Antisense Oligonucleotides for the Prevention of Genetic Prion Disease

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Abstract

Human prion disease is a fatal, currently untreatable neurodegenerative disease. Across subtypes – which include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker disease (GSS) – all cases are caused by the conformational corruption of the prion protein, or PrP, into a self-templating proteinaceous pathogen known as a prion. Strong genetic proofs of concept support the therapeutic strategy of reducing PrP expression in the brain, and plausible modalities to effect this reduction are now emerging. Pre-symptomatic carriers of high-risk genetic prion disease mutations can be identified in advance of symptoms, providing an opportunity for early therapeutic intervention to extend healthy life. Here we provide a set of tools to enable meaningful trials of PrP-lowering therapeutics in pre-symptomatic genetic prion disease mutation carriers.

First, we outline a regulatory strategy to support meaningful testing of PrP-lowering therapeutics in healthy carriers, leveraging PrP levels in cerebrospinal fluid (CSF) as a surrogate endpoint. The U.S. Food and Drug Administration (FDA) Accelerated Approval pathway provides a potentially appropriate mechanism, and we describe regulatory engagement to this effect.

Second, we report prophylactic efficacy of PrP-lowering ASOs in prion-infected mice. We show that administration of ASOs prior to prion infection extends life by delaying disease onset across a variety of paradigms. This benefit is dose-responsive and universal against all prion strains tested.

Third, we assess the technical and biological suitability of CSF to serve as a biomarker for therapeutic reduction of PrP in the brain. We show that PrP in human CSF can be reliably quantified, reflects the tissue of interest, and exhibits reasonable short-term within-subject stability.

Fourth, we describe a natural history study of pre-symptomatic genetic prion disease mutation carriers. We validate the short-term within-subject stability CSF PrP levels in this trial population of interest. We also show that pre-symptomatic carriers have normal levels of key prion disease-associated fluid biomarkers, supporting the need for a primary prevention strategy.

Our findings provide a therapeutic and clinical strategy for prevention of genetic prion disease.

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I came into my PhD unusually situated in almost every way: fatal genetic diagnosis in hand, all the wrong degrees stacked illustriously under my bed, husband and partner in all things by my side. It's fitting, then, that this PhD now comes to an unusual close, one that marks no particular conclusion in our work, mission or mandate. Instead, this thesis is an interim reflection on our race, very much ongoing, to rewrite the future by enabling the prevention of genetic prion disease.

We aren't remotely at the end. But it's fortifying to take this opportunity to acknowledge that we also aren't remotely at the beginning.

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I've never fought alone… And I never will.
– Harry Potter

"Harry Potter and the Cursed Child"
Part Two, Act IV, Scene 11
J.K. Rowling, Jack Thorne, and John Tiffany

Chapter 1: Introduction

Introduction to prion disease

Prion disease is a rapidly progressive neurodegenerative disease that afflicts humans and other mammals. It is universally fatal and currently untreatable. In humans, prion disease is relatively rare, with an incidence of 1-2 deaths per million people per year[1]. This gain-of-function disease results from the conformational corruption of the native prion protein (PrP), encoded by the prion protein gene (*PRNP* in humans) into a self-templating prion[2]. Such prions are unique in biology as naturally transmissible protein-only pathogens, capable of spreading across the brain through auto-catalytic conversion of native PrP molecules[2]. Through mechanisms still not well understood, this exponential spread of misfolded prions across the brain drives neuronal death and manifest disease. This chapter will describe how, despite the wide phenotypic and molecular diversity seen in the clinical phase of prion disease, the shared genetics and single causal protein underlying all cases offer a unifying theme that make prion diseases uniquely tractable in the difficult area of neurodegeneration.

The history of human prion disease is a cornucopia of varying etiologies, clinical names, presentations, molecular subtypes and neuropathological hallmarks. Uniquely, human prion disease can arise in three distinct ways. Roughly 85% of cases are sporadic, meaning they are not triggered or predicted by any known genetic or environmental factor, while about 15% of cases are genetic, caused by a handful of known pathogenic variants in *PRNP*[3]. Prion disease can also be acquired, as has been documented following use of prion-contaminated surgical implements, tissues or biologics[4], or following consumption of prion-infected tissue[5,6]. While transmission has accounted for few human cases in recent years, horizontal transmission appears to be the dominant etiology of some prion disease subtypes in other species[7,8], and the transmissible nature of prions has been a key asset for modeling prion disease in animals[9].

Clinically, human prion disease is most often termed Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), or Gerstmann-Straussler-Scheinker disease (GSS). These

subtypes were originally described as different conditions, each associated with a characteristic pathological fingerprint driving a distinct clinical presentation[10]. Sporadic CJD (sCJD) is the term applied to the vast majority of sporadic cases, and is associated most commonly with a "classic" presentation typified by cognitive decline and dementia, with prominent cortical pathology[11]. The term genetic CJD (gCJD) was first applied to a German kindred of Flemish descent in the 1920s, multiple generations of which presented with a classical CJD phenotype[12]. The name FFI was inspired by an Italian pedigree in which insomnia was a profound early symptom; thalamic pathology was reported to predominate and to drive a primarily autonomic early phenotype[13,14]. GSS, first described in an Austrian family, likewise refers to an inherited form of prion disease, but with a classical presentation involving early cerebellar ataxia, a somewhat more protracted course and pathology featuring multicentric extracellular plaques[15].

While the terms CJD, FFI and GSS are still in use, the clinical utility of these categories has long been under challenge[3,15]. With accumulation of cases and kindreds over time, heterogeneous clinical presentation has been described within each of these categories, as has considerable overlap between categories. One widely used scheme subdivides sporadic CJD into six molecular subtypes based in part on differential electrophoretic mobility of protease-resistance prion fragments found in diseased tissue[11]. Growing appreciation of the dizzying range of possible early symptoms has also led to a proliferation of new clinical subcategories of sCJD, including the cognitive, affective, Heidenhain, and Oppenheimer-Brownell subtypes[16]. The affective category broadens the spectrum to encompass early neuropsychiatric symptoms including mood lability, anxiety and mania. The Heidenhain variant resembles FFI, encompassing visual disturbances and hallucinations, while the Oppenheimer-Brownwell hinges on motor changes classically associated with GSS. Notably, these clinical categories do not map neatly onto the molecular subtypes described above, nor do they comprehensively account for all sCJD patients; in the retrospective study proposing their use, more than 10% of sCJD

cases were still categorized as indeterminate[16]. The subject of sCJD subclassification continues to generate debate and proposed reform[17–21].

Meanwhile, genetic prion disease subtypes have shown little more internal consistency. Reports abound of individuals in FFI kindreds presenting with classic CJD phenotypes[22–26], with the prominent ataxia characteristic of GSS[27,28], and with parkinsonism[29], while reporting absent or limited sleep disturbance. Likewise, individuals from GSS families have presented with parkonsinism, Alzheimer's-like changes, and overriding progressive dementia in the absence of more typical GSS symptoms[15,30]. Diverse phenotypic and pathological presentations have been reported not only within mutation, but within the same affected family[15,22,28] and even between affected monozygotic twins[31–33]. Meanwhile various subtypes of prion disease, both old and new, fail to fall neatly into any of three canonical bins. A distinct presentation of inherited prion disease in large pedigrees in the UK tends towards early onset, slow progression, multifocal dementia and ataxia – though here again, great diversity is seen between and within families, with psychiatric and Huntington's-like variants reported[34]. Sporadic fatal insomnia has been described as a non-familial phenocopy of FFI[35]. Most recently, the term variably protease-sensitive proteinopathy (VPSPr) has been applied to apparently sporadic prion disease cases that variably lack protease-resistant prion fragments, one of the traditional biochemical hallmarks of post-mortem prion tissue[36]. VPSPr cases present with phenotypic heterogeneity that draws from the profiles of both sCJD and GSS.

Arguably, the classical prion disease categories both fail to account for the diversity seen in early prion disease, and overstate differences in overall course. Dependent upon how long an affected individual survives, prion pathology appears to spread brain-wide over the course of disease[37], leading cases with diverse early symptoms to converge toward dementia and a physical state of akinetic mutism with myoclonus in advanced disease[38]. Today, it is more appropriate to conceive of CJD, FFI and GSS as part of a single pathophysiological spectrum, rather than as distinct diseases[3,39].

This convergence maps well onto the shared genetic basis of prion disease that has emerged in the interim. The classical naming scheme predates the central set of studies led by Dr. Stanley Prusiner in the 1980s that established the prion hypothesis. By purifying large amounts of the pathogenic agent from prion-infected hamster brains[40], Prusiner and others were able to determine the pathogen's amino acid sequence[41], trace its genomic origins using cDNA probes, and establish that the telltale gene was present in healthy as well as diseased brain[42,43]. Biochemical and structural analysis supported the hypothesis that post-translational conformational corruption led this normally benign host-encoded protein to misfold into a protein-only pathogen[44,45]. The proteinaceous infectious particle, or prion, had arrived, and went on to outlive the considerable accompanying controversy[46].

Oriented by this breakthrough, human genetics soon uncovered *PRNP* and its product, PrP, as unifying causal factors in all cases of prion disease. The variants underlying the disparate forms of genetic prion disease have since all been mapped to *PRNP*, as have all known modifiers of non-genetic forms of prion disease, including protective variants (Table 1-1). PrP's centrality to prion disease has also been compellingly confirmed through animal genetics, as described in detail in the next section.

Table 1-1: PRNP variants of note. *The listed pathogenic variants are the ten most common, together accounting for roughly 86% of genetic prion disease cases. A small handful of other variants seen only in one family also have strong evidence of Mendelian segregation, as described in Minikel 2018. OPRI refers to octapeptide repeat insertions; all other displayed variants are missense. Penetrance estimates are according to Minikel 2016.*

	***PRNP* variant**	**Historical clinical name**
Pathogenic, high penetrance	E200K[47,48]	gCJD
	D178N-129V[49]	gCJD
	D178N-129M[50]	FFI
	P102L[51]	GSS
	5-OPRI[52]	N/A
	6-OPRI[53,54]	N/A
	P105L[55]	GSS
	A117V[56,57]	GSS
Pathogenic, moderate to low penetrance	V180I[55]	gCJD
	V210I[58]	gCJD
	M232R[55]	gCJD
	***PRNP* variant**	**context**
Protective	M129V	Heterozygotes depleted in sporadic CJD cases[59,60]
	E219K	Heterozygotes depleted in sporadic CJD cases[61,62]
	G127V	V allele enriched in survivors of kuru[63]

Nearly four decades on from the coining of the term "prion"[64], this novel pathogen retains many of its mysteries. The molecular details of prion replication and neurotoxicity are just two of many areas where fundamentals of prion biology remain to be uncovered. But despite these open questions, the prion hypothesis provides a striking illustration of the explanatory power of genetics. It has allowed CJD, FFI and GSS, once unrelated medical curiosities, to be grouped definitely together under a single pathophysiological principle. It also enables action. By identifying PrP as the substrate essential to all prion disease subtypes, the prion hypothesis provides a single, shared target for rationally designed prion disease therapeutics.

Therapeutic development in prion disease

The dual nature of prion disease in the clinic – one of molecular and phenotypic heterogeneity juxtaposed with genetic simplicity –has implications for antiprion drug development. Indeed it is possible that the same progression from complexity to simplicity that has driven our overall conception of prion disease may hold lessons for how this disease may be most fruitfully intercepted.

The transmissibility of prions has long been leveraged to generate unique tools, among them the persistently prion-infected immortalized mouse cells that historically comprise the most widely used system for antiprion drug discovery. A handful of infected lines have been widely used for decades, all originally created by incubating cells with prion-infected tissue. Cells could then be assayed for propagation of prions either through bioassay[65], or through immunodetection of the protease-resistant prion fragments that survive limited protease digestion[66]. From the beginning, prion infection of cells has been idiosyncratic. For unknown reasons, at best only a minority of exposed cells take up prion infection[67]. Attempts to infect immortalized human cell lines with similar protocols have met with a mysterious lack of success, perhaps disadvantaged by the high proportion of protease-sensitive human prions[37] or by the relative rates of prion replication versus cell division[68]. In line with the latter possibility, successful infection of stem cell-derived post-mitotic human astrocytes was recently reported[69]; but by its nature, a non-dividing system may be challenging to scale for drug discovery.

In the absence of a human equivalent, mouse neuroblastoma cells infected with a mouse-adapted laboratory strain of prions have been used for antiprion phenotypic screening. Impressively, in the past decade, such screens have produced a handful of small molecules capable of dramatically delaying time to disease when administered to mice infected with the same prion strain[70–74]. However, when administered to mice expressing the human *PRNP* coding sequence and infected with human CJD prions, these molecules unanimously failed to

impact disease [70,71,74,75]. While the existence of distinct conformationally-encoded prion strains has long been recognized[76–78], these studies provided an ominous glimpse of their full implications for drug development.

Of the small molecules with *in vivo* efficacy against mouse prions, the 2-aminothiozoles (2-AMTs) showed the greatest effect, nearly quadrupling survival time if administered prophylactically[75], and have also undergone the most rigorous characterization. Optimized 2-AMTs showed differing efficacy profiles against mouse-adapted prions[75], were active against chronic wasting disease (CWD) prions[79], inactive against sheep scrapie prions[79], and ineffective against two human sCJD subtypes[70,75]. The mechanism of action is not presently known for 2-AMTs or any of the molecules that have shown *in vivo* antiprion activity, precluding systematic assessment of the factors underlying this pattern of this strain specificity.

