lack of power (see Discussion).

Discussion

Both our power calculation and simulation indicate that direct demonstration of clinical benefit in a randomized pre-approval prevention trial would require enrolling a number of *PRNP* mutation carriers that is not currently realistic. For instance, for a drug that reduces annual risk of onset by half (hazard ratio of 0.5), estimated to correspond to a 7-year delay in median age of onset, 80% power was reached only with 813 individuals randomized for 5 years (Table 3.2). Currently, only *N*=221 presymptomatic individuals in the U.S. have positive genetic test results for *PRNP* mutations, and we estimate that only ~60 of these have high penetrance mutations and fall in an age range (≥40 years) where their hazard is sufficiently high that they would contribute appreciable power to a randomized trial with a clinical endpoint. Randomized prevention trials might just barely achieve 80% power under the most wildly optimistic assumptions of an extremely effective drug (hazard ratio of 0.1, reducing annual risk of onset by ten-fold), along with some increase in predictive testing rates and a very successful trial recruitment effort. FDA has cautioned, however, that rare disease trials should not be designed around the hope of a huge effect size[31], and even if a drug were so profoundly effective, it is unlikely that a sponsor would have sufficient confidence in this *a priori* to invest in a trial that is underpowered for more moderate effect sizes.

At least three factors can explain why a randomized trial design following pre-symptomatic individuals to a clinical endpoint was deemed feasible for early-onset Alzheimer's disease, yet appears unviable for genetic prion disease. First, onset is less predictable in genetic prion disease. The standard deviation of age of onset ranges from ±10.0 to ±11.8 years for the three *PRNP* mutations we examined (Table 3.1), whereas estimates of the standard deviation of age of onset for *PSEN1* E280A Alzheimer's disease range from ±6.4 to ±8.6 years[42,43]. In addition, an individual's age of onset in genetic Alzheimer's disease is reported to be correlated with parental age of onset[43], and this property has been used to attempt to enrich for high-hazard individuals in trials[44], whereas we have found no evidence that parent and child age of onset are correlated in genetic prion disease (Supplemental Table S3.7 and ref. [32]). Second, genetic prion disease is rarer. The *PSEN1* preventive trial recruited from a single pedigree of ~5,000 individuals[45] from which 1,065 living individuals with the mutation have been enrolled in a registry[46]. There is no known genetic prion disease family this large. Third, genetic prion disease offers more limited financial incentives for a pharmaceutical sponsor. The cost of the *PSEN1* preventive trial has been estimated at $96 million[44,47], and while this price may be tenable for sponsors in view of potential for an expanded Alzheimer's indication, no similar potential exists for prion disease. Indeed, even in Alzheimer's disease, larger or longer primary prevention trials are likely to prove challenging for the private sector and may require public sector investment[48].

Preclinical proof-of-concept studies in mice have shown that some antiprion agents effective at delaying prion disease on a prophylactic basis become ineffective if given close to the time of clinical onset[16,19], suggesting that trials in symptomatic patients could fail to show a benefit that would have been realizable in preventive treatment. Yet our results here indicate that it would be difficult or impossible to design a well-powered randomized preventive trial with a clinical endpoint in genetic prion disease. Together, these observations argue for the characterization of biomarkers suitable as endpoints in presymptomatic genetic prion disease, and for their

evaluation by regulatory agencies as surrogate trial endpoints. Accompanying manuscripts describe one possible route to Accelerated Approval using a surrogate biomarker endpoint[49,50].

If Accelerated Approval could be achieved, then a post-marketing study would be required to confirm clinical benefit. We considered a model in which drug-treated individuals are enrolled in a surveillance program and their survival is compared to that of historical controls. We estimate that, compared to randomized pre-approval studies, such a program could reduce the number of individuals required for 80% power at the P=0.05 threshold by 3- to 20-fold. Meanwhile, conditional approval of a first prion disease drug may alter key parameters such as diagnosis, recruitment, and genetic testing rates, the last of which alone could increase participant availability by more than 2-fold. Thus, while power for any trial depends upon how effective the drug is, there exists a range of assumptions under which a post-marketing study could be adequately powered. There may be various formats through which the Accelerated Approval requirement of a post-marketing study to confirm clinical benefit could be met.

Under some assumptions, a post-marketing study might last a decade or longer and would benefit from following all mutation carriers taking the drug. With creative and careful planning, we propose that these goals could be achieved. In one model, a post-marketing study might take the form of a surveillance program, in which treated patients are followed long-term, perhaps in collaboration with existing prion specialist clinics and surveillance centers worldwide. In such a model, drug costs would be reimbursed by payors, in contrast to a more traditional sponsor-funded pivotal trial. While this model would be a departure from the more conventional design of most post-marketing studies required for recent Accelerated Approval drugs[2], precedents exist for regulatory innovation in this area. For example, FDA's Risk Evaluation and Mitigation Strategies (REMS) program for drugs with serious safety concerns entails indefinite post-market enrollment and monitoring of treated patients[51], and post-approval study

requirements for medical devices often include registries or surveillance efforts and are not always industry-funded[52,53].

Our study has several limitations. First, true age of onset distributions can only be obtained prospectively[54], whereas our data are largely retrospective. We have included asymptomatic individuals with pathogenic *PRNP* variants where possible, but our ascertainment of them is certainly incomplete due to limited uptake of predictive testing[23]. This bias may tend to make our estimates of age of onset overly pessimistic[32,55]. To the extent that true age of onset is older, or total lifetime risk lower, than our data suggest, randomized preventive trials with a clinical endpoint would require even greater numbers of individuals, and thus further increase our caution around this study design. Second, although our dataset is, to our knowledge, the largest ever reported for genetic prion disease age of onset, our statistical power to detect genetic modifiers, which might aid in age of onset prediction, is still limited. Third, although we have attempted to select a reasonable set of assumptions for modeling clinical trials, we have by no means exhaustively sampled the set of possible trial designs and parameters. Fourth, powering a post-marketing study will require a good historical control dataset to compare to, and our dataset, which was collected mostly retrospectively, may or may not be adequate. We found no evidence that our dataset overestimates the hazards facing prospectively followed individuals, but this could be due to a lack of power in our analysis. Fifth, the ascertainment of genetic prion disease by prion surveillance centers may be biased towards rapidly progressive phenotypes, meaning that the prevalence of more slowly progressive forms might be underestimated.

Our findings highlight two priorities for the prion field. First, the discovery and characterization of biomarkers capable of serving as trial endpoints may be essential to enable near-term presymptomatic trials in genetic prion disease. Second, a post-approval surveillance mechanism for age of onset merits consideration as one option for confirmation of clinical

benefit in the context of Accelerated Approval. The ability to access therapies that can prevent or delay prion disease, yet which are likely to be less effective or ineffective after symptom onset, could be greatly enhanced by success in these areas.

Methods

Literature annotation. We considered 70 reportedly pathogenic *PRNP* variants (Supplemental Table S3.1) and reviewed primary literature to determine which had evidence of at least one family with at least three affected individuals in a pattern consistent with Mendelian segregation, or had a documented case with a *de novo* mutation. We identified 27 such variants, deemed likely high penetrance variants. The remainder were seen in isolated patient(s) with a negative or unknown family history, and/or have population allele frequencies inconsistent with high penetrance[6]. These variants will include both benign and low-risk variants. It is possible that some genuinely high penetrance variants may also lack literature evidence for high penetrance due to missing family history information or an unavailability of family member DNA to confirm *de novo* status, but this issue will only affect variants with very low case counts and thus will have minimal impact on the results reported here.

Data collection. This study was performed under ethical approval from the Partners Healthcare Institutional Research Board (2014P000226/MGH) and the Broad Institute's Office of Research Subjects Protection (ORSP-2121 and NHSR-4190). Age of onset data were gathered from nine study centers: the UK National Prion Clinic, the German Reference Center for TSEs, the Memory and Aging Center at University of California San Francisco, the Australian National CJD Registry, the reference center for CJD at University of Bologna, the DOXIFF study at the Mario Negri Institute, the Japanese national prion surveillance network, the French national reference center for CJD, and the Spanish National Center for Epidemiology. The data include both previously reported and newly identified families and individuals. Data were collected

through clinical visits, reports to prion surveillance centers, and family histories, as previously described[32,56–61]. Age of onset was based on the earliest date of symptoms, determined by the patient or witnesses, that subsequently developed into prion disease. Data on the number of positive predictive genetic tests for *PRNP* mutations was provided by the National Prion Disease Pathology Surveillance Center for this study.

Life tables and hazard curves. We tabulated, for each *PRNP* mutation and for each age from 1-100, the number of individuals alive at the beginning of the interval (lives; l), becoming sick or dying within the interval (deaths; d), or being censored – alive and well at last followup or dead of a different cause – within the interval (withdrawals; w). The raw hazard (q) was computed as onsets divided by the mean number of people observed over the interval: $q = d/(l - w/2)$, and a smoothed hazard (q_smooth) was computed by passing a Gaussian filter (sd=3 years, maximum width=15 years) over the raw hazard. The proportion surviving for each interval (p) was 100% for the first year and was computed as (1-q) times the proportion surviving in the previous interval for every year thereafter. To compute the 95% confidence intervals on the smoothed hazard, we sampled each mutation's data, with replacement, 1000 times, generated life tables for iteration, and then chose the 2.5^{th} and 97.5^{th} percentile of the hazards in the bootstrapped distributions at each age.

Assumptions. To determine a reasonable assumption for withdrawal rate, we performed Google Scholar searches for preventive trials in neurology (N=2) or cardiology (N=6). The annual withdrawal rate was computed as $w = 1 - \exp(\log(A)/t))$, where A is the proportion of patients completing the trial at time t. Results are summarized in Supplemental Table S3.3.

Power calculation. The number of events (disease onsets, d) required was computed per Schoenfeld et al[30] (equation 1). The number of patients required in order to observe that number

of disease onsets was computed using an exponential model per Kohn et al[62]. Hazard in the placebo group was the baseline hazard specified in the text (4.6% for Table 3.2), and hazard for the drug group was the baseline hazard times the hazard ratio. The cumulative event rate in each group was computed as C = (h/(h+w)) * (1-exp(-(h + w)*t))), where h = hazard, w = withdrawal rate, and t = years of followup. The overall cumulative event rate C_{tot} was the average of the cumulative event rates for the two groups, weighted by proportion treated (in this case, 50/50). The number of randomized individuals required for d events to be observed was calculated as d /C_{tot}. To account for ignoring the first *g* years of data, we reasoned that the cumulative rate of events usable in the final dataset would be C_{usable} = (h/(h+w)) * (1-exp(-(h + w)*t))) - (h/(h+w)) * (1-exp(-(h + w)*g))), which simplifies to C_{usable} = (h/(h+w)) * (exp(-(h + w)*g)-exp(-(h + w)*t))

Simulations. Details of the simulations of randomized trials and historical control trials are in the Supplemental Discussion.

Source code and data availability. Raw data cannot be made available due to identifiability concerns, but life tables have been included in supplement (Supplementary Life Tables). All analyses were conducted in the R programming language. Life tables and R source code are presented in a public GitHub repository at https://github.com/ericminikel/prnp_onset and are sufficient to reproduce most analyses and figures herein.

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Chapter 4. Mass spectrometry-based quantification of prion protein in cerebrospinal fluid

Publication history:

This chapter is currently being prepared for pre-print posting to bioRxiv and submission to a peer-reviewed journal as:

Minikel EV*, Kuhn E* (*equal contribution) et al, Domain-specific quantification of prion protein in cerebrospinal fluid by targeted mass spectrometry.

Attributions:

I conceived and designed the study together with Sonia Vallabh, Eric Kuhn, Stuart Schreiber, and Steve Carr. I was primarily responsible for designing the experiments to answer our biological questions of interest, while Eric Kuhn was primarily responsible for the mass spectrometry methods development. Experiments were performed primarily by myself and Eric Kuhn, with contributions from Alexandra Cocco, Sonia Vallabh, Andrew Reidenbach, and Christina Hartigan. Eric Kuhn and Alexandra Cocco processed the raw data to yield peptide abundance measures in each sample; I performed all downstream data analysis. I created all of the main text display items. Eric Kuhn and I both created supplemental display items. Eric Kuhn and I wrote the manuscript together. Other authors contributed to data collection and/or interpretation, and revision of the manuscript. Stuart Schreiber and Steve Carr supervised the research.

Abstract

Therapies currently in preclinical development for prion disease seek to lower prion protein (PrP) expression in the brain. Trials of such therapies are likely to rely on quantification of PrP in cerebrospinal fluid (CSF) as a pharmacodynamic biomarker and possibly as a trial endpoint. Studies using PrP ELISA kits have reproducibly shown that CSF PrP is lowered in the symptomatic phase of disease, a potential confounder for reading out the effect of PrP-lowering drugs in symptomatic patients. To date it has been unclear whether the reduced abundance of PrP in CSF results from its incorporation into plaques, retention in intracellular compartments, downregulation as a function of the disease process, or other factors. Because misfolding or proteolytic cleavage could potentially render PrP invisible to ELISA even if its concentration were constant or increasing in disease, we sought to establish an orthogonal method for CSF PrP quantification. We developed a targeted mass spectrometry method based on multiple reaction monitoring (MRM) of nine PrP tryptic peptides quantified relative to known concentrations of isotopically labeled standards. Analytical validation experiments showed process replicate coefficients of variation below 15%, good dilution linearity and recovery, and

suitable performance for both CSF and brain homogenate and across humans as well as preclinical species of interest. In *N*=55 CSF samples from individuals referred to prion surveillance centers with rapidly progressive dementia, all six human PrP peptides, spanning the N- and C-terminal domains of PrP, were uniformly reduced in prion disease cases compared to individuals with non-prion diagnoses. This confirms the findings from ELISA studies, demonstrating that lowered CSF PrP concentration in prion disease is a genuine result of the disease process and not merely an artifact of ELISA-based measurement. We provide a targeted mass spectrometry-based method suitable for preclinical and clinical quantification of CSF PrP as a tool for drug development.

Introduction

Prion disease is a fatal and incurable neurodegenerative disease caused by misfolding of the prion protein (PrP), and may be sporadic, genetic, or acquired[1]. Therapies currently in preclinical development for prion disease seek to lower PrP levels in the brain, a genetically well-validated strategy[2]. Clinical trials of PrP-lowering agents will rely on quantification of PrP in cerebrospinal fluid (CSF) as, at a minimum, a pharmacodynamic biomarker[3]. This marker may, however, have even greater importance. Predictive testing of pre-symptomatic individuals harboring highly penetrant genetic mutations[4] that cause prion disease provides an opportunity for early therapeutic intervention to preserve healthy life, but randomization to a clinical endpoint in this population appears infeasible[5]. The U.S. Food and Drug Administration has indicated its willingness to consider lowered CSF PrP in this population as a potential surrogate endpoint for Accelerated Approval[2,6]. Precise quantification of PrP in CSF will be essential to the development of prion disease therapeutics.

PrP is an extracellular GPI-anchored protein that can be shed from the plasma membrane by

ADAM10 and other peptidases[7,8]. CSF PrP is predominantly soluble and full-length[9], suggesting that it originates chiefly from this proteolytic shedding near the C terminus, although lower molecular weight fragments of PrP have also been identified in CSF[10], which may originate from other endoproteolytic events[7,11], and anchored PrP is also released from cells on exosomes[12]. PrP is sufficiently abundant in CSF, at concentrations of tens or hundreds of nanograms per milliliter, to be readily quantified with enzyme-linked immunosorbent assay (ELISA). Studies using ELISA have reproducibly found that CSF PrP is decreased in the symptomatic phase of prion disease[3,13–16]. Therefore, even though CSF PrP is brain-derived and exhibits good within-subject test-retest reliability in individuals without prion disease[3], it might be difficult to use this biomarker to read out the effect of a PrP-lowering drug in symptomatic individuals, because it is unclear whether to expect that such a drug should cause a further decrease in CSF PrP as a direct pharmacodynamic effect, or an increase in CSF PrP due to alleviation of the disease process. This confounder could potentially limit the use of ELISA-based CSF PrP quantification as a pharmacodynamic biomarker to pre-symptomatic individuals only.

Prion disease is caused by a gain of function[1], and animal studies have shown that total PrP in the brain increases over the course of prion disease as misfolded PrP accumulates[17–19]. The paradoxical decrease in PrP in CSF during prion disease might be due to its incorporation into plaques[20], diversion into intracellular locations[21,22], or downregulation as a function of the disease process[23]. However, occlusion of epitopes due to misfolding[24] or upregulation of proteolytic cleavage in disease[7,23,25] could also render PrP invisible to ELISA even if its concentration were constant or increasing. We therefore sought to establish an orthogonal method for CSF PrP quantification.

Here, we describe quantification of CSF PrP using a form of targeted mass spectrometry — multiple reaction monitoring (MRM)[26]. We analyze *N*=55 clinical samples from prion and non-

prion disease patients by PrP MRM and find that six out of six PrP tryptic peptides, spanning N- and C-terminal domains of the protein, are uniformly decreased in prion disease. Thus, PrP concentration is genuinely lowered in prion disease CSF. Our findings supply an alternative method for validating the findings of ELISA-based studies of CSF PrP, and provide a potential assay for use as a pharmacodynamic biomarker in preclinical drug development and in human trials.

Methods

Cerebrospinal fluid and brain samples

This study was approved by the Broad Institute's Office of Research Subjects Protection (ORSP-3587). Written consent for research use of samples was obtained from patients or next of kin as appropriate.

All CSF samples in this study have been previously reported[3]. CSF samples for assay development were large volume normal pressure hydrocephalus samples provided by MIND Tissue Bank at Massachusetts General Hospital. Clinical CSF samples were from individuals referred to prion surveillance centers in Italy (Bologna) or Germany (Göttingen) with suspected prion disease and who were later either determined by autopsy or probable diagnostic criteria[27] including real-time quaking-induced conversion (RT-QuIC[28]) as prion disease, or confirmed as non-prion cases on the basis of autopsy, patient recovery, or definitive other diagnostic test. Individuals with non-prion diagnoses (*N*=21) included autoimmune disease (*N*=8), non-prion neurodegenerative disease (*N*=6), psychiatric illness (*N*=3), stroke (*N*=1), brain cancer (*N*=1), and other (*N*=2). Sporadic prion disease cases (*N*=23) included probable cases (*N*=10) and autopsy-confirmed definite cases (*N*=13, of subtypes: 6 MM1, 3 VV2 and 4 other/unknown). Genetic prion disease cases (*N*=11) included D178N (*N*=2), E200K (*N*=7), and V210I (*N*=2).

Samples were de-identified and broken into five batches (to be run on different days) randomly using an R script. Assay operators were blinded to diagnosis. PrP ELISA, hemoglobin, and total protein measurements on these CSF samples were previously reported[3].

Rat and cynomolgus monkey CSF were purchased from BioIVT. Human brain tissue was from a non-prion disease control individual provided by the National Prion Disease Pathology Surveillance Center (Cleveland, OH). Mouse brain tissue from Edinburgh PrP knockout mice[29] backcrossed to a C57BL/10 background[30], and matching tissue from wild-type C57BL/10 mice, were provided by Gregory J. Raymond (NIAID Rocky Mountain Labs, Hamilton, MT).

Recombinant protein preparation

Untagged recombinant HuPrP23-230 (MW=22,878) and MoPrP23-231 (MW=23,151), corresponding to full-length post-translationally modified human and mouse PrP without the signal peptide or GPI signal but retaining an N-terminal methionine, were purified by denaturation and Ni-NTA affinity from *E. coli* inclusion bodies as previously described[31,32], using a vector generously provided by Byron Caughey (NIAID Rocky Mountain Labs, Hamilton, MT). ^{15}N incorporation was achieved by growing the *E. coli* in ^{15}N cell growth medium (Cambridge Isotope Laboratories CGM-1000-N) induced with ^{15}N auto-induction medium (Millipore 71759-3). Protein concentration was determined by amino acid analysis (AAA, New England Peptide). Percent ^{15}N isotopic incorporation was estimated using LC-MS/MS. ^{15}N labeled human recombinant prion protein (10 µg) was digested and desalted following the procedure as described in *PrP MRM assay* and analyzed as described in *Pilot LC-MS/MS analysis*. Precursor masses for ^{15}N were extracted from the chromatograms using XCalibur software Qualbrowser software (Thermo) 3.0.63 with a 6 mz window of centered on the precursors and charge states listed in Supplemental Table S4.1. Isotopic envelopes between protein expressed in 15N containing media and standard media were compared visually. Summation of all observed mz

peak areas less than the ^{12}C monoisotopic mass peak were compared to summation of all expected isotope peak to estimate the overall completeness of ^{15}N incorporation (Supplemental Figure S4.1).

Pilot LC-MS/MS analyses of CSF and recombinant PrP

Samples of dried digested recombinant proteins or human cerebrospinal fluid (processed as described in *PrP MRM assay*) were reconstituted in 3% acetonitrile/5% acetic acid to a final concentration of approximately 1 µg total protein per 1 µL and analyzed in a single injection using a standard 2h reversed-phase gradient. LC-MS/MS was performed using a QExactive mass spectrometer (Thermo) equipped with a Proxeon Easy-nLC 1200 and a custom built nanospray source (James A. Hill Instrument Services). Samples were injected (1 to 2 µg) onto a 75 um ID PicoFrit column (New Objective) packed to 20 cm with Reprosil-Pur C18 AQ 1.9 um media (Dr. Maisch) and heated to 50°C. MS source conditions were set as follows: spray voltage 2000, capillary temperature 250, S-lens RF level 50. A single Orbitrap MS scan from 300 to 1800 m/z at a resolution of 70,000 with AGC set at 3e6 was followed by up to 12 MS/MS scans at a resolution of 17,500 with AGC set at 5e4. MS/MS spectra were collected with normalized collision energy of 25 and isolation width of 2.5 amu. Dynamic exclusion was set to 20 s and peptide match was set to preferred. Mobile phases consisted of 3% acetonitrile/0.1% formic acid as solvent A, 90% acetonitrile/0.1% formic acid as solvent B. Flow rate was set to 200 nL/min throughout the gradient, 2% - 6% B in 1 min, 6% - 30% B in 84 min, 30% - 60% B in 9 min, 60% - 90% B in 1 min with a hold at 90% B for 5 min. MS data were analyzed using Spectrum Mill MS Proteomics Workbench software Rev B.06.01.202 (Agilent Technologies). Similar MS/MS spectra acquired on the same precursor m/z within +/- 60 sec were merged. MS/MS spectra were excluded from searching if they failed the quality filter by not having a sequence tag length > 0 (i.e., minimum of two masses separated by the in-chain mass of an amino acid) or did not have a precursor MH+ in the range of 600-6000. All extracted spectra

were searched against a UniProt database containing human and mouse reference proteome sequences downloaded from the UniProt web site on October 17, 2014 with redundant sequences removed. A set of common laboratory contaminant proteins (150 sequences) were appended to this database and verified to contain the sequences for human and mouse major prion protein. Search parameters included: ESI-QEXACTIVE-HCD-v2 scoring, parent and fragment mass tolerance of 20 ppm, 40% minimum matched peak intensity and 'trypsin' enzyme specificity up to 2 missed cleavages. Fixed modification was carbamidomethylation at cysteine and variable modifications were oxidized methionine, deamidation of asparagine and pyro-glutamic acid. Database matches were autovalidated at the peptide and protein level in a two-step process with identification FDR estimated by target-decoy-based searches using reversed sequences. The list of identified proteins was further filtered to contain proteins and protein isoforms with at least 2 unique peptides and an aggregate protein score greater than 20. Protein-peptide comparison report comprised of all validated peptides was exported which included a ranked summary by intensity of all peptides unique to prion protein.

Selection of PrP peptides for MRM assay development

Nine peptides covering 4 species were selected from computational and empirical data (Supplemental Table S4.2 and Figures S4.2-S4.4). Peptides were prioritized based our criteria previously described[33] and outlined in detail in Supplemental Figure S4.2 as well as considerations based on PrP biology and desired assay applications described in Results (Figure 4.1). One peptide, PIIHFGSDYEDR, was included after being detected in CSF despite an N-terminal proline.

The nine peptides were synthesized (New England Peptide) using stable isotope labeled [$^{15}N_4{}^{13}C_6$]Arg or [$^{15}N_2{}^{13}C_6$]Lys at the C terminus and purified peptide specifications previously

outlined (>95% chemical purity, >99% isotopic purity, quantified by AAA) in order to qualify as standards for Tier 1 or Tier 2 assays[34].

PrP MRM assay

In devising a CSF sample preparation protocol, we drew upon our experience with MRM analysis of plasma[35] and published mass spectrometry protocols for prion studies[36,37].

Uniformly labeled ^{15}N-labeled recombinant HuPrP23-230 (starting concentration 2.42 mg/mL determined by AAA) with an estimated isotopic incorporation >97.5% (*see Recombinant Protein Preparation*) was diluted 1:5,000 in phosphate-buffered saline containing 1 mg/mL bovine serum albumin and 0.03% CHAPS. This solution was then further diluted 1:20 (1.5 µL added into 30 µL) into CSF samples (final concentration 24.2 ng/mL) prior to the denaturation and digestion workflow described below. ELISA analysis indicated that this concentration of carrier protein and detergent was sufficient to keep recombinant PrP in solution and avoid loss to plastic, without appreciably affecting CSF total protein content.

All concentrations listed below are final concentrations. For each replicate, 30 µL of CSF was incubated with 0.03% CHAPS with 6 M urea (Sigma U0631) and 20 mM TCEP (Pierce 77720) at 37°C while shaking at 800 rpm in an Eppendorf Thermomixer for 30 min to denature the protein and reduce disulfide bonds. 39 mM iodoacetamide was added for 30 min in the dark at room temperature to alkylate cysteine residues. Urea was diluted to 900 mM by the addition of 0.2 Trizma pH 8.1 (Sigma T8568) to permit trypsin activity. 1 µg of trypsin (Promega V5113) was added (final concentration of ~1.4 ng/µL), providing at least a 1:50 trypsin:substrate ratio for CSF samples with total protein content <1.6 mg/mL, which includes 97% of CSF samples we have analyzed[3]. Trypsin digestion proceeded overnight shaking at 800 rpm at 37°C. Digestion was stopped with 5% formic acid and transfer to 4°C. A mix containing 100 fmol of each ^{15}N/^{13}C-

labeled synthetic heavy peptide was then added to the CSF digests (3.33 nM peptide, equivalent to ~76 ng/mL full-length PrP based on an approximate molecular weight of 22.8 kDa).

To desalt the samples, StageTips[38] comprised of two punches of C18 material (Empore 66883-U) fitted into a 200 µL pipette tip using a 16 gauge needle with 90° blunt ends (Cadence Science 7938) and a PEEK tubing puncher (Idex 1567) were placed onto microcentrifuge tubes using an adapter (Glycen CEN.24). Tubes were centrifuged at 2,500*g* for 3 min after each step, as follows: conditioning with 50 µL 90% acetonitrile / 0.1% trifluoroacetic acid; equilibration with 50 µL 0.1% trifluoroacetic acid and priming with 10 µL 0.1% trifluoroacetic acid (no spin after priming); addition of CSF digest in increments of 150 µL; two washes with 50 µL of 0.1% trifluoroacetic acid; and two elutions into a new microcentrifuge tube with 50 µL of 40% acetonitrile / 0.1% trifluoroacetic acid. Eluates were frozen at -80°C.

Frozen samples were dried under vacuum centrifugation and resuspended in 12 µL 3% acetonitrile/5% acetic acid and placed into a vortexer for 5 minutes at room temperature. Samples were then centrifuged at 12,000*g* for 5 minutes and 10 µL of the supernatant was transferred to an HPLC vial (Waters 186000273). HPLC vials were centrifuged briefly (30 - 60s) at 1,200*g* to remove air bubbles and transferred into the nanoLC autosampler compartment set to 7°C. Samples were analyzed on a TSQ Quantiva triple quadrupole mass spectrometer installed with a Nanospray Flex source and Easy-nLC 1000 system (Thermo). Ion source was set to positive ion mode with capillary temperature of 300°C, spray voltage of 2,000 and sweep gas set to 0. The Easy-nLC 1000 system was primed with mobile phase A (3% acetonitrile / 0.1% formic acid), mobile phase B (90% acetonitrile / 0.1% formic acid). Samples were injected (2 µL, 20% of digested sample) onto a 0.075 mm ID PicoFrit (New Objective) column pulled to a 10 µm emitter and custom-packed to 20 cm with 1.9 µm 200Å C18-AQ Reprosil beads (Dr. Maisch). The LC gradient was 0% B to 30% B for 55 min, 30% B to 60% B in 5 min, 60% B to

90 % B in 1 min using a flow rate of 200 nL/min. Collision energies were optimized over 4 steps, 2.5 V per step in batches of less than 500 transitions per batch. Three to four transitions were monitored per peptide using the MRM transitions listed in Supplemental Table S4.1 using a 1.5s cycle time. In addition, even though the corresponding heavy peptides were not synthesized, we monitored for the transitions that corresponded to the oxidized methionine version of the peptide VVEQMCITQYER.

Data analysis

Extracted Ion chromatograms (XIC) of all transition ions were verified and integrated using a Skyline document as described[39] (Skyline version 4.1.0.11796, https://brendanx-uw1.gs.washington.edu/labkey/project/home/software/Skyline/begin.view) that contained all the selected peptides for the selected species of prion protein. After peak integration, the Skyline report file was exported as a text delimited file where the peak areas in the columns labeled as "Light", "Heavy" or "15N" for the single most intense, interference-free, reproducibly measured transition (Supplemental Table S4.1) were used for quantification and subsequent statistical analysis. Columns included for export were: Protein Name, Protein Gene, Protein Species, Peptide Sequence, Peptide Modified Sequence, File Name, Acquired Time, Replicate Name, SampleGroup, Peptide Retention Time, Precursor Mz, Fragment Ion, Area, Area Ratio, Total Area, Total Area Ratio.

In order to determine the response of each peptide in terms of L:^{15}N ratio as well as evaluate dilution linearity of the assay, we spiked 0, 2.4, 24, or 240 ng/mL of ^{15}N-labeled recombinant human PrP into a single control CSF sample (from an individual with normal pressure hydrocephalus) in triplicate. For each peptide, we then fitted a linear model correlating the (non-zero) spiked concentrations to the observed ^{15}N:light ratios with the intercept fixed at zero,

yielding slopes ranging from 39 to 448 ng/mL. Each peptide was then assigned a response factor equal to the highest slope observed for any peptide (448 ng/mL) divided by its own slope.

In N=12 individual replicates (out of 110) of the clinical samples, the oxidized methionine (met-ox) version of the VVEQMCITQYER peptide was more abundant than the reduced version, despite the inclusion of a reduction step in sample preparation. The VVEQMCITQYER peptide was omitted from analysis for these replicates.

Statistical analysis and data visualization were performed using R 3.5.1 in RStudio 1.1.456. Statistical tests are named throughout the text and are all two-sided. Reported *P* values are nominal.

Data and source code availability

All processed data and source code for this study are provided in a public GitHub repository at https://github.com/ericminikel/prp_mrm and are sufficient to reproduce the analyses and figures herein. This repository also includes a summary table for download containing the MRM results (light and ^{15}N peak areas, light:^{15}N ratio and normalized PrP concentration in ng/mL) for all clinical samples and all peptides.

Results

Design of the PrP MRM assay

PrP ranked number 8 in intensity out of 322 confidently detected proteins in single-shot, LC-MS/MS analysis of human CSF digested with trypsin (see Methods). This indicated that PrP was a good candidate for direct analysis by LC-MRM-MS in CSF without additional

fractionation[40] or enrichment methods[41]. PrP peptides with the highest MS intensities after digestion of recombinant human or mouse PrP as well as human CSF were preferentially ranked according to criteria described in Methods and Supplemental Figure S4.2. We selected six human peptides as well as three peptides specific to mouse, rat, and/or cynomolgus macaque PrP, to support assay application to preclinical drug development (Figure 4.1A, Supplemental Figure S4.4, and Supplemental Table S4.2). Peptides were chosen to span the N- and C-terminal domains of PrP, up- and down-stream of alpha and beta cleavage sites, allowing us to quantify proteolytic fragments of cleaved PrP (Figure 4.1A and Supplemental Figure S4.4).

We further designed a workflow for the PrP MRM assay (Figure 4.1B) incorporating an incubation in the presence of a strong chaotrope to denature both properly folded and misfolded forms of PrP. We then reduced and alkylated the protein mixture to break the disulfide bonds and prevent them from refolding, and thereby make the whole protein accessible to the enzymatic processing of r-trypsin. To permit quantification of endogenous, unlabeled (hereafter "light" or "L") PrP, we added a 9-plex mixture of synthetic $^{15}N/^{13}C$-stable isotope labeled (hereafter "heavy" or "H") internal standard peptides to CSF samples after digestion. In addition to properly identifying the endogenous light peptides by MRM, these heavy peptides control for variability in retention on the LC and the ionization on the MS, caused by the presence of a large number of peptides in the mixture, with over 4,000 peptides identified in CSF pilot study. To further control for the analytical variability that can occur during enzymatic proteolysis and solid phase extraction (SPE) using StageTips[38], we also added uniformly ^{15}N-labeled recombinant human PrP (hereafter "^{15}N") into clinical samples prior to analysis (Figure 4.1B).