More troublingly, even within initially susceptible strains, efficacy proved unstable. Prion strains in the brain appear to consist of a group of conformers, the proportions of which can shift in response to stimuli[78]. Human cases show evidence of this complexity, as molecular profiles within one patient may map to more than one canonical strain[19,21,80,81]. Strain adaptation can be seen in action upon passage of prions from one species to another – if able to convert the endogenous host PrP sequence, the infecting prions adapt to their host and on subsequent passage, drive more aggressive disease[78]. Heightening analogies between prions and viral and bacterial pathogens, such adaptation also appears to provide the basis for conformation-based drug resistance (Figure 1-1). Previously observed in the context of quinacrine treatment of prion-infected cells[82], drug-resistant prions with distinct strain properties eventually led to disease in 2-AMT treated mice[75], and naïve mice inoculated with these prions saw no benefit from 2-AMT treatment[70]. Perplexingly, further empirical testing uncovered that not all prion strains exposed to 2-AMTs develop drug resistance[79], and that not all antiprion compounds discovered through phenotypic screening give rise to drug resistance[74].

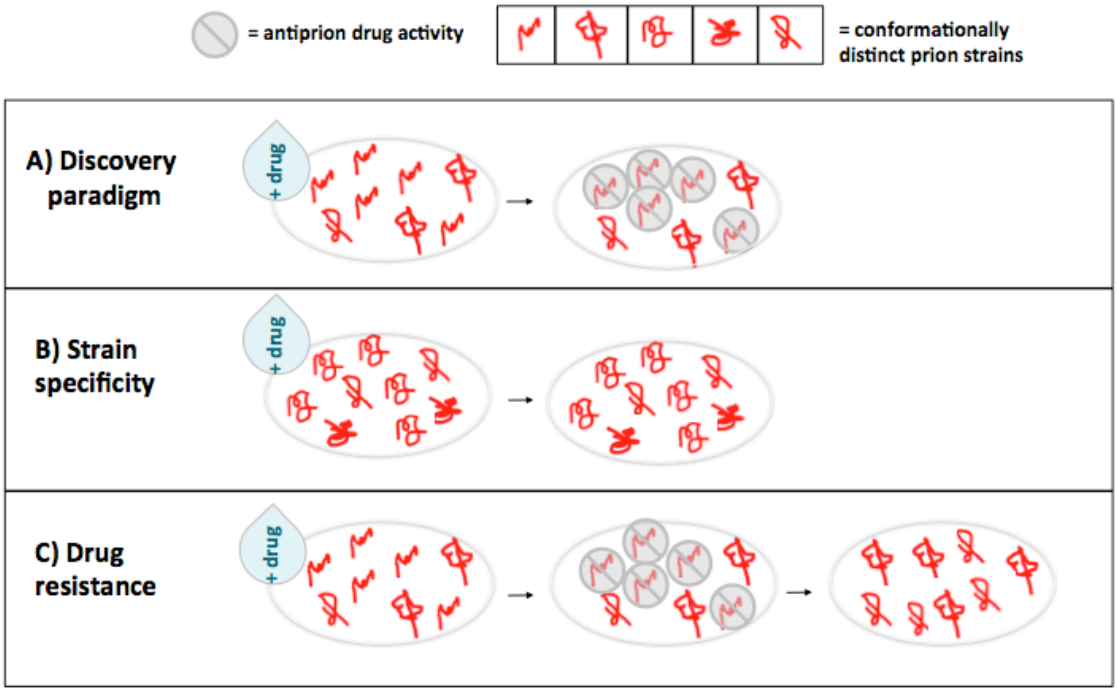

Figure 1-1: Schematic illustrations of two pitfalls of antiprion phenotypic screening. *A) Discovery paradigm: drug-treated, prion-infected cell culture shows reduced prion load when assayed at a fixed timepoint following treatment initiation. B) When tested against a different prion strain consisting of distinct prion conformations, the drug does not impact prion load. C) Efficacy against the initial strain wanes over time as the conformations not targeted by the drug repopulate the prion cloud.*

The above complexities caution that a facile human prion-infected cell system, though of biological interest for the study of prions, would likely not be a drug discovery panacea. Even were a compound found to reduce the prion load in a human cells, neither translation to other human prion strains nor continued efficacy against the original strain could be assumed. Strain specificity and drug resistance could only be assessed empirically, likely through bioassay, as cell models appear unable to fully predict *in vivo* development of drug resistance[70]. One implication is that a lead molecule would need to be developed to the point of reasonable *in vivo* pharmacokinetics and tolerability prior to first assessment of a potentially fatal strategic liability. Under foreseeable constraints, such a pipeline is not practical. Similarly, the structural determination of a human prion, while it could greatly enrich prion biology at large, might hold

surprisingly little immediate translational potential. A full roster of an unknown number of possible human prion structures would be needed to predict the pan-strain applicability of a conformationally targeted therapeutic, and even such a resource could not speak to the potential for adaptive emergence of alternate prion conformers.

In summary, as the biological mysteries of the strain phenomenon deepen, so would the prospects for a near-term advanceable therapeutic targeting misfolded prions, either directly or indirectly, seem in tandem to wane. As targets for primary drug discovery, prions draw too much advantage from their currently uncharted molecular diversity.

In contrast, genetics offers an alternate lens on prion disease therapeutics, one oriented around the prion protein as a shared target across all prion disease. Because we know unequivocally that PrP causes prion disease, one could aim to target this molecule before it begins to misbehave – while it exists as a comparatively well-defined, tractable and singular precursor, be it at the level of DNA, RNA or natively folded protein. By shifting our focus to PrP, we can choose to fight prion disease as one disease instead of many.

Over the past few decades, mouse genetic studies have convincingly demonstrated that endogenous PrP expression is required for prion disease. Full knockout animals do not develop disease following intracerebral inoculation with prions[83], reduced gene dosage extends time to disease, and overexpression accelerates time to disease[84]. In transgenic animals expressing mutant PrP sequences associated with genetic prion disease, time to symptoms is correlated with transgene copy number[85]. Conditional depletion of PrP using Cre[86] or Tet-off[87] systems recapitulates the protective effect of constitutive knockout, and if PrP-expressing tissue is grafted into a knockout mouse brain that is challenged with prions, only the graft is vulnerable to prion-induced degeneration[88]. These studies suggest that reduction of PrP should be an effective strategy for treatment or prevention of prion disease.

Multiple lines of evidence also suggest that reduction of PrP should be well tolerated. In 1992, PrP knockout mice were first reported, with evident surprise, to be grossly

developmentally and behaviorally normal[89]. Studies of additional independent PrP knockout mouse lines[90,91], PrP knockout cows[92], knockout goats[93], and goats with naturally occurring loss-of-function mutations in both *Prnp* alleles[94,95] have since corroborated this finding. Though many possible knockout phenotypes have been explored in the literature[96], it took more than twenty years for one such phenotype to robustly reproduce across knockout lines and trace to a plausible molecular mechanism[91]. It now appears that an N-terminal PrP fragment binds the G protein-coupled receptor Adgrg6, expressed on peripheral Schwann cells, to promote myelin maintenance[97]; under appropriate tests, full knockout mice display a corresponding age-dependent peripheral neuropathy[98]. This phenotype appears mild, has no known corollary in the central as opposed to peripheral nervous system, and is not seen in heterozygous knockout mice[98], supporting the overall tolerability of reduction of PrP gene dosage in the brain. Most recently, humans with one loss-of-function allele for *PRNP* have been identified; these individuals are healthy[99], and are seen at rates consistent with a lack of purifying selection against these mutational events[100].

These lines of evidence support PrP as a drug target in prion disease, and motivate the search for an appropriate modality. Efforts to discover small molecules that directly interact with the native prion protein have not yet generated reproducible monovalent binders, owing perhaps to the difficulty of discovering a small molecule that specifically binds a partially unstructured protein with no known biologically relevant cavities in its small globular domain[101]. Parenthetically, while it appears reasonably safe to conceive of the native prion protein as a single target based on high similarity between crystal and NMR structures of wild-type and mutant recombinant constructs[102], it remains possible that this is an oversimplification. Some studies have suggested that a larger proportion of mutant PrP molecules may occupy a partially unfolded intermediate state *in vitro*[103], with unknown implications for binding *in vivo*. Full clarification may await identification of a small molecule probe specific to PrP.

Phenotypic screens for small molecules that modulate PrP levels in cells to date have not resulted in compelling leads[104,105]. However, a growing number of platform technologies have the ability to sequence-specifically modulate DNA and/or RNA, including antisense oligonucleotides (ASOs)[106–108],RNA interference[109,110], zinc finger repressors[111], and CRISPRi[112]. These technologies could, in principle, be leveraged to reduce production of PrP, and are distinguished by varying levels of maturity in terms of practical near-term human application, particularly to whole-brain disorders like prion disease. For any of the above, a non-allele specific approach to globally reduce all PrP will likely be preferable to specifically targeting genetic prion disease associated mutant alleles, for both strategic and scientific reasons. Beyond the comparative feasibility, particularly in a rare disease, of advancing one drug rather than many, wild-type PrP is itself a liability, with evidence of conformational conversion not only in sporadic but also in some genetic cases[113–116].

Chapters 2 and 3 of this thesis will describe the practical advantages of ASOs as a potential first PrP-lowering therapeutic, as well as our ongoing efforts to develop this therapeutic modality for prion disease.

Patient populations in prion disease

As a therapeutic hypothesis, then, reduction of PrP powerfully unites the different subtypes of prion disease. However, looking ahead to therapeutic trials, there are still two distinct patient populations to account for – distinguished not by drug target, molecular pathology or phenotype, but by the practical clinical consideration of when in the disease course they can realistically be identified and treated. More so than in more slowly progressive diseases, in prion disease radically different considerations govern the prospects for trials in symptomatic patients compared to pre-symptomatic carriers of high-risk genetic mutations.

Symptomatic prion disease

Symptomatic prion disease is characterized by its blistering tempo: the average patient progresses from first, often minor symptoms to death in five months[117,118]. As this figure does not capture time spent of life support, it may yet overstate the window during which meaningful quality of life remains to be preserved[119]. Moreover, owing to disease rarity and heterogeneity of early symptoms, more than half this time is typically lost searching for a diagnosis[118], meaning that by the time they are identified patients are usually severely debilitated. At this advanced stage, secondary pathologies may limit life even if the primary disease pathology could be addressed.

The Medical Research Council (MRC) prion disease rating scale, which is used to track progression of symptomatic prion disease[119], provides an informative view into the typical course. The scale's development was exhaustively tailored to prion disease cases, incorporating data from almost 2,000 assessments of more than four hundred patients. Scores are assigned out of twenty possible points to broadly assess cognitive function, speech, mobility, personal care, feeding, and continence. Tellingly, in the authors' own use of the scale[120], any score less than a perfect 20/20 qualifies as "significant symptom onset"; there is no intermediate stage between a score consistent with perfect health and one reflecting relatively advanced disease. Across more than 200 symptomatic sporadic prion disease patients assessed with this scale, the median score at first assessment was 3 out of 20[119].

Most reports of human testing of prospective prion disease therapeutics describe case reports or observational studies in symptomatic patients, rather than randomized controlled trials[121–124]. Understandable resistance to administration of placebo, on the parts of both clinicians and patients, appears to play a major role in choice of study design[125]. Where patients have been offered the choice between randomization and open label drug access, only a small fraction opt for randomization[122,126]. However, open-label and observational studies suffer from

several known biases, including selection bias; patients with less advanced disease may be more willing to enroll[124,125].

Two relatively large-scale efforts have been made to conduct randomized controlled clinical trials in symptomatic prion disease patients, with an endpoint of survival. These examples highlight the importance of rigorous trial design, as both concluded that the tested agents were ineffective, overturning the positive results that smaller observational studies of the same drugs had previously reported[127,128]. At the same time, both studies illustrate the immense logistical challenges of conducting a randomized controlled trial in symptomatic prion disease patients. A single center randomized trial of quinacrine, reported in 2013[127], made an effort to exclude patients in an advanced state of disease, defined by inability to follow simple commands and swallow. Exclusion criteria, interim deaths, misdiagnosis, logistical difficulties and patient and family concerns combined to yield steep attrition. In the words of the authors, "Many potential or probable sCJD referrals did not want to participate in research, did not respond to follow-up, died before evaluation, were unable to travel, or did not wish to prolong life." Despite 425 referrals, 54 patients were ultimately randomized. At the two-month mark, 32 were alive.

A randomized trial of doxycyline in symptomatic prion disease patients, reported in 2014[128], prioritized broad recruitment. Trial investigators at collaborating centers in Italy and France widened their reach by including, where possible, neurologists able to treat small numbers of patients at local hospitals. Eligibility was not gated by symptom severity, and as a result, many patients were admitted with end-stage disease, including in states of obligate intubation and akinetic mutism[125,128]. Of 663 patients referred, roughly half appear to have been misdiagnosed, and an additional 109 died before randomization could be performed. Ultimately, 121 patients were randomized. As of the first interim analysis, 11 of 97 patients who had received at least one dose of drug or placebo were still alive. The trial was halted for futility. The authors conclude that future trials should make every effort to initiate treatment earlier, but

acknowledge the difficulties in doing so in a symptomatic paradigm in which most patients face "dramatic clinical deterioration and death within a few weeks."

What are the prospects for improving symptomatic trials going forward? The relatively recent development of the real-time quaking induced conversion (RT-QuIC) assay has changed the face of prion disease diagnosis, with RT-QuIC analysis of CSF providing the most specific and sensitive pre-mortem diagnostic test for prion disease[129–131]. Its adoption by national surveillance centers[132] may offer hope for earlier diagnosis of symptomatic prion disease in the future, but such progress is likely to be gradual in the face of substantial barriers to implementation. For RT-QuIC to be performed, a physician must suspect prion disease and order the test, but most neurologists will see few cases of such a rare disease the span of a career, and early symptoms can be misleadingly nonspecific and may not even appear neurological in nature[16,38,118]. In the meantime, the lack of any current standard of care de-prioritizes prion disease in the current differential diagnosis of rapidly progressive dementias[133], and while a treatment could raise the profile of prion disease in the differential diagnosis, such a boost can't be counted on to help reach the first treatment.