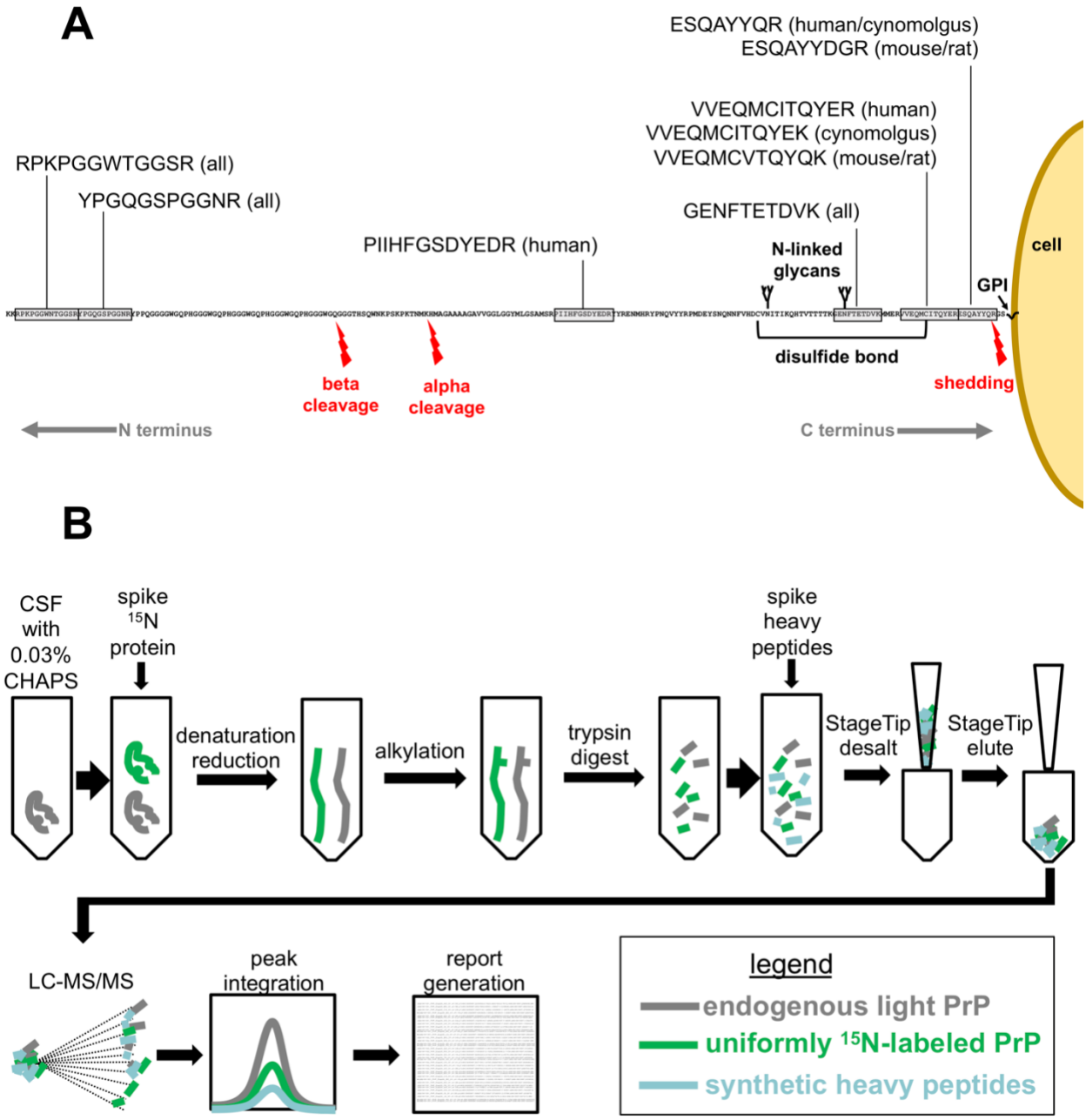

Figure 4.1 | Design of the PrP MRM assay. A) *Selection of PrP tryptic peptides for MRM. The full sequence of human PrP (residues 23-230) after post-translational modifications (removal of signal peptide residues 1-22 and GPI signal residues 231-253) is shown, GPI-anchored to the outer leaflet of the plasma membrane, with the position of selected peptides and their rodent or monkey orthologs shown relative to the positions of N-linked glycans, a disulfide bond, and endogenous proteolytic events*[7]*.* ***B)*** *PrP MRM workflow as described in Methods.*

Assessment of PrP MRM performance

We conducted a series of analytical validation experiments to assess the performance of the PrP MRM assay. To assess cross-species selectivity and sensitivity, we analyzed human, rat, and cynomolgus macaque CSF as well as mouse and human brain homogenate. For the six PrP peptides harboring sequence differences between species (Figure 4.1A, Supplemental Table S4.2), we observed excellent selectivity, with peptides consistently detected in sequence-matched species above the background level observed in non-sequence-matched species (Supplemental Figure S4.5A-B) and with technical replicate mean coefficients of variation (CVs) all <15% (Supplemental Table S4.3). In a dose-response experiment, ^{15}N-labeled recombinant human PrP added to human CSF in dose-response was recovered with good dilution linearity over at least the two orders of magnitude chosen for this experiment (Supplemental Figure S4.5C). Dilution linearity for endogenous CSF PrP was confirmed by mixing high-PrP and low-PrP human CSF samples in different proportions (Supplemental Figure S4.5D). We found that the total protein and lipid content of brain tissue precluded analysis of ≥1% brain homogenates, but 0.5% brain homogenates were technically tractable in PrP MRM. Using mixtures of wild-type mouse brain homogenates titrated into a background of PrP knockout mouse brain homogenate, we prepared samples to evaluate the specificity and dilution linearity across a PrP concentration range expected in CSF samples obtained from patients. MRM analysis revealed a linear response for three mouse sequence-matched peptides (Supplemental Figure S4.5E).

To support measurement of endogenous unlabeled PrP in *N*=55 human CSF clinical samples (see next section), we performed quality control analysis using the ^{15}N protein added into each sample before digestion as well as the cognate synthetic heavy peptides added after digestion. Clinical samples were divided into 5 batches run on separate days; each sample was processed and analyzed in duplicate within its day. A common control sample was also measured in duplicate on each day.

As expected, the mean absolute MS response, either from ^{15}N recombinant or from endogenous light PrP, varied by over an order of magnitude between the six PrP peptides (Figure 4.2A-B), primarily reflecting differences in electrospray ionization efficiencies[40,42,43]. The recovery of the six peptides from endogenous PrP relative to one another was preserved across CSF patient samples (Figure 4.2A), but differed from the recovery of the corresponding peptides derived from ^{15}N recombinant PrP (Figure 4.2B), resulting in a ~10-fold difference in mean light:^{15}N ratio between different peptides (Figure 4.2C and Supplemental Table S4.5). These differences between peptides were consistent between days (Supplemental Figure S4.6), and assessment of the ^{15}N:H ratio, which is expected to be the same in all samples, indicated that the analytical process was consistent between samples and days (Supplemental Table S4.4). The differences in peptide recoveries may reflect differences in proteolytic processing and/or post-translational modification (Figure 4.1A) of PrP in CSF relative to the bacterially expressed recombinant ^{15}N version used as reference. For example, a significant proportion of brain PrP is N-terminally truncated[11], and PrP cleavage products have been observed in CSF as well[10]. PrP is known to be variably glycosylated at residue N197, but our assay will only detect the non-glycosylated form of the GENFTETDVK peptide containing this site. This may account for the much lower response of this peptide in CSF vs. the ^{15}N standard (Figure 4.2). For the C-terminal peptide ESQAYYQR, our assay might not detect proteolytically shed PrP if the cut site for ADAM10, the predominant PrP sheddase[44], in human PrP is homologous to its reported cut site in rodent PrP[8,45]. For the most N-terminal peptide monitored, RPKPGGWNTGGSR, the presence of a retained N-terminal methionine three residues upstream of this sequence in bacterially expressed PrP, detected here (Supplemental Figure S4.3) consistent with reported N-terminal methionine excision patterns in *E. coli*[46], could alter its trypsin digest efficiency relative to brain and CSF PrP. Because we lacked access to purified full-length mammalian PrP to serve a reference standard, we cannot definitively dissect the reasons for the differences in recovery

between peptides. Accordingly, we assigned each peptide a response factor based on the slope of the light:^{15}N ratio observed in the ^{15}N dose-response experiment (Methods, Supplemental Figure S4.8). Applying these response factors to the light:^{15}N ratios brought each peptide's abundance into line with the highest-responding peptide, and yielded estimates of CSF PrP concentration in CSF that averaged 421 ng/mL across samples and all peptides (Supplemental Table S4.5, Supplemental Figure S4.8).

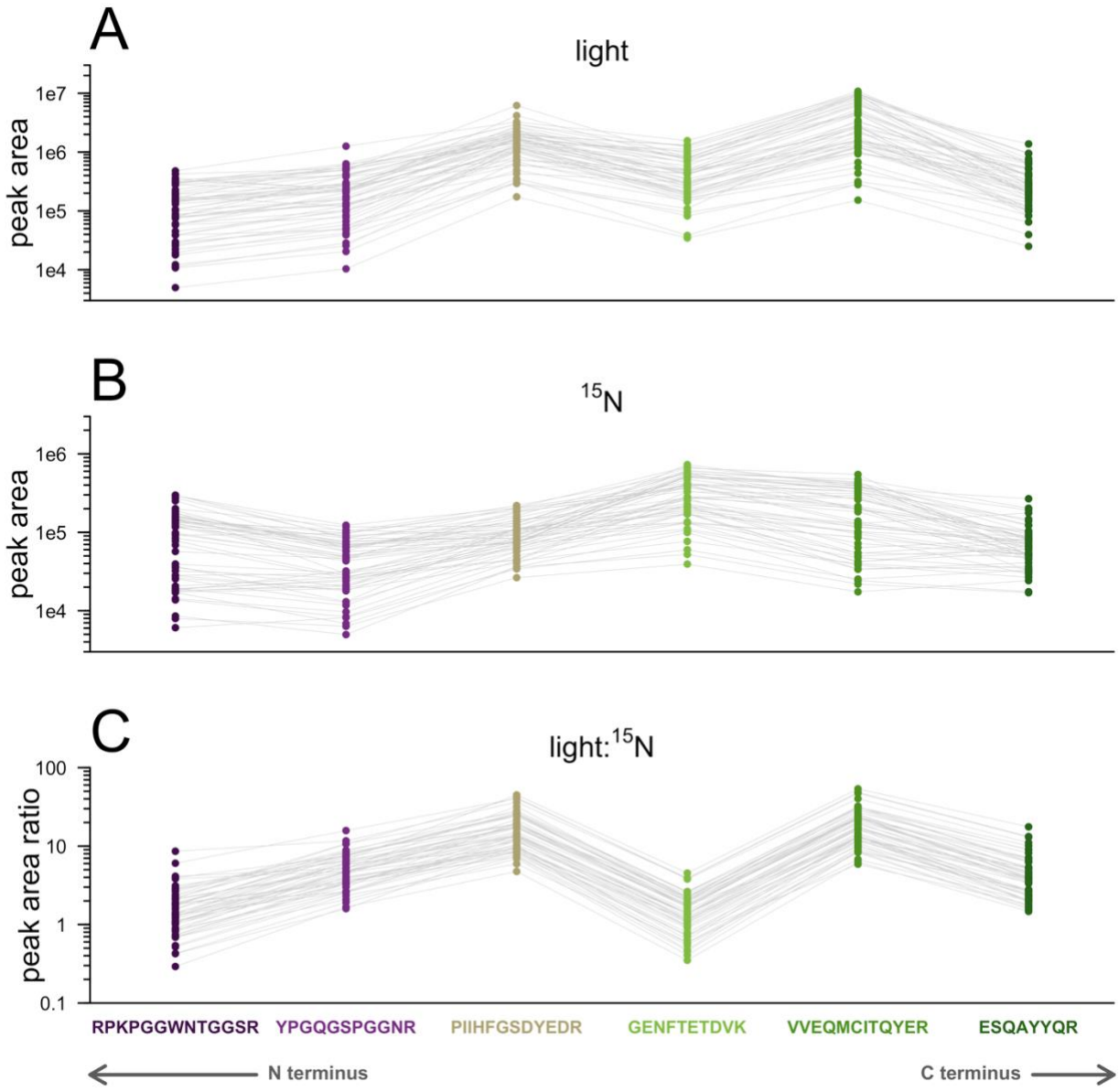

Figure 4.2 | Relative recovery of six human PrP peptides in CSF. *For each of N=55 clinical samples, panels show each peptide's* ***A)*** *light peak area,* ***B)*** *^{15}N peak area, and* ***C)*** *light:^{15}N ratio. Gray lines connect the dots representing distinct peptides from the same individual.*

All six peptides exhibited strong technical performance on par with other published MRM assays[35,40–42], with mean same-day technical replicate CVs <15% both overall (Table 4.1) and within quartiles across the range of low- to high-PrP samples (Supplemental Table S4.6), as well as inter-day technical replicate CVs <25%. These data suggest that PrP MRM is suitable for estimating the amount of PrP in CSF and how it changes within and across patients. In further support of the applicability of this multiplex assay to answering biological questions in clinical samples, we found that for every peptide, the variability in amount of PrP between patient samples was much larger than the analytical variability, with inter-individual CVs of 52-80% contrasting with the observed tight technical replicate agreement of ~10% CV (Table 4.1). Similar results were obtained when the L:H ratio was used instead (Supplemental Table S4.7, Supplemental Figure S4.9, S10). Given that analytical variability was much smaller than biological variability, all six peptides were deemed suitable for analysis in clinical samples, and, owing to their different positions within PrP's amino acid sequence (Figure 4.1A), each peptide was deemed able to inform independently upon the presence of its particular protein domain in CSF.

Table 4.1 | Recovery and performance of six human peptides in human CSF samples. *Mean intra-day CV (based on same-day process duplicates of N=55 samples); mean inter-day CV (based on a single inter-day control CSF sample analyzed in duplicate on N=5 separate days; and inter-individual CV among the 55 different samples.*

codons	peptide	mean intra-day CV	mean inter-day CV	inter-individual CV
25-37	RPKPGGWNTGGSR	10%	16%	80%
38-48	YPGQGSPGGNR	12%	22%	52%
137-148	PIIHFGSDYEDR	10%	12%	56%
195-204	GENFTETDVK	9%	12%	58%
209-220	VVEQMCITQYER	9%	12%	54%
221-228	ESQAYYQR	10%	18%	70%

PrP peptide abundance across diagnostic categories

We used PrP MRM to quantify CSF PrP peptides in *N*=55 clinical samples from individuals with rapidly progressive dementia referred to prion surveillance centers for testing and who ultimately either received non-prion disease diagnoses, or in whom sporadic or genetic prion disease was confirmed by autopsy (see Methods). All six human PrP peptides quantified by PrP MRM showed a marked decrease in abundance in prion disease patients compared to non-prion diagnoses, and all six peptides showed the same general pattern, with non-prion disease patients' CSF samples giving the highest mean peptide level, followed by sporadic prion disease, followed by genetic prion disease (Figure 4.3A). The results from MRM mirrored the previously reported PrP ELISA results for these same 55 individuals[3] (Figure 4.3B), but differed in the estimated absolute amounts of PrP by ~3-fold.

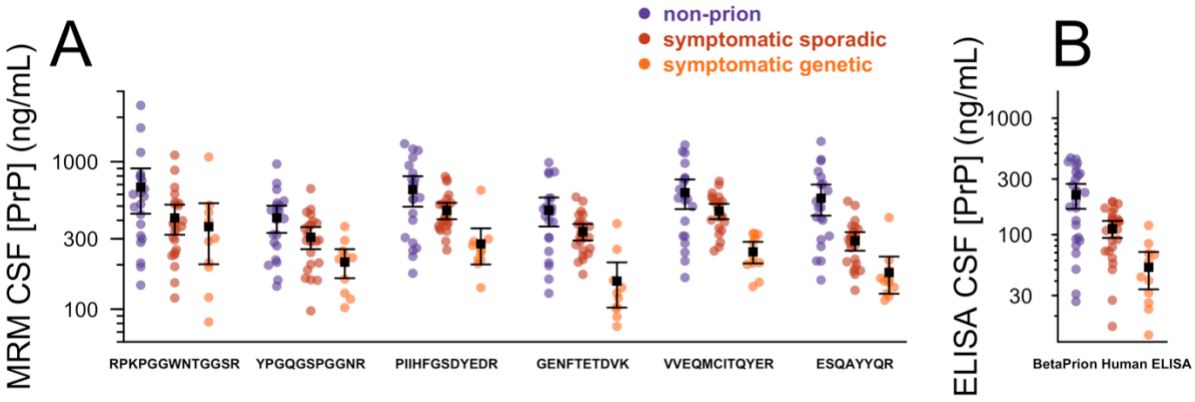

Figure 4.3 | All PrP MRM peptides are decreased in the CSF of prion disease patients. *CSF PrP concentrations in N=55 clinical CSF samples determined by* ***A)*** *PrP MRM for each of six peptides, arranged with the most N-terminal peptide at left and the most C-terminal peptide at right, compared with* ***B)*** *previously reported PrP ELISA results for the same samples, reproduced from Vallabh et al*[3]*. Black squares and bars show the mean and 95% confidence interval of the mean for each group.*

Relationship between PrP MRM and ELISA

Across the clinical samples, each peptide's abundance was positively correlated to the full-length PrP concentration determined by ELISA (Figure 4.4A). The coefficients of correlation, from 0.40 to 0.72, are within the ranges reported for other MRM assays compared to

corresponding immunoassays[41,42,47]. All peptides were strongly correlated to one another, with coefficients of correlation ranging from 0.67 to 0.96, and no obvious differences within versus between protein domains (N- and C-terminal; Figure 4.4B). The linear relationships between peptides were preserved across the range of samples analyzed and were similar in terms of both L:H as well as L:^{15}N ratios (Supplemental Figure S4.10). These results, together with the fact that the magnitude of decrease in abundance in prion disease cases was similar for all peptides (Figure 4.4A), suggested that PrP MRM and ELISA may be measuring the same analyte — predominantly full-length PrP. We therefore asked whether PrP MRM could serve as an orthogonal method to validate findings recently reported for ELISA.

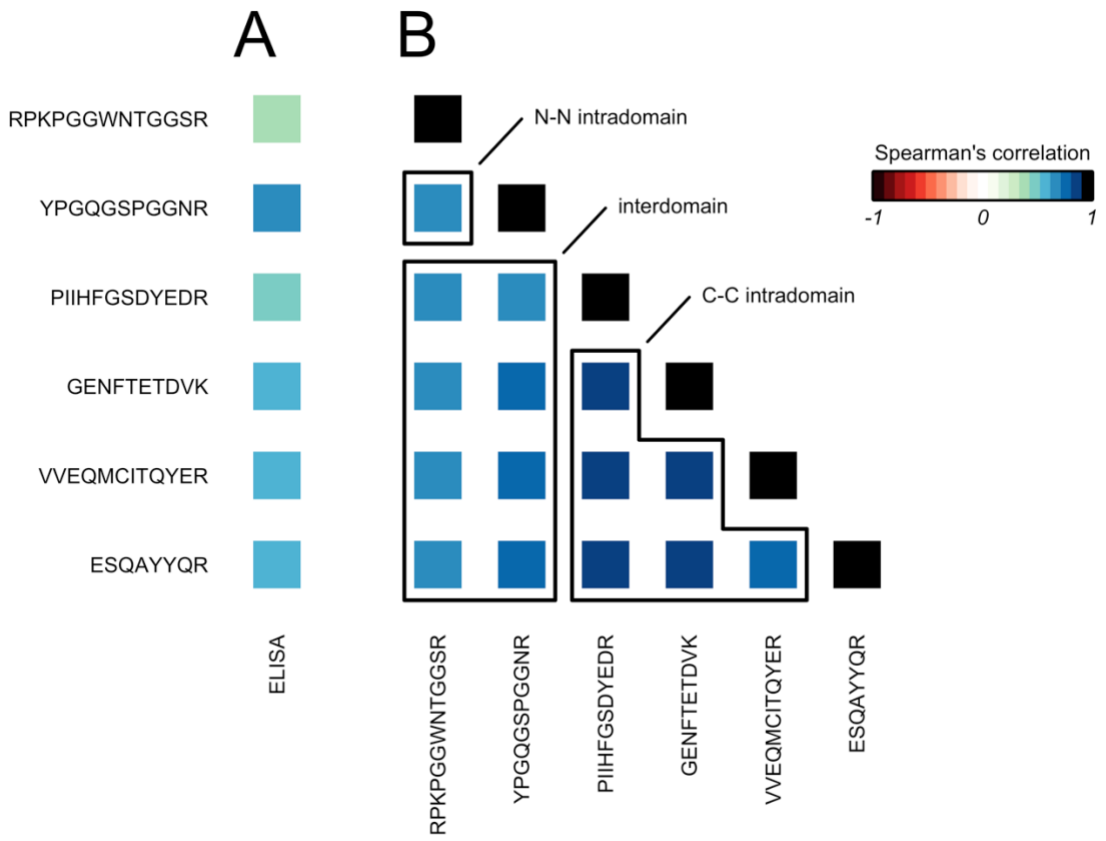

Figure 4.4 | Correlations among PrP MRM peptides and with ELISA. A) *Spearman's correlation between each peptide measured in MRM versus total PrP by ELISA.* ***B)*** *Spearman's correlation between every combination of peptides measured in MRM. All P < 0.01.*

Because plastic adsorption is reported to cause substantial loss of PrP in preanalytical handling, and detergent is reported to largely mitigate this[3], we analyzed replicates of one CSF sample by MRM with and without 0.03% CHAPS detergent. As with ELISA, we found that the addition of CHAPS increased PrP peptide recovery by an average of 51% (*P* = 2.3e-8, Type I ANOVA).

To compare PrP MRM and ELISA results while introducing covariates, we calculated a final estimated PrP concentration from MRM for each CSF sample by averaging the normalized PrP concentration across the six peptides. The estimated PrP concentrations obtained by MRM and by ELISA were correlated across CSF samples (r = 0.61, Spearman's correlation, *P* = 1.3e-6). MRM PrP concentration was uncorrelated with CSF hemoglobin (*P* = 0.85, Spearman's correlation), supporting the conclusion that blood contamination is not a source of CSF PrP[3].

The concentration of PrP in CSF measured by ELISA is correlated with the total protein concentration in CSF[3]. This could reflect true biology, or it could reflect pre-analytical factors, if other proteins serve a blocking function, mitigating PrP loss to plastic during handling[3]. A potential concern, however, is that such a correlation could also arise if non-specific binding of other proteins in the human CSF matrix contributes to PrP ELISA background signal. If true, this would call into question the ability of ELISA-based PrP measurement to accurately quantify a pharmacodynamic decrease in PrP concentration. To distinguish between these possibilities, we tested the relationships between ELISA PrP concentration, MRM PrP concentration, and total protein concentration among our clinical samples. The correlation between ELISA PrP concentration and total protein concentration was marginal but observable among the 55 samples analyzed here (+94 ng/mL PrP per 1 mg total protein, *P* = 0.043, linear regression: ELISA PrP ~ total protein), but this relationship vanished completely when MRM PrP concentration was included as a covariate (*P* = 0.60 for total protein in linear regression: ELISA PrP ~ MRM PrP + total protein). Likewise, MRM PrP concentration was itself correlated to total

protein (+238 ng/mL PrP per 1 mg/mL total protein, P = 0.017, linear regression: MRM PrP ~ total protein). Together, the observations that the relationship between PrP and total protein was replicated in MRM, and that total protein did not explain any residual variance in ELISA-measured PrP after controlling for MRM-measured PrP, suggest that the correlation between CSF PrP and total protein in CSF is a genuine property of the samples analyzed, and that ELISA is specifically measuring PrP in human CSF.

Discussion

Here we describe a targeted mass spectrometry assay for measuring CSF PrP. Six of six human PrP peptides we quantified, from the N to the C terminus, were lowered in prion disease patients compared to non-prion disease patients. Thus, the highly reproducible finding that CSF PrP concentration decreases in prion disease[3,13–16] appears to represent genuine disease biology and is not merely an ELISA measurement artifact. This confirms that CSF PrP will be difficult to interpret as a pharmacodynamic biomarker in symptomatic prion disease patients, because the direct effect of a PrP-lowering drug and the effect of disease process alleviation would be expected to push CSF PrP in opposing directions. Instead, trials to demonstrate target engagement and perform dose-finding for a PrP-lowering drug may need to be conducted in pre-symptomatic individuals at risk for genetic prion disease[2,3].

We also validate other findings from PrP ELISA studies. We confirm that the correlation between CSF PrP and total protein is genuine, and not just a result of matrix interference in ELISA. We also confirm that CSF PrP is not correlated with CSF hemoglobin, further supporting the brain and not blood origin of CSF PrP. Our data provide supportive evidence for the existing literature indicating that CSF PrP can be meaningfully quantified by ELISA.

Our study has several limitations. First, we have only compared samples between prion and non-prion disease patients to examine the effect of the disease state on CSF PrP. Determining the effect of PrP-lowering drug treatment on CSF PrP is a priority for future work. Second, we still cannot exclude the possibility that protein misfolding contributes somewhat to the decrease in CSF PrP that we observe, because the chaotrope used here — 6 M urea — has not been proven to denature all misfolded PrP. This concentration of urea was shown to abolish 99.99% of hamster prion infectivity[48], but prion strains differ in their conformational stability[24]. Human prions unfold at ~3 M guanidine hydrochloride[49,50], but urea is a less potent denaturant[51]. Third, while our assay appears to perform very well, we have not undertaken the full bioanalytical method validation that would be expected if the assay is to be used in clinical decision-making[52], and the LC/MS gradient used here, at 45 minutes, is longer than the ~5 minutes expected for high-throughput clinical biomarker assay. For clinical use, the feasibility and performance of the assay would likely need to be assessed at a faster gradient under microflow conditions using commercially available C18 columns. This increase in assay throughput may come at the cost of some sensitivity, but because all PrP peptides in this study demonstrated comparable behavior across this set of clinical samples, a future implementation of PrP MRM might choose to monitor fewer or even a single peptide, facilitating the implementation of a chromatographically faster procedure. Fourth, because bacterially expressed recombinant PrP is an imperfect standard by which to quantify mammalian PrP, our data do not support any firm conclusions about the baseline composition of PrP in terms of different cleavage products in human CSF generally. Nevertheless, by comparing the abundance of each PrP peptide between diagnostic categories — individuals with and without prion disease — we do establish that any changes driven by the disease state apparently affect all domains of PrP equally. This finding is not inconsistent with existing literature: for example, the PrP C2 fragment resulting from beta cleavage is known to be increased in brain parenchyma during prion disease[25], but if C2 is then retained in intracellular aggregates rather than being shed, while its counterpart N2 is

rapidly degraded, then increased beta cleavage might result in both N- and C-terminal PrP peptides being decreased in prion disease CSF, as observed here. Our principal finding, that all PrP peptides move in concert with one another in the disease state, contrasts with the more complex situation reported for tau isoforms in CSF[53,54], and should simplify the use of CSF PrP quantification as a tool in drug development.

As PrP-lowering therapies progress towards the clinic, MRM and ELISA both appear suitable as tools for measuring PrP in CSF. ELISA is cheaper and less equipment-intensive. MRM offers a wide dynamic range without dilution, and applicability of a single assay both to humans and to multiple preclinical species of interest. Regardless of whether MRM or ELISA is ultimately used in preclinical development and clinical testing of PrP-lowering drugs, the concordance between the two methods builds confidence in CSF PrP as an analyte, and supports its use as a pharmacodynamic biomarker and, perhaps, as a trial endpoint.

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Chapter 5. Antisense oligonucleotide treatment of established brain prion infection in mice

Publication history:

A subset of studies reported in this chapter have been submitted for publication in:

Raymond GJ et al. Antisense oligonucleotides extend survival of prion-infected mice after prophylactic or near-clinical treatment. In review.

The remainder of studies reported here will be published in a separate manuscript.

Attributions:

This chapter includes experiments performed at Ionis Pharmaceuticals (PI: Holly Kordasiewicz), NIAID Rocky Mountain Labs (PI: Byron Caughey), and McLaughlin Research Institute (PI: Deborah E. Cabin) for which I was closely involved in study conception, design, and interpretation. Experiments at the Broad Institute were performed jointly by myself and Sonia Vallabh with assistance from the Comparative Medicine team, particularly veterinarian Tyler Caron and veterinary technicians Jason Le and Samantha Graffam

Abstract

Prion disease is a fatal, incurable neurodegenerative disease of humans and other mammals caused by conformational conversion of the prion protein (PrP). Lowering of PrP expression in the brain is a genetically well-validated therapeutic strategy. Intrathecally delivered antisense oligonucleotides (ASOs) offer a practical modality to sequence-specifically reduce a target protein in the human brain, thus encouraging preclinical proof-of-concept studies of ASOs in prion disease. Studies have established the efficacy of PrP-lowering ASOs delivered prophylactically against intracerebral prion infection in mice. To complement these studies, here we evaluate the efficacy of PrP-lowering ASOs against already-established brain prion infections in mice. Single ASO treatments given immediately upon the detection of neuropathology by bioluminescent live animal imaging (83 days post-infection) or near the time of clinical symptoms (120 days post-infection) extend median survival time of animals by 46 – 68% while delaying clinical onset, mitigating body weight loss, and ameliorating reactive astrogliosis. Chronic ASO treatment is more effective the earlier it is initiated, with late treatment delaying a symptomatic endpoint by 14-24% while early treatment timepoints have resulted in delays of at least 125%. Our results support further development of ASOs for prion disease.

Introduction

Lowering of prion protein (PrP) expression in the brain is a well-validated therapeutic strategy for prion disease[1]. Genetically engineered mouse models show that reduced PrP dose-dependently increases time to disease after intracerebral inoculation of prions[2–4], and conditionally knocking out or lowering PrP is protective even after the disease process is well underway[5,6]. Meanwhile, homozygous PrP knockout mice are grossly normal[7], with only a very mild phenotype[8,9] not observed at all in heterozygotes[8]. Knockout cattle and goats are reported as normal[10–12], and heterozygous loss-of-function mutations are found in healthy humans[13,14]. Thus, PrP lowering is expected to be safe and effective against prion disease.

Antisense oligonucleotides (ASOs) appear to provide a realistic route to lowering a target protein in the human brain[15]. ASOs consist of 15-30 nucleotides, incorporating one or more chemical modifications to improve potency and/or pharmacokinetics, sequence-targeted to bind an RNA of interest[16]. Upon binding, ASOs can recruit the enzyme RNAse H1 to degrade the target RNA[17–19]. Preclinical data show that intrathecally delivered ASOs distribute widely across the non-human primate brain[20,21]. An intrathecally delivered splice-modulating ASO for spinal muscular atrophy, nusinersen, demonstrated uptake in human brain, months-long duration of action, and striking clinical benefit leading to FDA approval[22–24]. Another intrathecally delivered ASO now in clinical trials, RG6042 for Huntington disease, has so far demonstrated potent target engagement, lowering mutant huntingtin by 40% in cerebrospinal fluid[25].

The potential for ASOs to treat prion disease has been anticipated for over twenty years[26]. The first *in vivo* study of PrP-lowering ASOs demonstrated some benefit in prion-infected mice[27], but with several limitations. The osmotic pump-based delivery system in use at that time[27] appeared

less practical for human use than the bolus injection paradigm that has since been established both preclinically[28] and clinically[22]. Oligonucleotides can non-sequence-specifically bind PrP and antagonize prion propagation in cell culture[27,29,30], and the omission of a non-PrP-targeting ASO from the *in vivo* studies[27] left doubt as to whether the PrP-targeting ASO acted by RNAse H1 or by an aptameric mechanism. Finally, the ASO used in the initial study was not tolerated in mice with established prion infections[27], leaving unanswered the question of how late in disease ASOs could be effective. Recently, we have revisited the question of ASO efficacy in prion disease, delivering multiple new ASOs with new sequences and chemistries into the mouse CNS by bolus intracerebroventricular (ICV) injection on a prophylactic basis, before prion infection[1]. We have demonstrated that prophylactic ASOs dramatically extend survival after intracerebral challenge with prions, by a PrP-lowering mechanism of action, in a dose-dependent but prion strain-independent manner[1]. In parallel, we have worked to establish a surrogate biomarker[31] and regulatory pathway[1,32] that might allow PrP-lowering drugs to be tested in pre-symptomatic individuals at risk for genetic prion disease. The efficacy of prophylactic ASOs in mice suggests that such early intervention in humans may be beneficial.

Here, we assess to what degree ASOs can be effective after prion infection is already established in the mouse brain. Incorporating studies of several different ASO molecules at three collaborating sites, with multiple ASOs, and in multiple mouse backgrounds, we characterize the effects of treatments initiated throughout the silent incubation period, including after the detection of neuropathological changes, and up to timepoints near the onset of frank neurological symptoms.

Results

Selection of ASOs for *in vivo* efficacy studies

Screening against the mouse *Prnp* gene (see Methods) yielded eight ASOs selected for advancement into studies in prion-infected mice, including six active, PrP-lowering ASOs found to reduce cortex PrP mRNA to 31-57% of normal levels, plus two non-targeting control ASOs (Table 5.1). Six of these molecules have been described previously[1]. Targeted regions include *Prnp* intron 2, coding sequence, and 3'UTR (Figure 5.1), consistent with the reported activity of ASOs in both the cytoplasm and nucleus, including against pre-mRNA[19].