Moreover, even if RT-QuIC were leveraged to recruit patients within days of first symptoms, symptomatic trials would still face the confounder of heterogeneity in disease progression. Careful efforts to stratify sporadic prion disease subtypes by *PRNP* codon 129 genotype and molecular signature still offer only imperfect predictions of any one patient's trajectory[119]. In a rare disease where large trials cannot be expected, this heterogeneity may compromise power to detect a therapeutic effect[119]. Genetic prion disease subtypes with slower than average progression may seem to offer a strategic opportunity to ascertain early symptomatic individuals whose courses may be more predictable. But here again, averages disguise a world of variation. For example, the 6-OPRI mutation conveys an average disease duration of 10 years, but disease course has been seen to range unpredictably from two to fifteen years even within one pedigree[34]. In both well documented monozygotic twin pairs with

the relatively slowly progressive P102L mutation, one twin's disease course was twice as long as the other's [31,33].

It would appear that symptomatic trials play to the heterogeneity that has emerged as precisely the prion's greatest weapon. If a treatment capable of stabilizing disease progression already existed, likely some symptomatic prion disease patients would have factors in their favor – early testing by RT-QuIC, slower than average progression – to enable diagnosis early enough for quality of life to be preserved. But the process of conducting a trial to get a first effective drug approved in the first place requires more than anecdotal success – it requires early identification of sufficient numbers of patients facing similar enough prognoses that the expected effect size of a drug can hope to rise above the noise of phenotypic variability. While early and accurate diagnosis is an important priority for many reasons – clarity for families not least among them – improvements in this domain are unlikely to deliver the homogenous symptomatic patient population that would best support a well-powered clinical trial.

Symptomatic trials thus suffer from a host of limitations even without reaching the difficult question of whether extension of life in a state of profound neurological impairment would do a service to patients or families. But there may exist an alternative – another opportunity to leverage the genetic simplicity of prion disease, by intervening before prion disease begins. This proposal amounts to a human-level allegory for the therapeutic hypothesis outlined in the previous section. To avoid fighting an unknowable number of different prion conformations on the molecular level, we can shift our target upstream to native PrP; to avoid fighting an unmanageably diverse set of prion disease manifestations in the clinic, we can shift our treatment population upstream, to pre-symptomatic individuals at risk (Figure 1-2).

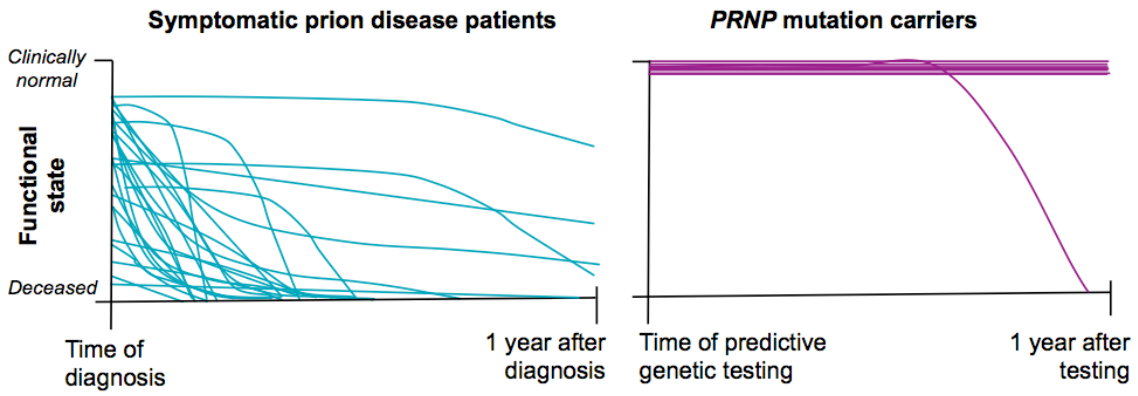

Figure 1-2: Schematic of patient and carrier trajectories. *Schematic of functional decline of symptomatic prion disease patients versus PRNP mutation carriers identified by predictive genetic testing, over one year following diagnosis or testing. Hypothetical symptomatic patient trajectories are informed by Thompson 2013, and assume that the majority of symptomatic patients identified in a given time frame are suffering from sporadic CJD, as has been observed in recent clinical trials*[127,128]*. Age of onset distributions in Minikel 2018 and prospective carrier cohort studies*[120,134,135] *support the assumption that over a 1 year period following a given set of fifty pre-symptomatic PRNP mutation carriers drawn from the adult population, 0-1 symptom onset events would be expected.*

Is it feasible to focus trials on pre-symptomatic individuals? At present, there is no way to identify sporadic prion disease patients prior to symptomatic onset, so this population is not amenable to pre-symptomatic trials. But genetic prion disease may offer such an opportunity. Individuals carrying high-penetrance *PRNP* mutations can be identified years or decades in advance of disease through predictive genetic testing[99]. Chapter 2 will present a regulatory strategy for trials in pre-symptomatic carriers, and propose that such a model offers the most meaningful opportunity to rigorously and swiftly test PrP-lowering therapeutics for prion disease. As pre-symptomatic carriers have no phenotype, such trials may be facilitated by incorporation of biomarker endpoints, a topic further explored in Chapters 4 and 5. Below, I review what is currently known about pre-symptomatic genetic prion disease mutation carriers.

Pre-symptomatic genetic prion disease mutation carriers

Age of onset in genetic prion disease is highly variable, and is not predicted by any known genetic or environmental factors[136]. In other neurodegenerative diseases, including

Alzheimer's disease, Huntington's disease, and frontotemporal dementia, a progressive prodromal period preceding symptom onset by as much as 15-20 years has been characterized by brain imaging or biochemical detection of pathological biomarkers[137–142]. No such changes have been consistently reported in prion disease. Though analogies to other neurodegenerative diseases may tempt the assumption that prion disease onset is likewise foreshadowed years in advance, it is also possible that the whole disease process is as uniquely rapid as its post-onset kinetics may suggest.

While prion-infected animals provide excellent models for some aspects of human disease, the experimental paradigm of horizontal transmission brooks limited analogy to genetic prion disease in humans. Wild-type mice experience a months-long silent incubation period following intracerebral inoculation with prions, during which no signs of disease are manifest despite biochemical evidence that prion titers are exponentially rising in their brains[143]. The earliest detectable pathology in animals with wild-type PrP levels has been reported around 55 days post injection, in a transgenic line that supports monitoring of astrocytic gliosis through live-animal bioluminescence imaging[144]. These findings are difficult to apply to human genetic prion disease, as it is not known presently when prions first form, and over what term they replicate, relative to the decades of healthy life that typically precede genetic prion disease onset. Animal models of genetic prion disease have not been characterized for prodromal changes in advance of symptom onset, but even were changes to be identified in such a model, the translation from mouse to human lifespan would not be obvious.

Available data from human studies suggest that the pre-symptomatic structural and metabolic changes that have been reported in other genetic neurodegenerative diseases are not reliably detectable before symptom onset in genetic prion disease. Most reports of imaging changes prior to disease onset come from incidental case reports of individuals who happened to have an MRI performed for other reasons prior to developing prion disease, and the reported changes were identifiable as prion-related only retrospectively[145–148]. The largest prospective

imaging study reported to date performed annual scans on 101 individuals at-risk for the he *PRNP* E200K mutation, including 50 carriers, for nine years, and observed five onsets. Investigators concluded that the technique and visit frequency did not enable prediction of onset[134]. In another prospective study using serial ^{18}FDG-PET scans on D178N mutation carriers, nine carriers were followed for thirteen years, with four onsets observed. The majority of reported metabolic changes were seen only after symptomatic onset, with indication of pre-symptomatic change reported for only one individual roughly a year before onset[135]. A recent report followed twenty-three P102L mutation carriers for 12 years and observed six onsets. MRI abnormalities, peripheral reflex changes and sensory thermal threshold defects were noted after conversion and could contribute to earlier diagnosis of symptomatic onset, but were not observed in advance of symptoms[120]. Overall, these studies do not provide evidence for a detectable prodrome in healthy carriers.

Fluid biomarkers have not been prospectively studied in genetic prion disease mutation carriers, and mounting evidence from other neurological diseases suggests that biochemical markers may offer early, perhaps the earliest, evidence of prodromal disease process[137,140,149]. It is known that total tau (t-tau) and neurofilament light chain (NfL) levels are elevated in the CSF and serum of symptomatic prion disease patients when compared groupwise to non-dementia controls, and even when compared to patients with non-prion dementias[150–156], However, it is not known when levels of these proteins begin to rise relative to the clinical course. In addition to these general markers of neuronal damage, the RT-QuIC assay provides a prion-specific fluid biomarker. In the context of horizontal prion transmission, either in experimentally inoculated hamsters[157] or naturally scrapie-infected sheep[158], RT-QuIC seeding is known to rise in advance of symptom onset, but as discussed above, the applicability of incubation phase studies to human carriers is not clear. In published reports to date, RT-QuIC has near-perfect sensitivity to detect sporadic prion disease in CSF, and can also detect symptomatic genetic prion disease, albeit with sensitivity that appears to vary by mutation[131,159,160]. Two existing reports describe a

genetic prion disease patient converting from negative to positive by RT-QuIC between clinical lumbar punctures. One was said to convert around the time of symptom onset[155]; the other appeared to convert between 2 and 4 months after onset[159]. To date prospective testing of a cohort of pre-symptomatic genetic mutation carriers has not been reported.

Characterization of genetic prion disease mutation carriers will be an important element of enabling pre-symptomatic trials, which may in turn be critical to meaningful testing of PrP-lowering therapeutics currently under development. In Chapter 5, we describe a clinical cohort of genetic prion disease mutation carriers and controls that we have established at Massachusetts General Hospital, with the goal of characterizing natural history and key biomarker dynamics in this population longitudinally.

Present work in context

Though prion disease today remains universally fatal, focus on the single genetic target that unites all cases of prion disease will soon yield rational therapeutics. Among the many potential technologies that could seek to reduce PrP in the human brain, antisense oligonucleotides have emerged as a practical and well tolerated modality, and the work presented in this thesis supports the advancement of PrP-lowering ASOs as a therapeutic for prion disease. As prion disease faces unique clinical challenges, including its rarity and aggressive pace, meaningful clinical assessment of such drugs may hinge on thoughtful exploration of non-standard clinical trial designs. To that end, this thesis also lays out a clinical and regulatory framework to facilitate meaningful testing of ASOs and future PrP-lowering therapeutics in healthy genetic prion disease mutation carriers.

In Chapter 2, we describe a regulatory strategy to facilitate efficient and meaningful biomarker-based clinical trials of PrP-lowering therapeutics in healthy genetic prion disease mutation carriers. We propose that in a relatively swift and small trial, the ability of an ASO to lower PrP in the brain could be assessed by quantifying PrP in CSF. We further describe

regulatory engagement with FDA to assess whether CSF PrP could serve as an appropriate surrogate endpoint for trials in this population under the Accelerated Approval program. In Chapter 3, we show prophylactic efficacy of PrP-lowering ASOs in prion-infected mice. ASOs are capable of extending healthy life and delaying disease, an effect that is robust across study sites, mouse genetic backgrounds, and prion strains. We further show that ASO-mediated reduction of PrP dose-dependently extends survival. In Chapter 4, we provide evidence that CSF PrP as measured by an available ELISA assay is technically and biologically suitable biomarker of brain PrP with levels suitably stable in one individual over time to enable a drug-dependent decrease to be measured. In Chapter 5, we describe preliminary characterization of a clinical cohort of genetic prion disease mutation carriers, and confirm the within-subject stability of CSF PrP over time in this potential trial population. We further provide evidence that, as a group, genetic prion disease mutation carriers do not harbor consistently detectable prodromal changes that distinguish them from healthy controls, suggesting that preventive strategies will be best informed by genetic risk rather than biochemical signatures of incipient onset.

Together, these advances provide a framework for testing genetically targeted PrP-lowering therapeutics in pre-symptomatic individuals at risk for genetic prion disease, and nominate PrP-lowering ASOs as a possible first such therapeutic.

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Chapter 2: Regulatory strategy for the prevention of genetic prion disease

Publication history:

This chapter is adapted from a white paper presented to 25 scientists at the U.S. Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) at a Critical Path Innovation Meeting on November 11, 2017. This chapter is also being adapted for publication.

Attributions:

I wrote this chapter in collaboration with Eric Minikel, with guidance from Eric Lander and Stuart Schreiber.

Abstract

Prion disease is a rare, fatal, untreatable neurodegenerative disease caused by misfolding of the prion protein (PrP). Most human cases arise spontaneously and are not diagnosed until a state of profound dementia. Roughly 15% of cases are genetic, and predictive genetic testing creates an opportunity for early therapeutic intervention to delay or prevent disease. Direct demonstration of clinical benefit in the presymptomatic population would be impractical. Congruent lines of evidence from biochemistry, human genetics, and mouse models agree that PrP is central to prion disease pathophysiology. Preclinical proofs of concept suggest that a reduction in PrP levels in the brain, potentially achievable using antisense oligonucleotides, would delay disease onset in individuals with pathogenic PrP mutations. We present a proposal made to scientists at the U.S. Food and Drug Administration's Center for Drug Evaluation and Research that PrP load in human cerebrospinal fluid (CSF) merits evaluation as a surrogate endpoint in the context of the Accelerated Approval program, as quantitative demonstration of reduced PrP levels in human CSF is reasonably likely to predict clinical benefit in prion disease. Such an approach could enable, for the first time, rigorously controlled trials in the presymptomatic population in the strongest position to benefit from an anti-prion therapeutic.

Introduction

Prion disease, though currently untreatable, follows a clear pathogenic mechanism, in which a single gene gives rise to a single protein capable of converting into the sole causal disease agent. This mechanistic clarity will soon yield rational therapies. Disease rarity and tempo pose stark and foreseeable challenges to conducting a clinical trial with a clinical endpoint in this indication. Below, we provide evidence that the biology of prion disease is well suited to use of an on-pathway surrogate endpoint to address these challenges while still enabling rigorous and informative trials. The field's ability to advance life-saving therapeutics may critically depend on the thoughtful deployment of such an alternative approach.