Table 5.1 | Composition and potency of ASOs used in this study. *Color coding and lowercase letters indicate ASO chemistries described previously[16]. Black: unmodified deoxyribose (2'H); orange: 2' methoxyethyl (MOE); blue: 2'-4' constrained ethyl (cET). Unmarked linkages: phosphorothioate (PS); linkages marked with o: normal phosphodiester (PO). mC: 5-methylcytosine. PrP mRNA indicates mouse ipsilateral cortex Prnp mRNA level quantified by qPCR 8 weeks after a single 700 µg ICV dose and normalized to the mean of saline-treated animals, mean±sd, N=3 to 4 each. n.d., not done.*

treatment	sequence and chemistry	PrP mRNA
saline	—	100% ± 6%
active ASO 1	mCToAoTTTAATGTmCAoGoTmCT	52% ± 4%
active ASO 2	TToGomCAATTmCTATmComCoAAA	34% ± 13%
control ASO 3	mCGomCoTTATAmCTAATmCoAoTAT	n.d.
control ASO 4	CCoToAoTAGGACTATCCAoGoGoAA	n.d.
active ASO 5	TToGoCoAATTCTATCCAAoAoTAA	56% ± 16%
active ASO 6	CToToCoTATTTAATGTCAoGoTCT	47% ± 5%
active ASO 7	TAoGoCoCTTTGTACCTTAoAoCCA	57% ± 6%
active ASO 8	GmCoCoAAGGTTCGCCoAoTGA	31% ± 6%

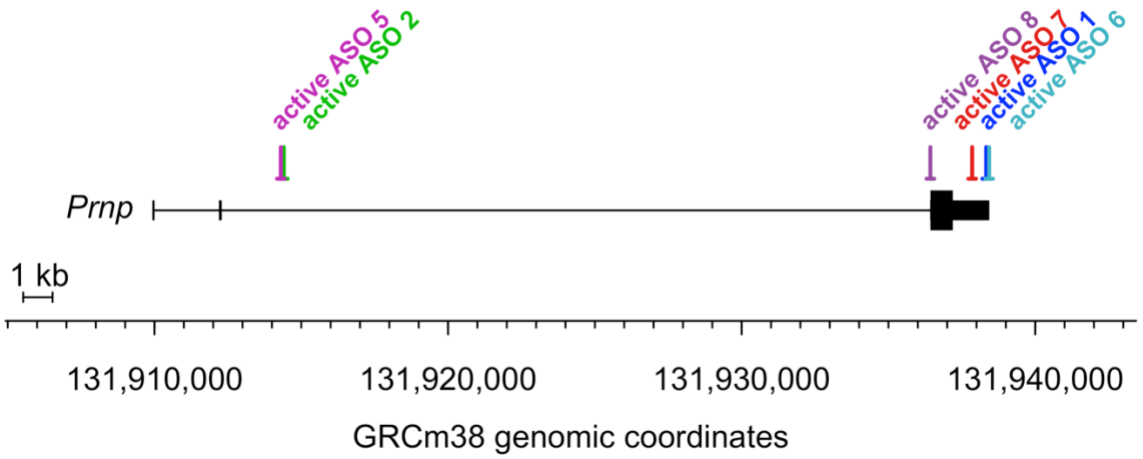

Figure 5.1 | Positions in mouse Prnp targeted by active ASOs in this study. *Positions of targeted sequences in Prnp on mouse chromosome 2 (GRCm38 coordinates) for each active ASO. Sequences overlap for active ASOs 1 and 6 and for active ASOs 2 and 5. Targets are Prnp intron 2 (ASOs 2 and 5), 3'UTR (ASO 1, 6, and 7), or coding sequence, overlapping the start codon (ASO 8).*

Preliminary tolerability screening in naïve mice

Proof-of-concept ASOs are not subject to the intensive toxicological evaluation afforded clinical candidates, but limited tolerability studies were conducted in naïve wild-type mice (Figure 5.2). At a 700 µg ICV bolus dose, all active ASOs caused at least some acute side effects, assessed at 3h post-surgery, and three of them were fatal to at least one mouse (Figure 5.2A). Nonetheless, body weight trajectories out to eight weeks post-surgery were similar to saline-injected animals (Figure 5.2B). After eight weeks, cortical Aif1, a marker of microglial activation, was largely normal (Figure 5.2C), while Cd68 in the thoracic spinal cord, another neuroinflammatory marker, was only modestly increased (Figure 5.2D). On the basis of these studies, the six active ASOs were deemed suitable to evaluate in prion-infected mice.

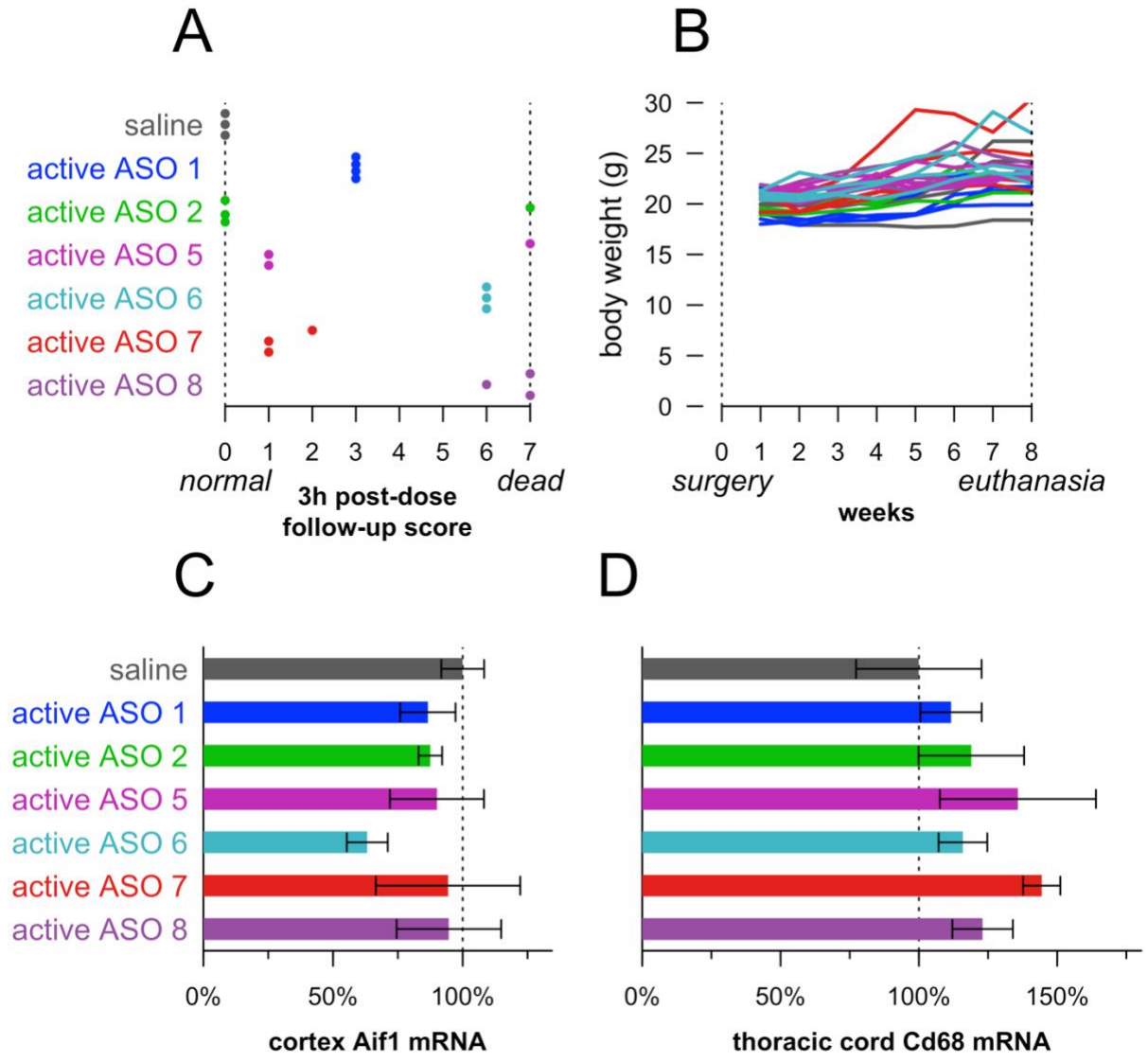

Figure 5.2 | Results of basic tolerability screening of active ASOs. *Groups of N=3-4 naïve wild-type C57BL/6N mice were given 700 µg ICV doses of the indicated ASOs at Ionis Pharmaceuticals.* ***A)*** *Acute tolerability indicated by a follow-up score at 3 hours post-dose. Mice are scored on seven vital signs and receive one point for each sign that is absent (see Methods); a score of zero indicates normal behavior while seven indicates death.* ***B-D)*** *Medium-term tolerability measures assessed over 8 weeks.* ***B)*** *Body weight trajectories for individual animals up to sacrifice at 8 weeks post-dose.* ***C)*** *Cortex Aif1 mRNA and* ***D)*** *thoracic cord Cd68 mRNA, two neuroinflammatory markers, measured by qPCR and normalized to the saline group mean at 8 weeks post-dose. Error bars indicate 95%CI of the mean.*

Pilot near-clinical assessment of efficacy

In the first late treatment study, mice intracerebrally infected with the RML strain of prions at NIAID Rocky Mountain Laboratories were treated with a single 300 µg ICV dose of ASO at 120

days post-infection (dpi), a timepoint when neuropathology is expected to be prominent but mice are grossly phenotypically normal (Figure 5.3). Active ASO 1 delayed the onset of disease by 33% compared to saline (median 189 vs. 142 dpi) while control ASO 3 was ineffective (Figure 5.3A), confirming that PrP lowering is the mechanism of action for ASOs *in vivo* against prion disease. Active ASO 1 also slowed the progression from onset of symptoms to terminal disease by more than three-fold (53±7 vs. 15±4 days, mean±sd; Figure 5.3B), thus increasing overall survival time by 55% compared to saline (median 244 vs. 157 dpi; Figure 5.3C).

Active ASO 2, however, was not tolerated at this timepoint, and all treated mice experienced sudden neurological decline (within ~16h) leading to death or euthanasia approximately 8-9 days post-surgery (Figure 5.3C). Some (2/9) mice treated with control ASO 3 experienced a similar rapid decline, while the remainder (7/9) had survival consistent with saline-treated animals (Figure 5.3C).

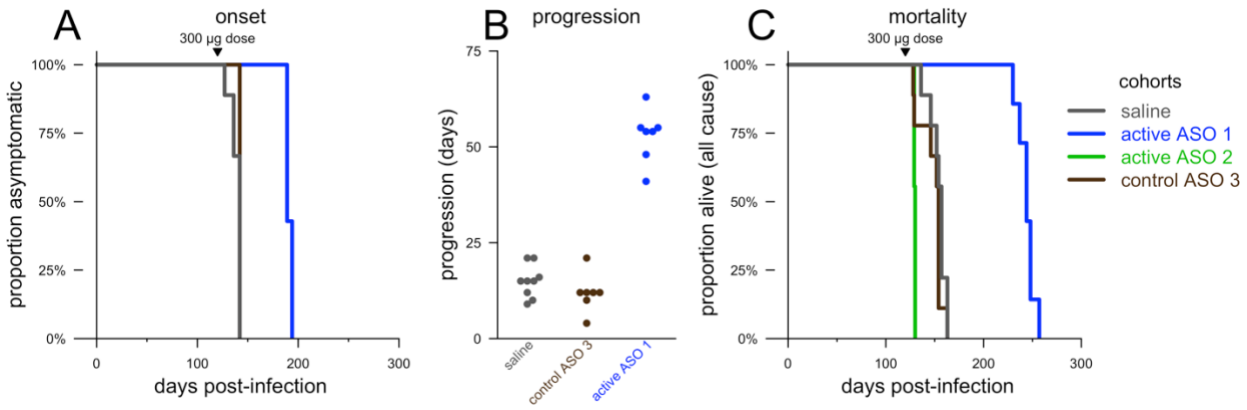

Figure 5.3 | Survival of prion-infected mice given a single ASO dose at 120 dpi. *Studies conducted at NIAID Rocky Mountain Laboratories in the RML strain of mice, intracerebrally infected with RML prions and treated with a single 300 µg ICV dose.* ***A)*** *Time to onset of clinical signs of prion disease.* ***B)*** *Progression from onset to terminal disease.* ***C)*** *All-cause mortality.*

Bioluminescence imaging in mice treated at the onset of reactive gliosis

Reactive gliosis associated with increased expression of the astroglial intermediate filament gene *Gfap* is one of the earliest neuropathological changes in prion-infected mice[33]. Using Tg(Gfap-luc) mice[34], which express luciferase under the Gfap promoter, it is possible to track the progression of gliosis by live animal bioluminescence imaging (BLI) throughout the course of prion disease[35] and to obtain time-series data on the effect of drug treatment[36].

We designed an experiment at McLaughlin Research Institute to evaluate the effects on survival and pathological progression of an ASO treatment initiated upon the onset of pathology detectable by increased BLI. We monitored RML prion-infected and uninfected mice by BLI every 7-11 days, and pre-specified that a single 500 µg dose of ASO would be administered only after two consecutive imaging sessions showed a nominally significant ($P < 0.05$) difference in BLI between infected and uninfected mice. Significant differences were observed at 73 and 81 dpi, triggering the ASO injections to be performed at 83-84 dpi (Figure 5.4A).

A sharp increase in BLI was observed in both saline- and ASO-treated mice after ICV injections, perhaps reflecting an inflammatory reaction to the surgical intervention (Figure 5.4A). By 109 dpi, more than three weeks post-surgery, the BLI in mice treated with active ASO 1 had declined to below the level in saline-treated animals (Figure 5.4A). Thereafter, BLI in saline-treated animals increased sharply up through terminal disease, while BLI in active ASO 1-treated animals remained low through their terminal disease stage 46% later (median 234 vs. 160 dpi; Figure 5.4A-B).

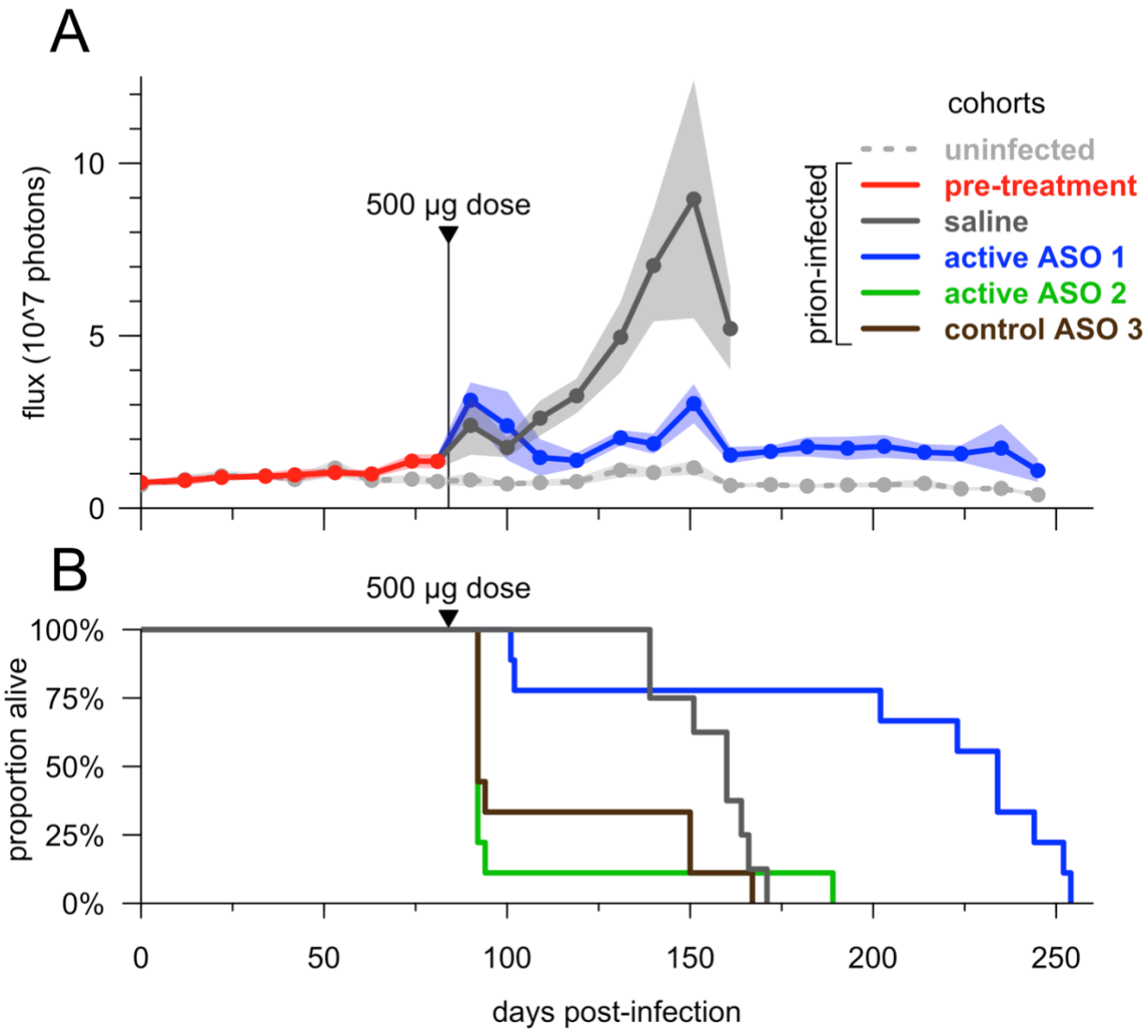

Figure 5.4 | Bioluminescence and survival in prion-infected mice given a single ASO dose after the onset of reactive gliosis. *Studies conducted at McLaughlin Research Institute using Tg(Gfap-luc) mice on a FVB/N background, intracerebrally infected with RML prions and treated with a single 500 µg ICV dose.* ***A)*** *Bioluminescent signal, with lines indicating means and shaded areas indicating 95%CI of the means, and* ***B)*** *all-cause mortality. Animals treated with active ASO 2 and control ASO 3 are excluded from the bioluminescence plot due to low N.*

In this experiment, none of the three ASOs was perfectly tolerated by all treated animals. 8/9 animals treated with active ASO 2 and 6/9 treated with control ASO 3 died or were euthanized 8-11 days post-surgery. In the active ASO 1 cohort, 2/9 animals died 17-19 days post-surgery, yielding a bimodal survival distribution (Figure 5.4B) such that all-cause survival was more

modestly increased when considering the mean rather than median (205±61 dpi for active ASO 1 vs. 156±12 dpi for saline, P = 0.012, two-sided Kolmogorov-Smirnov test).

Across treatment groups, all of the mice that survived the three-week period after surgery eventually developed progressive neurological signs consistent with prion disease. Half (9/18) of these mice, however, never reached terminal disease endpoint because they died acutely within minutes following an intraperitoneal luciferin injection for live animal imaging. This was surprising because such adverse reactions have not been reported in Tg(Gfap-luc) mice before[34], have never been observed in McLaughlin Research Institute's extensive experience using luciferin injections and BLI for other, non-prion, mouse studies (Deborah E. Cabin, personal communication), and were not observed in any of the uninfected controls in this study. This was not due to an interaction between luciferin and ASOs, because N=3 of these sudden deaths occurred in saline-treated animals.

Tolerability testing in prion-infected mice

The tolerability limitations identified in the initial late treatment studies (Figures 3 and 4) were generally not observed in naïve mice (Figure 2) or in prophylactic treatment against prion infection[1]. We therefore initiated two studies specifically to evaluate the tolerability of our full panel of ASOs (Table 5.1) in prion-infected mice.

In one study, at the Broad Institute, groups of N=5-6 mice infected with RML prions received ICV injections of 500 µg ASO or saline at 120 dpi and were then closely monitored for four weeks for the development of sudden death or neurological decline requiring euthanasia (Table 5.2). Active ASO 8 caused acute death (within hours of surgery) in 3/6 mice, consistent with its poorer performance in initial tolerability screening (Table 5.1). In each of the remaining

treatment groups, including the saline group, all mice survived the day of surgery but some died or became acutely sick within a few weeks.

Table 5.2 | Four-week tolerability testing of ASOs in prion-infected mice at 120 dpi. *Studies conducted at the Broad Institute in wild-type C57BL/6N mice intracerebrally infected with RML prions and treated with a single 500 µg ICV dose. Active ASO 2 was not evaluated in this study due to an insufficient number of animals available for dividing into treatment groups.*

treatment	same-day deaths	four-week deaths	days post-treatment
saline	—	2/6	12-17
active ASO 1	—	2/6	9
control ASO 3	—	5/6	9-18
control ASO 4	—	1/6	22
active ASO 5	—	2/5	22
active ASO 6	—	1/6	12
active ASO 7	—	5/6	6-7
active ASO 8	3/6	3/3	6-7

Of animals that died within the four-week window (N=21 total), most were either euthanized upon advanced but non-specific neurological signs (N=11), or were found dead (N=7). In a few animals (N=3, one each from active ASOs 5, 7, and 8), hindlimb paralysis was noted to develop rapidly over the course of approximately 24 hours. The development of rapidly progressive symptoms in multiple mice within a narrow interval post-surgery, particularly for ASOs 1, 3, 7, and 8 is consistent with observations from studies by collaborators (Figures 5.3 and 5.4) and suggests ASO-related sequelae. Complications of prion disease or of ICV surgery are also possible explanations for deaths in this time period, however, and two saline-treated animals also succumbed — one was euthanized with sudden neurological signs at 12 days post-surgery and one was found dead at 17 days post-surgery. Based on the data from this study, we identified active ASOs 5 and 6 and control ASO 4 as relatively better-tolerated than the other ASOs at 120 dpi, and these compounds were selected for further survival studies (Figure 5.5).

In order to confirm these results, and to assess the disease stage dependence of adverse reactions to ASOs, we also launched a time-series study of ASO tolerability at McLaughlin Research Institute (Table 5.3). In this study, groups of *N*=4 mice received ICV injections of 300 µg ASO or saline at five different timepoints throughout the course of prion disease. As soon as

any mouse in a treatment group died or exhibited sudden decline, the remainder of its whole cohort of four animals were euthanized; otherwise, animals were monitored up through 150 dpi, when symptoms of prion disease are advanced in untreated mice, before brains were collected for comparative histology.

Table 5.3 | Time series tolerability testing of ASOs in prion-infected mice. *Studies conducted at McLaughlin Research Institute in cohorts of N=4 wild-type C57BL/6N mice each, intracerebrally infected with RML prions and treated with a single 300 µg ICV dose at the indicated timepoints. The data in each cell indicate the number of days until the first animal died or developed sudden neurological signs requiring euthanasia. — indicates no animals developed such signs through at least 150 dpi.*

treatment	-14 dpi	30 dpi	60 dpi	90 dpi	123 dpi
saline	—	—	—	—	—
active ASO 1	—	—	—	—	—
active ASO 2	—	—	8 days	5 days	6 days
control ASO 3	—	—	—	—	14 days
control ASO 4	—	—	—	—	—
active ASO 5	—	—	—	32 days	16 days
active ASO 6	—	—	—	—	—
active ASO 7	—	—	—	8 days	8 days
active ASO 8	—	—	7 days	6 days	7 days

The ASOs associated with acute decline with the highest penetrance in previous studies (active ASOs 2 and 8, see Figure 5.3, Figure 5.4, and Table 5.2) here yielded adverse reactions that occurred both more rapidly (in as few as 5-6 days post-surgery) and at an earlier disease stage (60 dpi) than other ASOs. Some ASOs were tolerated much later in disease than others, and in no case was an ASO better tolerated at a later timepoint than at an earlier timepoint. Overall, the results suggested that tolerability is both ASO- and disease stage-dependent (Table 5.3).

Replication study of near-clinical efficacy with new ASOs

Following the first round of tolerability studies in prion-infected mice (Table 5.2), we initiated an ASO treatment study at a near-clinical timepoint at the Broad Institute in an attempt to replicate the findings of the initial study at NIAID Rocky Mountain Labs (Figure 5.3). Mice infected with RML prions received a single ICV dose of 500 µg ASO or saline at 120 dpi. In this and all

subsequent studies at the Broad, individuals responsible for caretaking and endpoint determination in the animals were blinded to treatment status.

Survival in mice treated with control ASO 4 was indistinguishable from saline-treated animals (Figure 5.5A). Active ASO 5 caused acute deaths in 4/10 animals due to seizures, later determined to be the result of some of the dose being injected into brain parenchyma rather than CSF because of a misaligned tooth bar on the stereotactic apparatus. In the remaining 6/10 mice, active ASO 5 also slightly decreased survival time (Figure 5.5A), consistent with the time-series study at McLaughlin Research Institute, in which this ASO was associated with sudden decline, albeit at a longer interval post-surgery than for other ASOs (Table 5.3). It may be that the four-week window had not provided sufficient follow-up time to observe this effect in our own tolerability studies (Table 5.2). Active ASO 6, however, resulted in a 68% increase in survival time compared to saline (median 277 vs. 165 dpi; Figure 5.5A), with all mice surviving beyond the point when all of the saline-treated animals had died.

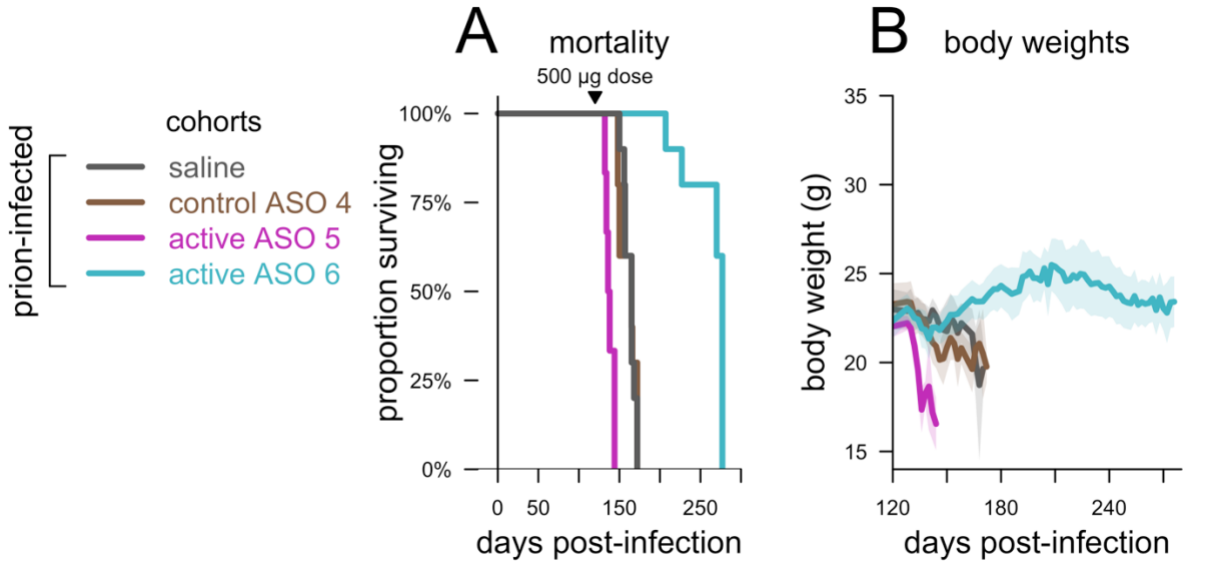

Figure 5.5 | Survival of prion-infected mice given a single dose of new ASOs at 120 dpi. *Studies conducted at the Broad Institute in wild-type C57BL/6N mice intracerebrally infected with RML prions and treated with a single 500 µg ICV dose.* ***A)*** *All-cause mortality and* ***B)*** *body weights. Note that active ASO 5 curve excludes 4/10 animals that died of acute complications post-surgery, see text for details.*

Body weights in animals treated with active ASO 6 declined in tandem with saline- and control ASO 4-treated animals for approximately three weeks post-surgery before beginning to rebound (Figure 5.5B), consistent with the prior observation that gliosis measured by BLI did not begin to be alleviated until at least three weeks after ASO administration (Figure 5.4). Afterwards, active ASO 6-treated animals gained weight for an additional ~60 days (through 210 dpi) before they began to decline again as the mice developed progressive neurological symptoms (Figure 5.5B).

Based on these results, we selected active ASO 6 to advance into survival efficacy studies at a broader range of timepoints throughout the course of prion disease. For subsequent experiments, we also adjusted our endpoint criteria (see Methods) so that mice would be euthanized before becoming moribund, if blinded technicians observed at least five of eight pre-defined symptoms for two consecutive behavioral monitoring sessions, or if stricter thresholds of physical deterioration were met (15% instead of 20% body weight loss, or body condition score ≤2 instead of <2).

Timepoint dependence of ASO treatment efficacy

Several antiprion therapeutics previously shown effective in intracerebrally prion-infected mice exhibited a time dependence, with earlier treatment initiation resulting in a larger effect on survival time[37–40]. To determine the relationship between treatment initiation timepoint and survival time, we initiated an experiment at the Broad Institute in which we are treating cohorts of RML prion-infected mice with 500 μg active ASO 6 or saline (*N*=8 each), chronically every 90 days until endpoint, beginning at seven different timepoints ranging from 7 days before infection to 120 dpi.

This experiment is ongoing as of this writing, but preliminary results (Figure 5.6) indicate that ASO benefit is disease timepoint-dependent. Animals treated beginning at 105 or 120 dpi survived only slightly longer than saline-treated controls (Figure 5.6A), not long enough to receive a second dose. This contrasts with the results of our first 120 dpi treatment study (Figure 5), perhaps on account of the tightened endpoint criteria employed here (see Methods). *N*=2 mice in the 105 dpi and *N*=1 mouse in the 120 dpi cohort also developed hindlimb paralysis and died suddenly in the weeks following ASO treatment. When only the mice that reached disease endpoint criteria are considered (Figure 5.6B), the mean increase in survival time at these timepoints was 24% (+35 days for 105 dpi treated animals) and 14% (+20 days for 120 dpi treated animals). In contrast, in each cohort of animals treated at timepoints up through 78 dpi, at least half of animals are still surviving today, more than one year after prion infection.

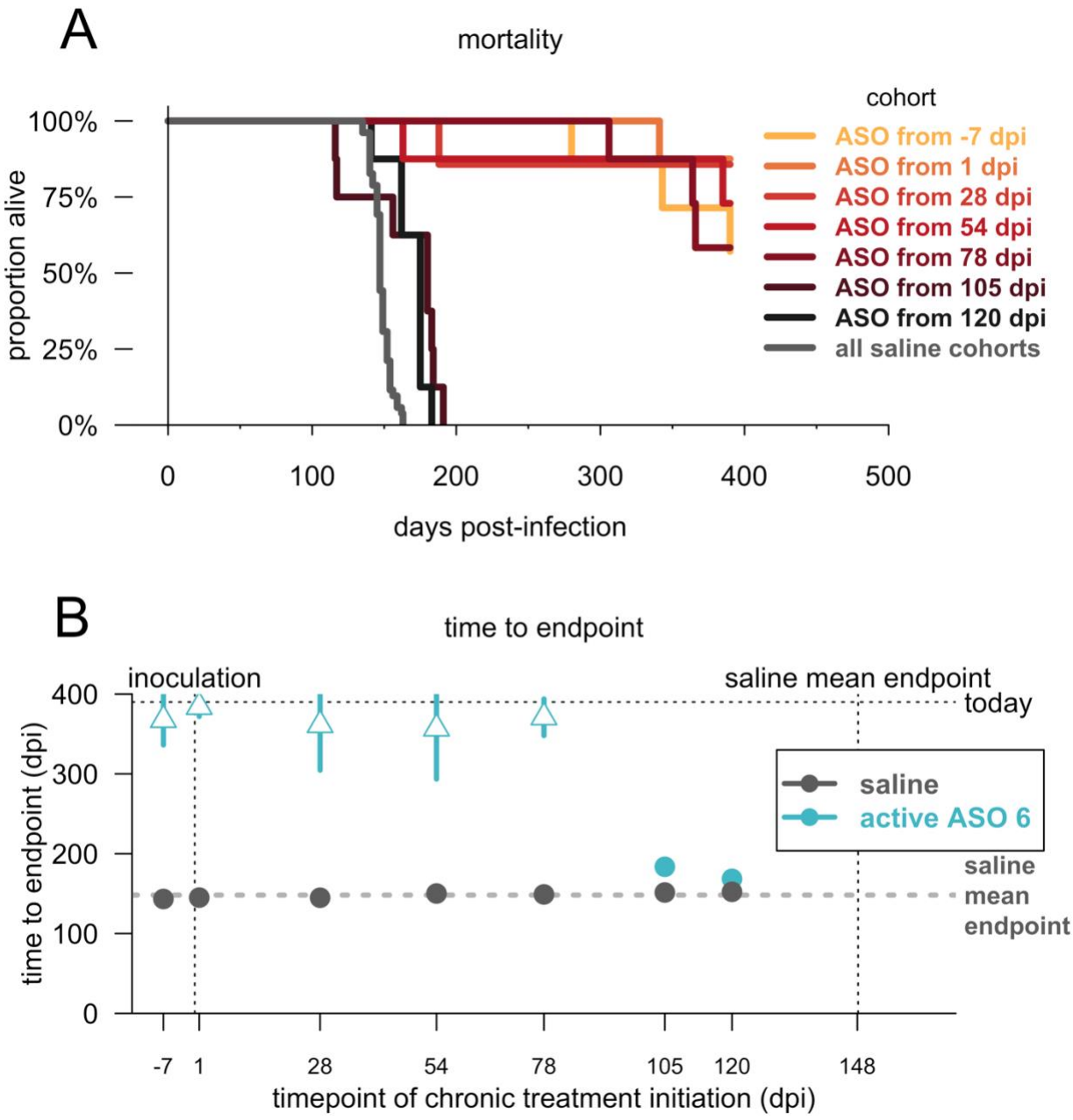

Figure 5.6 | Timepoint dependence of the efficacy of chronic ASO treatment. A) *All-cause mortality survival curves, and* ***B)*** *time to meeting endpoint criteria, as a function of the time at which chronic treatment was initiated. Dots represent the mean and error bars the 95%CI of the mean. Open triangles represent cohorts in which some animals are still surviving, and the position of the triangles on the y axis represents what the mean would be if all remaining animals died today.*

Discussion

Here we evaluated the therapeutic effect of ASO treatment against established brain prion infections in mice. We identified two active, PrP-lowering ASOs that increased median survival time by 46 – 68% when administered at 83 – 120 dpi, and which also delayed clinical onset, mitigated body weight loss, or ameliorated reactive astrogliosis measured through bioluminescent live animal imaging. Our finding of ASO efficacy even at late timepoints is consistent with studies in which PrP expression in transgenic mice was conditionally reduced or turned off near the time of disease onset[5,6]. In agreement with prophylactic treatment studies[1], neither of two control ASOs extended survival time, confirming that ASOs are effective against prion disease only if they lower PrP, not through an aptameric mechanism.

Our prion-infected mice up to 120 dpi appeared grossly normal, even though neuropathology is prominent at this timepoint[35,41]. With careful monitoring, however, some investigators have documented subtle behavioral changes in prion-infected mice at timepoints well before the onset of clear neurological signs[42,43]. Indeed, in some laboratories, mice receiving the same dose of the same prion strain used here (30 µL of a 1% RML brain homogenate) are routinely euthanized at 120 dpi or earlier[36,40]. We therefore allow the possibility that our mice were subtly symptomatic in ways we did not observe. At a minimum, our findings establish that, in mice, PrP-lowering ASO intervention can be effective even very close to the time of symptom onset. Studies are now underway to determine whether ASO intervention can still increase survival time if initiated after the development of obvious clinical symptoms.

A previous study observed sudden deaths in mice treated with an ASO at 60 dpi[27]. Here we replicate this phenomenon, observing sudden neurological decline and death in a subset of mice in the weeks following ASO treatment. This occurred with multiple active ASOs but also

with one control ASO, demonstrating that this is not an on-target effect of PrP lowering. The mechanism behind this phenomenon is not yet clear. We also observed sudden death in prion-infected animals injected with luciferin for bioluminescence imaging, raising the possibility that neuropathology in mice with advanced prion infections may render them more susceptible to otherwise tolerable insults. Indeed, one of our collaborators reports having separately identified similar prion disease stage-dependent reactions in mice to injections of granulocyte colony-stimulating factor and pentobarbital (Katsumi Doh-Ura, personal communication). Our data also indicate that the phenomenon is both ASO-dependent and disease stage-dependent, because in our time-series study, some ASOs were generally tolerated throughout the prion disease incubation period, whereas others began to be associated with adverse events as early as 60 dpi or as late as 123 dpi. Histology on the brains collected from animals in this study may help to illuminate the mechanism at work here. In the meantime, it is important to bear in mind that the ASOs used here were proof-of-concept compounds — not clinical candidates — and had passed only limited tolerability screening.

Although PrP-lowering ASOs can be effective even in delayed treatment against prion infection, they are more effective if given earlier. First, while we observed substantial increases in survival time in experiments with endpoint criteria corresponding to more advanced disease, such as 20% body weight loss, experiments with the same ASO, same prion strain, and same timepoint but tighter endpoint criteria based on symptomatology appeared to show only marginal benefit. Second, the increases in survival time that we observed following prophylactic treatment[1] were generally larger than those reported here, and were driven primarily or entirely by increase in healthy lifespan, whereas a more gradual symptomatic phase appears to have contributed to the survival benefit in some studies reported here. Third, in a chronic treatment experiment with the dosing regimen and endpoint criteria held constant, and only the time of intervention varied between cohorts, we observed that the latest treatment (beginning at 120 dpi) delayed mean

time to disease endpoint by just 14%, while the earlier treatments have each delayed endpoint by at least 125% as of this writing. This is in line with findings for several other antiprion agents tested in mice[37–40].

Overall, our results encourage the further development of PrP-lowering ASOs by showing that they can be effective after neuropathology is detectable and close to the time of symptom onset. Our observation that efficacy is greater if treatment is initiated earlier on suggests that ASOs may need to be evaluated separately for ability to delay or prevent disease, and for ability to treat symptomatic disease.

Methods

Animals

Experiments were approved by respective Institutional Animal Care and Use Committees (Ionis Pharmaceuticals IACUC P-0273, Broad Institute IACUC 0162-05-17, Rocky Mountain Labs IACUC 2015-061, and McLaughin Research Institute IACUC 2017-GAC22 and 2018-MPK29). Studies at Ionis, Broad, and McLaughlin used C57BL/6N females purchased from either Taconic (Germantown, NY) or Charles River Labs (Wilmington, MA), with the exception of Tg(Gfap-luc) mice[34], which were provided by Jasna Kriz (Université Laval, Quebec City, Canada) and bred to homozygosity and maintained on an FVB/N background at McLaughlin. Studies at Rocky Mountain Labs used a strain of C57BL/10 mice inbred at Rocky Mountain Labs for many generations. All of these mouse strains harbor the *Prnp*^{a} (MoPrP-A) haplotype[44] as found in the mouse reference genome.

ASO discovery

Methods and pipelines for ASO screening have been described broadly elsewhere[27,45]. For these studies, ~500 ASOs were synthesized and screened in electroporated HEPA1-6 cells at 7 μM with qPCR quantification of *Prnp* mRNA 24h later. Potent ASOs advanced to four-point dose response in HEPA1-6 cells, followed by preliminary *in vivo* screening with a single 300 μg dose followed by tissue collection 2 weeks later and brain *Prnp* mRNA quantification by qPCR. Hits validated in this *in vivo* screen were advanced into the further potency studies described previously[1] and into the tolerability studies described in Results.

qPCR

General procedures for qPCR used here have been described elsewhere[1]. Results for *Prnp*, *Iba1* (Aif1), and *Cd68* were normalized to the housekeeping gene *Ppia* (cyclophilin) and then further normalized to the mean of saline-treated animals. Primers were as follows: *Prnp* forward: TCAGTCATCATGGCGAACCTT, reverse: AGGCCGACATCAGTCCACAT, probe: CTACTGGCTGCTGGCCCTCTTTGTGACX; *Aif1* forward: TGGTCCCCCAGCCAAGA, reverse: CCCACCGTGTGACATCCA, probe: 5'-Fam-AGCTATCTCCGAGCTGCCCTGATTGGX-Tamra-3'; *Ppia* forward: TCGCCGCTTGCTGCA, reverse: ATCGGCCGTGATGTCGA, probe: CCATGGTCAACCCCACCGTGTTCX; *Cd68* forward: TGGCGGTGGAATACAATGTG, reverse: GATGAATTCTGCGCCATGAA, probe: CCTTCCCACAGGCAGCACAGTGGX.

Acute tolerability rating

Functional observational battery (FOB) scoring was performed 3 hours after a bolus ICV dose of ASO. Mice were scored on seven different signs: bright, alert, and responsive (BAR); standing or hunched absent stimulus; movement absent stimulus; forward movement if lifted; any movement if lifted; response to tail pinching; and regular breathing. The score was the number of these signs that were absent. A score of 7 corresponded to death.

Intracerebroventricular injection

ASOs and saline were delivered into CSF by intracerebroventricular (ICV) injection on a stereotactic apparatus. The same general procedure was used at Ionis Pharmaceuticals, the Broad Institute, and McLaughlin Research Institute and is described in detail here; procedures at Rocky Mountain Laboratories differ slightly and have been described elsewhere[1].

Anesthesia was achieved with 3.0-3.5% isoflurane for induction and 2.5-3.0% for maintenance. Heads were shaved. Animals were placed into a stereotactic apparatus (ASI Instruments, SAS-4100), with 18° ear bars inserted into the ear canals and incisors inserted into the mouse adapter tooth bar, which was adjusted to -8mm to make the bregma and lambda landmarks on the skull level with the ground. Animals received prophylactic meloxicam for pain relief, and heads were swabbed with betadine. A centimeter-long incision in the scalp allowed subcutaneous tissue and periosteum to be scrubbed from the skull using sterile cotton-tipped applicators to reveal the bregma. Hamilton syringes (VWR 60376-172) coupled to 22-gauge Huber needles (VWR 82010-236) were filled with 10 µL of saline with or without ASO (diluted from 100 mg/mL in sterile dPBS, Gibco 14190). The needle was first positioned over bregma and then moved to coordinates 0.3 mm anterior and 1.0 mm right. The needle was then usually advanced ventral (downward) until the bevel of the needle disappeared into the skull and then an additional 3.0 mm further. In later studies at the Broad Institute this procedure was revised so that the needle was advanced until it touched the skull, and then an additional 3.5 mm further; this obviates being able to see the bevel, which can be difficult because for prion-infected animals the stereotaxis is inside a biosafety cabinet. With the needle at the correct coordinates, 10 µL of saline was manually injected over ~10 seconds and the needle was allowed to sit in place for 3 minutes. The needle was then backed out of the skull while applying downward pressure on the skull with a sterile cotton-tipped applicator. The incision was closed with a

horizontal mattress stitch using 5-O Ethilon suture (Ethicon 661H). Animals recovered from the anesthesia in their home cages on a warming pad.

Intracerebral prion inoculation

Brains from terminally sick prion-infected animals were obtained from Deborah E. Cabin at McLaughlin Research Institute (RML prion strain[46]).

Brains were homogenized at 10% wt/vol in dPBS (Gibco 14190) in 7 mL tubes pre-loaded with zirconium oxide beads (Precellys no. KT039611307.7) by 3x 40 sec pulses on high on a MiniLys tissue homogenizer (Bertin EQ06404-200-RD000.0). Homogenates were then diluted to 1% wt/vol in dPBS and extruded through progressively smaller-gauge blunt needles (Sai Infusion B18, B21, B24, B27, B30) to ensure homogeneity and then loaded into 2mL amber sealed sterile glass vials (Med Lab Supply) using a 31G needle (BD 328449). Homogenates were frozen at -80°C and irradiated (~7.0 kGy) on dry ice to kill nucleic acid-containing pathogens[47]. On the day of inoculation, homogenates were thawed and 31G needles (BD 328449) were each loaded with 30 µL of brain homogenate for each animal.

Inoculations were performed in animals aged 7-10 weeks, when the skull is still cartilaginous enough to allow this procedure. Animals were induced and maintained at 3% isoflurane, received prophylactic meloxicam for pain relief, and heads were wiped with betadine. The needle was manually inserted through the skull, the brain homogenate was ejected, and after three seconds, the needle was removed. Animals recovered from anesthesia in home cages.

Inoculations at McLaughlin Research Institute were performed in the same manner described above, except that mice were induced for 7 minutes at 3.5% isoflurane and then the inoculation was conducted within ~30 seconds without nosecone maintenance of anesthesia; mice remain

on a surgical plane of anesthesia for this time period. Inoculations at Rocky Mountain Labs differed in particulars and have been described elsewhere[1].

Bioluminescence imaging

Each mouse received a 100 µL intraperitoneal injection of 50 mg/mL D-luciferin (GoldBio) dissolved in PBS for a final dose of 5 mg/mouse. After ~7 minutes to allow drug distribution and another ~7 minutes of induction with 3.5% isoflurane, each animal was positioned onto a platform fitted with a nosecone for isoflurane maintenance and placed inside a Lumina II in vitro imaging system (IVIS; Perkin Elmer) to be imaged for 1 minute before returning to its home cage.

Three control Tg(Gfap-luc) animals were imaged for each session to confirm luciferin injection and equipment functionality: two mice injected with intraperitoneal lipopolysaccharide (LPS; positive control inducing CNS gliosis), and one mouse injected with PBS (negative control), 16 hours prior.

Data were extracted with Living Image Software 4.5 (Perkin Elmer) from a single region of interest (ROI) for all animals, defined based on an LPS control animal. Bioluminesence was quantified in units of photons/sec/cm^2/sr, meaning the number of photons per second emitted from one square centimeter of tissue radiating into a solid angle of one steradian (sr). This metric, often abbreviated as radiance units, or simply photons, is a calibrated measure that controls for charge coupled device (CCD) camera settings including exposure, binning, and F-stop. This contrasts with an absolute measurement of photos incident on the CCD, and has the advantage that camera settings can be adjusted if needed without compromising the comparability of results.

Animal monitoring and endpoint criteria

Animals at the Broad Institute were housed four per cage on a 12-hour light/dark cycle with food and water ad libitum, in disposable cages incinerated after each cage change. Animals were checked daily for any acute health conditions, and were weighed and subjected to individual behavioral tests 3-4x per week beginning at 120 dpi. In the first two studies (Table 2 and Figure 5), animals were euthanized if any of the following criteria were met: body condition score <2, body weight loss ≥20% from baseline, inability to reach food or water, severe respiratory distress, or severe neurological deficits. In subsequent experiments (Figures 6 and 7), animals were scored 0 or 1 every other day for each of eight behavioral tests: scruff / poor grooming, poor body condition, reduced activity, hunched posture, irregular gait / hindlimb weakness, tremor, blank stare, and difficulty righting. Animals were euthanized if they met any of the following criteria: ≥5 symptoms for 2 consecutive behavioral sessions, body condition score ≤2, ≥15% body weight loss from baseline, or unable to reach food or water. Cages were also scored for nest material utilization every other day, using a scheme described previously[1]. Because ours were the first prion-infected animals ever housed at Broad, early experiments were unblinded as staff learned the signs and symptoms associated with disease progression. Thereafter (for Figures 5, 6, and 7), all behavioral monitoring and all determinations of endpoint criteria were undertaken by veterinary technician staff blinded to the animals' drug treatment conditions.

At McLaughlin Research Institute, mice were monitored every other day by unblinded raters and euthanized upon 20% body weight loss from a baseline taken at 3 months of age, or when deemed moribund due to severe neurological signs or inability to reach food or water. At Rocky Mountain Labs, mice were monitored every other day by unblinded raters, with disease onset determined based on seven criteria: progressive deterioration of ataxia, tremors, myoclonus,

weight loss, somnolence, kyphosis and poor grooming, and with euthanasia upon determination of advanced clinical signs.

In some cases, the weight loss or moribund status leading to euthanasia may have been due to intercurrent illness or to adverse reactions to ASO treatment. In addition, some animals died before meeting endpoint criteria. To avoid selective reporting of only those deaths subjectively attributed to prion disease, all survival curves reported herein include all causes of death except for the following: death prior to any ICV treatment (meaning prior to experimental treatment group being assigned); acute deaths within 1 day post-surgery due to surgical complications; and euthanasia due to experimental error (such as incorrect dosing or inability to position animal in stereotaxis).

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Chapter 6. Conclusions

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The road to prevention

Most clinical trials in neurodegenerative disease have treated symptomatic patients[1,2]. To the extent that any focus has shifted upstream, trials have enrolled asymptomatic individuals with biomarker evidence of neuropathology — so-called "secondary prevention" trials[3–5]. In this dissertation and the accompanying dissertation by Sonia Vallabh[6], we seek to enable even earlier intervention in prion disease — "primary prevention" before the disease process has even begun at the molecular level.

It may be uncontroversial to argue that what patients want is primary prevention — additional years of healthy life are sure more valuable than additional years of disease. The fact that, despite this, so much effort is focused on symptomatic populations is likely driven simply by practical considerations, such as the ability to adequately power trials for a clinical endpoint that will be convincing to regulators, prescribers, and payors. Some drugs are only effective in prevention, and not treatment, of a condition — consider statins and heart attack — and in such cases, efficacy could only be demonstrated through prevention trials. In other cases, the same drug may be effective in both symptomatic and presymptomatic populations[7,8], and the regulatory approval and broad labeling achieved through a symptomatic trial ultimately do enable preventive use. Thus, symptomatic trials can in some cases represent a relatively expeditious route towards the goal of disease prevention. To what extent symptomatic trials in common, adult-onset neurodegenerative diseases will ultimately serve to enable prevention remains to be seen.

Prion disease has unique attributes that make it a strong test case for pursuing primary prevention in clinical trials. On one hand, its biology is extraordinarily well-understood. Because the disease is both monogenic and transmissible, and naturally afflicts animals other than humans, there is an exceptional convergence of diverse lines of evidence to implicate PrP as the agent of disease. At the same time, the clinical picture of symptomatic prion disease is extraordinarily challenging. The rapid progression of disease means that many patients are not diagnosed until a state of deep dementia, with no quality of life left to preserve. The rarity, unpredictable age of onset, and lack of detectable prodrome rule out many of the "secondary prevention" models employed in trials in other neurodegenerative diseases. Yet, there exists an opportunity for prevention, as people with predictive genetic testing for *PRNP* mutations can know their status decades in advance of any symptoms.

This combination of attributes led us to propose that PrP-lowering therapeutics for prion disease could be tested in pre-symptomatic *PRNP* mutation carriers, and evaluated for approval based on a surrogate endpoint of lowered PrP in cerebrospinal fluid (CSF)[6]. This is not business as usual. Indeed, if we are successful in launching such a trial, it will be to our knowledge the first trial ever to combine all three of the following attributes: 1) recruitment of asymptomatic individuals based on predictive genetic testing results, 2) treatment with a targeted therapy, and 3) measurement of a novel surrogate biomarker endpoint as primary endpoint for approval.

In a disease where all completed trials to date have focused on symptomatic patients with a survival endpoint, re-orienting around primary prevention is a dramatic change of course, and successful execution will require tremendous effort.

One front of effort is the identification and recruitment of presymptomatic patients for trials. The ability to quantify the penetrance of different *PRNP* mutations (Chapter 2) to identify individuals at high risk is one important step, but much work remains. Because these individuals are pre-symptomatic, most do not see a neurologist, and there is no existing referral network. Our online registry (PrionRegistry.org) offers a venue for such individuals to volunteer for research, and in the year and half since launch, *N*=120 people self-identifying as "healthy but at-risk" have joined. Meanwhile, our clinical research study at Massachusetts General Hospital has begun to build a cohort of such individuals, complementing pre-symptomatic cohorts that have been followed at University of California San Francisco[9], at the U.K. National Prion Clinic[10], and elsewhere. Still, only approximately one quarter of individuals at risk for genetic prion disease choose predictive testing[11], suggesting that ultimately, making sure a drug is available to all who could benefit from it will require a considerable outreach effort. Individuals at risk are often counseled not to pursue testing because the results are "not medically actionable," yet developing a drug in order to make these results actionable is only possible if enough people do get tested and choose to participate in research. The meaning of actionability in predictive genetic testing will be an ongoing conversation.

In thinking through the details of recruiting for a clinical trial, and ultimately prescribing a new drug, another problem emerges: it is important to have some idea not just of who will develop disease, but when. Age of onset (Chapter 3) turns out to be highly variable and unpredictable. Nonetheless, even if only probabilistic in nature, the life tables we have assembled should be useful in determining when to begin drug treatment. For example, since the earliest disease onset ever observed for the E200K mutation was at age 31, and most individuals survive into their 60s, and it might not make sense to treat individuals with this mutation beginning in their 20s. Decisions about when to begin treatment might ultimately take into account both the age-dependent survival curve for each individual's mutation and the tolerability of the drug. Even if

provisional approval can be achieved based on a biomarker, such as through the Accelerated Approval program, one would still ultimately want to confirm that age of onset was delayed, and so future work might also consider the design of a post-approval surveillance program. The life tables presented here may be useful as a historical comparator in such a program.

A major area of ongoing effort is the establishment of CSF PrP as a biomarker that could merit provisional drug approval. The technical performance of CSF PrP ELISA[6,12], the good test-retest reliability of CSF PrP in the pre-symptomatic population[6], and the cross-validation of ELISA findings using an orthogonal mass spectrometry assay (Chapter 4) are all promising. However, future work will need to confirm, in preclinical animal models, that CSF PrP does indeed drop in response to drug-mediated lowering of brain PrP. More extensive analytical validation of the assay to be used clinically might also be desired. Finally, reproducible measurement of CSF PrP depends upon very careful sample handling to minimize pre-analytical variability[12], and while we have succeeded in implementing such a handling protocol at Massachusetts General Hospital[6], adequately powering a trial may necessitate implementation of this protocol at other sites as well.

Most critically, pre-symptomatic trials will depend upon having a plausible targeted therapy to test. Antisense oligonucleotides (Chapter 5) appear to fit the bill, and are currently under preclinical development[13]. Much further preclinical work will be required in order to launch this therapy into clinical trials. Meanwhile, based on studies to date, ASOs appear to delay the onset of prion disease in animals by a considerable margin, in keeping with the known benefit of heterozygous PrP knockout[14], but ASOs may not delay disease indefinitely. True prevention — delaying prion disease onset well beyond the human lifespan — will likely require additional PrP-lowering drugs, an area for future research which should be catalyzed by the development of the clinical pathway and biomarker we have described.

Although an enormous amount of work remains, we have begun to glimpse a future in which genetic prion disease can be delayed or prevented using a targeted, PrP-lowering therapy tested clinically in pre-symptomatic people for the ability to lower PrP. The details of this strategy all rest on the particulars of prion disease, which is perhaps unique in all of biology. Nevertheless, at a higher level, we hope that the thought process outlined in these two dissertations can be useful to others seeking to enable primary prevention in their diseases of interest, and that this work will ultimately serve the goal of preventing disease and extending healthy life.

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Appendix

Supplement to *Quantifying penetrance in prion disease*

Additional variants

Of the 63 reportedly pathogenic variants (Supplemental Table S2.2), 10 are discussed in the main text. Of those 10, our data and our analysis of the literature indicate high penetrance for 4 (P102L, A117V, D178N, and E200K), intermediate penetrance for 3 (V180I, V210I, and M232R), and suggest that 3 others may be benign (P39L, E196A, and R208C). In this section we discuss four additional variants that we cannot conclusively reclassify but which are unlikely to be highly penetrant, and we also provide a brief discussion of interpretation for remaining variants.

R148H has been reported in a two isolated patients with a sporadic Creutzfeldt-Jakob disease phenotype and negative family history [1,2] and appears one additional time in our case cohorts (Supplemental Table S2.1). Based on its rarity in cases, lack of familial segregation and presence on 3 alleles in ExAC, it is unlikely to be a highly penetrant Mendelian variant. It might be benign or it might slightly increase prion disease risk.

T188R has been reported in two cases in the literature. One German individual presented with a sporadic Creutzfeldt-Jakob disease phenotype but no autopsy was performed; family history was negative [3,4]. One Mexican-American individual had autopsy-confirmed prion disease and an ambiguous family history[5]. This variant appears 12 times in our case cohort (all in the United States) and 3 times in ExAC (all in Latino populations). Based on its allele frequency in controls, rarity in cases and lack of any clear evidence for segregation in families, T188R is unlikely to be a highly penetrant Mendelian disease variant. It is not clear whether it is benign or increases prion disease risk.

V203I has been reported in three heterozygous patients - one Italian [6], one Korean [7], and one Chinese [8], as well as in one Japanese homozygote [9]. Family history is negative in all of these reported patients as well as in two additional V203I cases in our Japanese case cohort (Supplemental Table S2.10). In our cohorts, this variant appears in a total of 16 cases from several countries; in ExAC, it appears in 3 European individuals. Based on its allele frequency in controls, rarity in cases and lack of any clear evidence for segregation in families, V203I is unlikely to be a highly penetrant Mendelian disease variant, and could be benign or could increase prion disease risk. The report of prion disease in a V203I homozygote makes us slightly inclined to favor the interpretation that V203I does increase prion disease risk.

R208H has been reported in several isolated cases of varied ancestries, all with a negative family history [10–16]. In our cohorts, it appears in 13 prion disease cases, 9 ExAC individuals and 22 individuals in the 23andMe database. Given its high frequency in controls, this variant may be benign or may slightly increase prion disease risk.

Other variants. Excluding variants discussed in the main text and above, 0.8% (87 / 10460) of individuals in our case series harbor other rare *PRNP* missense variants, some of which have been reported as pathogenic (Supplemental Table S2.2) and others of which have not. Because most of these variants are very rare both in cases and in population controls, comparisons of case and control allele frequency are not well powered to evaluate the pathogenicity of most individual variants. Collectively, our data indicate that this category includes at least some variants that increase prion disease risk, because only 0.3% (187 / 60706) of ExAC individuals harbor a rare missense variant other than those discussed in the main text or above, whereas 0.8% (87 / 10460) of prion disease cases harbor one of these variants, a significant enrichment ($p = 1 \times 10^{-12}$, Fisher's exact test). Indeed, Mendelian segregation has been demonstrated for

some of these variants, such as T183A and F198S [17,18]. However, the fact that, in the aggregate, we observe only modest (~3-fold) enrichment of such variants in cases versus controls suggests that this category also includes many neutral or very low-risk variants, consistent with our expectation that sporadic prion disease cases should, by chance, harbor some rare variants unassociated with disease. We also cannot exclude the possibility that some specific rare variants, particularly those observed in controls and not in cases, could be protective.

Future novel missense variants. Additional novel missense variants in *PRNP* are sure to be observed in prion disease patients in the future. Our findings that some reportedly pathogenic variants are either benign or exhibit low penetrance, together with our observation that ~4 in 1000 controls harbor a rare *PRNP* missense variant, urge caution in the interpretation of novel variants in prion disease patients. This is consistent with current guidelines [19,20], which indicate that novel protein-altering variants, even in established disease genes, should not be assumed to be causal or highly penetrant until evidence, such as Mendelian segregation, or significant enrichment in cases over controls, can be established.

Dominant versus allelic models. Virtually all patients ever reported with genetic prion disease have been heterozygous for the putative pathogenic variants. Five individuals homozygous for E200K [21] were reported to have a younger age of onset than heterozygotes (mean 50 vs. 59 years, $p = .03$), suggesting some degree of codominance. There have been individual case reports of homozygotes for Q212P [22] and V203I [9], both without a family history among heterozygote relatives, which might suggest that dosage of the mutant allele is important. We are not aware of any other reports of individuals homozygous for potentially pathogenic variants in *PRNP*. Regardless of whether a dominant or allelic model is assumed, our formula for lifetime

risk (Methods) gives identical point estimates of penetrance and virtually identical 95% confidence intervals.

Supplemental Table S2.1 | Allele counts of rare PRNP variants in 16,025 definite and probable prion disease cases in 9 countries. *Abbreviations: OPRD, octapeptide repeat deletion; OPRI, octapeptide repeat insertion. *V203I in Japan: two heterozygotes and one homozygote, four alleles total. All other individuals are heterozygotes.*

	Australia	France	Germany	Italy	Japan	Netherlands	Spain	U.K.	U.S.	TOTAL
Start year	1993	1991	1993	1993	1999	1993	1993	1990	2000	
End year	2014	2013	2015	2013	2014	2013	2013	2013	2014	
Definite plus probable cases	553	2383	2690	1684	2144	409	1280	1963	2919	16025
Of which PRNP sequenced	152	1774	1307	1054	1533	163	749	1088	2640	10460
Proportion sequenced	27%	74%	49%	63%	72%	40%	59%	55%	90%	65%
Cumulative allele count of rare variants	31	196	125	396	464	22	127	173	361	1895
2-OPRD						3				3
1-OPRI		2	1						4	7
2-OPRI							1		5	6
3-OPRI		1	1							2
4-OPRI		1	3				2	13	4	23
5-OPRI		2	10			1	1	13	12	39
6-OPRI		2						35	15	52
7-OPRI		1	1			1		2		5
8-OPRI		10								10
9-OPRI									4	4
10-OPRI								1		1

	Australia	France	Germany	Italy	Japan	Netherlands	Spain	U.K.	U.S.	TOTAL
OPRI (length unspecified)				9	8					17
A2V			1							1
G54S								1	4	5
P84S								1		1
G88A							1			1
G94S									1	1
H96Y							1			1
P102L	2	10	7	59	83		1	34	25	221
P105L					12			1		13
P105S									1	1
P105T	3		2							5
G114V									1	1
A117V		3				8	1	12	9	33
G131V						1				1
S132I								1		1
A133V	1								1	2
R148H			1						2	3
Q160X									1	1
Y163X								2		2
D167G								1		1
V176G	1									1
D178N	3	34	32	18	5	4	65	12	36	209
V180I		1		1	218				5	225
T183A									3	3
Q186X									1	1
H187A									1	1

	Australia	France	Germany	Italy	Japan	Netherlands	Spain	U.K.	U.S.	TOTAL
H187R									7	7
T188A	1									1
T188K			2						1	3
T188R									12	12
E196A									1	1
E196K		3	8	2						13
F198S									5	5
E200G									1	1
E200K	11	101	28	123	63	2	52	38	153	571
V203I		5		3	4*				5	17
R208H		1	2	7	1				4	15
V210I	4	13	19	171	1			3	36	247
E211Q		5	2	3				1		11
E211D		1								1
Q212P								2		2
I215V							1			1
Y218N							1			1
A224V									1	1
Y226X						1				1
Q227X						1				1
M232R					63					63
V180I *trans* M232R					4					4
Variant not specified	5		5		2					12

Supplemental Table S2.2 | Rare PRNP variants reported in peer-reviewed literature to cause prion disease. *Note: this table represented a complete review of the literature as of 2015 when this chapter was submitted for publication. A review of reportedly pathogenic PRNP variants that is complete up through February 2019 can be found in Supplemental Table S3.1.*

Variant	First report	See also
P39L	Bernardi 2014 [23]	
2-OPRD	Beck 2001 [24]	Capellari 2002 [25]
1-OPRI	Laplanche 1995 [26]	Pietrini 2003 [27]
2-OPRI	Hill 2006 [28]	
3-OPRI	Nishida 2004 [29]	
4-OPRI	Laplanche 1995 [26]	Campbell 1996 [30], Kaski 2011 [31]
5-OPRI	Goldfarb 1991 [32]	
6-OPRI	Owen 1990 [33]	Mead 2006 [34]
7-OPRI	Goldfarb 1991 [32]	Lewis 2003 [35]
8-OPRI	Goldfarb 1991 [32]	Laplanche 1999 [36]
9-OPRI	Krasemann 1995 [37]	
12-OPRI	Kumar 2011 [38]	
P84S	Jones 2014 [39]	
S97N	Zheng 2008 [40]	
P102L	Goldgaber 1989 [41]	Hsiao 1989 [42]
P105L	Yamada 1993 [43]	Yamada 1999 [44]
P105S	Tunnell 2008 [45]	
P105T	Polymenidou 2011[46]	
G114V	Rodriguez 2005 [47]	Liu 2010 [48]
A117V	Tateishi 1990 [49]	Hsiao 1991 [50]
129insLGGLGGYV	Hinnell 2011 [51]	
G131V	Panegyres 2001 [52]	Jansen 2012 [53]
S132I	Hilton 2009 [54]	
A133V	Rowe 2007 [55]	
Y145X	Kitamoto 1993 [56]	
R148H	Krebs 2005 [1]	Pastore 2005 [2]
Q160X	Finckh 2000 [57]	Jayadev 2011 [58]

Variant	First report	See also
Y163X	Revesz 2009 [59]	Mead 2013 [60]
D167G	Bishop 2009 [61]	
D167N	Beck 2010 [22]	
V176G	Simpson 2013 [62]	
D178Efs25X	Mastuzono 2013 [63]	
D178N	Goldfarb 1991 [64]	Medori 1992 [65], Goldfarb 1992 [66]
V180I	Hitoshi 1993 [67]	Chasseigneaux 2006 [68]
T183A	Nitrini 1997 [17]	Grasbon-Frodl 2004 [69]
H187R	Butefisch 2000 [70]	
T188A	Collins 2000 [71]	
T188K	Finckh 2000 [57]	Roeber 2008 [4]
T188R	Windl 1999 [3]	Roeber 2008 [4], Tartaglia 2010 [5]
T193I	Kotta 2006 [72]	
E196A	Zhang 2014 [73]	
E196K	Peoc'h 2000 [6]	
F198S	Farlow 1989 [74]	Hsiao 1992 [18]
F198V	Zheng 2008 [40]	
E200G	Kim 2013 [75]	
E200K	Goldgaber 1989 [41]	Hsiao 1991 [76]
D202G	Heinemann 2008 [77]	
D202N	Piccardo 1998 [78]	
V203I	Peoc'h 2000 [6]	
R208C	Zheng 2008 [40]	
R208H	Mastrianni 1996 [79]	Capellari 2005 [11], Roeber 2005 [12]
V210I	Ripoll 1993 [80]	Pocchiari 1993 [81], Mouillet-Richard 1999 [82]
E211D	Peoc'h 2012 [83]	
E211Q	Peoc'h 2000 [6]	
Q212P	Piccardo 1998 [78]	
I215V	Munoz-Nieto 2013 [84]	
Q217R	Hsiao 1992 [18]	

Variant	First report	See also
Y218N	Alzualde 2010 [85]	
Y226X	Jansen 2010 [86]	
Q227X	Jansen 2010 [86]	
M232R	Hitoshi 1993 [67]	Hoque 1996 [87]
M232T	Bratosiewicz 2000 [88]	
P238S	Windl 1999 [3]	

Supplemental Table S2.3 | Allele counts of rare PRNP variants in 60,706 individuals in ExAC. *Chromosomal positions are given in GRCh37 coordinates and HGVS notations are given relative to Ensembl transcript ENST00000379440. Mean read depth across the PRNP coding sequence was 55.21. Call rate is the proportion of ExAC individuals with a genotype call of genotype quality (GQ) ≥20 and a depth (DP) of ≥10 reads.*