The necessity and feasibility of prevention in genetic prion disease

1. The pathogenesis of human prion disease is well understood.

Prion disease is an untreatable, uniformly fatal neurodegenerative disease. Various forms of prion disease in humans and other mammalian natural hosts are noted in Table 2-1. All cases of prion disease trace to the same molecular event, a misfolding of the native prion protein (PrP), encoded by the prion protein gene (*PRNP*). The misfolded protein, known as a "prion," is capable of autocatalytic conformational templating of other PrP molecules. Through such templated misfolding, prions spread exponentially throughout the brain in a conformational cascade recognized for decades as the molecular mechanism driving PrP's disease-state gain-of-function[1,2].

Table 2-1. Other names for prion disease. *Several mammalian species besides humans are natural hosts of prion disease. Different species and different clinical presentations of prion disease are associated with historical names, most of which date to before the molecular mechanism of disease was known.*

species	**name**
humans	Creutzfeldt-Jakob disease (CJD) fatal familial insomnia (FFI) Gerstmann-Straussler-Scheinker disease (GSS) variant Creutzfeldt-Jakob disease (vCJD) kuru Huntington disease-like 1 variably protease-sensitive prionopathy PrP cerebral amyloid angiopathy
sheep and goats	scrapie
cattle	bovine spongiform encephalopathy (BSE or "mad cow")
deer and elk	chronic wasting disease (CWD)
any	transmissible spongiform encephalopathy

Human prion disease is rare: the true annual incidence is estimated at 1-2 deaths per million population[3], although due to under-diagnosis only 200-300 cases are diagnosed and reported in the United States each year[4]. Although prion disease is infamous for the small minority (<1%) of cases acquired through infection[5], the majority (~85%) of cases occur spontaneously, with no known environmental or genetic trigger (these cases are referred to as *sporadic*). The remainder (~15%) arise from dominant, gain-of-function, protein-altering variants in *PRNP* (Figure 2-1A)[6]. Some of these variants are highly penetrant, with lifetime risk approaching 100%, and three such variants account for the majority of genetic cases[6]. Age of onset is highly variable (Figure 2-1B).

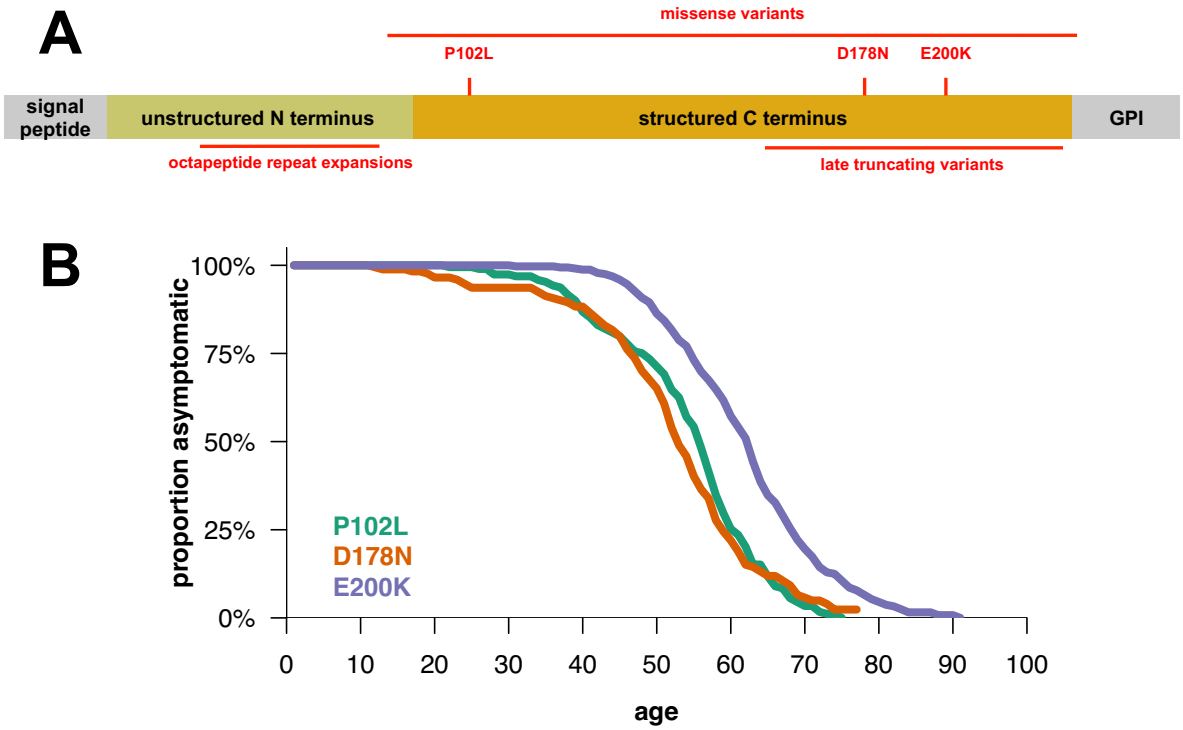

Figure 2-1. Genetic prion disease. *A)* PRNP *contains a single protein-coding exon, with the mature protein of 208 amino acids comprising an unstructured N terminus and a structured C terminus. Over 60 mutations have been identified in patients with prion disease, though only a subset are highly penetrant; three high-penetrance missense mutations (top) account for >50% of cases*[6]*. In addition to missense, pathogenic variants also include expansions of the N-terminal octapeptide repeat region, and late frameshift or stop variants that leave most of the protein while causing a gain-of-function through change in localization*[6]*. B) Survival curves for the three most common genetic prion disease missense mutations*[7]*.*

Among neurodegenerative diseases, prion disease is exceptionally rapid. Patients progress from first symptom to death in a median time of only 5 months[8]. In this short time they rapidly descend into profound dementia, losing the ability to perform all activities of daily living, and typically spending the last weeks of life in a state of akinetic mutism. Throughout, the brain is the epicenter of destruction and the only tissue with a known phenotype. Compounding the rapid course of disease, diagnosis is not reached until, on average, two-thirds of the way through the symptomatic phase[9].

Although rare, prion disease is well understood at the molecular level, with all lines of evidence pointing to the centrality of PrP in prion disease. PrP is unique in all of human biology,

as the only protein ever demonstrated to form a naturally transmissible proteinaceous pathogen devoid of nucleic acid[1], and biochemical, human genetic, and model organism approaches are in agreement that PrP is absolutely required for prion disease (Table 2-2).

Table 2-2. Evidence that PrP is central to prion disease pathophysiology.

category	evidence
biochemical	• Prions, the infectious agent in prion disease, are composed of PrP[10]. • Prion "strains" are encoded in distinct conformations of PrP[9,11–13]. • Prion infectivity can be generated *in vitro* from bacterially expressed recombinant PrP[14].
human genetics	• All multiplex prion disease families possess protein-altering variants in *PRNP*[15]. • Certain missense variants in *PRNP* confer protection against prion disease[16–18]. • *PRNP* is the only locus to exhibit genome-wide significant association to prion disease risk[19].
animal genetics	• PrP is required for prion propagation[20]. • PrP is required for prion neurotoxicity[21]. • PrP dosage and incubation time are inversely correlated[22,23]. • PrP amino acid sequence governs the "species barrier"[24–26].

2. Substantially lowering PrP levels is likely to be a safe and effective strategy to prevent or treat prion disease.

The study of prion disease benefits from excellent animal models, where intracerebral inoculation of wild-type animals with prions leads to fatal disease after a highly predictable incubation time. Experiments in these models have shown that (i) PrP knockout confers complete resistance to prion disease[20], (ii) prion neurotoxicity only affects cells expressing PrP[21], and (iii) postnatal suppression of PrP expression can delay or halt the progression of prion disease[27,28]. Moreover, PrP gene dosage is correlated with the pace of disease across a wide range of expression levels[23], with heterozygous PrP knockout mice surviving prion infection 2.5 times as long as wild-type mice[22]. Similarly, in transgenic mouse models expressing PrP with mutations that cause genetic prion disease in humans, PrP dosage is inversely correlated with age of onset of spontaneous illness[29].

PrP knockout mice are viable, fertile, have normal lifespans, and exhibit normal behavior, initially defying efforts to identify a knockout phenotype[30]. It has recently been found that, in the

peripheral nervous system, PrP undergoes proteolytic cleavage to release a signaling peptide that promotes myelin maintenance[31]. PrP knockout mice develop a slowly progressing demyelinating polyneuropathy, which leads to mild sensorimotor deficits late in life[32]. Heterozygotes are unaffected[32]. No native function has yet been identified in the central nervous system. Knockout cattle[33] and naturally occurring knockout goats[34] are described as phenotypically normal. The few humans with heterozygous loss-of-function variants identified in *PRNP* are healthy[6], indicating that a reduction in *PRNP* gene dosage is well-tolerated in humans.

The above data suggest that lowering PrP levels would be a safe and effective therapeutic strategy for delaying or preventing prion disease. Multiple therapeutic strategies could, in principle, reduce PrP levels, by targeting the *PRNP* gene, RNA, or the mature protein itself. Despite this strong therapeutic hypothesis, however, little drug development has occurred in this area. The four agents that have been advanced to clinical trials in prion disease to date were all existing drugs with no strong preclinical evidence to support advancement into humans[35–41].

3. Prevention of disease in of pre-symptomatic mutation carriers is likely feasible based on preclinical precedent, and would allow extension of healthy life.

No therapeutic intervention administered after onset of symptoms has ever convincingly extended survival in an animal model. In contrast, there do exist proofs of concept for dramatically delaying prion disease by intervening before symptom onset.

Phenotypic screens in prion-infected cells have identified several small molecules that inhibit prion replication by an unknown mechanism of action, and extend survival in animals intracerebrally infected with prions. None are effective against human prion strains[42–45], precluding their advancement to the clinic. Certain high molecular weight, sulfated sugar polymers are also known to inhibit prion replication, but are limited by the infeasibility of broad

delivery to the brain parenchyma[46,47]. Despite their lack of prospects for clinical advancement, these molecules have nonetheless provided important insights into the time dependence of antiprion therapeutic efficacy.

Four of these compounds have been tested in mice with treatment beginning at a battery of different timepoints during the disease course[44,46,48,49]. In each case, the compound was less effective the later it was administered. None was effective after the onset of symptoms. For example, the most thoroughly studied molecule, IND24, quadrupled survival time when administered before prion infection, increased survival time by about 60% when administered after infection but before symptom onset, and had no effect on survival when given after symptom onset. More than 100 other candidate therapeutics have been tested in prion-infected mice[45,50,51], and while most have proven ineffective regardless of disease stage, the few that have shown convincing evidence of efficacy did so only when administered before symptom onset. For example, monoclonal antibodies to PrP[52] and certain metallated porphyrins[53,54] poor at crossing the blood-brain barrier have delayed the neuroinvasion of peripherally acquired prions, but have been ineffective after onset. Intracerebral infusion of polythiophenes nearly doubled survival when given prophylactically, but had marginal effects when initiated around the time of symptom onset[55].

These results are consistent with the understanding of prion disease kinetics as established in animal models. From transgenic mice expressing varying levels of PrP, it is known that after prion infection begins, prion titers in the brain rise exponentially during a clinically silent incubation phase. The rate of prion load accumulation during this incubation phase corresponds to the PrP expression level of the animal, with higher expression resulting in more rapid accumulation and a shorter time to maximum titers of prions in the brain. Critically, symptoms emerge only when prion titers have plateaued[1,56,57]. Thus, not only does post-symptom intervention face the challenge that neuronal loss is irreversible, it also faces a

disease stage that is fundamentally different at the molecular level, compared to pre-symptomatic treatment.

4. Antisense oligonucleotides represent the most feasible near-term strategy for treating prion disease.

While the vision set forth in this chapter should apply to any molecule capable of lowering PrP levels in the brain, the most realistic near-term therapeutic of this nature is likely to be antisense oligonucleotides (ASOs). ASOs are short (17-20 base) single-stranded oligonucleotides, chemically modified for pharmacokinetic stability, that specifically bind a complementary target RNA and can trigger its degradation[58–60].

Efforts are currently underway to develop ASOs against the PrP RNA, involving scientists at NIH (led by Dr. Byron Caughey), McLaughlin Research Institute (led by Dr. Deborah Cabin), the Broad Institute, and Ionis Pharmaceuticals. Survival studies in prion-infected mice have now been conducted at two sites with lead ASOs against mouse *Prnp* capable of reducing PrP mRNA levels by about 50% in the mouse cortex and spinal cord following a single intraventricular dose. Across both study sites, ASO dosing paradigms, mouse genetic backgrounds, ASO sequences and backbone chemistries, ASO molecules that reduce PrP show potent extension of survival (Chapter 3). The protective effect of ASOs appears dose-dependent and has replicated across all prion strains tested (Chapter 3). While additional studies are ongoing, these results suggest that ASOs that reduce PrP are capable of protecting against prion disease, effectively pharmacologically mimicking the protective effect of reduced *Prnp* gene dosage.

Antisense oligonucleotides are uniquely modular drugs. The nucleotide sequences of ASOs specify target binding through Watson-Crick base pairing, yet these sequences are orthogonal to the classes of backbone chemistry that determine many pharmacokinetic and pharmacodynamic parameters[61]. ASOs may modulate their target RNAs by a variety of mechanisms, including RNAse H-mediated degradation of target RNA. At present two ASO

drugs – mipomersen for homozygous *LDLR* mutant hypercholesterolemia, and nusinersen for spinal muscular atrophy (SMA) – have full FDA approval of a New Drug Application (NDA). Another, eteplirsen for exon 51-skippable Duchenne muscular dystrophy, has Accelerated Approval of an NDA. ASOs for neurological applications have been under intensive study, including the recently successfully completed Phase I, II and III trials of nusinersen in children with SMA[62,63] and Phase I/II trial of an anti-Huntingtin ASO (ASO-HTT Rx) in adults with Huntington's Disease (HD)[64,65]. These drug development programs have compiled a wealth of knowledge regarding the behavior of intrathecally delivered ASOs in the CNS.

1. **Delivery.** In nonhuman primates, ASOs delivered by intrathecal infusion or intrathecal bolus injection achieved broad distribution across the brain and 25 percent to 67 percent knockdown of target mRNA across brain regions including the cortex, striatum, hippocampus, pons, and spinal cord[66,67]. Both the SMA and HD clinical studies relied on bolus ASO delivery by intrathecal injection.
2. **Safety and tolerability.** Published results from the Phase I escalating dose study of intrathecally delivered nusinersen show no safety or tolerability concerns[62]. Notably, this trial was performed in a population of 2-14 year old symptomatic SMA patients, in whom scoliosis and spinal abnormalities are common, making lumbar punctures more challenging than in healthy adults such as presymptomatic *PRNP* mutation carriers. Across five ascending dose cohorts, ASO-HTT Rx was likewise reported to be well tolerated in individuals with early symptomatic Huntington's disease; the mostly mild observed adverse events were unrelated to the drug[64].
3. **Time to effect.** ASO activity is reflected in target mRNA levels within 14 days of treatment in rodents and declines by 4 months[66]. Lag in protein levels depends upon the half-life of the protein in question. PrP has an estimated *in vivo* half-life of 18 hours[28], indicating that ASO-based mRNA depletion could quickly impact PrP at the protein level.

4. **Wash out period.** The *in vivo* half life of nusinersen in CSF was estimated at 132-166 days[62]. The ASO-HTT-Rx Phase I/II study was designed to dose once a month[64], and the planned Phase III study will contain monthly and bimonthly treatment conditions[68]. Such periodic dosing offers the opportunity to discontinue administration should adverse events arise.

Together, these findings suggest that intrathecal ASOs have been sufficiently de-risked that, provided preclinical toxicity studies are favorable, a trial in healthy pre-symptomatic individuals would not expose subjects to unreasonable risk.

5. It is appropriate for trials in pre-symptomatic genetic prion disease mutation carriers to use PrP levels in cerebrospinal fluid as a surrogate endpoint, on the strength of PrP's role as the sole necessary and sufficient precursor of the infectious agent in prion disease.

In some cases, one can test a therapeutic against a neurodegenerative disease by conducting a trial in pre-symptomatic individuals with the goal of demonstrating a direct clinical benefit. For example, the Alzheimer's Prevention Initiative is following 300 randomized participants with the *PSEN1* E280A mutation being treated with crenezumab, a monoclonal antibody against amyloid beta, with a cognitive endpoint after five years[69,70].

However, there is no currently realistic route to conduct a trial to directly demonstrate clinical benefit in pre-symptomatic individuals with *PRNP* mutations. Designing such a trial would be infeasible for several reasons.

1. **Recruitment numbers.** Recruitment for the crenezumab trial was made possible by the existence of a single extended family of more than 5,000 individuals; as of October 2016, 1,065 living mutation carriers from this extended kindred had been genotyped through the Columbian Alzheimer's Prevention Initiative Registry[71,72]. No comparably large family exists for any genetic prion disease mutation. Indeed, for the three most common high-

penetrance *PRNP* mutations combined, only 1,001 cases have ever been ascertained in the U.S., Europe, Japan, and Australia in total over the 15-20 years that prion surveillance networks have been in effect[6].

2. **Predictability of age of onset.** Age of onset for *PSEN1* E280A has a standard deviation of ~6.4 to 8.6 years[73,74], whereas for the three highly penetrant *PRNP* mutations presented in Figure 1, the standard deviation of age of onset ranges from 10.0 to 11.8 years[75,76]. The crenezumab trial also relies on a correlation between parent and child age of onset that has been reported in *PSEN1* Alzheimer's disease[74], but such a correlation is not observed in prion disease[75]. Both of these factors mean that a larger or longer trial would be required to assess clinical benefit in genetic prion disease.
3. **Economic incentives for drug development.** The economic return on genetic prion disease is low. The cost of the crenezumab trial in Alzheimer's disease is estimated at $96 million[69]. The sponsor has surely weighed the cost of this trial against the prospects for widespread off-label use and/or eventual expanded labeling in late-onset Alzheimer's disease if crenezumab were approved. A drug to lower PrP would have no prospects for off-label or expanded use outside of prion disease, making such a costly trial untenable.

Some have sought to design smaller or shorter prevention trials in Alzheimer's disease by using biomarkers to selectively enroll individuals whose onset can be predicted[70]. At present, however, no reliable imaging or biochemical biomarkers exist that could serve this purpose for prion disease. Three prospective cohort studies of pre-symptomatic genetic prion disease mutation carriers have reported changes detected concurrently with or shortly after symptomatic onset, including changes in brain structure[77], brain metabolism[78], and neurophysiological measurements[79]. But these changes were not consistently seen prior to symptom onset. At most, despite regular pre-symptomatic monitoring of cohorts ranging in size from nine to fifty carriers over terms up to twelve years, suggestive changes were noted in single individuals, no

earlier than about one year prior to symptom onset[77,78]. A handful of case reports describe individuals who underwent brain MRI scans for other reasons before going on to develop prion disease; these likewise report abnormalities only less than or roughly one year before symptoms, on balance too subtle to be notable except in hindsight[80–83].

A set of cerebrospinal fluid biomarkers are known to be elevated in symptomatic prion disease, including two markers of neuronal damage, total tau and neurofilament light chain, and prion "seeds", operationally defined by their ability to trigger fibrillization of recombinant PrP *in vitro*.[84–86]. We are prospectively evaluating these potential biomarkers in a cohort of pre-symptomatic genetic prion disease mutation carriers, but data to date suggest that these tests do not reliably distinguish carriers from non-carrier controls (Chapter 5). Even if such a biomarker could be identified, establishing its predictive value would be logistically challenging given the rarity of genetic prion disease combined with the variability in age of onset. Validation would require observation of at least several onsets, an exercise likely to take well upwards of a decade in a cohort realistically sized at tens of carriers[77–79]. While illuminating the natural history of genetic prion disease is a valuable long-term project, today's exploratory biomarkers, even should they prove to predict disease onset, are not likely to be validated in time for use in near term clinical trials.

Most importantly, even if a biomarker to enrich for individuals close to onset were available, preclinical proofs of concept indicate that this approach would specifically **enrich for those individuals least likely to benefit from a drug**, as prion amplification and neuropathology have already begun.

In view of the infeasibility of demonstrating clinical benefit in pre-symptomatic people, we propose that PrP levels in cerebrospinal fluid should be considered as a surrogate endpoint for evaluating the efficacy of PrP-lowering therapeutics.

Background on the U.S. FDA Accelerated Approval program

Since passage of the Food Drug and Cosmetic Act (FDCA) in 1938, the U.S. Food and Drug Administration has evaluated new drugs for approval based on safety. In 1962, the Kefauver-Harris Drug Amendments created the requirement for affirmative pre-marketing approval based not just on safety but also on efficacy, as supported by "substantial evidence"[87]. By law, "substantial evidence" is defined as "evidence consisting of adequate and well-controlled investigations, including clinical investigations" by qualified experts[87]. The modern stages of drug development, including the three traditional clinical trial phases, have evolved on the foundation of these requirements[88].

Under traditional approval mechanisms, a drug's efficacy is established in a clinical trial through demonstration of direct benefit to patients, as captured by a clinical endpoint. A clinical endpoint "directly measures a therapeutic effect of a drug – an effect on how a patient feels (e.g., symptom relief), functions (e.g., improved mobility), or survives"[89]. Such endpoints directly report on the patient experience. Alternatively, trials can employ a validated surrogate endpoint – a laboratory measurement that is not directly perceived by the patient, but is "known to predict clinical benefit," such as blood pressure for cardiovascular disease[89,90]. In practice, validated surrogate endpoints are typically informed by previous clinical trials in a given indication, which tracked the surrogate marker in parallel to a traditional clinical endpoint[91].

In the context of rising costs and timelines per approved drug[92] as well as increasingly sophisticated insights into disease biology, FDA has created a number of expedited drug approval programs in recent decades. All focus on serious diseases with unmet medical needs, motivated by the recognition that lack of a meaningful existing standard of care informs how risks and benefits are weighed both by patients and physicians[89]. Three such programs – the Priority Review, Fast Track and Breakthrough Therapy designations – prioritize qualifying projects for earlier, more flexible, and more frequent review with FDA scientists[88,89]. The

Accelerated Approval program is unique in that it establishes a new kind of approvable endpoint, allowing for provisional approval of new drugs on the basis of a surrogate biomarker that lacks previous clinical validation, but is deemed "reasonably likely" to predict clinical benefit[93]. Clinical benefit is subsequently assessed in a post-marketing, so called “phase 4” trial[93].

Accelerated Approval was established through FDA regulation in 1992 in the context of the HIV/AIDS crisis, before being codified by Congress in the FDA modernization Act (FDAMA) in 1997[93]. Inspired by concerted advocacy by HIV/AIDS patients, the mechanism was used to speed patient access to new AIDS drugs based first on the surrogate endpoint of blood CD4 white cell count, then on PCR-based measurement of HIV “viral load.” The latter biomarker provided the backbone for approval of the protease inhibitors used in highly active retroviral therapy (HAART) drug cocktails, and is still used to evaluate AIDS drugs today[94]. Patient advocacy subsequently fueled expansion of the mechanism into cancer indications, with tumor shrinkage and a number of other measures serving as surrogate endpoints in different cancer subtypes[90,95].

To date, the majority of drugs approved through the Accelerated Approval pathway have pertained to either HIV/AIDS or cancer[96]. With the 2012 passage of the FDA Safety and Innovation Act (FDASIA), Congress urged FDA to enhance its application of this mechanism to rare diseases, leveraging the "unprecedented understanding of the underlying biological mechanism and pathogenesis of disease" provided by modern scientific tools[93]. In light of the unique constraints governing rare disease drug development, Congress envisioned a "broad range of surrogate or clinical endpoints" used to conduct "fewer, smaller, or shorter clinical trials" without reducing FDA's standards for safety and efficacy. FDASIA specifies that a lack of viable alternative clinical trial designs should be considered in review of surrogate endpoints: "[FDA] shall consider 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"[93]. FDA has indicated that its

criteria for increased flexibility on acceptance of surrogate biomarker endpoints will include the "severity [and] rarity" of the disease, as well as "the extent to which the pathophysiology of a disease is understood" and how the biomarker fits into that disease pathway[89]. Rare disease advocates have noted that the Accelerated Approval pathway is a particularly good fit for rare neurological diseases, as their clinical presentations can be difficult to predict or quantify, while their progression is irreversible[97]. Indeed, it has been argued that in some such cases, rather than a compromise, a biomarker endpoint may represent a more precise, quantitative and immediate readout of treatment efficacy than a clinical endpoint could avail[97]. Genetic prion disease, with its genetic and molecular clarity and clinical heterogeneity, offers precisely one such case.

Application to prion disease

PrP lies directly on the sole pathway of prion disease pathophysiology (Figure 2-2), with all available lines of evidence agreeing that PrP is required for disease initiation and progression, in a dose-dependent manner. Our understanding of prion disease biology is sufficiently strong that a reduction in PrP abundance in pre-symptomatic people can be deemed very likely to predict clinical benefit.

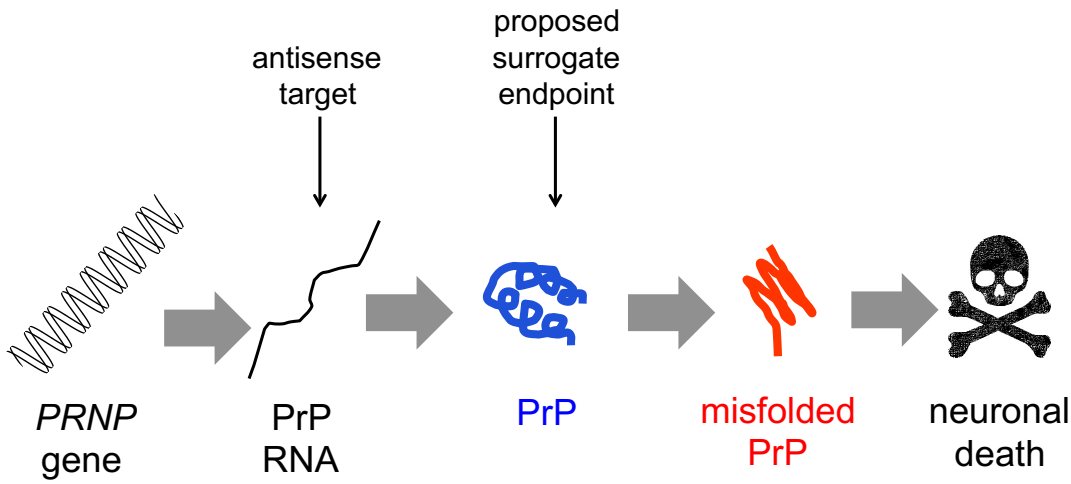

Figure 2-2. Drug target and proposed surrogate endpoint within the pathophysiological pathway of prion disease. *PrP lies directly on the sole pathway of prion disease.*

Prions are, to date, the only established naturally transmissible protein-only pathogen. Conceptually, prion disease resembles an infectious disease, driven by the formation and replication of this discrete infectious agent. The native prion protein, PrP, is the pivotal molecule on this disease pathway, as the sole necessary and sufficient precursor for pathogen formation. PrP levels dose-dependently predict time to prion disease onset, as documented by decades of genetic studies in mammalian hosts of prion disease. “PrP load” as a surrogate endpoint for prion disease is therefore analogous to “viral load” in HIV. Prion disease is no less deadly today than HIV in the early 1990s, and its causality is no less clear.