Chrom	Pos	Ref	Alt	HGVS	Variant	Class	Call rate	AC
20	4679863	C	T	c.-4C>T		non-coding	97%	1
20	4679871	C	T	c.5C>T	A2V	missense	97%	2
20	4679877	T	A	c.11T>A	L4H	missense	98%	3
20	4679877	T	G	c.11T>G	L4R	missense	98%	1
20	4679888	A	G	c.22A>G	M8V	missense	98%	1
20	4679901	T	C	c.35T>C	F12S	missense	98%	1
20	4679916	G	C	c.50G>C	S17T	missense	98%	10
20	4679920	C	A	c.54C>A	D18E	missense	98%	2
20	4679920	C	T	c.54C>T	D18D	synonymous	98%	18
20	4679927	C	A	c.61C>A	L21I	missense	98%	1
20	4679932	C	T	c.66C>T	C22C	synonymous	98%	2
20	4679935	G	A	c.69G>A	K23K	synonymous	98%	2
20	4679939	C	T	c.73C>T	R25C	missense	98%	2
20	4679944	G	A	c.78G>A	P26P	synonymous	98%	6
20	4679967	G	T	c.101G>T	G34V	missense	98%	1
20	4679969	G	A	c.103G>A	G35S	missense	98%	1
20	4679975	C	T	c.109C>T	R37X	nonsense	98%	1

Chrom	Pos	Ref	Alt	HGVS	Variant	Class	Call rate	AC
20	4679982	C	T	c.116C>T	P39L	missense	98%	3
20	4679983	G	A	c.117G>A	P39P	synonymous	98%	8
20	4679986	G	A	c.120G>A	G40G	synonymous	98%	12
20	4680005	A	G	c.139A>G	N47D	missense	98%	1
20	4680026	G	A	c.160G>A	G54S	missense	97%	78
20	4680028	T	C	c.162T>C	G54G	synonymous	97%	5
20	4680038	G	T	c.172G>T	G58W	missense	97%	1
20	4680045	C	T	c.179C>T	P60L	missense	96%	1
20	4680055	T	A	c.189T>A	G63G	synonymous	96%	1
20	4680077	G	A	c.211G>A	G71S	missense	96%	1
20	4680089	C	T	c.223C>T	Q75X	nonsense	96%	1
20	4680091	G	A	c.225G>A	Q75Q	synonymous	96%	2
20	4680093	C	G	c.227C>G	P76R	missense	96%	1
20	4680129	G	C	c.263G>C	G88A	missense	98%	1
20	4680134	G	A	c.268G>A	G90S	missense	98%	1
20	4680145	T	G	c.279T>G	G93G	synonymous	99%	1
20	4680151	C	T	c.285C>T	T95T	synonymous	99%	1
20	4680172	G	A	c.306G>A	P102P	synonymous	99%	21
20	4680185	A	G	c.319A>G	T107A	missense	99%	1
20	4680199	C	T	c.333C>T	H111H	synonymous	99%	2
20	4680202	G	A	c.336G>A	M112I	missense	99%	1
20	4680231	T	G	c.365T>G	V122G	missense	99%	1
20	4680232	G	T	c.366G>T	V122V	synonymous	99%	3
20	4680244	C	A	c.378C>A	G126G	synonymous	99%	1
20	4680244	C	T	c.378C>T	G126G	synonymous	99%	3
20	4680250	C	T	c.384C>T	Y128Y	synonymous	100%	22
20	4680252	T	C	c.386T>C	M129T	missense	100%	1

Chrom	Pos	Ref	Alt	HGVS	Variant	Class	Call rate	AC
20	4680257	G	T	c.391G>T	G131X	nonsense	100%	1
20	4680258	G	T	c.392G>T	G131V	missense	100%	1
20	4680259	A	G	c.393A>G	G131G	synonymous	100%	3
20	4680262	T	C	c.396T>C	S132S	synonymous	100%	1
20	4680274	G	A	c.408G>A	R136R	synonymous	100%	2
20	4680274	G	T	c.408G>T	R136S	missense	100%	2
20	4680279	T	C	c.413T>C	I138T	missense	100%	1
20	4680289	C	T	c.423C>T	F141F	synonymous	100%	2
20	4680292	C	T	c.426C>T	G142G	synonymous	100%	1
20	4680299	T	G	c.433T>G	Y145D	missense	100%	1
20	4680308	C	T	c.442C>T	R148C	missense	100%	1
20	4680309	G	A	c.443G>A	R148H	missense	100%	3
20	4680311	T	C	c.445T>C	Y149H	missense	100%	1
20	4680316	T	C	c.450T>C	Y150Y	synonymous	100%	1
20	4680317	C	T	c.451C>T	R151C	missense	100%	2
20	4680318	G	A	c.452G>A	R151H	missense	100%	3
20	4680324	A	G	c.458A>G	N153S	missense	100%	1
20	4680328	G	A	c.462G>A	M154I	missense	100%	1
20	4680342	A	G	c.476A>G	N159S	missense	100%	1
20	4680349	G	A	c.483G>A	V161V	synonymous	100%	1
20	4680359	C	T	c.493C>T	P165S	missense	100%	2
20	4680362	A	G	c.496A>G	M166V	missense	100%	2
20	4680364	G	A	c.498G>A	M166I	missense	100%	2
20	4680373	C	T	c.507C>T	Y169Y	synonymous	100%	1
20	4680382	G	A	c.516G>A	Q172Q	synonymous	100%	1
20	4680385	C	T	c.519C>T	N173N	synonymous	100%	5
20	4680394	G	A	c.528G>A	V176V	synonymous	100%	2

Chrom	Pos	Ref	Alt	HGVS	Variant	Class	Call rate	AC
20	4680397	C	G	c.531C>G	H177Q	missense	100%	1
20	4680397	C	T	c.531C>T	H177H	synonymous	100%	4
20	4680403	C	T	c.537C>T	C179C	synonymous	100%	1
20	4680404	G	A	c.538G>A	V180I	missense	100%	6
20	4680412	C	G	c.546C>G	I182M	missense	100%	2
20	4680429	C	G	c.563C>G	T188R	missense	100%	3
20	4680429	C	T	c.563C>T	T188M	missense	100%	4
20	4680443	A	G	c.577A>G	T193A	missense	100%	2
20	4680445	C	A	c.579C>A	T193T	synonymous	100%	1
20	4680449	G	C	c.583G>C	G195R	missense	100%	3
20	4680451	G	A	c.585G>A	G195G	synonymous	100%	3
20	4680453	A	C	c.587A>C	E196A	missense	100%	9
20	4680462	C	A	c.596C>A	T199N	missense	100%	1
20	4680463	C	T	c.597C>T	T199T	synonymous	100%	2
20	4680467	A	T	c.601A>T	T201S	missense	100%	1
20	4680469	C	T	c.603C>T	T201T	synonymous	100%	3
20	4680470	G	A	c.604G>A	D202N	missense	100%	1
20	4680472	C	T	c.606C>T	D202D	synonymous	100%	8
20	4680473	G	A	c.607G>A	V203I	missense	100%	3
20	4680488	C	T	c.622C>T	R208C	missense	100%	1
20	4680489	G	A	c.623G>A	R208H	missense	100%	9
20	4680490	C	T	c.624C>T	R208R	synonymous	100%	4
20	4680491	G	A	c.625G>A	V209M	missense	100%	1
20	4680494	G	A	c.628G>A	V210I	missense	100%	2
20	4680501	A	C	c.635A>C	Q212P	missense	100%	1
20	4680502	G	A	c.636G>A	Q212Q	synonymous	100%	2
20	4680520	C	T	c.654C>T	Y218Y	synonymous	100%	17

Chrom	Pos	Ref	Alt	HGVS	Variant	Class	Call rate	AC
20	4680534	A	T	c.668A>T	Q223L	missense	100%	1
20	4680539	T	C	c.673T>C	Y225H	missense	99%	1
20	4680540	A	G	c.674A>G	Y225C	missense	99%	1
20	4680541	T	C	c.675T>C	Y225Y	synonymous	99%	3
20	4680552	G	A	c.686G>A	G229E	missense	98%	1
20	4680553	A	G	c.687A>G	G229G	synonymous	98%	1
20	4680561	T	G	c.695T>G	M232R	missense	97%	10
20	4680566	C	T	c.700C>T	L234F	missense	95%	29
20	4680590	C	T	c.724C>T	L242F	missense	87%	1
20	4680598	C	G	c.732C>G	I244M	missense	84%	1
20	4680598	C	T	c.732C>T	I244I	synonymous	84%	1
20	4680626	T	G	c.760T>G	X254G	read-through	66%	1

Supplemental Table S2.4 | Summary of rare PRNP variants by functional class in ExAC.

Class	Total AC
missense	236
non-coding	1
nonsense	3
read-through	1
synonymous	180

Supplemental Table S2.5 | Allele counts of 16 reportedly pathogenic PRNP variants in >500,000 23andMe research participants. *To protect the privacy of 23andMe research participants, allele count (AC) values between 1 and 5 inclusive are displayed as "1-5" and are rounded up to 5 for the purposes of plotting. These alleles were seen almost exclusively in a heterozygous state, with fewer than 5 homozygous individuals total across all 16 variants.*

Variant	dbSNP id	23andMe id	Called genotypes	AC	Comments
P102L	rs74315401	i5004359	502075	1-5 total	
A117V	rs74315402	i5004358	501820		
D178N	rs74315403	i5004357	502450		
E200K	rs28933385	rs28933385	531370		
M232R	rs74315409	i5004352	502475	78	AC=29 in 2,685 individuals with >90% Japanese ancestry
V180I	rs74315408	i5004353	502125	15	AC=1-5 in 2,670 individuals with >90% Japanese ancestry
V210I	rs74315407	i5004354	502290	13	AC=8 in 385,030 Europeans
R208C	rs55826236	rs55826236	501850	8	
R208H	rs74315412	i5004349	501775	22	AC=19 in 384,645 Europeans
P105L	rs11538758	rs11538758	531575	1-5 total	
G131V	rs74315410	i5004351	499455		
A133V	rs74315415	i5004347	502520		
T183A	rs74315411	i5004350	502295		
F198V	rs55871421	rs55871421	501540		
F198S	rs74315405	i5004356	502460		
G217R	rs74315406	i5004355	502385		

Supplemental Table S2.6 | Phenotypes investigated in studies in which ExAC individuals with reportedly pathogenic PRNP variants were ascertained. *Note that we do not have access to phenotypic data to indicate whether a particular individual was ascertained as a case or a control. Therefore "cardiovascular" simply means an individual was ascertained in a cardiovascular disease cohort, not necessarily that the individual has cardiovascular disease. "Mixed" cohorts include controls, cardiovascular and pulmonary phenotypes.*

Cohort phenotype	**Total in ExAC**	**Number with reportedly pathogenic *PRNP* variants**
Autoimmune	1675	4
Cancer	7601	3
Cardiovascular	14622	14
Metabolic	15327	19
Mixed	3936	2
Population controls	2215	6
Psychiatric	15330	4
Total	**60706**	**52**

Supplemental Table S2.7. Inferred ancestry and codon 129 genotypes of ExAC individuals with reportedly pathogenic variants. *Three-letter HapMap ancestry codes are defined in Supplemental Table S2.8.*

Variant	Populations	Codon 129 genotypes
P39L	1 PJL, 2 TSI	2 M/M, 1 M/V
G131V	1 TSI	1 M/V
R148H	1 CEU, 1 IBS, 1 PJL	3 M/M
V180I	1 CHB, 2 JPT, 3 PJL	4 M/M, 1 M/V, 1 V/V
T188R	1 CLM, 2 MXL	1 M/V, 2 V/V
E196A	3 CHB, 6 CHS	9 M/M
D202N	1 TSI	1 M/V
V203I	1 IBS, 2 TSI	1 M/M, 2 M/V
R208C	1 ACB	1 M/M
R208H	1 ACB, 2 ASW, 1 CLM, 2 IBS, 1 MSL, 2 TSI	4 M/M, 5 M/V
V210I	2 TSI	2 M/M
Q212P	1 CEU	1 M/V
M232R	5 CHB, 5 JPT	10 M/M

Supplemental Table S2.8 | Inferred ancestry of all ExAC individuals. *Ancestry assignment is described in Methods.*

Population code	Description	Super population	N in ExAC
ACB	African Caribbeans in Barbados	AFR	2267
ASW	Americans of African Ancestry in SW USA	AFR	2151
BEB	Bengali from Bangladesh	SAS	483
CDX	Chinese Dai in Xishuangbanna, China	EAS	19
CEU	Utah Residents (CEPH) with Northern and Western European ancestry	EUR	14185
CHB	Han Chinese in Beijing, China	EAS	1553
CHS	Southern Han Chinese	EAS	1733
CLM	Colombians from Medellin, Colombia	AMR	870
ESN	Esan in Nigeria	AFR	89
FIN	Finnish in Finland	EUR	3977
GBR	British in England and Scotland	EUR	10358
GIH	Gujarati Indian from Houston, Texas	SAS	79
GWD	Gambian in Western Divisions in The Gambia	AFR	102
IBS	Iberian population in Spain	EUR	3534
ITU	Indian Telugu from the UK	SAS	1089
JPT	Japanese in Tokyo, Japan	EAS	663
KHV	Kinh in Ho Chi Minh City, Vietnam	EAS	369
LWK	Luhya in Webuye, Kenya	AFR	72
MSL	Mende in Sierra Leone	AFR	189
MXL	Mexican Ancestry from Los Angeles USA	AMR	2658
PEL	Peruvians from Lima, Peru	AMR	1900
PJL	Punjabi from Lahore, Pakistan	SAS	6300
PUR	Puerto Ricans from Puerto Rico	AMR	579
STU	Sri Lankan Tamil from the UK	SAS	460

TSI	Toscani in Italia	EUR	4795
YRI	Yoruba in Ibadan, Nigeria	AFR	232

Supplemental Table S2.9 | Inferred ancestry of 23andMe research participants

Ancestry	**Minimum called genotypes**	**Maximum called genotypes**	**Total allele count of reportedly pathogenic *PRNP* variants**
European	382865	408475	≥35
Latino	42425	44480	≥10
African	22945	23795	≥10
East Asian	20255	21710	≥75
All others	30975	33125	≥20
TOTAL	499455	531575	141

Supplemental Table S2.10 | Details of Japanese prion disease cases. EE = glutamic acid homozygosity; EK = glutamic acid/lysine heterozygosity; KK = lysine homozygosity; MM = methionine homozygosity; MV = methionine/valine heterozygosity; PSWCs = periodic synchronous wave complexes

Variant	N	Male/Female	Age at onset*	(range)	Positive family history (%)
Insertion	8	4/4	51.0 ± 12.0	(26-68)	5 (63)
P102L	83	38/45	55.5 ± 10.3	(22-75)	69 (83)
P105L	12	7/5	46.9 ± 8.4	(31-61)	11 (92)
D178N-129M	4	3/1	54.5 ± 5.5	(46-61)	None
D178N-129V	1	1/0	74		None
V180I	218	84/134	77.4 ± 6.8	(44-93)	5 (2)
E200K	63	30/33	61.1 ± 9.9	(31-83)	28 (44)
V203I	3	2/1	73		None
R208H	1	0/1	74		None
V210I	1	0/1	55		None
M232R	63	32/31	64.4 ± 10.9	(15-82)	2 (3)
V180I+M232R	4	2/2	71.3 ± 3.6	(65-74)	None

*Age at onset is expressed as the mean ± SD (range) years.

Variant	Duration**	(range)	Codon 129	Codon 219
Insertion	27.8 ± 17.7	(3-57)	MM 6; MV 1	EE 6; KK 1
P102L	48.4 ± 35.8	(2-186)	MM 67; MV 6	EE 70; EK 2
P105L	90.2 ± 40.4	(25-184)	MV 11	EE 7
D178N-129M	8.5 ± 4.4	(2-13)	MM 4	EE 4
D178N-129V	24		MV 1	EE 1
V180I	16.4 ± 14.5	(0-70)	MM 162; MV 54	EE 210
E200K	5.0 ± 6.0	(1-32)	MM 58; MV 3	EE 58; EK 3

V203I	3.7 ± 2.1	(1-6)	MM 3	EE 3
R208H	3		MM 1	EE 1
V210I	3		MM 1	EE 1
M232R	8.6 ± 12.7	(0-78)	MM 60; MV 2	EE 61; EK 1
V180I+M232R	21.8 ± 17.7	(1-47)	MM 4	EE 4

**Duration between the onset and akinetic mutism or death without akinetic mutism. Duration is expressed as the mean ± SD (range) months.

Variant	PSWCs on EEG (%)	Hyperintensities on MRI (%)	Positive 14-3-3 protein (%)
Insertion	3/8 (38)	2/7 (29)	0/1 (0)
P102L	11/72 (15)	32/76 (42)	13/34 (38)
P105L	1/10 (10)	1/11 (9)	1/2 (50)
D178N-129M	0/4 (0)	1/4 (25)	1/2 (50)
D178N-129V	0/1 (0)	0/1 (0)	1/1 (100)
V180I	19/203 (9)	212/213 (99)	110/140 (79)
E200K	56/63 (89)	56/59 (95)	29/31 (94)
V203I	3/3 (100)	2/2 (100)	1/1 (100)
R208H	1/1 (100)	1/1 (100)	1/1 (100)
V210I	1/1 (100)	1/1 (100)	not done
M232R	46/61 (75)	55/60 (92)	31/43 (72)
V180I+M232R	0/4 (0)	4/4 (100)	0/1 (0)

Supplemental Table S2.11 | Phenotypes of individuals with N-terminal PrP truncating variants.

HGVS	Variant	Zygosity	Sex	Age	Available phenotype information
c.59_60insC	G20Gfs84X	Het	F	79	Ascertained as part of the Rotterdam Study [89], a prospective cohort study of middle-aged and elderly persons. In good health and free of dementia as of at least age 78, at last in-person examination completion. Has 5 siblings and 2 children. Only family history noted is that one sibling has had a stroke before age 65.
c.109C>T	R37X	Het	M	73	Ascertained as a control for the Swedish schizophrenia study. Underwent heart bypass surgery in 2008, has a family history of heart problems. 4 siblings. Reports no family history of neurodegeneration or neuropathy.
c.223C>T	Q75X	Het	M	52	Ascertained in a study of type 2 diabetes. Has mild type 2 diabetes treated with metformin. Has children.
c.391G>T	G131X	Het	F		None available.

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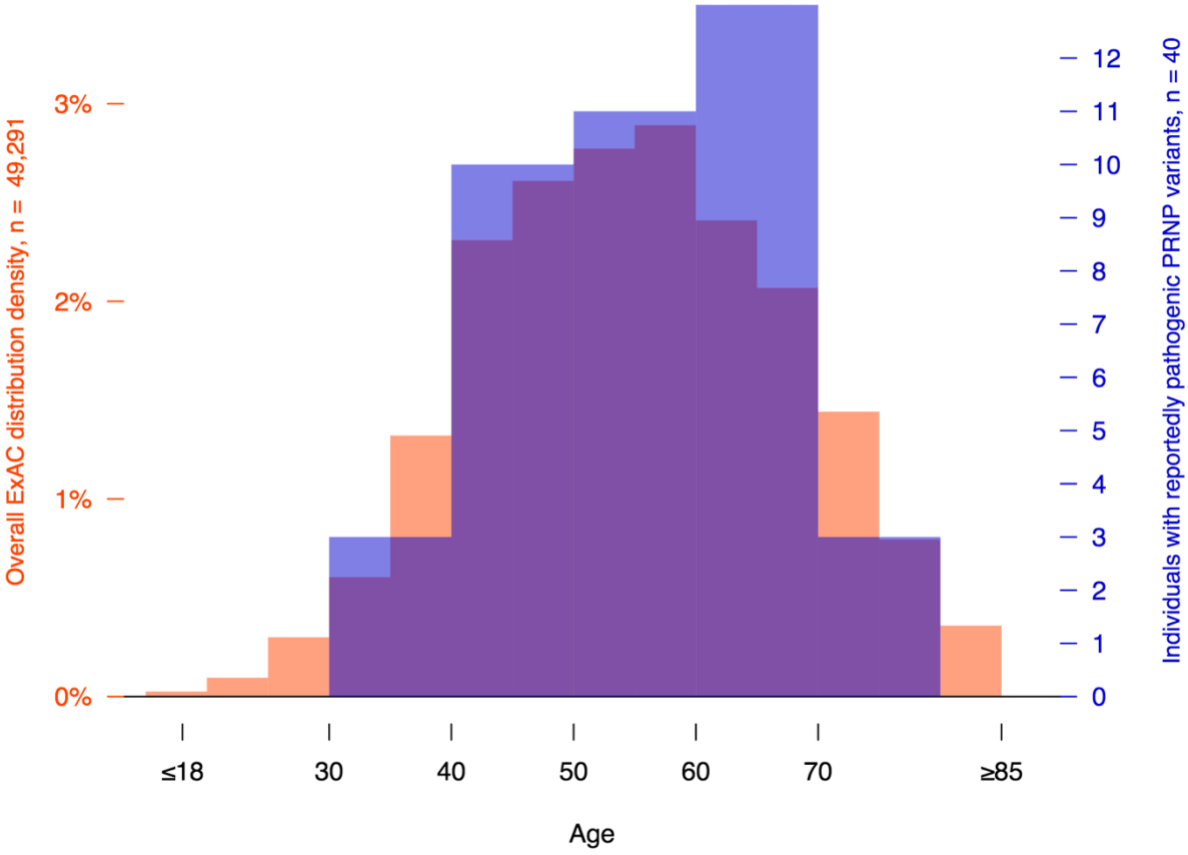
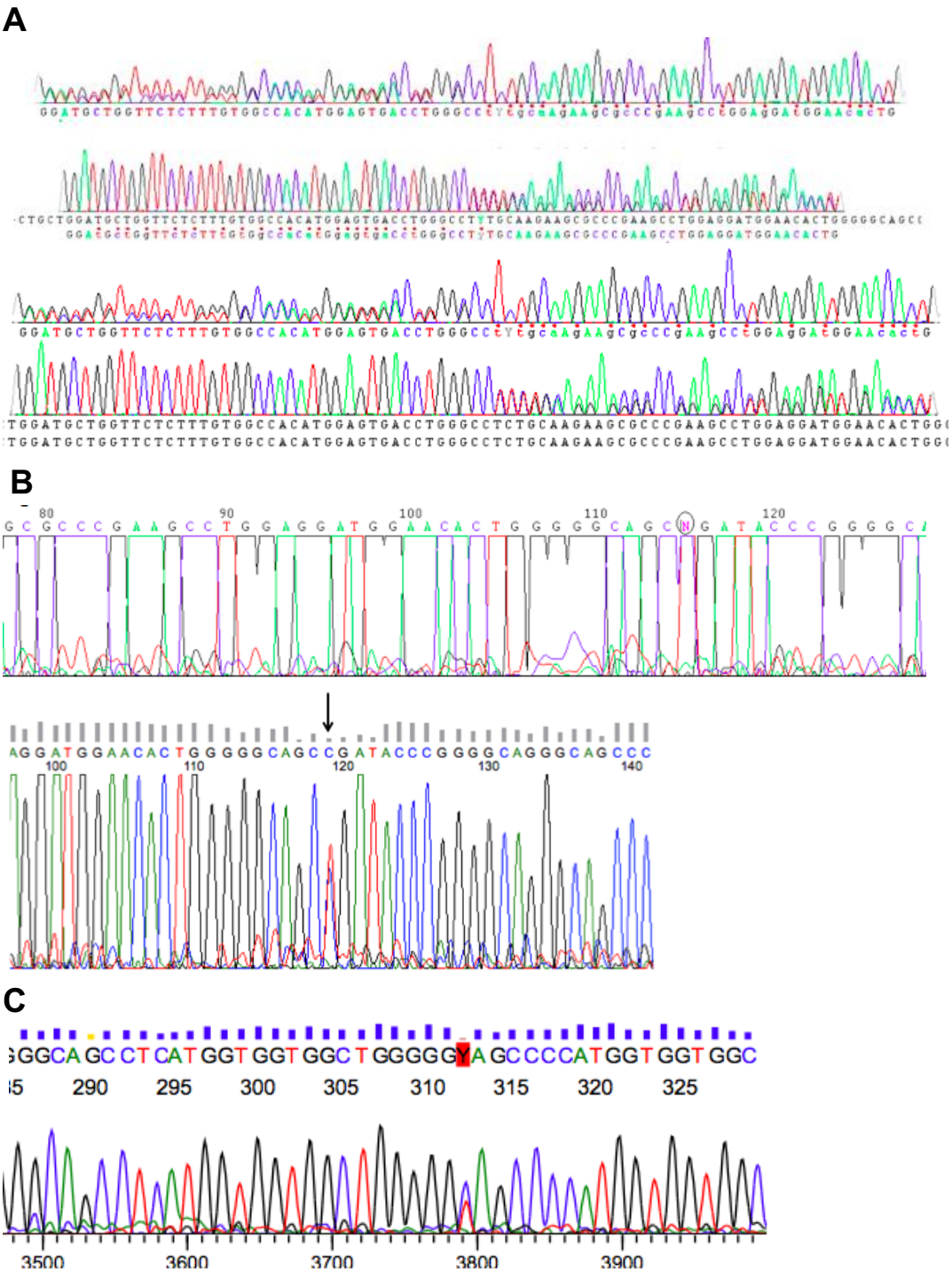


Supplemental Figure S2.1 | Age of ExAC individuals with reportedly pathogenic PRNP variants versus all individuals in ExAC. *The distribution of ages, available for 40 of 52 individuals with reportedly pathogenic PRNP variants, did not differ from the distribution overall (p = .69, Wilcoxon rank-sum test; p = .69, student's t test) nor after controlling for cohort (p = .15, linear regression)*



Supplemental Figure S2.2 | Sanger sequencing results for individuals with N-terminal truncating variants. A) *G20Gfs84X. Reverse (top) and forward (bottom). Primers: 2a-forward: AACTTAGGGTCACATTTGTCCTTGG; 2a-reverse: GGTAACGGTGCATGTTTTCACG. 2b forward: GTGGTGGCTGGGGTCAAGG; 2b reverse: TTTCCAGTGCCCATCAGTGC. B) R37X. DNA from whole blood (top) and fibroblasts (bottom). Primers: PrP2-F: TGGGACTCTGACGTTCTCCT; PrP2-R: GGTGAAGTTCTCCCCCTTGG* ***C)*** *Q75X. Primers: PRNP_EX2-M13-F [TGTAAAACGACGGCCAGT] CCATTGCTATGCACTCATTCA; PRNP_EX2-M13-R [CAGGAAACAGCTATGACC] CCATGTGCTTCATGTTGGTT.*

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Supplement to *Age of onset and preventive trial design in genetic prion disease*

Supplemental Discussion

Estimation of number of individuals available for trials

It is possible to estimate the true number of high penetrance *PRNP* mutation carriers based on disease prevalence. Using data from recent case series, 1,176 prion disease cases harbored a *PRNP* variant classified here as highly penetrant, out of 10,460 sequenced cases or 16,025 total cases[1]. Thus, 7 - 11% of prion disease cases have a high penetrance *PRNP* variant. Prion disease is responsible for ~1 in 5,000 deaths[1], suggesting that ~1 in 45,000 to 71,000 deaths are due to a high penetrance *PRNP* variant. The carrier rate among the living population will be somewhat lower because these variants reduce life expectancy, but it is reasonable to suppose that ~1 in 100,000 people harbors a high penetrance *PRNP* variant. This is in line with recent population control data, where out of 138,632 individuals in the gnomAD database as of December 2017 (http://gnomad.broadinstitute.org/)[2], there is one individual with the E200K mutation and no others with any variant classified here as high penetrance. Similarly, out of ~531,575 individuals genotyped by 23andMe, between 1 and 5 harbored one of four well-known high penetrance variants (P102L, A117V, D178N, and E200K) and between 1 and 5 harbored one of an additional set of variants which includes three classified here as high penetrance (P105L, T183A, and F198S). If the true carrier rate is 1 in 100,000, then there may exist 3,000 people in the United States with high penetrance *PRNP* variants. However, this figure greatly overestimates the number of people available for trials, as most of these individuals have not undergone predictive testing. Indeed, many are likely not even aware that they are at risk, perhaps because a family history is absent or a family member was not diagnosed correctly.

The National Prion Disease Pathology Surveillance Center in Cleveland, Ohio, as the provider of the vast majority of *PRNP* gene testing in the U.S., has near-exhaustive ascertainment of individuals who have chosen predictive testing for genetic prion disease in this country. In the period from 1996 through January 2017, it provided *N*=221 positive predictive test results, for any *PRNP* variant, to individuals who are not known to have developed disease as of 2017. Privacy concerns prevent publication of a breakdown of this number by age and specific *PRNP* mutation, but estimates can be made based on other cohorts. Among U.S. symptomatic prion disease cases with a rare *PRNP* variant, 75% (271/362) of individuals had a mutation classified here as high penetrance (Supplemental Table S3.1), and in the reported U.K. predictive testing cohort[3], 36% (37/104) of individuals who chose predictive testing were age 40 or older. Thus, a conservative estimate that there are only 221× 75%×36% = ~60 individuals alive in the United States today who meet the criteria we use in our power calculations.

The above estimate is conservative in that it reflects individuals who currently know their genetic status. In the U.K. predictive testing cohort, only 23% of individuals at 50/50 risk chose predictive testing[3], similar to reported figures in Huntington's disease, another incurable neurological disease (see refs in [3]). In contrast, 60% of individuals at risk for *BRCA1* or *BRCA2* mutations, for which preventive measures are available, chose predictive testing[4]. Thus, it is possible to imagine a 60%/23% = ~2.6X increase in the uptake of predictive testing if a preventive therapy for prion disease were available. Thus, a more generous estimate of the number of individuals age ≥40 available in the U.S. is 60×2.6 = 156. Such an estimate is probably more realistic when considering an approved prevention measure (as in post-marketing studies) than when considering an experimental drug entering randomized pre-approval trials (see below).

Although we contemplated worldwide trials with multiple international sites, we did not have adequate data to estimate the number of genetically tested presymptomatic individuals worldwide. The NHS National Prion Clinic in the U.K. has seen 72 presymptomatic individuals with *PRNP* mutations since 1990, and the French surveillance center in Paris has delivered 18 positive *PRNP* predictive test results since 2004, but the other centers involved in this report did not have comprehensive data on predictive testing in their respective countries analogous to that available for the U.S. We also note that a large number of E200K mutation carriers are suspected to exist in Slovakia and Israel due to founder mutations, although fewer than 100 carriers appear to have been identified in each country to date[5,6].

We also considered estimates based on the incidence of genetic prion disease. U.S. prion surveillance reported 271 individuals dying of prion disease with high penetrance mutations, suggesting that at least a comparable number of carriers in the U.S. are currently healthy and will have onset with a correct diagnosis within the next 15 years. The comparable figure including Europe, Australia, and Japan is 1,176. These last two figures are still lower than the true number of carriers in existence due to underdiagnosis, yet they overestimate the number of individuals actually reachable for trials because they ignore the question of how many individuals would choose predictive testing, and they include individuals who would be difficult to ascertain prospectively because they lack a known family history of prion disease, either due to *de novo* mutations, incorrect or incomplete information about previous family illnesses, or <100% penetrance.

For all of the above estimates, an important caveat is that the number of individuals successfully recruited, screened, and enrolled for a trial will be only a fraction of the number who meet the most basic enrollment criteria such as genetic status and age. Willingness, geography, and various exclusion criteria will dramatically lower the number actually enrolled.

Finally, it is worth noting that all of our calculations and assumptions are based upon the present moment, when there exists no drug for prion disease. It is likely that approval of a first prion disease drug would increase the number of patients available for future trials. A drug could improve diagnosis rates, as prion disease is not currently prioritized in the differential diagnosis of rapidly progressive dementia due to its being untreatable[7]. The U.S. observes an incidence of ~1 prion disease case per million population per year, but up to twice that incidence has been observed in countries with more intense surveillance systems[8]. Because many prion disease patients die undiagnosed, their relatives may never learn that they are at risk for a *PRNP* mutation. A drug might also increase the uptake of predictive genetic testing among those who do learn that they are at risk. The 23% uptake observed for prion disease[3] is consistent with other currently "medically inactionable" indications such as Huntington's disease[9], while as noted in the main text, "actionable" indications such as *BRCA1/2* mutations appear to have much higher uptake[4]. Finally, the existence of a drug may promote general awareness of the disease and improve the infrastructure for surveillance, registries, and patient ascertainment.

Simulation of power for randomized preventive trials with a clinical endpoint

Individuals were assigned one of the three *PRNP* mutations and a starting age distributed between 40 and 80, weighted by mutation prevalence and by the proportion of individuals surviving at each age. As above, we assigned half of individuals to drug and half to placebo, and assumed a w=15.2% annual withdrawal rate, a P=0.05 statistical threshold, and a 5-year trial duration with a 1-year "run-in" period. For each year of the trial, each individual withdraws with probability w, becomes sick with a probability corresponding to the hazard function for their particular *PRNP* mutation and age at the time, multiplied by the simulated hazard ratio if drug treated, or else continues on in the trial. At the end of each simulated trial, we analyzed the censored trial data to determine a P value. For non-stratified simulations, drug/placebo status

was assigned without regard to mutation, and survival status was regressed on drug/placebo status alone using a log-rank test, with the overall *P* value as the readout. For stratified trials, drug/placebo status was assigned 50/50 within each mutation, and mutation was included as a covariate in a Cox proportional hazards regression, with the *P* value for the "drug" parameter as the readout.

We then compared this model to the power calculation results by taking the calculated required numbers of individuals for 80% power (Table 3.2) and then running the simulation (500 iterations) to determine the power for this number of individuals. The results (Supplemental Table S3.6) show overall good agreement between the power calculation and the simulation — for most scenarios tested, the power is indeed close to 80%, with or without stratification. Stratification actually reduces statistical power for the conditions with low *N* and low hazard ratios. Under such conditions, it is a common occurrence that there may be zero disease onsets either in one randomized group (usually the drug-treated group) or in one mutation, resulting in an infinite regression coefficient or beta in the Cox model. Thus, the regression never converges, and the simulated trial results turn out statistically non-significant.

Codon 129 effects on age of onset and disease duration

To determine whether codon 129 affects age of onset for the three most prevalent mutations considered here, we used a log-rank model based on codon 129 diplotype (phased genotype) where available (Supplemental Table S3.7). In this model, only D178N showed clear evidence for genetic modification of age of onset and disease duration, with *P* values significant after multiple testing correction. To determine the nature of this genetic modification, we plotted survival curves by codon 129 diplotype and, because phase was unknown for many codon 129 heterozygous individuals, we also considered phaseless genotypes. In pairwise tests for D178N, M/M was not significantly different from M/V (nominal $P = 0.14$) nor from V/M (nominal $P = 0.69$),

and in the phaseless survival curve, MV was overall similar to MM (Supplemental Figure S3.4D). These results suggest that the significant codon 129 effect on D178N age of onset is most likely driven primarily by a younger age of onset in V/V individuals compared to other diplotypes. Despite the strong statistical significance of this difference, the small number of D178N-129VV individuals means that codon 129 does not add any explanatory power for age of onset in the dataset as a whole. As noted in the main text, mutation alone explains limited variance in age of onset (adjusted R^2 = 0.15, *P* = 1.3e-33). Adding *cis* and *trans* codon 129 to this model decreases the variance explained (adjusted R^2 = 0.14, *P* = 3.6e-18).

We also investigated in further detail previously reported associations. For disease duration, D178N M/M and V/V were significantly more rapid than either heterozygous diplotype, consistent with previous reports. Although codon 129 diplotype did not have a significant effect on E200K disease duration overall (nominal *P* = 0.10), a phaseless genotypic model was suggestive (nominal *P* = 0.031), with MV heterozygotes appearing to have a slightly longer disease duration than MM homozygotes, a direction of effect consistent with previous reports[10,11]. Whereas P102L age of onset was reported to be higher for M/V than M/M individuals[12], here we find no evidence for this and, ignoring phase, the non-significant trend is towards younger onset in MV than MM individuals (nominal *P* = 0.056).

Potential age of onset confounders

Because our data were gathered from a variety of study centers using a variety of methodologies, we asked whether any confounders might affect age of onset (Supplemental Table S3.7). There was no difference in age of onset between directly and indirectly ascertained individuals (*P* = 0.78). Age of onset was correlated with year of birth after controlling for mutation (*P* < 1e-48), which is a previously reported artifact caused by our relatively limited ability to ascertain individuals whose onset has not yet arrived (though we ascertain some of

them through predictive testing) or whose onset occurred before genetic diagnosis of prion disease was possible (though we ascertain some of them through family histories)[11]. This correlation does not affect estimation of overall age of onset distributions. Age of onset appeared to differ slightly among the nine contributing study centers after controlling for mutation, although it was not significant after multiple testing correction (nominal P = 0.012, Bonferroni P = 0.26, two-way ANOVA), and it only marginally increased variance explained (adjusted R^2 = 0.16) compared to mutation alone (adjusted R^2 = 0.15, see above). Year of onset showed evidence of positive correlation with age of onset after controlling for study center and mutation (nominal P = 0.00032, Bonferroni P = 0.008, linear regression), although the effect size was small (+0.12 years of age per calendar year, or in other words, cases in 2010 have on average an age of onset 1.2 years older than cases in 2000) and, again, the impact on variance explained was minimal (adjusted R^2 = 0.18). This slight positive correlation might be due to improved ascertainment of older-onset cases as prion surveillance strengthens over time.

Justification for trial duration assumptions

In the main text, we argued that a longer trial duration could be considered for a post-marketing study because it would run concurrently with, rather than reducing, the drug's effective market exclusivity period (the period before generic equivalents can be approved). In the U.S., new drugs may be protected by patent exclusivity granted by the Patent and Trademark Office and/or by market exclusivity measures granted by FDA; these exclusivity periods are not additive. Patents last 20 years beginning from their filing, which is usually during the preclinical development phase. The 1984 Hatch-Waxman Act allows sponsors to recover up to 5 years of additional exclusivity, not to exceed a total of 14 years of market exclusivity, to make up for time the drug spends in FDA review[13]. FDA can offer varying periods of market exclusivity depending upon the indication and treatment modality, including 12 years for new biologics[14] and 7 years for rare disease drugs granted Orphan Drug designation[15]. In practice, new drugs receive on

average about 12 years of effective market exclusivity[16,17]. The vast majority of pivotal trials supporting new drug approvals last less than one year[18]. While there are rare examples of 5-year trials[19], a 10- or 15-year prevention trial would exhaust most or all of a drug's effective market exclusivity period. In contrast, as noted in the Discussion, there do exist precedents for very long-term surveillance of patients receiving a drug after approval.

Historical control trial simulation

As for the simulation of randomized trials, individuals were assigned one of the three *PRNP* mutations and a starting age distributed between 40 and 80, weighted by mutation prevalence and by the proportion of individuals surviving at each age. Again, we assumed a w=15.2% annual withdrawal rate (Supplemental Table S3.5), a *P*=0.05 statistical threshold, and a 1-year "run-in" period where disease events are ignored. Distinct from the randomized trial simulation, here all simulated individuals are treated with the drug. For each year of the trial, each individual withdraws with probability w, becomes sick with a probability corresponding to the hazard function for their particular *PRNP* mutation and age at the time, multiplied by the simulated hazard ratio, or else continues on in the trial. At the end of each simulated trial, the censored trial data on treated individuals are compared to our original dataset as historical controls (Supplementary Life Tables). To determine a *P* value we used a Cox proportional hazards counting model accounting for different left-truncation times[20]: for untreated individuals in the original dataset, we assumed age 0 as a start time, while for treated individuals, we assumed left truncation at the age at trial enrollment, plus one year to account for the "run-in" year.

While we cannot currently rule out the possibility that our dataset is biased relative to the *true* hazards facing mutation carriers in real life (see main text Discussion), we sought to confirm that our simulation method is not itself biased. We reasoned that if our simulation was unbiased, then for a drug with hazard ratio equal to 1 (a completely ineffective drug), even long trials with

large numbers of individuals should have power equal to alpha, by the definition that alpha is the false positive rate when the null hypothesis (no efficacy) is true. We therefore ran 1000 iterations of a simulation with a hazard ratio of 1 and 1000 individuals followed for 20 years. We observed a significant result at $P < 0.05$ in only 5.5% of iterations, consistent with the expected 5%.

In contrast to the result for randomized trials (see discussion above and Supplemental Table S3.6), we found that stratification by mutation in the analysis of historical control trial simulation did just slightly increase statistical power. For example, with *N*=156 individuals followed for 15 years with a hazard ratio of 0.5, power was 90.6% (906/1000 iterations) without stratification and 94.1% (941/1000 iterations) with stratification. This difference from the randomized trial simulation may be a property of the Cox counting model, combined with the fact that our historical comparison dataset has *N*=1,000 individuals, and we considered follow-up periods of up to 15 years, meaning that the dataset was large enough for the small explanatory power of different *PRNP* mutations to matter. Nevertheless, for consistency with the methods used for the randomized trial simulations, we chose not to stratify in the simulations used for Table 3 and Supplemental Figure S3.6.

We performed power calculations for post-marketing studies using historical controls under a range of assumptions in addition to those explored in Table 3 in the main text. In one set of experiments, we considered the effects of varying the length of the follow-up period. For a hazard ratio of 0.5, 80% power could be achieved within 9 years for *N*=156 participants, but is never achieved for *N*=60 participants (Supplemental Figure S3.6A). This is because statistical power eventually plateaus for lack of participants: our assumption of a 15.2% withdrawal rate compounded annually means that after 10 years, only 19% of the original participants remain in the trial. If the set of drug recipients followed in a post-marketing study were fixed shortly after

approval, then this is a realistic concern. If, on the other hand, study design allows new individuals who are prescribed the drug to be added to the monitored cohort continually, the number of individuals in the trial could stay constant or even grow. To simulate this possibility, we also considered a zero withdrawal rate scenario. Under this assumption, even with *N*=60 individuals, 80% power is achieved in 10 years (Supplemental Figure S3.6A).

In another set of experiments, we compared the power for post-marketing studies with historical controls, with or without modeling withdrawal, in comparison to pre-approval randomized trials, for a range of hazard ratios (Supplemental Figure S3.6B). For the same hazard ratio and level of statistical power, post-marketing trials generally required only about one fifth as many individuals, and if withdrawal is set to zero, simulating continuous enrollment, only one twentieth as many, as pre-approval randomized trials.

Certainly, a post-marketing study is not a panacea, and under certain assumptions even this trial design is not well-powered: for instance, for a drug of marginal efficacy (hazard ratio 0.9, delaying onset by ~1 year) even a 15-year trial with no withdrawal could not achieve 80% power with 1,000 participants. But, under a range of moderate assumptions, a post-marketing study is more feasible than randomized pre-approval trials with a clinical endpoint.

Supplementary Tables

Supplemental Table S3.1 | Literature review to identify probable high penetrance variants. *"Mendelian segregation" indicates the presence of at least one family with at least three affected individuals in a pattern consistent with Mendelian segregation. "De novo" indicates a case with a confirmed de novo mutation. — indicates neither of these criteria was present.*

variant	evidence for high penetrance	comments
P39L	— [21]	
2-OPRD	— [22,23]	
1-OPRI	— [24,25]	
2-OPRI	— [26]	
3-OPRI	— [27]	
4-OPRI	— [28]	most cases have a negative family history
5-OPRI	Mendelian segregation[29]	
6-OPRI	Mendelian segregation[30]	
7-OPRI	Mendelian segregation[31]	
8-OPRI	Mendelian segregation[31,32]	
9-OPRI	Mendelian segregation[33], *de novo*[34]	
12-OPRI	Mendelian segregation[35]	
P84S	— [36]	
S97N	— [37]	
P102L	Mendelian segregation[12]	
P105L	Mendelian segregation[38]	2 sibs affected & genotyped, 1 ungenotyped parent likely affected
P105S	— [39]	
P105T	Mendelian segregation[40]	
G114V	Mendelian segregation[41,42]	pedigree suggests penetrance high though not 100%
A117V	Mendelian segregation[43]	
129insLGGLGGYV	*de novo*[44]	
G131V	— [45,46]	positive family history in one case
S132I	Mendelian segregation[47]	extensive family history, only proband genotyped
A133V	— [48]	
Y145X	— [49]	
R148H	— [31]	
R156C	— [50]	
Q160X	Mendelian segregation[51]	
Y162X	Mendelian segregation[52]	
Y163X	Mendelian segregation[53,54]	
D167G	— [55]	
D167N	— [56]	
Y169X	Mendelian segregation[54]	
V176G	— [57]	
D178Efs25X	Mendelian segregation[58]	only proband genotyped

variant	evidence for high penetrance	comments
D178N	Mendelian segregation[59], *de novo*[60]	
V180I	— [61]	
T183A	Mendelian segregation[62]	
H187R	Mendelian segregation[63]	
T188A	— [64]	
T188K	— [65]	some patients have a positive family history[65–67]
T188R	— [65,68]	
V189I	— [69]	
T193I	— [70]	
K194E	— [71]	
E196A	— [72]	
E196K	Mendelian segregation[73]	only proband genotyped
F198S	Mendelian segregation[74,75]	
F198V	— [37]	
E200G	— [76]	
E200K	Mendelian segregation[77]	
T201S	— [78]	
D202G	Mendelian segregation[79]	only proband genotyped
D202N	— [80]	
V203I	— [81]	
R208C	— [37]	
R208H	— [82]	
V210I	— [83,84]	
E211D	Mendelian segregation[85]	supplement describes 1 family with 3 affected
E211Q	— [73]	2 sibs affected
Q212P	— [56]	
I215V	— [86]	
Q217R	— [75]	2 affected
Y218N	Mendelian segregation[87]	
A224V	— [88]	
Y226X	— [89]	
Q227X	— [89]	
M232R	— [61]	
M232T	— [90]	
P238S	— [91]	

Supplemental Table S3.2 | Descriptive statistics regarding sources of age of onset data.

study center		***N***
Japanese national prion surveillance network (Shimotsuke & Kanazawa, Japan)		215
MRC Prion Unit (London, U.K.)		211
French national reference center for CJD (Paris, France)		168
UCSF Memory and Aging Center (San Francisco, U.S.)		147
Spanish National Center for Epidemiology (Madrid, Spain)		114
German Reference Center for TSEs (Göttingen, Germany)		101
DOXIFF study at the Mario Negri Institute (Milan, Italy)		65
Reference Center for CJD at University of Bologna (Bologna, Italy)		49
Australian National CJD Registry (Melbourne, Australia)		24
	total	*1094*
method of ascertainment		***N***
direct (clinical visit, autopsy, or surveillance report)		843
indirect (family history)		251
	total	*1094*
vital status		***N***
censored — died due to intercurrent illness without developing prion disease		4
censored — alive and well at last follow-up		101
symptomatic with prion disease at last follow-up		81
died of prion disease		908
	total	*1094*

Supplemental Table S3.3 | Age of onset statistics on supplemental variants.

	without censored data		**survival curve including censored data**		
mutation	**mean ± sd**	***N***	**median (IQR)**	**range**	***N***
5-OPRI	46.8 ± 6.0	14	49 (44 - 53)	34 - 56	18
6-OPRI	35.1 ± 5.8	31	35 (32 - 39)	23 - 47	34
P105L	46.5 ± 8.5	13	47 (40 - 51)	31 - 61	13
A117V	41.2 ± 7.8	26	41 (37 - 45)	25 - 58	28

Supplemental Table S3.4 | Withdrawal rates in preventive clinical trials. *w, annual withdrawal rate. CHD, coronary heart disease. NSAID, non-steroidal anti-inflammatory drug. See Methods for details.*

category	trial	description	w
cardiology	WOSCOPS[92]	pravastatin for CHD	6.9%
cardiology	AFCAPS/TexCAPS[93]	lovastatin for CHD	7.1%
cardiology	OSLER[94]	evolocumab for CHD	9.0%
cardiology	JUPITER[95]	rosuvastatin for CHD	14.1 %
neurology	ADAPT[96]	NSAIDs for Alzheimer's	16.3 %
cardiology	ODYSSEY LONG TERM[97]	alirocumab for CHD	19.0 %
cardiology	NCT00607373[98]	mipomersen for homozygous *LDLR* hypercholesterolemia	22.1 %
neurology	PRECREST[99]	creatine for Huntington's disease	54.9 %

Supplemental Table S3.5 | Power calculations under alternative assumptions. *Each block of this table is equivalent to Table 3 but with different assumptions as indicated (except where stated, other assumptions are identical to those in Table 3.2). A) Best case scenario: overall average hazard is 4.8% (the higher figure including the less common mutations shown in Supplemental Table S3.2 and Supplemental Figure S3.1), the withdrawal rate is 6.9% per year (the lowest rate in any of the trials we reviewed, see Supplemental Table S3.4), and there is no run-in period — the drug is effective immediately and so disease onsets within the 1st year of the trial are included. B) Worst case scenario: overall average hazard is only 3.5% — one quarter lower than calculated in this manuscript, because our data are biased due to under-incluson of asymptomatic individuals, and/or because predominantly younger people enroll in a trial — and the withdrawal rate is 54.9% per year (the highest rate in any trial we reviewed, see Supplemental Table S3.4). C) Targeted trial scenario: only the mutations with higher hazards — 5-OPRI, 6-OPRI, P105L, and A117V — are targeted for recruitment, resulting in a higher baseline hazard of 5.2%. Although the enrollment requirements for this scenario are lower than in Table 3.2, these mutations are also approximately one order of magnitude rarer[1], making achievement of these enrollment numbers yet more unlikely. D) Long follow-up scenario: trial duration is 15 years. This reduces the required numbers somewhat, but this benefit is limited by the withdrawal rate, which means that few individuals are still enrolled after 15 years. E) Zero withdrawal scenario: withdrawal rate is set to zero.*

alternate scenario	hazard ratio	years of life added	onsets required	participants required
A (best case)	0.1	undefined*	6	59
	0.2	21	12	110
	0.3	13	22	182
	0.4	9	37	291
	0.5	7	65	475
	0.6	5	120	821
	0.7	3	247	1,589
	0.8	2	631	3,850

alternate scenario	hazard ratio	years of life added	onsets required	participants required
	0.9	1	2,828	16,434
B (worst case)	0.1	undefined*	6	357
	0.2	21	12	666
	0.3	14	22	1,097
	0.4	10	37	1,757
	0.5	7	65	2,866
	0.6	5	120	4,955
	0.7	4	247	9,586
	0.8	2	631	23,196
	0.9	1	2,828	98,897
C (targeted trial)	0.1	undefined*	6	92
	0.2	undefined*	12	171
	0.3	10	22	280
	0.4	7	37	449
	0.5	5	65	732
	0.6	4	120	1,267
	0.7	3	247	2,457
	0.8	2	631	5,958
	0.9	1	2,828	25,471
D (long follow-up)	0.1	undefined*	6	59
	0.2	21	12	108
	0.3	14	22	178
	0.4	10	37	285
	0.5	7	65	465
	0.6	5	120	806
	0.7	4	247	1,568
	0.8	2	631	3,816
	0.9	1	2,828	16,384
E (zero withdrawal)	0.1	undefined*	6	66
	0.2	21	12	123
	0.3	14	22	202
	0.4	10	37	323
	0.5	7	65	527
	0.6	5	120	912
	0.7	4	247	1,767
	0.8	2	631	4,285
	0.9	1	2,828	18,314

Supplemental Table S3.6 | Comparison of power calculation and simulation results. *The first four columns are reproduced from Table 3.2 for ease of comparison. The number of participants required was calculated to yield 80% power; the final two columns show the power for this number of participants, at P=0.05, indicated by simulation. See Supplementary Discussion above for details of the method.*

hazard ratio	years of life added	onsets required	participants required	calculated power	simulated power without stratification	simulated power with stratification
0.1	undefined*	6	101	80.0%	62.2%	35.0%
0.2	21	12	189	80.0%	71.8%	69.2%
0.3	14	22	311	80.0%	78.6%	76.6%
0.4	10	37	498	80.0%	80.0%	81.2%
0.5	7	65	813	80.0%	80.0%	81.8%
0.6	5	120	1406	80.0%	83.2%	79.8%
0.7	4	247	2,724	80.0%	80.4%	80.8%
0.8	2	631	6,602	80.0%	81.4%	77.6%
0.9	1	2,828	28,204	80.0%	80.6%	78.0%

Supplemental Table S3.7 | Tests for modifiers and confounders of age of onset. *All p-values are two-tailed. As explained in Supplementary Discussion, diplotypes (phased genotypes) are indicated with a slash (cis/trans to the mutation) while unphased genotypes have no slash. We were unable to obtain phase data for many 129MV individuals, so the genotypic tests represent not only a different grouping of data but also include more data points than the corresponding diplotypic tests. Thus, we considered them as independent tests for the purposes of multiple testing correction. p (raw) indicates the raw p value; p (bc) is Bonferroni-corrected for 22 tests. For parent-child comparisons n is the number of pairs. For linear regressions, child year of birth was included in the model as a covariate. The prior column represents the prior expectation of whether there would be a significant difference in each test based on previous reports in the literature.*

variable	mutation	comparison	n	test	*P* (raw)	*P* (bc)	prior evidence
onset	P102L	M/M vs. M/V vs. V/V	125 vs. 13 vs. 1	log-rank	0.18	1	mixed[12,30,100]
onset	P102L	MM vs. MV vs. VV	125 vs. 32 vs. 1	log-rank	0.057	1	
onset	D178N	M/M vs. M/V vs. V/M vs. V/V	133 vs. 18 vs. 9 vs. 13	log-rank	0.000062	0.0014	none[100–103]
onset	D178N	MM vs. MV vs. VV	133 vs. 58 vs. 13	log-rank	0.000018	0.00040	
onset	E200K	M/M vs. M/V vs. V/M vs. V/V	286 vs. 33 vs. 10 vs. 5	log-rank	0.13	1	none for *trans* allele[11,100,103,104],
onset	E200K	MM vs. MV vs. VV	288 vs. 92 vs. 5	log-rank	0.30	1	suggestive for *cis* allele[11]
duration	P102L	M/M vs. M/V	89 vs. 8	log-rank	0.55	1	none[12]
duration	P102L	MM vs. MV	89 vs. 21	log-rank	0.93	1	
duration	D178N	M/M vs. M/V vs. V/M vs. V/V	62 vs. 13 vs. 8 vs. 10	log-rank	0.000081	0.0018	yes[102]
duration	D178N	MM vs. MV vs. VV	62 vs. 33 vs. 10	log-rank	0.00000010	0.0000022	
duration	E200K	M/M vs. M/V vs. V/M vs. V/V	208 vs. 21 vs. 6 vs. 5	log-rank	0.10	1	yes[5,10,11]
duration	E200K	MM vs. MV vs. VV	210 vs. 50 vs. 5	log-rank	0.031	0.68	
onset	P102L	parent vs. child	32	linear regression	0.44	1	suggestive[105]
onset	D178N	parent vs. child	15	linear regression	0.12	1	none
onset	E200K	parent vs. child	40	linear regression	0.68	1	none[11]
onset	top three	men vs. women	446 vs. 492	Cox	0.22	1	none
duration	top three	men vs. women	264 vs. 281	linear regression	0.02	0.44	yes[10]
onset	top three	direct vs. indirect ascertainment	843 vs. 251	Cox	0.78	1	none
duration	top three	direct vs. indirect ascertainment	544 vs. 90	linear regression	0.64	1	none
onset	top three	year of birth	697	linear regression	3.6E-49	7.9E-48	yes[11]
onset	top three	study centers	973	two-way ANOVA	0.012	0.26	none
onset	top three	year of onset	697	linear regression	0.00032	0.0070	none

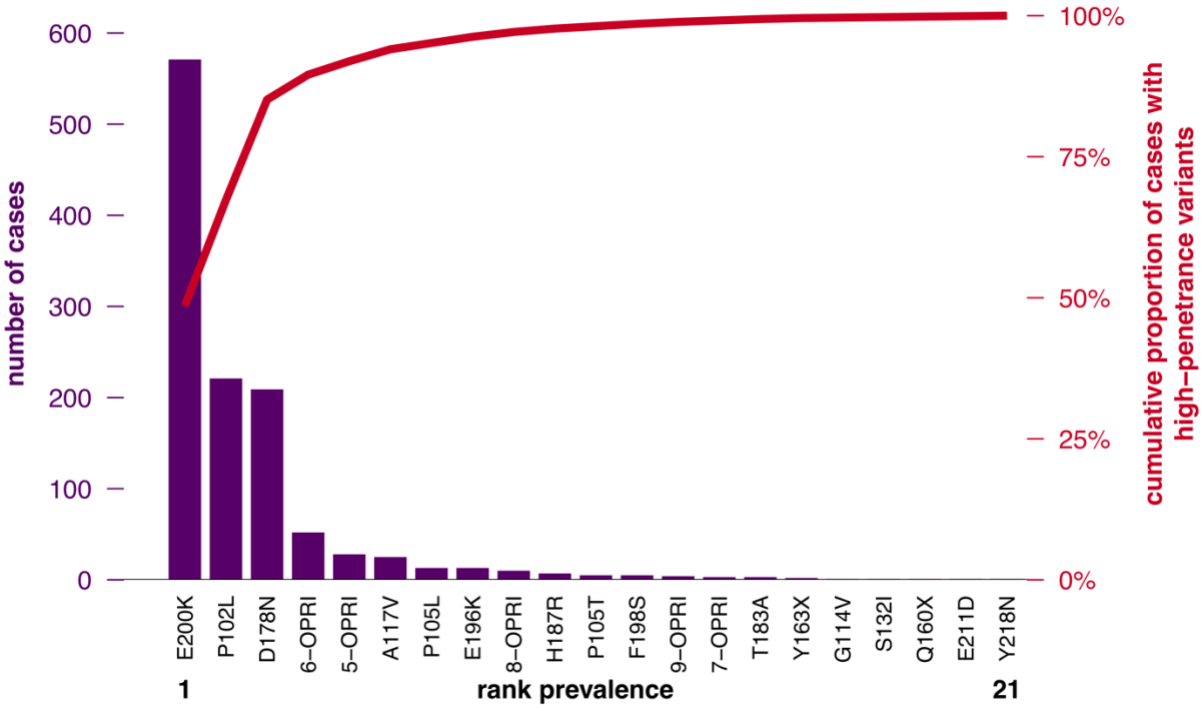
Supplementary Life Tables

These tables are made available as .tsv and .xls files in the code and data repository for this manuscript: https://github.com/ericminikel/prnp_onset

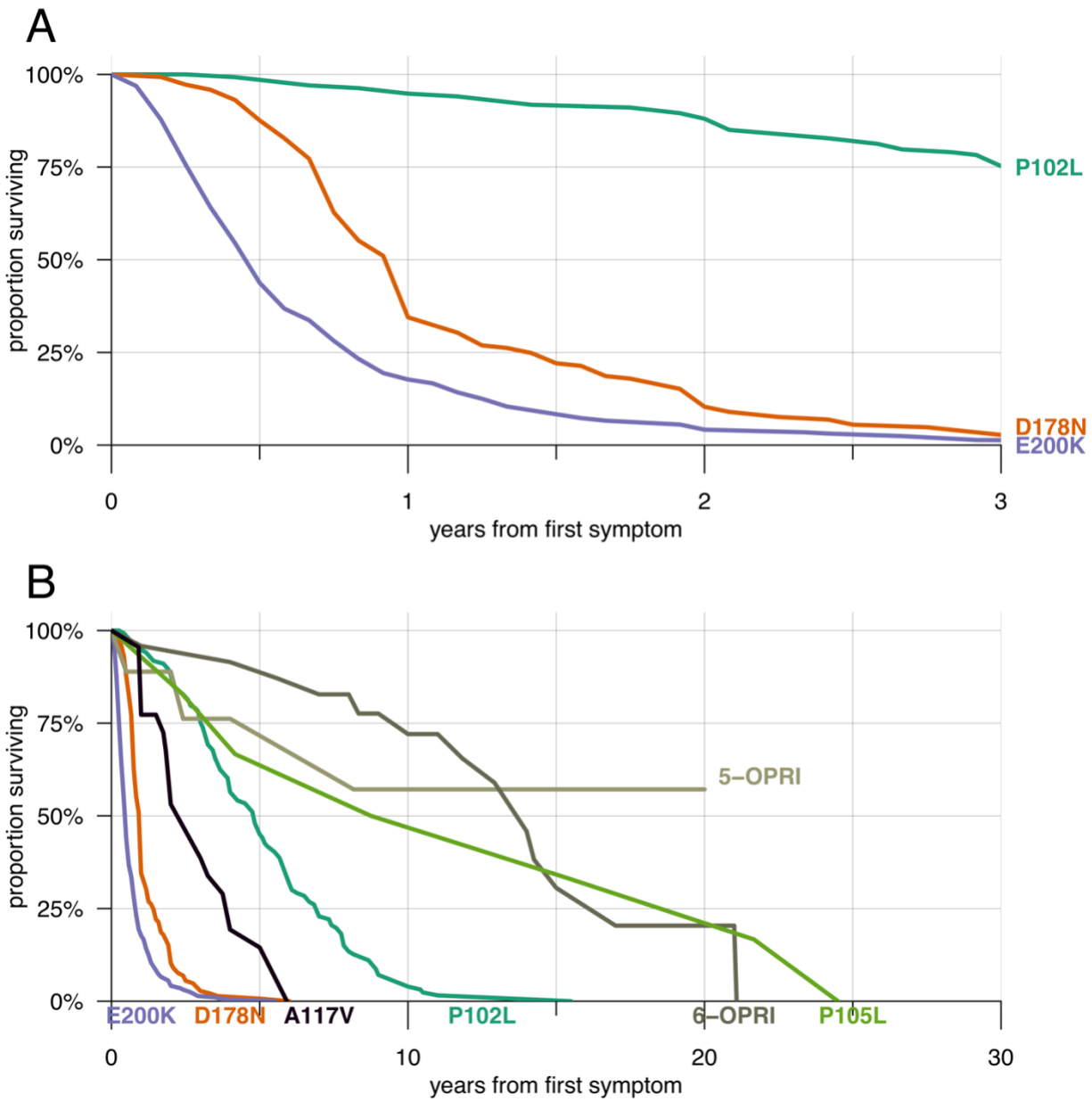
Supplementary Duration Tables

These tables are made available as .tsv and .xls files in the code and data repository for this manuscript: https://github.com/ericminikel/prnp_onset

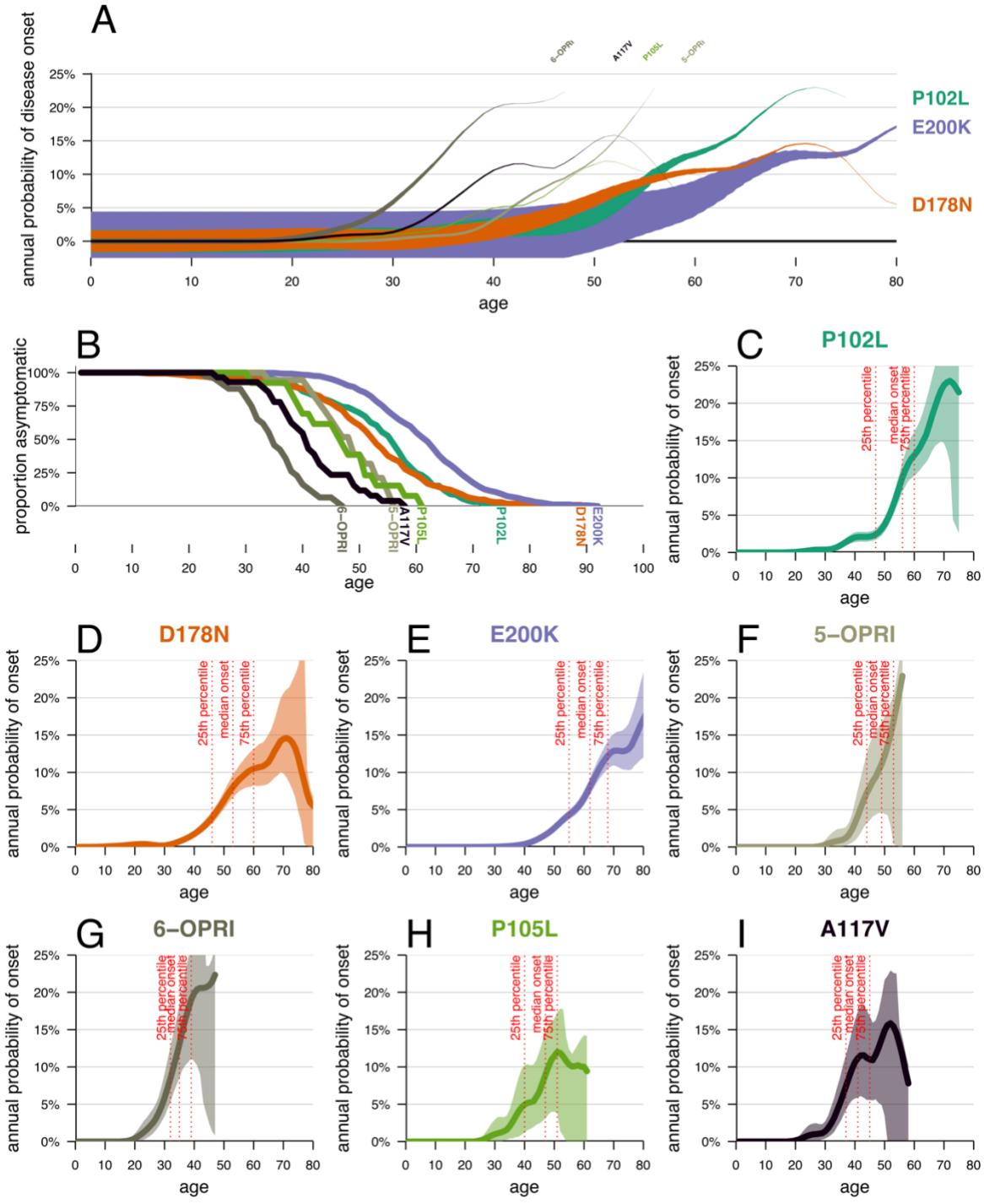
Supplementary Figures



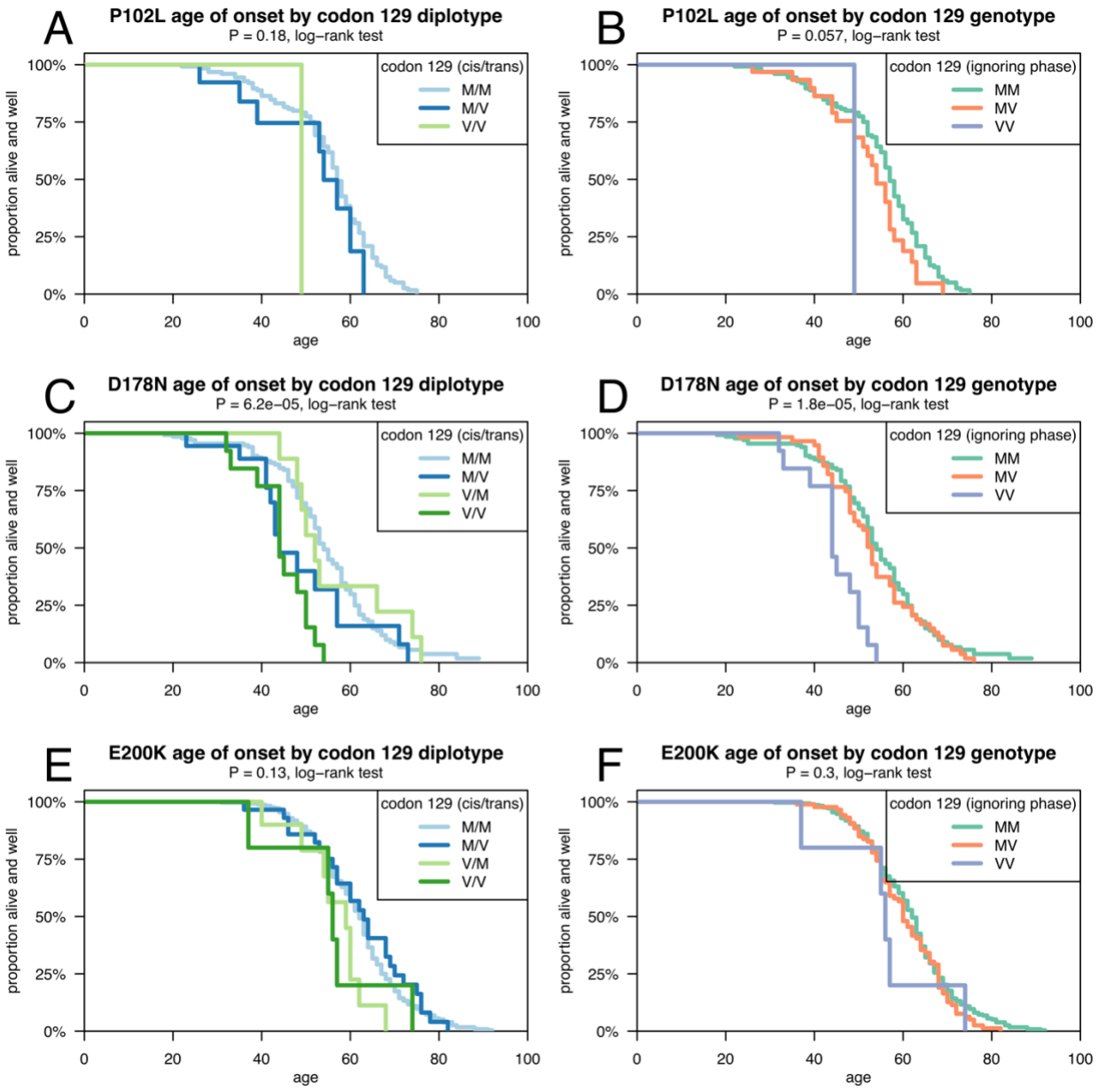
Supplemental Figure S3.1 | Variant prevalence among prion disease cases with a high penetrance variant. *Genetic variants deemed highly penetrant based on the literature review in Supplemental Table S3.1 are plotted by the rank (x axis) versus number (left axis) and cumulative proportion (right axis) of high penetrance cases they explain in a recent case series*[1].