Direct sampling of brain, the tissue of interest for prion therapeutics, is impractical, but PrP is abundant in human cerebrospinal fluid (CSF), with a concentration on the order of tens to hundreds of nanograms per milliliter detected using commercially available ELISA kits[98–101]. Levels vary by as much as an order of magnitude between individuals[101], but intra-individual variability appears lower. We have found a mean coefficient of variation of only 8.2% in uniformly processed pairs of serial within-subject CSF samples donated by pre-symptomatic genetic prion disease mutation carriers at 2-4 month intervals (Chapter 5). PrP is much less

abundant in blood than in cerebrospinal fluid, suggesting that the detected PrP is primarily brain- rather than blood-derived, and therefore more likely to reflect any therapeutic reduction in brain PrP (Chapter 4). Corroborating data from a mass spectrometry-based method we have developed for orthogonal quantification of CSF PrP supports the ELISA assay's ability to specifically quantify total PrP in CSF[102].

CSF PrP levels are reduced in individuals with symptomatic prion disease compared to controls or patients with other dementias[101], perhaps reflecting the sequestration of normally extracellular PrP within the endosomal-lysosomal pathway where prions are formed[103–105]. For this reason, a reduction in CSF PrP could be difficult to interpret in symptomatic patients, as it could reflect either an intended effect of treatment or simple progression of disease. This provides another motivation for initiating treatment in pre-symptomatic individuals who are, on expectation, years from onset of disease. Our natural history study supports the test-retest stability of CSF PrP in carriers across ages and mutations, even up to the appearance of prion seeding activity in CSF (Chapter 5). It is possible that despite being probabilistically far from onset as a group, some of these individuals may convert to symptomatic during the course of a biomarker-based preventative trial. The clinical onset of prion disease is a rapid event in which individuals progress from first symptoms to dementia on a timescale of weeks[8]. Therefore, symptomatic individuals can be readily identified and excluded from analyses of treatment-based PrP reduction, to ensure that changes in PrP levels are attributable to treatment as opposed to symptom onset.

FDA engagement

As we considered an Accelerated Approval strategy, we took advantage of the FDA's Critical Path Innovation Meeting (CPIM) mechanism to request an in-person meeting with FDA scientists regarding the prospects for Accelerated Approval for a genetic prion disease drug that lowers CSF PrP, based on the considerations above. In November 2017, we traveled to the

FDA's headquarters in Silver Spring, Maryland and met with 25 of the agency's scientists. The FDA scientists were supportive of the concept, offered constructive questions and ideas about appropriate biomarker and preclinical data that would be needed, and generously offered to provide continued input. While the FDA does not make commitments under the CPIM mechanism, we felt the process was a model for regulatory partnership in rare-disease drug development, and we are continuing to work closely with FDA as we gather further data in support of this biomarker and clinical strategy.

Next steps

We are expanding our efforts to collect samples from pre-symptomatic individuals with *PRNP* mutations in order to continue characterizing CSF PrP levels in this population, including over a longer term. We have performed a prophylactic ASO dose response experiment in prion-infected mice, and observed that even the lowest ASO dose, corresponding to a 20% reduction in brain *Prnp* RNA, significantly delayed onset of disease (Chapter 3.) We are currently developing a rodent PrP ELISA assay to enable protein-level quantification of the brain PrP reduction corresponding to each ASO dose. This assay will also be used to correlate changes in brain and CSF PrP levels following ASO treatment. Together, these experiments will help to establish the effect size threshold that would merit Accelerated Approval.

Pending the results of these further experiments, it is our hope that a meaningful reduction in CSF PrP in pre-symptomatic *PRNP* mutation carriers, demonstrated through an adequate and well-controlled trial (such as a double-blinded, placebo-controlled study) could constitute a basis for Accelerated Approval. Confirmatory post-marketing studies could then further assess the clinical benefit of a PrP-lowering therapeutic. To ready the field for preventive trials, we must work now to lay appropriate groundwork. This means:

1. **Offering forward-looking advice on predictive genetic testing.** At-risk individuals are routinely counseled against learning their mutation status, and less than a quarter of individuals at risk currently pursue predictive genetic testing[106]. But in this rapidly progressive disease, being able to make an informed decision in advance of symptoms may be essential to accessing and benefiting from plausible near-term therapeutics. As ASOs continue to move towards the clinic, it is more important than ever to counsel the pros as well as cons of predictive testing for prion disease mutations.
2. **Referring healthy mutation carriers to prionregistry.org.** Healthy prion disease mutation carriers and those at risk, unlike symptomatic patients, are not typically ascertained by hospitals or clinics. In July 2017, in collaboration with the patient organizations CJD Foundation and CJD International Support Alliance, we launched a simple online portal called the Prion Registry (prionregistry.org) in part to fill this gap. The registry aims to be a location-agnostic, researcher-agnostic resource that provides information about research studies and trials to patients, carriers and families on an opt-in basis. In the process of registering, individuals fill out a simple survey that helps researchers and trialists estimate how many carriers fall within, for example, certain age ranges or geographical areas. Referring at-risk individuals to this centralized platform in advance of preventive trials will help to motivate drug development partners across sectors, and to facilitate swift trial recruitment when the time comes.

Today, there exists no disease-modifying therapeutic for any adult-onset neurodegenerative disease. Calls are mounting for therapeutic efforts to aim earlier in the disease process[107–110], but the tractability of doing so varies across indications based on disease biology and available tools. Genetic prion disease has unique assets: a single causal gene and protein, robust genetic proofs of concept supporting a knockdown therapeutic strategy, and faithful animal models in which this strategy can be honed. These insights and resources

align to present a unique opportunity to shift therapeutic intervention upstream, beyond early symptoms or preclinical pathology, to genetically informed primary prevention. The unique constraints of genetic prion disease make it an ideal test case for a concept with resonance well beyond this one rare indication: the leveraging of predictive genetics to rewrite the future, and keep healthy people healthy.

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Chapter 3: Prophylactic treatment of prion-infected mice with prion protein-lowering antisense oligonucleotides

Publication history:

Some experiments included in this chapter are also included in a manuscript recently submitted for publication*: Raymond GJ et al. Antisense oligonucleotides extend survival of prion-infected mice after prophylactic or near-clinical treatment.* The remaining experiments will be published in a future manuscript.

Attributions:

This chapter includes experiments performed at the following three sites, in collaboration with a number of scientists:

1. The Broad Institute. Sonia Vallabh, Eric Minikel, Tyler Caron (veterinarian), Jason Le (veterinary technician), Samathan Graffam (veterinary technician), Broad Comparative Medicine team.
2. Ionis Pharmaceuticals. Holly Kordasiewicz (Executive Director, Neuroscience), Hien Zhao (Associate Director, Neuroscience), Curt Mazur (animal lead).
3. NIH/Rocky Mountain Labs. Byron Caughey (PI), Greg Raymond (staff scientist), Lynne Raymond (staff scientist), Brent Race (staff scientist).

I led design and interpretation of experiments conducted across sites. I performed experiments at the Broad Institute jointly with Eric Minikel. I supported data analysis and visualization, which were led by Eric Minikel.

Abstract

Human prion disease is a currently untreatable neurodegenerative disease that is swiftly fatal following the onset of symptoms. All cases are caused by post-translational misfolding the prion protein, or PrP, into a proteinaceous pathogen called a prion. Genetic proofs of concept support the therapeutic strategy of reducing PrP expression in the brain. Here we show prophylactic efficacy of antisense oligonucleotides (ASOs) that reduce brain PrP by targeting the RNA precursor of PrP. When administered to mice beginning prior to intracerebral prion inoculation, PrP-lowering ASOs extended survival by 61% - 108% by delaying symptom onset, an effect replicated across different ASO chemistries, mouse genetic backgrounds, dosing regimens, and study sites. ASO-mediated PrP lowering conveyed dose-responsive protection, showed efficacy against five mouse-adapted prion strains and did not appear to cause drug resistance. These data support the hypothesis that PrP-lowering ASOs may delay onset of genetic prion disease in pre-symptomatic carriers of high-risk genetic prion disease mutations.

Introduction

Human prion disease is a rapid, universally fatal neurodegenerative disease that can be sporadic in origin, genetic, or in rare cases, acquired. Different clinical names are used for human prion disease, including Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker disease (GSS)[1]. Regardless, all cases are caused by the autocatalytic spread of misfolded prion protein, PrP, encoded by the gene *PRNP*[2]. Reduction of PrP is a longstanding therapeutic hypothesis in prion disease. Genetic knockout and conditional depletion of PrP are protective against disease[3–5]. In both exogenously infected and genetic models, *Prnp* gene dosage in animals correlates with time to onset of prion disease[6–8]. Meanwhile multiple lines of genetic evidence, including from heterozygous knockout humans, suggest that reduction of PrP dosage should be well tolerated[9–12].

Antisense oligonucleotides (ASOs) represent one potential therapeutic modality for reduction of a single disease-causing protein in the brain. ASOs are chemically modified nucleotide sequences that can be targeted to bind an RNA of interest through Watson-Crick base-pairing[13,14]. While ASOs can be designed to modulate target RNAs in a variety of ways, their canonical mechanism of action is degradation of the target mediated by recruitment of RNAse H[15–17]. ASOs targeting the prion protein RNA have been investigated in one previous study. Though a 40% extension of survival was observed when mice were dosed 1 day following prion infection, multiple complications arose surrounding poor tolerability of the implanted osmotic pump used for dosing, as well as the single ASO tested *in vivo*[18]. In light of reports in the prion literature of phosphorothioated oligonucleotides sequence-independently binding PrP[19,20], further ambiguities arose as to mechanism of action, and the project was terminated.

Since that time, ASOs have matured as a therapeutic modality for the central nervous system (CNS), and have been rigorously characterized in both non-human primates (NHPs) and

humans in the context of ASO drugs currently approved or in trials for multiple CNS indications including spinal muscular atrophy, Huntington's disease, amyotrophic lateral sclerosis, and tauopathies[21–26]. Periodic bolus dosing has emerged as a superior delivery method of ASOs to the CNS, and additional data gathered in this paradigm support ASO tolerability, broad brain distribution, and stability up to several weeks in the brain[27]. Clinical approaches to prion disease are also evolving. Advances in prion disease genetics[12] and a trend over time towards increased predictive genetic testing[28] have sparked interest in the potential for pre-symptomatic intervention in cases where individuals at high genetic risk for prion disease can be identified in advance of symptoms.

Informed by these developments, here we revisit ASOs as a therapeutic for prion disease. Specifically, we assess bolus-delivered PrP-lowering ASOs in a prophylactic paradigm to assess their ability to delay or prevent the onset of prion disease. We demonstrate prophylactic efficacy of ASOs at two study sites following treatment prior to infection with prions, corresponding to extension of both survival and healthy life. We demonstrate that the benefit of PrP-targeting ASOs is mediated by sequence-specific reduction of PrP RNA, confirming their relevant mechanism of action *in vivo*. We further show a continuous dose-dependent relationship between ASO-mediated PrP-lowering and delay of disease. Finally, we show that ASO efficacy is consistent across multiple prion strains, and that treatment does not give rise to drug dependent prions.

Results

Discovery and characterization of candidate ASOs

ASOs complementary to mouse *Prnp* RNA were designed using a previously characterized set of chemical modifications[14] (Figure 3-1) chosen for their established pharmacological profiles and history of use in the central nervous system[27,29]. A mix of phorphorothioate (PS) and phosphidiester (PO) linkages were incorporated into the nucleotide

backbone to promote stability, while two 2' sugar modifications, (S)-constrained ethyl (cET) and 2'-O-methoxyethyl (MoE), were incorporated at strategic positions to balance their conformational benefits with their inhibition of RNAse H cleavage[14]. Potent ASOs with both cET/MOE and MOE-only sugar modifications (hereafter referred to as cET and MOE ASOs, respectively) were selected for *in vivo* screening, as was one non-targeting control of each chemistry (Table 3-1). Selected ASOs variously target *Prnp* intron 2, the PrP coding sequence, and the *Prnp* 3'UTR, as described in a companion study[30].

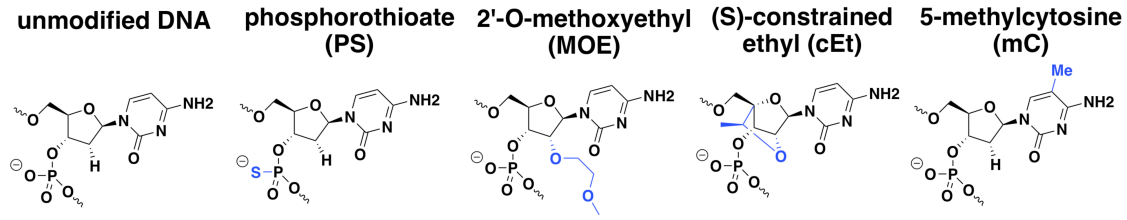

Figure 3-1: ASO chemistries used in this study. *Schematics of nucleotide backbone, base and sugar chemistries incorporated into ASO molecules used this study. Chemical modifications, compared to unmodified DNA, are shown in blue.*

Table 3-1. ASOs designed against the mouse Prnp RNA. *Normal phosphodiester (PO) nucleotide linkages are indicated with (o). All other linkages are phosphorothioate (PS). Sugar chemistry follows the following color scheme: 2' methoxyethyl (MOE), 2' H (unmodified DNA), 2'-4' constrained ethyl (cET). mC indicates 5-methylcytosine. ASO chemistries have been described previously*[14].