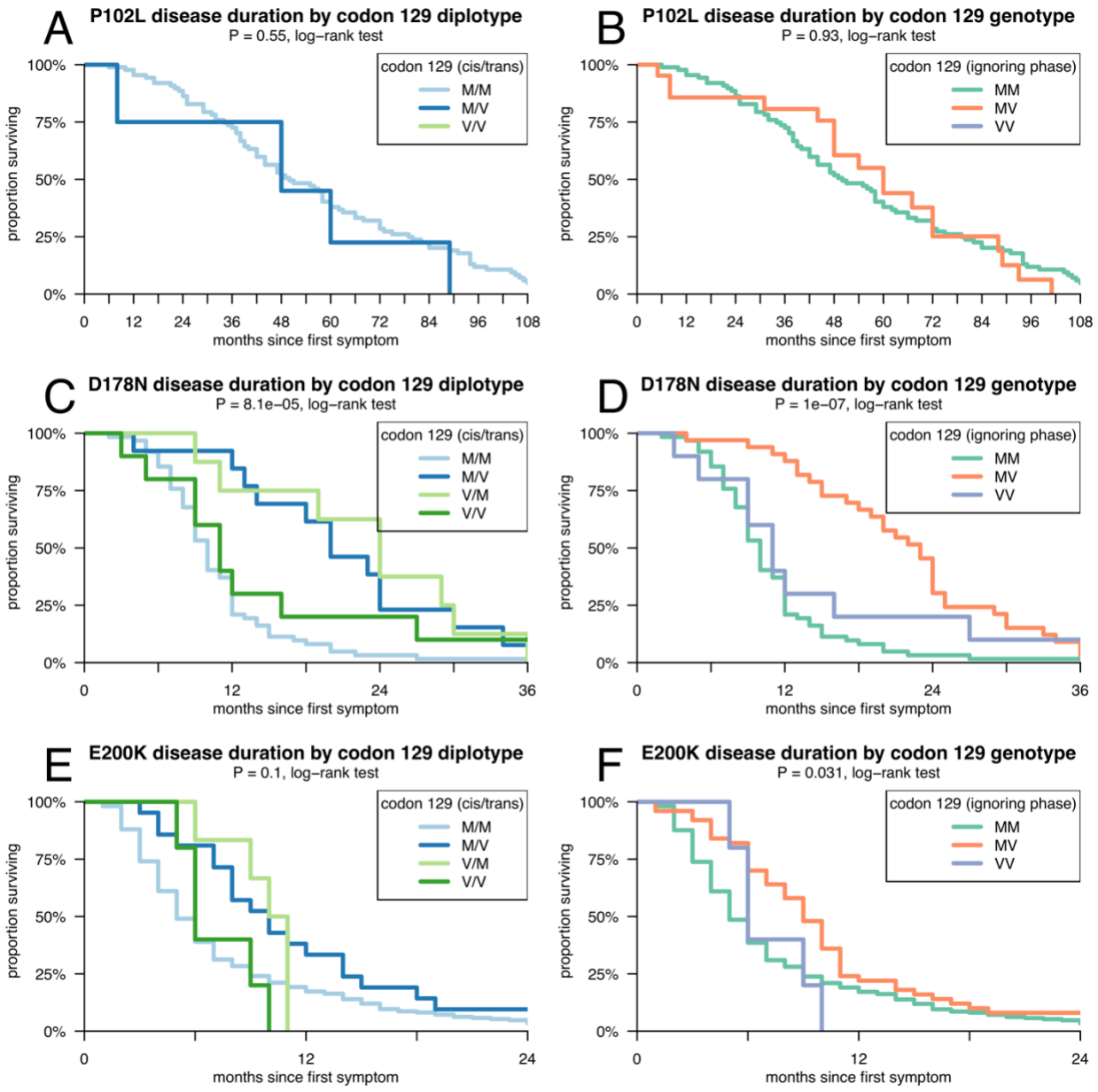
Supplemental Figure S3.2 | Disease duration by mutation. A) *Disease duration (time from first symptom to death) in genetic prion disease. D178N and E200K are classified as rapidly progressive mutations, with >50% of individuals dying within one year of first symptom.* ***B)*** *Zoomed out to 30 years (note y axis) and including supplemental mutations. Disease duration data are provided in the Supplementary Duration Tables.*



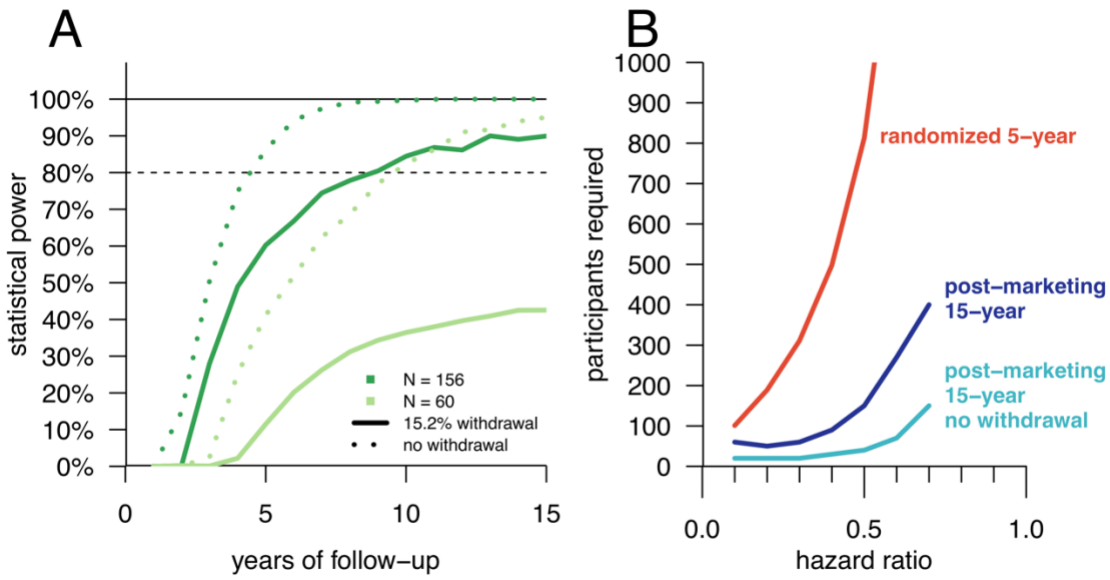
Supplemental Figure S3.3 | Survival and hazard curves. A) *Hazard vs. time with line thickness representing survival, as Figure 3.1 but including the top 7 mutations.* ***B)*** *Survival curves for the 7 mutations.* ***C-I)*** *Hazard vs. age with 95% confidence intervals displayed in 50% transparency.*



Supplemental Figure S3.4 | Age of onset and codon 129. *Survival curves for age onset or death in P102L* ***(A-B)****, D178N* ***(C-D)****, and E200K* ***(E-F)*** *genetic prion disease stratified by codon 129 diplotype* ***(A, C, E)*** *or phaseless genotype* ***(B, D, F)****.*



Supplemental Figure S3.5 | Disease duration and codon 129. *Survival curves for disease duration (time from first symptom to death) in P102L* ***(A-B)****, D178N* ***(C-D)****, and E200K* ***(E-F)*** *genetic prion disease stratified by codon 129 diplotype* ***(A, C, E)*** *or phaseless genotype* ***(B, D, F)****.*



Supplemental Figure S3.6 | Power increases with long follow-up periods in simulations using historical controls. A) *Simulated trial power under the Cox proportional hazards model as a function of the number of individuals randomized and the number of years of follow-up with (solid line) or without (dotted line) modeling withdrawal, assuming a hazard ratio of 0.5 and a run-in period of one year.* ***B)*** *Number of participants required for 80% power at P < 0.05, as a function of hazard ratio (x axis) and trial design (different curves). Numbers for randomized trials (red curve) are taken directly from Table 3.2, while numbers for post-marketing studies (dark and light blue curves) are obtained by simulation (Supplementary Discussion).*

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Supplement to *Mass spectrometry-based quantification of prion protein in cerebrospinal fluid*

Supplemental Table S4.1 | Precursor and product characteristics for all peptides monitored. *Best fragment ions were chosen as transition ions with the highest peak area that were also interference-free and reproducibly measured in pilot studies.*

peptide modified sequence	precursor charge	fragment ion	product charge	precursor mz [light]	precursor mz [heavy]	precursor mz [^{15}N]	product mz [light]	product mz [heavy]	product mz [15n]	best fragment ion
PIIHFGSDYEDR	3	b4	1	483.57	486.90	489.21	461.29	461.29	467.27	
	3	y9	2	483.57	486.90	489.21	563.23	568.24	570.21	
	3	y4	1	483.57	486.90	489.21	582.25	592.26	589.23	y4
	3	y7	1	483.57	486.90	489.21	841.33	851.34	851.30	
RPKPGGWNTGGSR	3	y4	1	457.24	460.58	464.55	376.19	386.20	383.17	y4
	3	y5	1	457.24	460.58	464.55	477.24	487.25	485.22	
	3	y6	1	457.24	460.58	464.55	591.28	601.29	601.25	
YPGQGSPGGNR	2	y5	1	545.26	550.26	553.23	500.26	510.27	509.23	y5
	2	y7	1	545.26	550.26	553.23	644.31	654.32	655.28	
	2	y9	1	545.26	550.26	553.23	829.39	839.40	843.35	
GENFTETDVK	2	y4	1	570.26	574.27	576.25	462.26	470.27	467.24	
	2	y5	1	570.26	574.27	576.25	591.30	599.31	597.28	
	2	y6	1	570.26	574.27	576.25	692.35	700.36	699.33	
	2	y7	1	570.26	574.27	576.25	839.41	847.43	847.39	
VVEQMC[+57]ITQYER	2	y5	1	778.37	783.37	786.84	696.33	706.34	705.30	y5
	2	y7	1	778.37	783.37	786.84	969.45	979.45	980.41	
	2	y8	1	778.37	783.37	786.84	1100.49	1110.49	1112.45	
VVEQM[+16]C[+57]ITQYER	2	y5	1	786.36	791.37	794.84	696.33	706.34	705.30	y5
	2	y7	1	786.36	791.37	794.84	969.45	979.45	980.41	
	2	y8	1	786.36	791.37	794.84	1116.48	1126.49	1128.45	
ESQAYYQR	2	y3	1	522.74	527.75	529.22	466.24	476.25	473.22	y3
	2	y4	1	522.74	527.75	529.22	629.30	639.31	637.28	
	2	y5	1	522.74	527.75	529.22	700.34	710.35	709.31	
VVEQMC[+57]VTQYQK	2	y5	1	756.86	760.87	n/a	667.34	675.36	n/a	y5
	2	y7	1	756.86	760.87	n/a	926.44	934.45	n/a	
	2	y8	1	756.86	760.87	n/a	1057.48	1065.49	n/a	
VVEQM[+16]C[+57]VTQYQK	2	y5	1	764.86	768.87	n/a	667.34	675.36	n/a	y5
	2	y7	1	764.86	768.87	n/a	926.44	934.45	n/a	
	2	y8	1	764.86	768.87	n/a	1073.48	1081.49	n/a	

peptide modified sequence	precursor charge	fragment ion	product charge	precursor mz [light]	precursor mz [heavy]	precursor mz [^{15}N]	product mz [light]	product mz [heavy]	product mz [15n]	best fragment ion
ESQAYYDGR	2	y4	1	544.74	549.74	n/a	510.23	520.24	n/a	y4
	2	y5	1	544.74	549.74	n/a	673.29	683.30	n/a	
	2	y6	1	544.74	549.74	n/a	744.33	754.34	n/a	
VVEQMC[+57]ITQYEK	2	y5	1	764.36	768.37	n/a	668.32	676.34	n/a	y5
	2	y7	1	764.36	768.37	n/a	941.44	949.45	n/a	
	2	y8	1	764.36	768.37	n/a	1072.48	1080.49	n/a	
VVEQM[+16]C[+57]ITQYEK	2	y5	1	772.36	776.37	n/a	668.32	676.34	n/a	y5
	2	y7	1	772.36	776.37	n/a	941.44	949.45	n/a	
	2	y8	1	772.36	776.37	n/a	1088.48	1096.49	n/a	

Supplemental Table S4.2 | Species sequence matching, sequence context, and pilot study detection of all peptides monitored.

previous AA	sequence	next AA	species	MS peak area in recombinant PrP	peak area in CSF
(LCKK)	RPKPGGWNTGGSR	(YPGQ)	human, cyno, mouse, rat	5.49E+09	1.24E+08
(GGSR)	YPGQGSPGGNR	(YPP)	human, cyno, mouse, rat	4.90E+10	8.62E+08
(AMSR)	PIIHFGSDYEDR	(YYR)	human		1.06E+09
(TTTK)	GENFTETDVK	(MME)	human, cyno, mouse, rat	5.11E+10	2.48E+08
(MMER)	VVEQMCITQYER	(ESQ)	human	3.46E+09	1.12E+09
(MMER)	VVEQMCVTQYQK	(ESQA)	mouse, rat	5.21E+10	
(MMER)	VVEQMCITQYEK	(ESQ)	cyno		
(QYER)	ESQAYYQR	(GSS)	human, cyno	2.32E+10	
(QYQK)	ESQAYYDGR	(RSS)	mouse, rat	6.85E+10	

Supplemental Table S4.3 | Characteristics and performance of the nine PrP MRM peptides in assay development samples. *Analytical validation experiments were performed without ^{15}N protein internal controls and instead utilized the light:heavy peptide area under the curve ratio as described in Methods. Data from N=19 samples (N=4 cynomolgous macaque CSF, N=10 human CSF, N=1 human brain, N=1 mouse brain, and N=4 rat CSF) in a total of N=35 replicates were analyzed to determine the basic performance characteristics of each peptide (this table) as well as the sensitivity and selectivity of the assay (Supplemental Figure S5). Here, only data from samples where the peptide is sequence-matched to the species in question are shown. *Reduced peptides only; met-ox versions were not monitored in these runs. Retention time is shown as mean±sd in minutes for a 45-minute gradient. Mean L:H ratio is the mean light:heavy area ratio. Mean CV is calculated across the subset of samples run in technical duplicate or triplicate within the same run, and N indicates the number of unique samples.*

peptide	retention time (min)	mean L:H ratio	mean CV
RPKPGGWNTGGSR	16.9±0.7 (N=28)	0.1 (N=21)	11% (N=12)
YPGQGSPGGNR	18.6±0.7 (N=28)	0.6 (N=21)	5.2% (N=12)
PIIHFGSDYEDR	36.5±1.8 (N=19)	3.9 (N=13)	6.5% (N=9)
GENFTETDVK	26.7±1.3 (N=28)	0.4 (N=21)	7.2% (N=12)
VVEQMCITQYER*	37.0±1.5 (N=19)	7.0 (N=13)	5.7% (N=9)
VVEQMCVTQYQK*	33.7±0.6 (N=3)	1.0 (N=4)	7.2% (N=1)
VVEQMCITQYEK*	35.3±0.3 (N=6)	1.4 (N=2)	4.3% (N=2)
ESQAYYQR	19.9±0.8 (N=25)	2.2 (N=17)	3.3% (N=11)
ESQAYYDGR	23.3±0.3 (N=3)	0.3 (N=4)	0.92% (N=1)

Supplemental Table S4.4 | Analytical process variability assessment. *Because the spiked ^{15}N protein and synthetic heavy peptide concentrations were the same for every sample and every day, we used the ^{15}N:H peak area ratio (PAR) to assess analytical process variability. Mean PARs varied by no more than 22% per day (largest variability: VVEQMCITQYER, 0.28 for day 4 vs. 0.23 for day 3) and mean CVs among all samples, all replicates within each day was <20% for all days and <15% for all days except YPGQGSPGGNR day 3. These data provide supporting evidence for the technical validity of our analytical process, and suggest that the assay is suitable to measure the biological variability among our clinical samples. *Excludes N=12 met-ox samples.*

	^{15}N:H PAR mean CV by day					^{15}N:H mean PAR by day				
peptide	1	2	3	4	5	1	2	3	4	5
RPKPGGWNTGGSR	5.7%	8.2%	11.0%	10.4%	6.9%	0.04	0.05	0.04	0.03	0.03
YPGQGSPGGNR	8.0%	13.9%	18.4%	13.5%	9.4%	0.08	0.08	0.08	0.07	0.08
PIIHFGSDYEDR	13.5%	10.6%	10.7%	9.1%	12.5%	0.13	0.13	0.13	0.13	0.14
GENFTETDVK	7.4%	9.4%	9.5%	4.5%	8.2%	0.24	0.23	0.24	0.21	0.21
VVEQMCITQYER*	6.9%	8.2%	13.9%	7.8%	9.4%	0.26	0.25	0.23	0.28	0.25
ESQAYYQR	7.4%	5.7%	12.1%	10.5%	9.0%	0.30	0.28	0.31	0.32	0.25

Supplemental Table S4.5 | Normalization of peptide responses. *The L, ^{15}N, and L:^{15}N columns summarize the data from Figure 2. Across N=55 clinical samples, the mean L:^{15}N ratio for each peptide varied by >10-fold. If these ratios are simply multiplied by the known concentration of ^{15}N protein spiked in (24 ng/mL), they correspond to "raw" PrP concentrations here, ranging from 39 – 478 ng/mL. We calculated a response factor for each peptide as described in Methods and Supplemental Figure S8, which, for the one CSF sample used in the dose-response experiment, serves to bring each peptide up to equal abundance as the highest-responding peptide (VVEQMCITQYER). Multiplying the L:^{15}N ratio by the response factor and the spiked ^{15}N PrP concentration in clinical samples yields normalized PrP concentrations that are within ±50% of one another. Note that this small residual difference between peptides in terms of normalized PrP concentration reflects the fact that the single CSF sample used in the dose-response experiment did not have exactly the same ratio among the different peptides as the average clinical sample.*

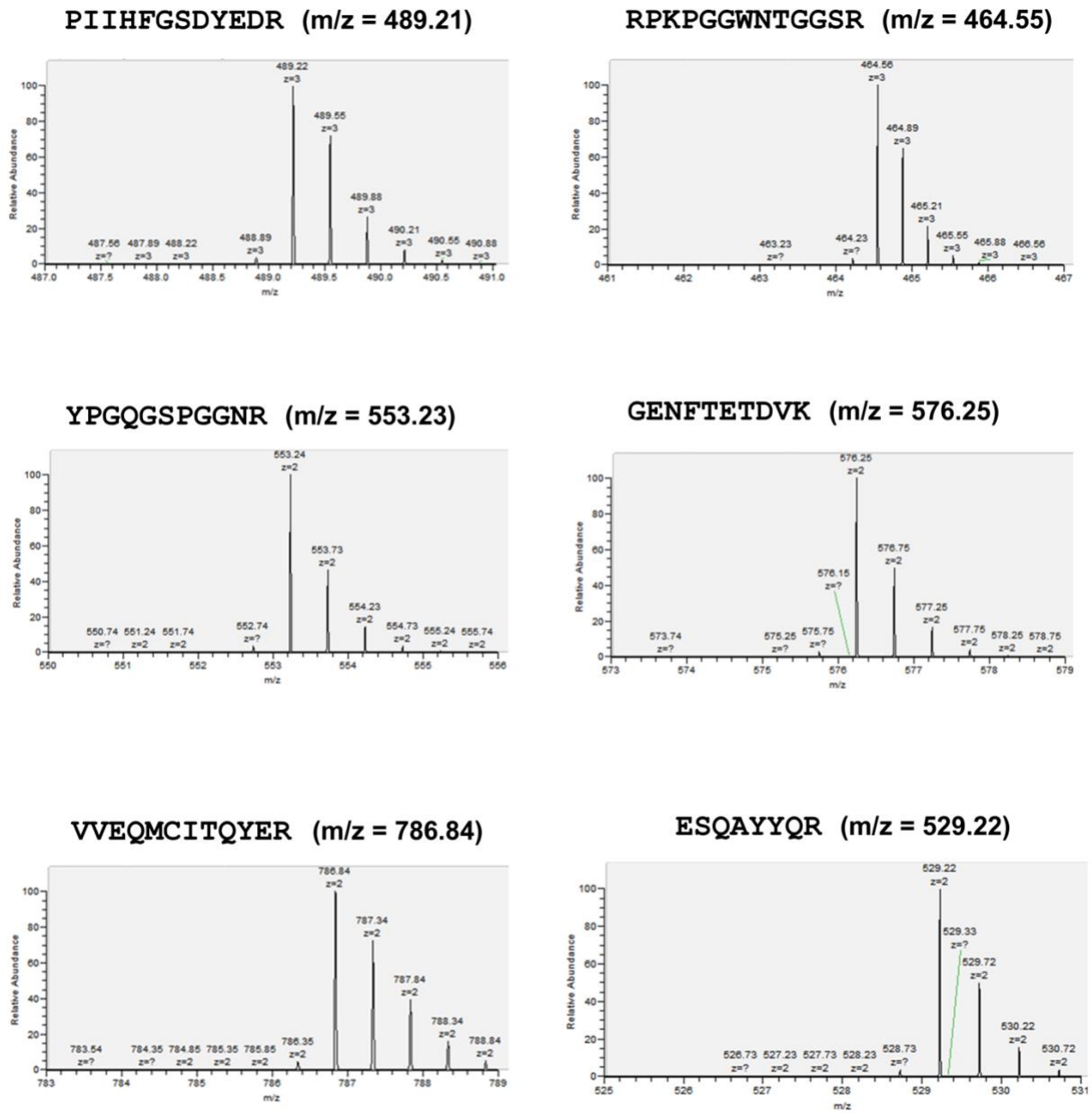
peptide	mean ± sd L peak area (millions)	mean ± sd ^{15}N peak area (millions)	mean ± sd L:^{15}N ratio	mean raw [PrP] (ng/mL)	response factor	mean normalized [PrP] (ng/mL)
RPKPGGWNTGGSR	0.14±0.12	0.10±0.08	1.8±1.4	43	11.6	503
YPGQGSPGGNR	0.26±0.22	0.05±0.03	5.3±2.7	130	2.5	329
PIIHFGSDYEDR	1.59±1.04	0.10±0.05	16.7±9.3	409	1.2	497
GENFTETDVK	0.49±0.36	0.33±0.19	1.6±0.9	39	9.0	350
VVEQMCITQYER	3.67±3.17	0.20±0.15	19.7±10.7	478	1.0	478
ESQAYYQR	0.35±0.28	0.08±0.05	4.8±3.3	116	3.2	373

Supplemental Table S4.6 | Characteristics and performance of the six PrP MRM human peptides in clinical samples by quartile. *For each peptide, the N=55 samples were broken into quartiles of L:^{15}N ratio. The mean coefficient of variation (CV) of technical duplicates and the mean L:^{15}N ratio of samples was calculated within each quartile. Note that the rank order used for binning is similar (Figure 3B) but not identical between peptides. Also note that for VVEQMCITQYER, because 12 replicates (including both replicates of one sample) with methionine oxidation were thrown out, sample size is N=44 for CV calculations (using only those samples with N=2 valid process replicates) and N=54 for mean L:^{15}N ratio calculations (including all samples with N≥1 valid process replicate). The results show that all six peptides, across all four quartiles, had mean CV ≤15% and mean L:^{15}N ratio ≥0.6. Because our data suggest assay linearity extending at least as low as 0.1x of the ^{15}N PrP concentration we used (estimated to be 0.24 ng/mL, Supplemental Figure S4.5C), this suggests that PrP MRM had acceptable performance in all quartiles and that all measurements in clinical samples were within the dynamic range of the assay.*

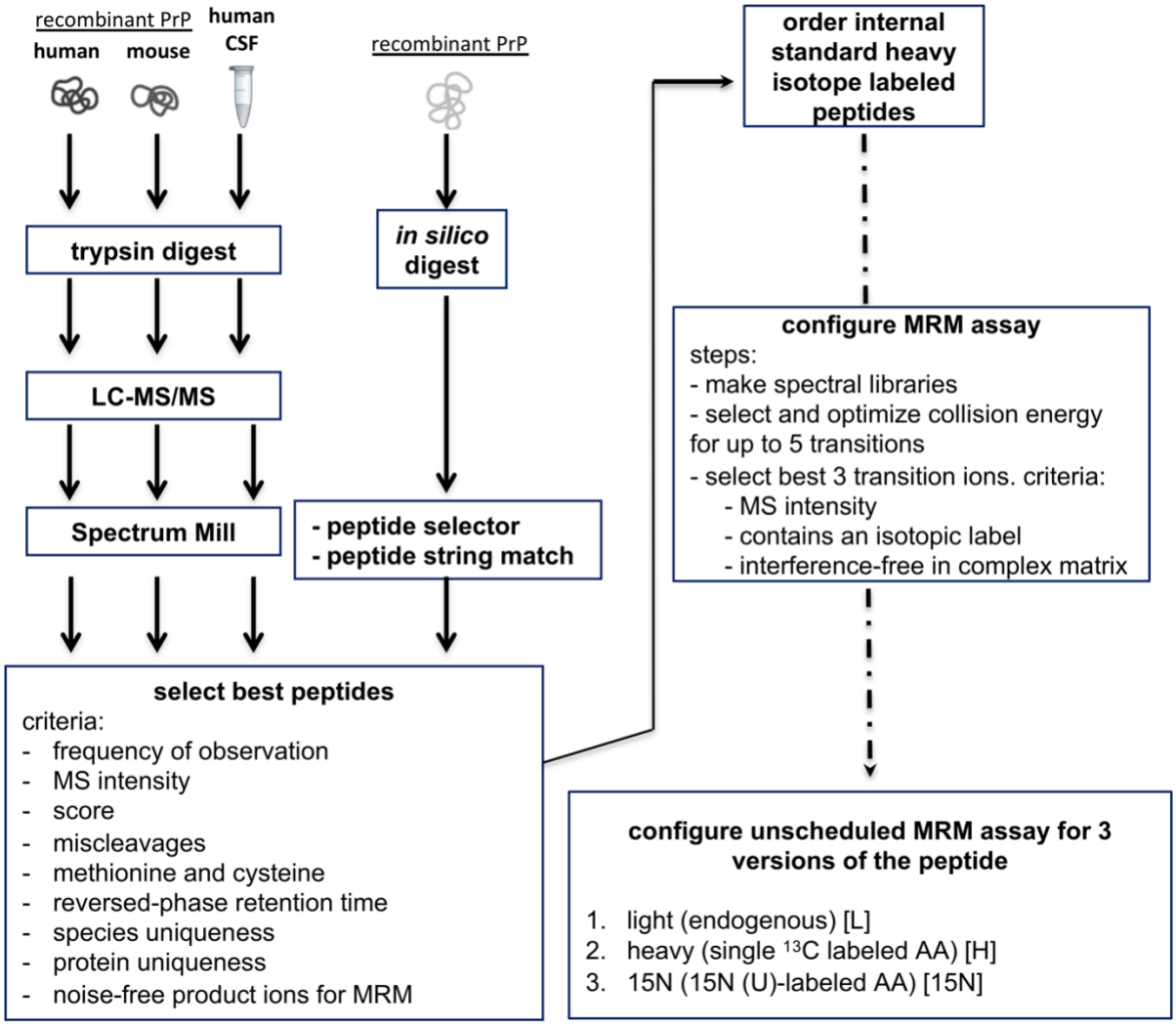
	mean CV by quartile				mean L:^{15}N ratio by quartile			
peptide	0-24%	25-49%	50-74%	75-100%	0-24%	25-49%	50-74%	75-100%
RPKPGGWNTGGSR	9.8%	14.5%	11.7%	3.9%	0.7	1.2	1.8	3.7
YPGQGSPGGNR	12.5%	11.9%	14.0%	9.5%	2.5	4.1	5.9	9.3
PIIHFGSDYEDR	10.9%	11.3%	5.4%	11.1%	7.9	11.9	17.8	30.9
GENFTETDVK	12.1%	9.9%	8.8%	6.6%	0.6	1.2	1.8	2.9
VVEQMCITQYER	12.8%	12.0%	3.4%	6.4%	9.0	14.9	21.7	35.6
ESQAYYQR	9.2%	9.3%	10.7%	9.1%	2.0	3.1	4.8	9.6

Supplemental Table S4.7 | Recovery and performance of six human peptides quantified in human CSF samples using L:H ratio. *This table is identical to Table 4.1 except using the L:H peak area ratio rather than the L:^{15}N peak area ratio.*

codons	peptide	mean intra-run CV	mean inter-run CV	inter-individual CV
25-37	RPKPGGWNTGGSR	6%	25%	63%
38-48	YPGQGSPGGNR	7%	13%	55%
137-148	PIIHFGSDYEDR	7%	8%	59%
195-204	GENFTETDVK	5%	11%	57%
209-220	VVEQMCITQYER	5%	9%	58%
221-228	ESQAYYQR	4%	6%	66%



Supplemental Figure S4.1 | Extracted MS intensities for human PrP peptides used for estimation of isotopic purity of ^{15}N-labeled protein. *Isotopic envelopes of each peptide identified by MS/MS after digestion of the ^{15}N protein with trypsin. Minimal or lack of observed mz peak areas less than the ^{12}C monoisotopic mass peak (highest signal for the mz of these peptides) indicates near complete ^{15}N incorporation. Lower mass peaks corresponding to incomplete ^{15}N incorporation were unquantifiably small, consistent with >97.5% isotopic purity for all peptides.*



Supplemental Figure S4.2 | Assay development workflow. *Schematic outline of steps described in Methods to select peptides based on empirical and bioinformatic data, and optimize and configure a 9-plex MRM assay.*

A human CSF digest

1	MANLGCWMLV	LFVATWSDLG	LC**KKRPKPGG**	**WNTGGSRYPG**	**QGSPGGNRYP**	50	not expected (cleaved)
51	**PQGGGGWGQP**	**HGGGWGQPHG**	**GGWGQPHGGG**	**WGQPHGGGWG**	**QGGGTHSQWN**	100	**not detected**
101	**KPSKPKTNMK**	**HMAGAAAAGA**	**VVGGLGGYML**	**GSAMSRPIIH**	**FGSDYEDRYY**	150	**detected**
151	**RENMHRYPNQ**	**VYYRPMDEYS**	**NQNNFVHDCV**	**NITIKQHTVT**	**TTTKGENFTE**	200	
201	**TDVKMMERVV**	**EQMCITQYER**	**ESQAYYQRGS**	SMVLFSSPPV	ILLISFLIFL	250	
251	IVG						

66% covered (138/208 amino acids, considering mature protein only)

B recombinant HuPrP23-230 digest

1	**MKKRPKPGGW**	**NTGGSRYPGQ**	**GSPGGNRYPP**	**QGGGGWGQPH**	**GGGWGQPHGG**	50	**not detected**
51	**GWGQPHGGGW**	**GQPHGGGWGQ**	**GGGTHSQWNK**	**PSKPKTNMKH**	**MAGAAAAGAV**	100	**detected**
101	**VGGLGGYMLG**	**SAMSRPIIHF**	**GSDYEDRYYR**	**ENMHRYPNQV**	**YYRPMDEYSN**	150	
151	**QNNFVHDCVN**	**ITIKQHTVTT**	**TTKGENFTET**	**DVKMMERVVE**	**QMCITQYERE**	200	
201	**SQAYYQRGS**						

71% covered (149/209 amino acids)

Supplemental Figure S4.3 | Sequence coverage map. *Map of sequence coverage of PrP in pilot LC-MS/MS analyses of* ***A)*** *human CSF and* ***B)*** *recombinant HuPrP23-230. A peptide containing a retained N-terminal methionine (MKKRPKPGGWNTGGSR) was detected in the recombinant digest with intensity* 6.9×10^9.