ASO name	sequence and chemistry	target
active ASO 1	mCToAoTTTAATGTmCAoGoTmCT	*Prnp* 3'UTR
active ASO 2	TToGomCAATTmCTATmComCoAAA	*Prnp* intron 2
control ASO 3	mComGomCTATAmCTAATomCoATAT	none
control ASO 4	CCoToAoTAGGACTATCCAoGoGoAA	none
active ASO 5	TToGoCoAATTCTATCCAAoAoTAA	*Prnp* intron 2
active ASO 6	CToToCoTATTTAATGTCAoGoTCT	*Prnp* 3' UTR

Intracerebral ventricular (ICV) injections of all active ASOs were performed in groups of N=4 wild-type C57BL/6 mice. Reduction PrP RNA was observed by RT-PCR in microdissected cortex and thoracic cord 2 weeks following injection of either 300 µg or 700 µg of all active ASOs (Figures 3-2A and 3-2B). Serial RT-PCR assessment following single bolus injections of

500 µg of a subset of ASOs showed suppression of cortical *Prnp* RNA for 84 days after dosing followed by a return roughly to baseline levels by 112 days (Figure 3-2C).

A companion study assessed tolerability of this set of ASOs by single ICV bolus injection of 700 µg ASO in naïve wild-type C57BL/6, and found on analysis 8 weeks later that body weight change from baseline and glial markers were similar to PBS-injected mice[30]. Two active ASOs and one inactive ASO of each chemistry were advanced to further studies. In light of slightly better tolerability of some ASOs at 500 µg versus 700 µg, we selected a dosing regimen of 500µg every 90 days for survival studies.

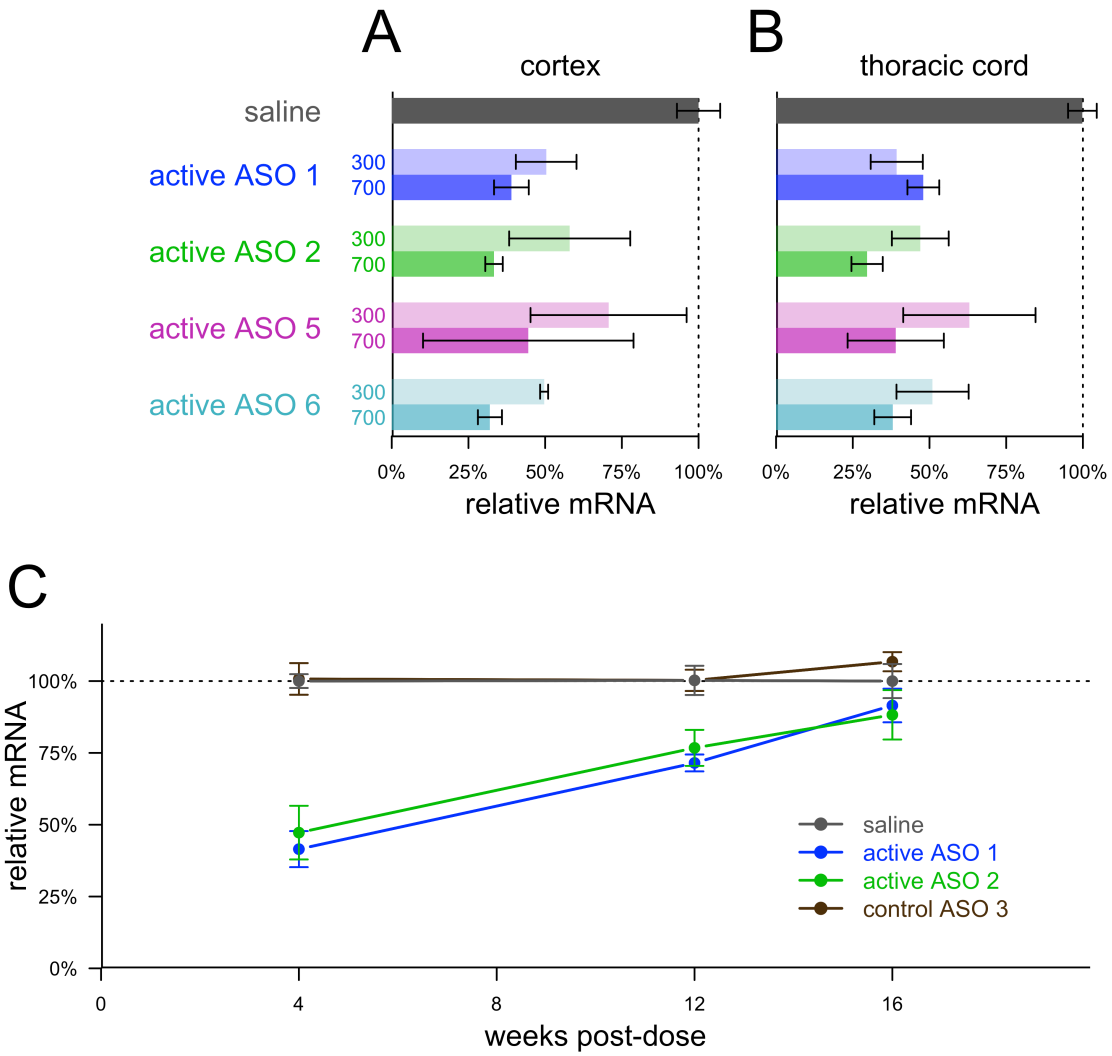

Figure 3-2. Potency and persistence of anti-PrP ASOs. *A-B) Four active ASOs were administered ICV at 300 and 700 µg to groups of N=4 mice. Ipsilateral hemispheres were collected 2 weeks later and microdissected for quantification of PrP RNA in cortex (A) and thoracic cord (B) by RT-PCR. Data were normalized to the mean value for saline-treated animals. Error bars indicate 95% confidence intervals (CI) of the mean. C) For a subset of ASOs, RT-PCR quantification of PrP RNA was performed on ipsilateral cortex 4, 12, and 16 weeks after a single 500 µg ASO dose. Data were normalized to the mean value for saline-treated animals. Error bars indicate 95% CI of the mean.*

Benefit of prophylactic ASO treatment against prion disease in mice

We next sought to determine the efficacy of *Prnp*-targeting ASOs in a standard mouse model of prion disease: wild-type animals intracerebrally infected with the RML strain of prions. To assess the ability of PrP-lowering to delay onset of disease, we pursued a prophylactic dosing regimen, with the first ASO treatment 14 days prior to infection and a second dose

administered 90 days later, at 76 days post injection (dpi). In a first experiment at the Broad Institute, we tested active and inactive cET oligos, with groups of N=8 C57BL/6N mice receiving two doses of ASO 1, ASO 2, control ASO 3, or saline by stereotactic intracerebroventricular (ICV) injection. Animals were monitored for prion disease symptoms with a primary endpoint of terminal prion disease. Active ASOs 1 and 2 delayed median all-cause mortality by 61% and 76% respectively compared to saline (median 274 and 300 dpi vs. 170 dpi) (Figure 3-3A), with control ASO 3 providing no benefit. Treated animals eventually developed and succumbed to prion disease, and followed a standard clinical course. The survival benefit in treated animals corresponded to delayed disease onset rather than a protracted symptomatic phase, as reflected by their delayed trajectory of weight loss (Figure 3-3B).

The prophylactic efficacy of ASOs 1-3 was independently assessed at a separate site, using a different laboratory strain of mice and distinct dosing regimen. In experiments conducted at NIH, an in-house strain of wild-type mice (see Methods) were divided into the following treatment groups: no treatment, saline, control ASO, active ASO 1, or active ASO 2. Compared to the study above, mice received three smaller (300 μg) doses of ASO, with ICV injections performed 14 days before infection with the RML strain of prions and again at 46 and 106 dpi. Active ASOs 1 and 2 delayed the onset of prion disease clinical signs by 82% and 99% respectively compared to saline (median 250 and 272 vs. 137 dpi), while the control ASO had no effect (Figure 3-3C). As above, ASO-treated animals ultimately developed terminal prion disease, and followed a delayed but standard symptomatic course of duration similar to that seen in controls (Figure 3-3D). Several intercurrent losses were observed in this study, including that 4/8 animals treated with control ASO 3 died suddenly 14-15 days after the third ICV surgery. Even including all deaths, however, the benefit of active ASO treatment was evident, with ASOs 1 and 2 delaying all-cause mortality by 81% and 98% respectively (median 259 and 283 dpi vs. 143 dpi, Figure 3-3E).

We next repeated the Broad Institute prophylactic experiment with ASOs 4-6 with the alternative MOE chemistry. As in the first experiment, ASO treatments were administered at 14 days prior to and 76 days following infection with RML prions. Groups of N=8 C57BL/6N mice received two doses of ASO 5, ASO 6, control ASO 4, or saline by stereotactic intracerebroventricular (ICV) injection. Active ASOs 5 and 6 closely replicated the survival benefit seen with active ASOs 1 and 2, extending median all-cause mortality by 108% and 80% respectively compared to saline (314 and 270 vs. 150 dpi) (Figure 3-3F). Again, the control ASO conveyed no benefit (Figure 3-3F). ASOs 5 and 6 delayed onset of disease as reflected by delayed weight loss (Figure 3-3G) and delayed decline in nest-building activity (Figure 3-3H) in treated animals.

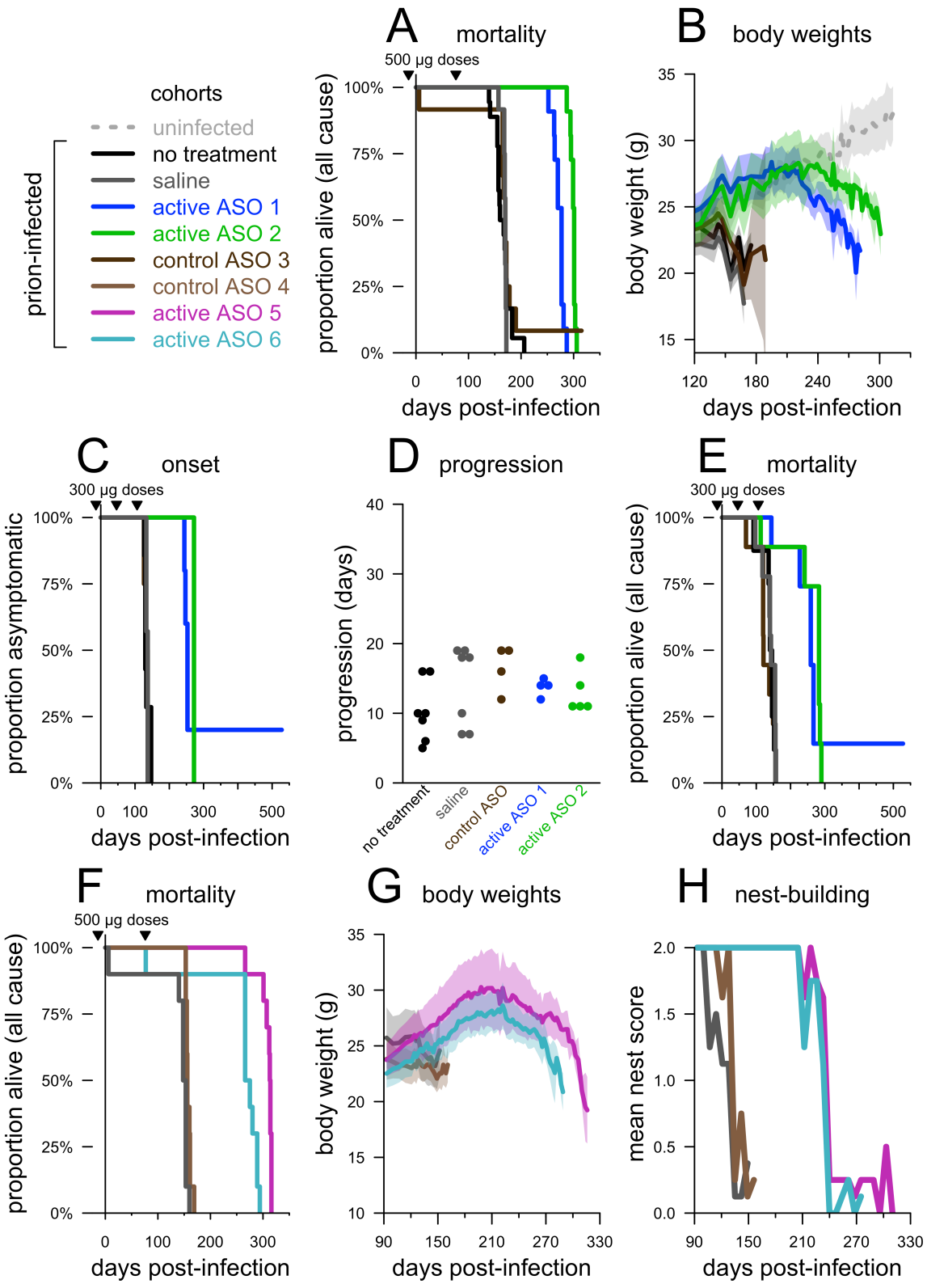

Figure 3-3: Prophylactic ASO treatment delays disease and extends survival of prion-infected mice. *A) All-cause mortality of prion-infected animals treated with cET ASOs*

Figure 3-3 (Continued) *(ASOs 1-3) at the Broad Institute*. Arrows indicate timing of 500 µg ICV doses. B) Body weights of animals treated with cET ASOs (ASOs 1-3) at the Broad Institute. Lines indicate means and shaded areas indicate 95% CI of the means. Only timepoints with ≥2 animals are included. C) Delay of onset of clinical signs characteristic of prion disease in animals treated with cET ASOs (ASOs 1-3) at NIH*. Arrows indicate timing of 300 µg ICV doses. D) Disease duration (onset to end stage) in animals treated with cET ASOs at NIH. E) All-cause mortality in animals treated with cET ASOs (ASOs 1-3) at NIH*. F) All-cause mortality in animals treated with MOE ASOs (ASOs 4-6) at the Broad Institute. Arrows indicate timing of 500 µg ICV doses. G) Body weights of animals treated with MOE ASOs (ASOs 4-6) at the Broad Institute. Lines indicate means and shaded areas indicate 95% CI of the means. Only timepoints with ≥2 animals are included. H) Next building score for animals treated with MOE ASOs (ASOs 4-6) at the Broad Institute. *At both sites, one mouse showed no prion disease symptoms prior to euthanasia at 315 dpi (Broad, Figure 3-3A) or 527 dpi (NIH, Figure 3-3C and 3-3E). This phenomenon is further discussed below.*

To assess end-stage brain prion titers across treatment cohorts, we performed real-time quaking induced conversion (RT-QuIC)[31] on brain homogenates prepared from terminal brain tissue of a subset of animals that received ASO or saline treatment at the Broad Institute. At the terminal stage of prion disease, prion titers in brains of all animals were roughly comparable by RT-QuIC analysis regardless of treatment cohort. One mouse treated with control ASO 3 did not show symptoms of prion disease prior to euthanasia at 315 dpi (Figure 3-4A), at which point it had survived beyond the mean endpoint for its cohort by >15 standard deviations. Upon sacrifice, this animal's brain was positive by RT-QuIC analysis and with prion titers at or slightly below levels in terminal mice with endpoint at a 10^7 dilution (Figure 3-4B). The original prion inoculation of this animal may have delivered an incomplete dose of prion infectivity.