>sp|P04156|PRIO_HUMAN Major prion protein OS=Homo sapiens GN=PRNP PE=1 SV=1 MW = 27,661

							amino acids	peptide sequence
1	MANLGCWMLV	LFVATWSDLG	LC**KKRPKPGG**	**WNTGGSRYPG**	**QGSPGGNRYP**	50	25-37	**RPKPGGWNTGGSR**
51	**PQGGGGWGQP**	**HGGGWGQPHG**	**GGWGQPHGGG**	**WGQPHGGGWG**	**QGGGTHSQWN**	100	38-48	**YPGQGSPGGNR**
101	**KPSKPKTNMK**	**HMAGAAAAGA**	**VVGGLGGYML**	**GSAMSR**PIIH	FGSDYEDR**YY**	150	137-148	PIIHFGSDYEDR
151	**RENMHRYPNQ**	**VYYRPMDEYS**	**NQNNFVHDCV**	**NITIKQHTVT**	**TTTK**GENFTE	200	195-204	**GENFTETDVK**
201	TDVK**MMERVV**	EQMCITQYER	**ESQAYYQRGS**	SMVLFSSPPV	ILLISFLIFL	250	209-220	**VVEQMCITQYER**
251	IVG						221-228	**ESQAYYQR**

>sp|P04925|PRIO_MOUSE Major prion protein OS=Mus musculus OX=10090 GN=Prnp PE=1 SV=2, MW = 27,977

							amino acids	peptide sequence
1	MANLGYWLLA	LFVTMWTDVG	LC**KKRPKPGG**	**WNTGGSRYPG**	**QGSPGGNRYP**	50	25-37	**RPKPGGWNTGGSR**
51	**PQGGTWGQPH**	**GGGWGQPHGG**	**SWGQPHGGSW**	**GQPHGGGWGQ**	**GGGTHNQWNK**	100	38-48	**YPGQGSPGGNR**
101	**PSKPKTNLKH**	**VAGAAAAGAV**	**VGGLGGYMLG**	**SAMSRPMIHF**	**GNDWEDRYYR**	150	194-203	GENFTETDVK
151	**ENMYRYPNQV**	**YYRPVDQYSN**	**QNNFVHDCVN**	**ITIKQHTVTT**	**TTK**GENFTET	200	208-219	**VVEQMCITQYER**
201	DVK**MMERVVE**	QMCVTQYQKE	**SQAYYDGRRS**	**S**STVLFSSPP	VILLISFLIF	250	220-228	**ESQAYYDGR**
251	LIVG							

>sp|P67992|PRIO_MACFA Major prion protein OS=Macaca fascicularis OX=9541 GN=PRNP PE=2 SV=1, MW = 27,676

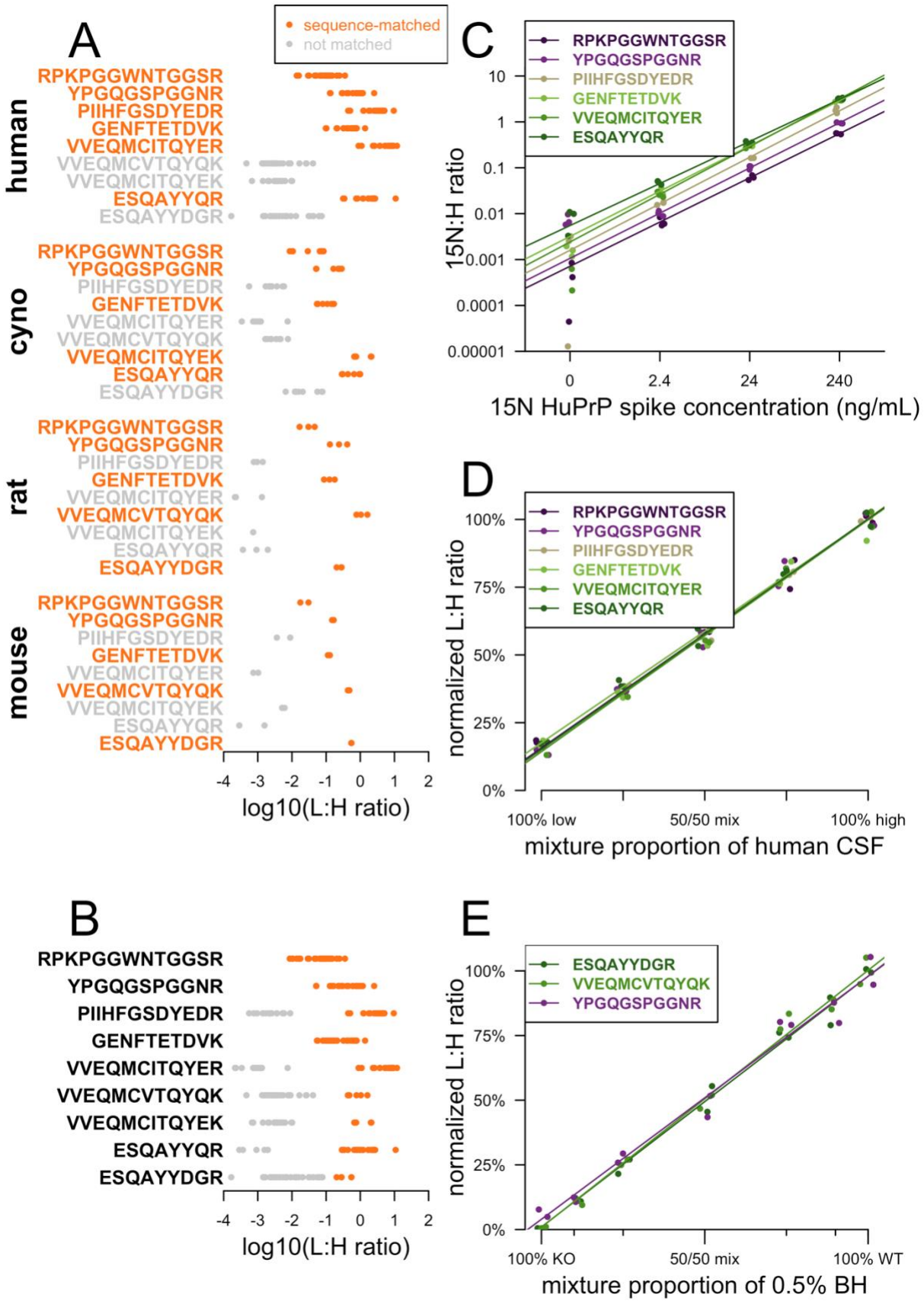
							amino acids	peptide sequence
1	MANLGCWMLV	LFVATWSDLG	LC**KKRPKPGG**	**WNTGGSRYPG**	**QGSPGGNRYP**	50	25-37	**RPKPGGWNTGGSR**
51	**PQGGGGWGQP**	**HGGGWGQPHG**	**GGWGQPHGGG**	**WGQPHGGGWG**	**QGGGTHNQWH**	100	38-48	**YPGQGSPGGNR**
101	**KPSKPKTSMK**	**HMAGAAAAGA**	**VVGGLGGYML**	**GSAMSRPLIH**	**FGNDYEDRYY**	150	195-204	GENFTETDVK
151	**RENMYRYPNQ**	**VYYRPVDQYS**	**NQNNFVHDCV**	**NITIKQHTVT**	**TTTK**GENFTE	200	209-220	**VVEQMCITQYEK**
201	TDVK**MMERVV**	EQMCITQYEK	**ESQAYYQRGS**	SMVLFSSPPV	ILLISFLIFL	250	221-228	**ESQAYYQR**
251	IVG							

>sp|P13852|PRIO_RAT Major prion protein OS=Rattus norvegicus OX=10116 GN=Prnp PE=1 SV=2, MW = 27,804

							amino acids	peptide sequence
1	MANLGYWLLA	LFVTTCTDVG	LC**KKRPKPGG**	**WNTGGSRYPG**	**QGSPGGNRYP**	50	25-37	**RPKPGGWNTGGSR**
51	**PQSGGTWGQP**	**HGGGWGQPHG**	**GGWGQPHGGG**	**WGQPHGGGWS**	**QGGGTHNQWN**	100	38-48	**YPGQGSPGGNR**
101	**KPSKPKTNLK**	**HVAGAAAAGA**	**VVGGLGGYML**	**GSAMSRPMLH**	**FGNDWEDRYY**	150	195-204	GENFTETDVK
151	**RENMYRYPNQ**	**VYYRPVDQYS**	**NQNNFVHDCV**	**NITIKQHTVT**	**TTTK**GENFTE	200	209-220	**VVEQMCVTQYQK**
201	**TDVKMMERVV**	**EQMCVTQYQK**	**ESQAYYDGRR**	**S**SAVLFSSPP	VILLISFLIF	250	221-228	**ESQAYYDGR**
251	LIVG							

Supplemental Figure S4.4 | Selected PrP peptides in protein context. *Full amino acid sequences of PrP with UniProt identifiers for the four species of interest with locations of PrP MRM peptides noted. Bold indicates residues present in the mature, post-translationally modified protein. The non-bold N terminus is an ER signal peptide, and the non-bold C terminus is a GPI signal, and both are cleaved before the protein reaches the cell surface. Molecular weights pulled from UniProt do not account for these post-translational modifications; the mature protein is ~23 kDa, see Methods in main text. For human and mouse PrP, the bold text also corresponds to the residues present in the recombinant PrP constructs used in this study.*

Supplemental Figure S4.5 | Partial analytical validation of the PrP MRM assay. A) *Sensitivity and selectivity across species. Data from N=19 samples (N=4 cynomolgous macaque CSF, N=10 human CSF, N=1 human brain, N=1 mouse brain, and N=4 rat CSF) in a total of N=35 replicates were analyzed. L:H peptide ratios are shown for peptides expected in each species (sequence-matched, orange) versus not expected (non-matched, gray).* ***B)*** *Results from panel A collapsed across species. This shows that all species-specific peptides were observed in the sequence-matched species at least an order of magnitude above the noise observed in non-sequence-matched species, with the exception of ESQAYYDGR (sequence-matched species: mouse, rat), for which the separation was only about half an order of magnitude.* ***C)*** *Assay linearity. ^{15}N HuPrP23-230 was spiked into the same human CSF sample in duplicate at three concentrations plus a zero (x axis) and quantified relative to heavy (H) peptides. Best fit lines for each peptide are shown. Peptides vary in absolute recovery (different y-intercepts), as also shown in Table 1, but exhibit similar strong linearity (slopes range 0.96 – 1.04 and adjusted R^2 values range 99.5% - 99.8%, linear regression), suggesting that normalization relative to ^{15}N internal control should provide at least 2 orders of magnitude dynamic range for endogenous PrP. The ^{15}N:L ratio in this experiment was used to calculate response factors for each peptide, see Methods.* ***D)*** *Two human CSF samples previously measured to have high (240 ng/mL) and low (12 ng/mL) PrP by ELISA were mixed in different proportions (all low, 25/75, 50/50, 75/25, and all high) and assayed by PrP MRM. Each peptide's light:heavy ratio is normalized to the average value of the two "all high" replicates, and best-fit lines are shown. Individual replicates are jittered slightly along the x-axis so that separate points are visible. Each peptide exhibits good linearity. Note that because the low-PrP CSF sample still has non-zero PrP, the fact that the y-intercepts are non-zero is expected. Best fit lines for each peptide have adjusted R^2 values ranging 97.6% - 99.8% (linear regression).* ***E)*** *10% brain homogenate from wild-type mice (WT) or Edinburgh PrP knockout mice[29] (KO) were mixed in seven different proportions (all KO, 10/90, 25/75, 50/50, 75/25, 90/10, and all WT), further diluted to 0.5% brain homogenate in saline and 0.03% CHAPS, and assayed by PrP MRM. Of the five peptides sequence-matched to mouse PrP, the three with best performance in this experiment (mean process replicate CV <10%) are shown here, again with individual replicates jittered along the x axis so that separate points are visible. Each peptide's L:H ratio is normalized to the average value of the two "all WT" replicates, and best-fit lines are shown. All three peptides exhibit good linearity, with y-intercepts very close to zero, as expected for PrP knockout mice, and adjusted R^2 values ranging 98.2% - 99.0% (linear regression).*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sequence-matched
not matched
human
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YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
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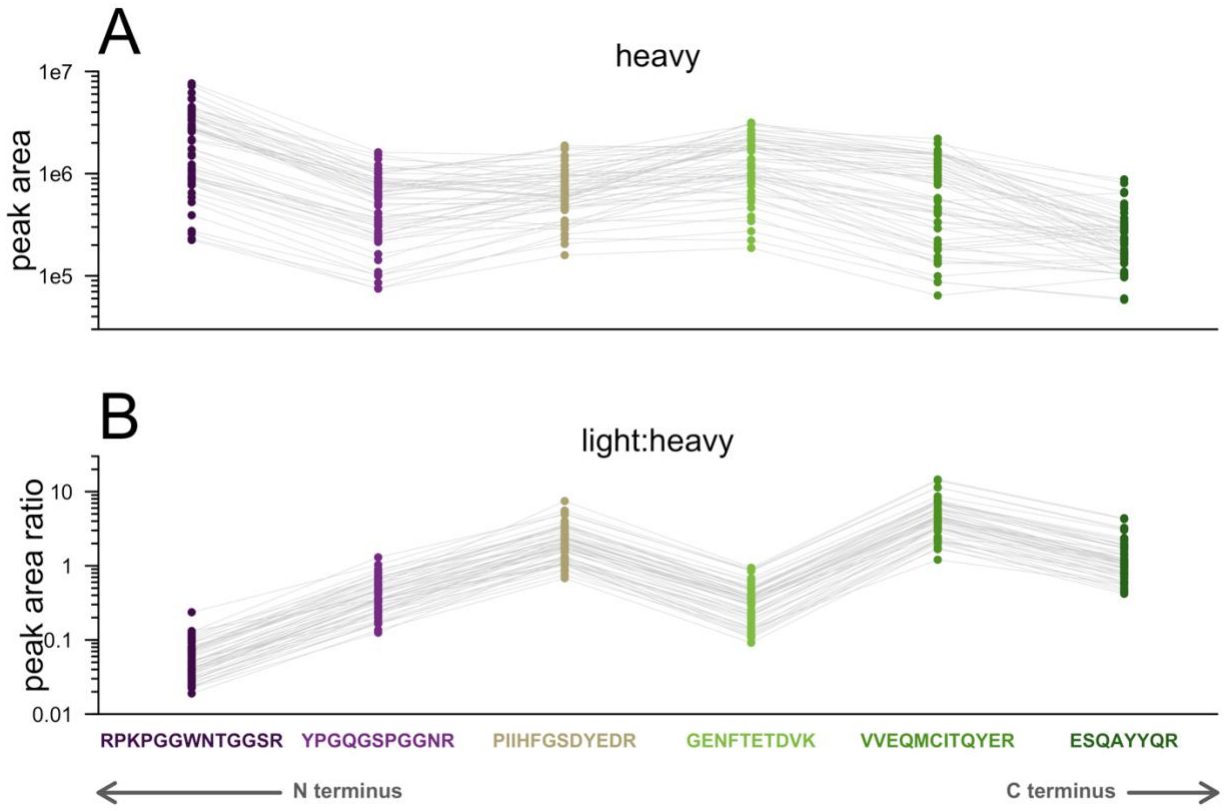
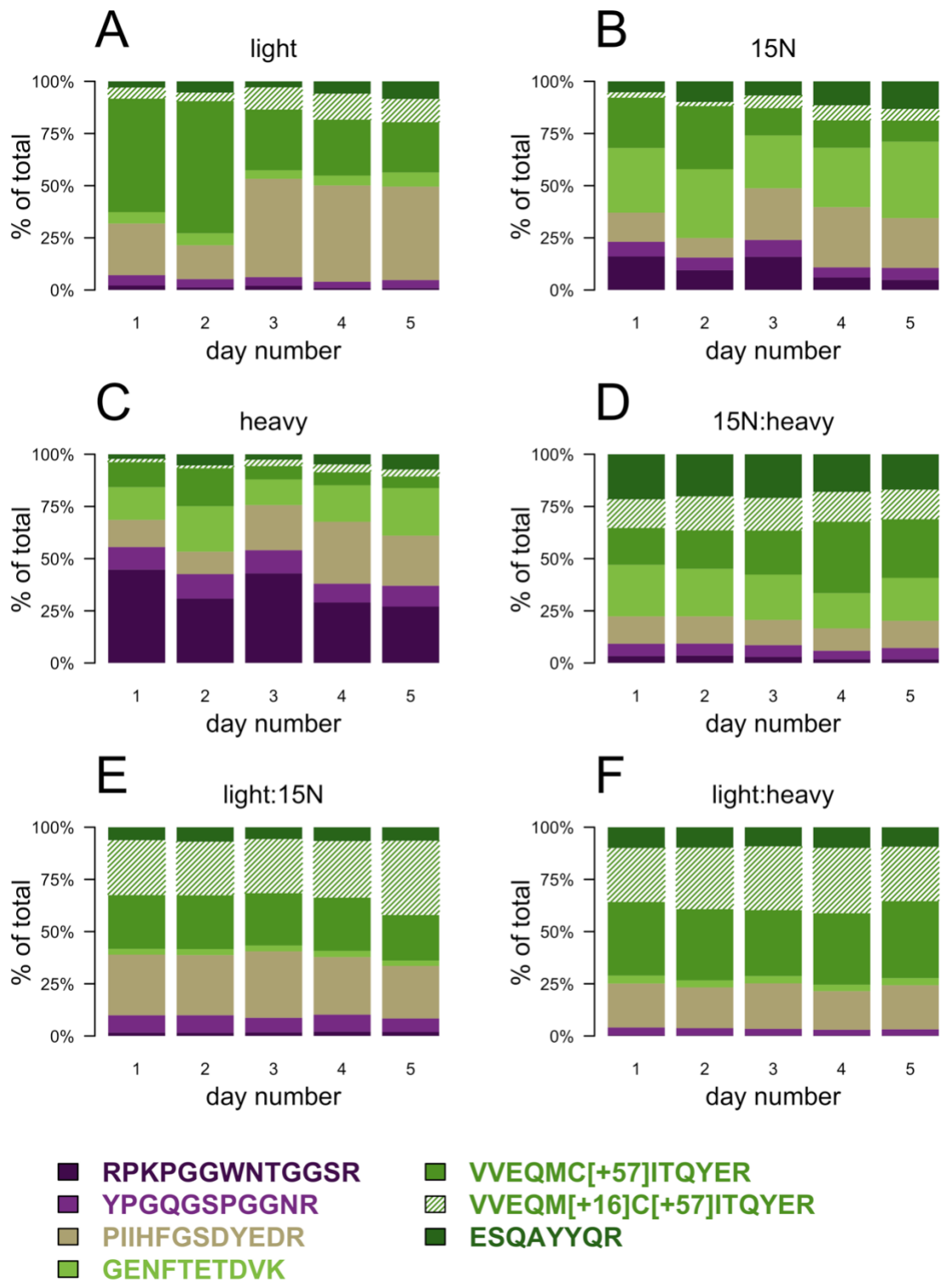
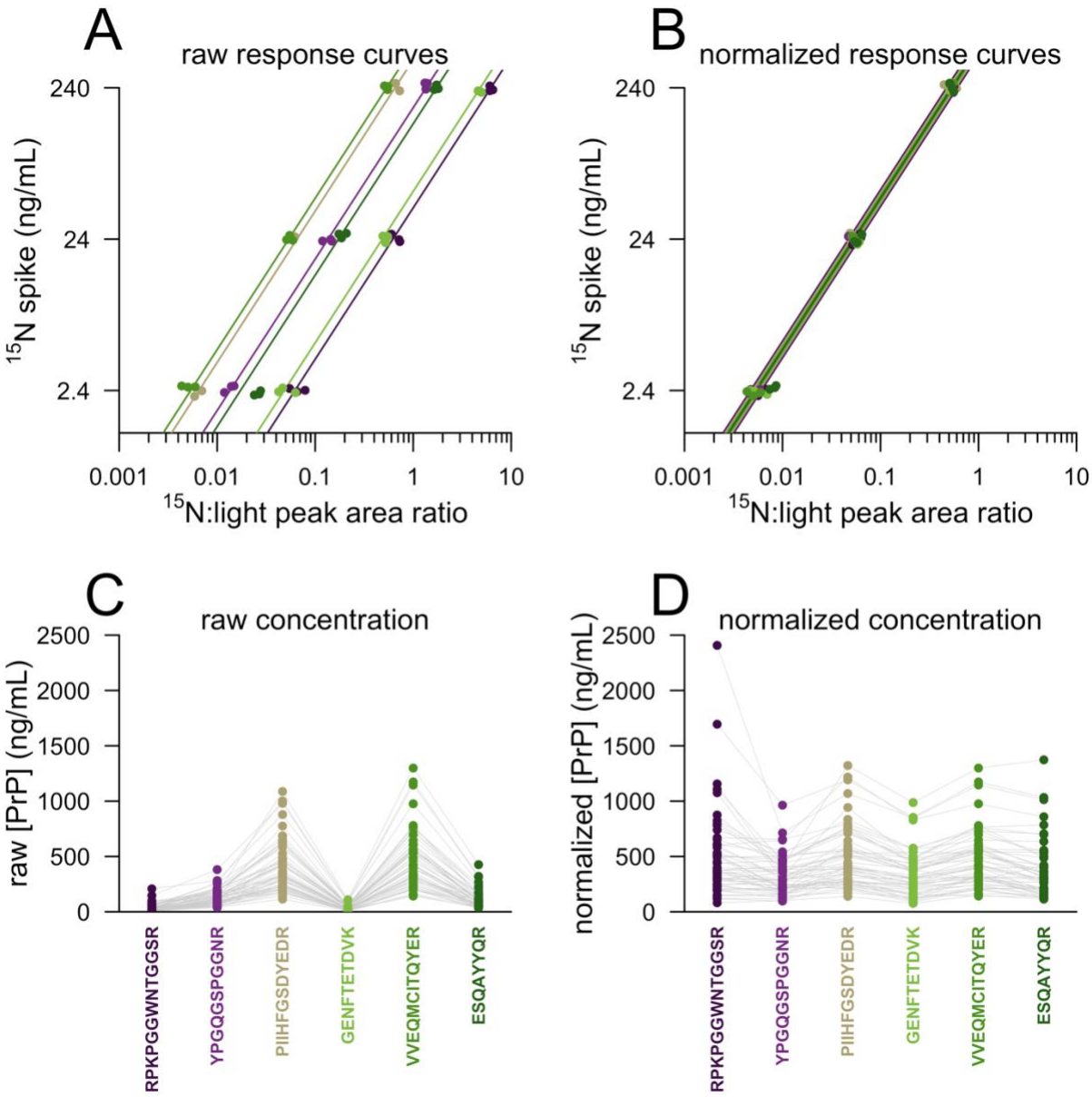
VVEQMCITQYEK
ESQAYYQR
ESQAYYDGR
cyno
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
VVEQMCVTQYQK
VVEQMCITQYEK
ESQAYYQR
ESQAYYDGR
rat
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
VVEQMCVTQYQK
VVEQMCITQYEK
ESQAYYQR
ESQAYYDGR
mouse
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
VVEQMCVTQYQK
VVEQMCITQYEK
ESQAYYQR
ESQAYYDGR
-4 -3 -2 -1 0 1 2
log10(L:H ratio)
C
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
ESQAYYQR
15N:H ratio
10
1
0.1
0.01
0.001
0.0001
0.00001
0
2.4
24
240
15N HuPrP spike concentration (ng/mL)
D
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
ESQAYYQR
normalized L:H ratio
100%
75%
50%
25%
0%
100% low
50/50 mix
100% high
mixture proportion of human CSF
B
RPKPGGWNTGGSR
YPGQGSPGGNR
PIIHFGSDYEDR
GENFTETDVK
VVEQMCITQYER
VVEQMCVTQYQK
VVEQMCITQYEK
ESQAYYQR
ESQAYYDGR
-4 -3 -2 -1 0 1 2
log10(L:H ratio)
E
ESQAYYDGR
VVEQMCVTQYQK
YPGQGSPGGNR
normalized L:H ratio
100%
75%
50%
25%
0%
100% KO
50/50 mix
100% WT
mixture proportion of 0.5% BH



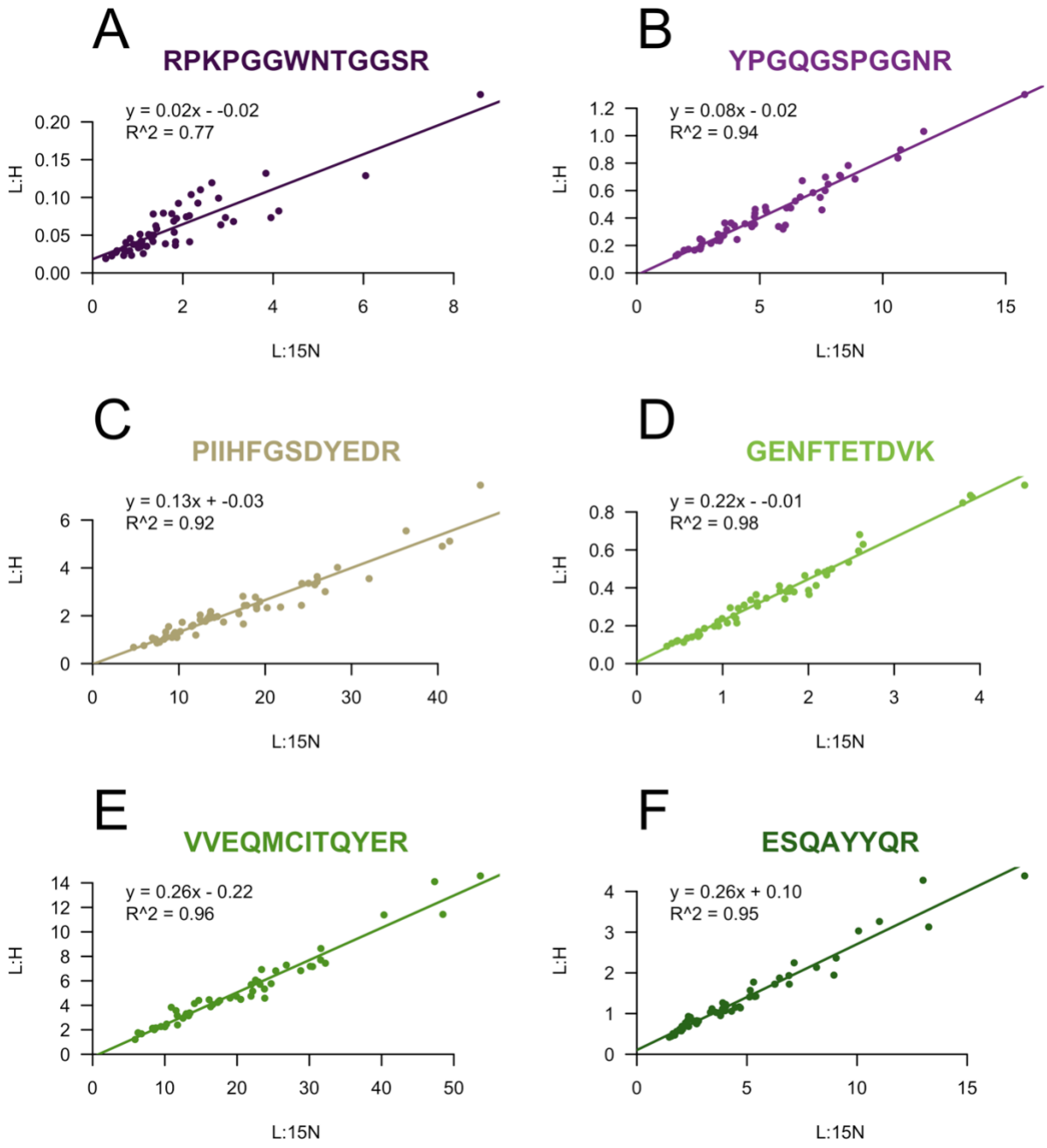
Supplemental Figure S4.6 | Relative recovery of synthetic heavy peptides in CSF. *This plot is the same as Figure 4.2 but showing* ***A)*** *heavy peak area and* ***B)*** *light:heavy ratio across the 55 clinical samples.*



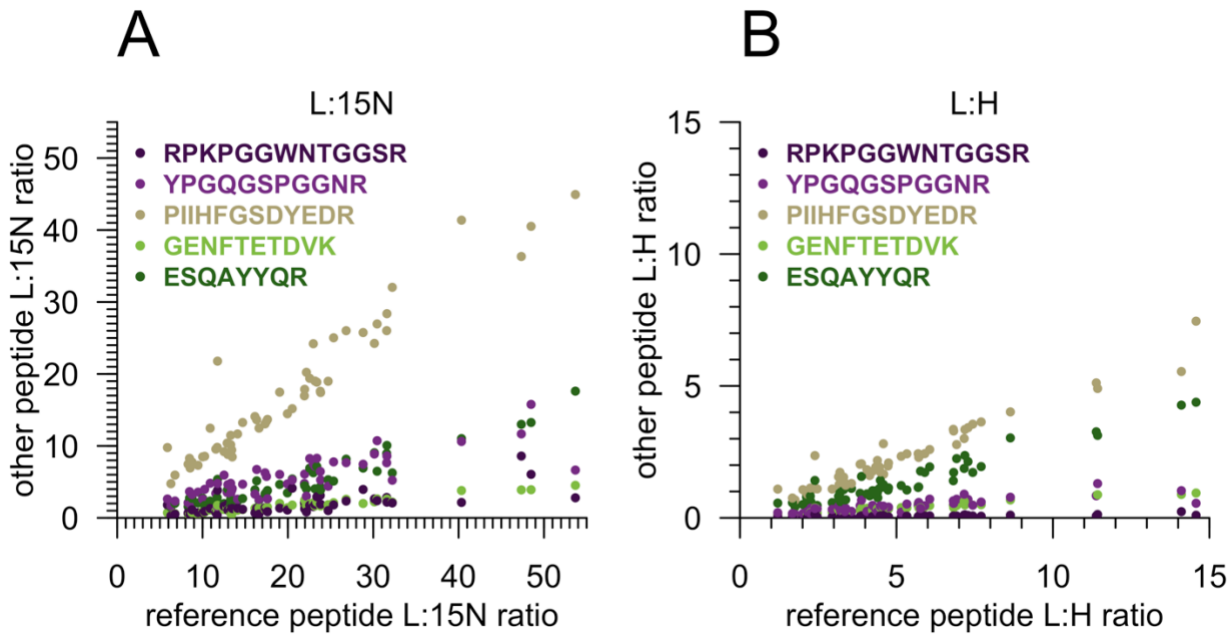
Supplemental Figure S4.7 | Peptide abundance and ratios by day. *Each stacked barplot shows the percent of total PrP peptide abundance contributed by each peptide in clinical samples across five different days.*



Supplemental Figure S4.8* | *Normalization based on dose-response data. *Normalization was performed as described in Methods using data from the same ^{15}N dose-response experiment depicted in Supplemental Figure S4.5C.* ***A)*** *Spiked ^{15}N PrP concentration versus observed ^{15}N:L ratio for each peptide. Each point is one replicate, and points are jittered along the y axis so that each point is visible. We fit linear models correlating spike ~ ^{15}N:L ratio with the y-intercept fixed at zero, and each peptide yielded a different slope. Note that this figure is plotted in log-log space, so the different slopes appear as different intercepts. We assigned each peptide a response factor equal to the maximum observed slope (that for VVEQMITQYER, top left) divided by its own slope.* ***B)*** *Same data from panel A but with response factors applied.* ***C)*** *Raw PrP concentrations in clinical samples (simply ^{15}N:L ratio times the known ^{15}N concentration of 24 ng/mL).* ***D)*** *Normalized PrP concentrations in clinical samples (^{15}N:L ratio times 24 ng/mL times peptide response factor). In C and D, gray lines connect the dots representing distinct peptides from the same sample.*



Supplemental Figure S4.9 | Correlation between L:^{15}N and L:H ratios across clinical samples, for each peptide. *The correlations exhibit different slopes, consistent with the different observed area of heavy vs. ^{15}N across peptides (Figure 4.2 and Supplemental Figure S4.6) and likely arising from differences in recovery from trypsin digestion or other analytical factors prior to heavy peptide addition. Nonetheless, all correlations show good linearity ($R^2 \geq 0.77$), indicating that the two normalization approaches — using synthetic heavy peptides or uniformly ^{15}N-labeled recombinant — give similar results.*



Supplemental Figure S4.10 | Scatterplot correlations between peptides across clinical samples. A) *L:^{15}N ratio and* ***B)*** *L:H ratio. The most abundant peptide, VVEQMCITQYER, is used as reference (x axis) versus all other peptides (y axis). The slopes differ, consistent with different response of different peptides (Supplemental Table S4.5 and Figure 4.2 and S4.5), but linear correlations are observed for each, across the full dynamic range of samples analyzed. This provides supporting evidence that our assay is technically able to measure the biological variability among samples, and that all peptides move together according to changes in disease state.*

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