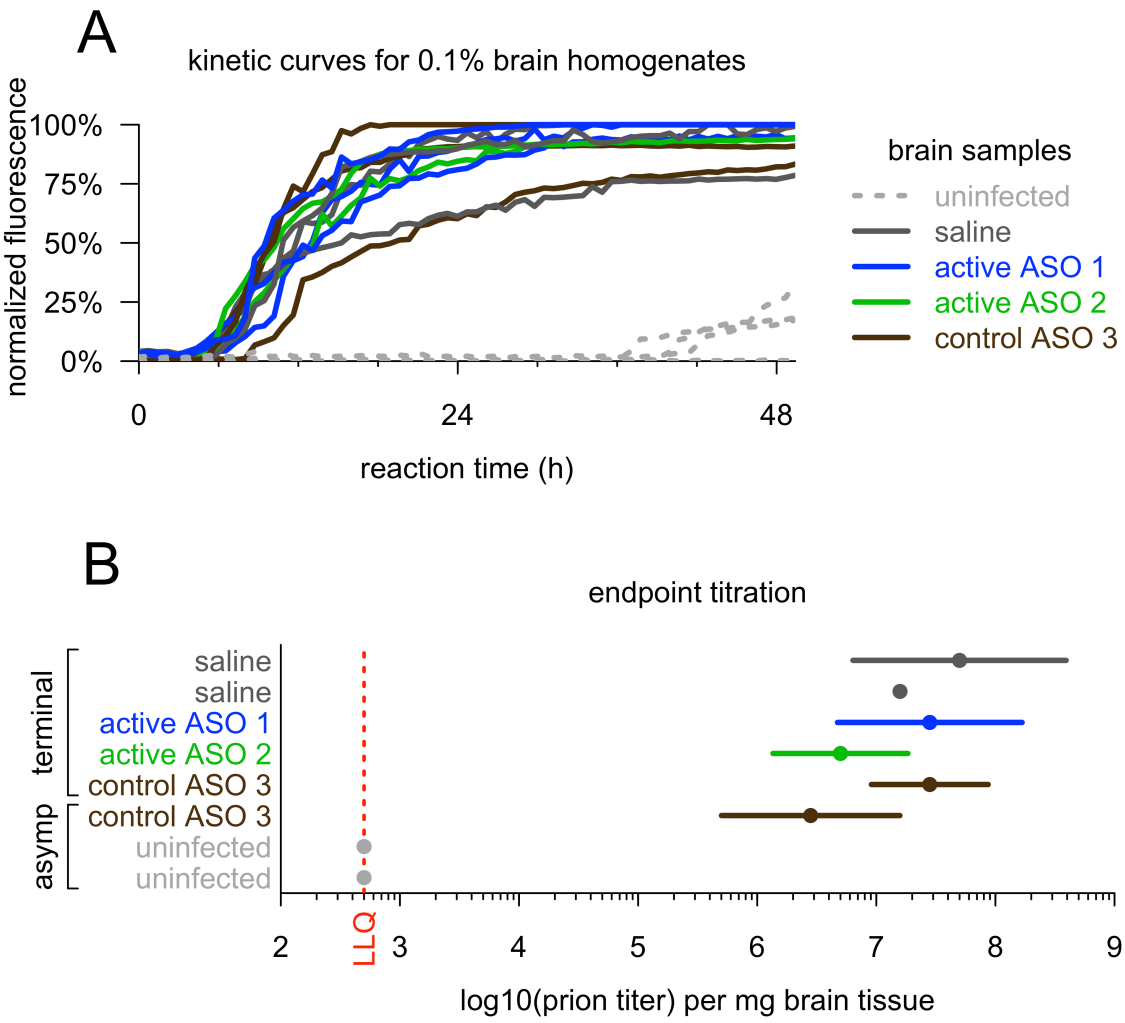

Figure 3-4: Prion titer is comparable in end-stage prion brains regardless of treatment. *A) To assess the presence of prions in the brains of terminally prion-sick animals, RT-QuIC was performed using 2 uL seeds of 0.1% brain homogenate. Kinetic curves show normalized fluorescence over 48 hours of reaction monitoring. B) Endpoint titration was performed by testing serial log dilutions of brain homogenate ranging from 10^{-3} to 10^{-9}. Prion titers were determined by Spearman-Karber analysis[32]. Dots represent best estimates of titer and error bars represent 95% CIs.*

ASOs extend survival of prion-infected mice in a dose-dependent manner

Based on this preliminary demonstration of efficacy, we set out to investigate the threshold of effect and dose-responsiveness of ASO activity. First, multiple doses of ASO 1 ranging from 30 µg to 700 µg were administered to uninfected mice. Across this range, single bolus ICV injections of escalating doses of ASO 1 dose-dependently reduced *Prnp* RNA in

cortex as measured by RT-PCR 2 weeks post dose (Figure 3-5A). Next, we performed a survival study in RML prion-infected mice using prophylactic doses of 0 (saline), 30, 100, 300, or 500 μg of ASO 1. As above, mice were dosed at -14 and 76 dpi. All ASO doses resulted in extension of survival, with PrP reduction closely correlating with survival benefit in animals that ultimately succumbed to prion disease (Figure 3-5B). When intercurrent deaths were included in the analysis, we similarly observed dose-dependent extension of all cause mortality (Figure 3-5C). This survival benefit was foreshadowed by dose-dependent delays in weight loss (Figure 3-5D), accumulation of prion disease symptoms (Figure 3-5E), and decline in nest-building (Figure 3-5F).

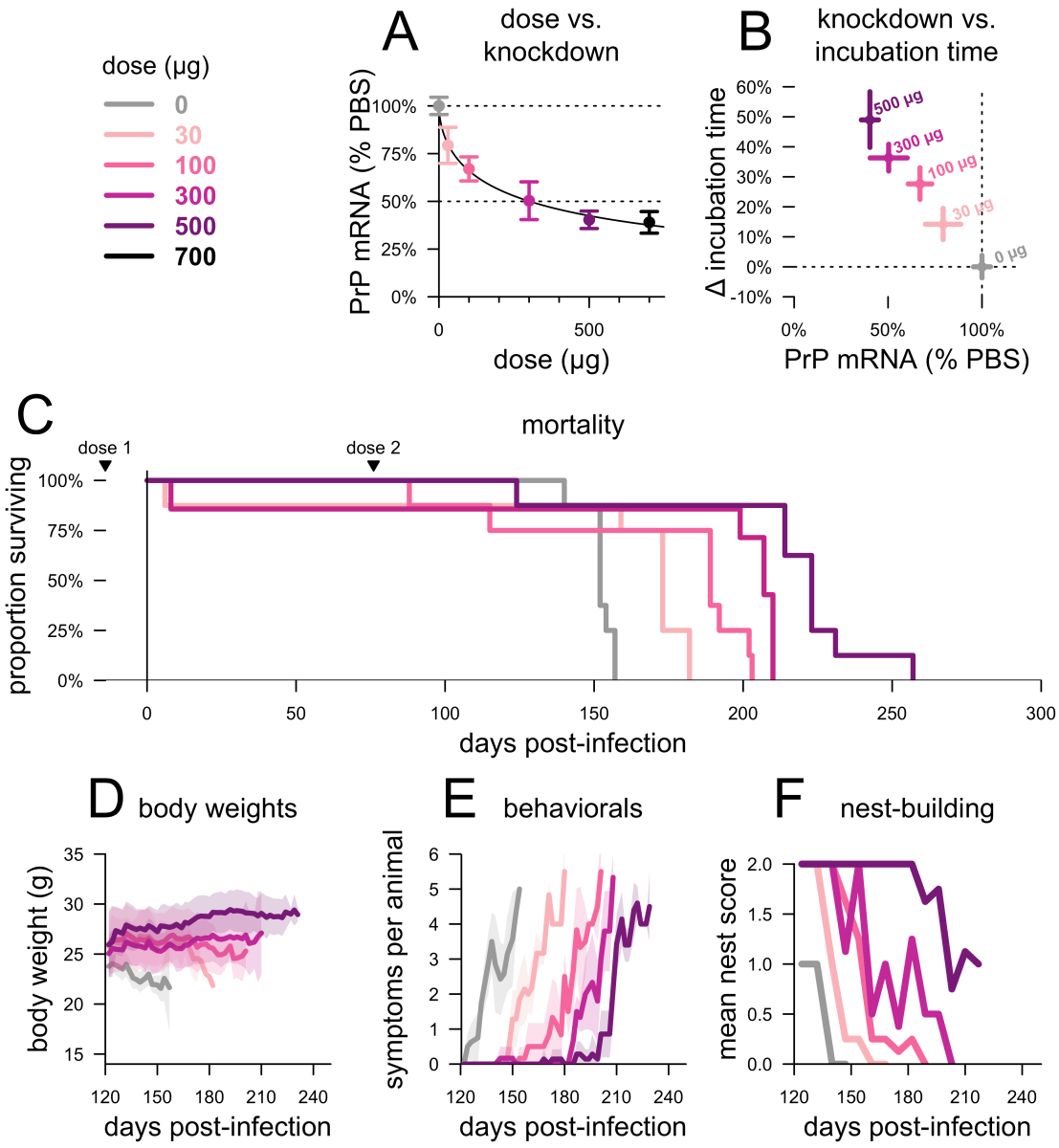

Figure 3-5: ASO-mediated PrP lowering dose-dependently delays disease and extends survival of prion-infected mice. *A) Five doses of ASO 1 (30, 100, 300, 500 and 700 ug) or saline were administered by single ICV bolus injection to N=4 mice. Ipsilalateral hemispheres were collected 2 weeks later for microdissection and cortical PrP RNA was quantified by RT-PCR. Data were normalized to the mean value for saline-treated animals. Error bars indicate 95% CI of the mean. Note that for the 300 and 700 ug doses, the same RT-PCR data are shown in Figure 3-2A (blue bars). C) Cortical PrP mRNA knockdown measured for each dose of ASO 1 plotted against survival of treated animals that ultimately died of prion disease. These data exclude intercurrent non-prion deaths. For each, dots represent means and error bars indicate 95% CI of the mean. C) All-cause mortality of animals treating with differing doses of ASO 1. Arrows represent dosing timepoints. D) Body weights of ASO1 treated animals by dose. Lines indicate means and shaded areas indicate 95% CI of the means. Only timepoints with ≥2 animals surviving are included. E) Number of recorded symptoms for ASO1 treated animals by*

Figure 3-5 (Continued) *dose, as recorded by a blinded rater. Lines indicate means and shaded areas indicate 95% CI of the means. Only timepoints with ≥2 animals surviving are included. F) Nest-building scores, assigned per cage by a blinded rater, for ASO 1-treated animals by dose. Only timepoints with >1 cage surviving are included.*

ASOs show efficacy across a battery of distinct prion strains

Previous prion disease drug discovery efforts have shown that strain specificity can limit the generalizability of antiprion compounds, and that prions are capable of adapting to drug treatment, giving rise to new drug-resistant prion strains[33,34]. As all prion strains share the common substrate of PrP, we hypothesized that ASO-mediated reduction of PrP would not suffer from these limitations. To test this hypothesis, we performed a survival experiment testing ASO 1 against five different previously characterized mouse-passaged laboratory prion strains: RML, ME7, Fukuoka-1, OSU, and 22L[35–37]. Groups of N=8 C57BL/6N mice received two doses (first dose 14 days before infection and second dose 90 days later) of 500 µg ASO 1 or saline delivered ICV, and each group was infected intracerebrally with a different prion strain. Within each strain, disease was delayed and survival extended in ASO-treated animals compared to untreated controls (Table 3-2).

To test whether ASO treatment gives rise to drug-resistant prion strains, we prepared RML[ASO1] brain homogenate from terminally sick, RML prion-infected, active ASO 1-treated animals included in our initial prophylactic experiment at the Broad Institute (Fig 3-3A). New groups of N=8 C57BL/6N mice were administered either 500 µg ASO 1 or saline, delivered ICV (first dose 14 days before infection and second dose 90 days later). Following the first treatment, both groups were intracerebrally inoculated with the RML[ASO1] prion brain homogenate. In an ongoing experiment, ASO 1 has delayed the onset of prion disease clinical signs by at least 45% compared to saline animals (Table 3-2), indicating that ASO 1 has retained its efficacy.

Table 3-2. ASO-mediated PrP lowering is efficacious across strains, and does not give rise to drug-resistant prions. *Median survival time ± standard deviation is shown for mice challenged with six prion strains compared to untreated controls. Data includes animals that reached prion endpoint. For each strain, the difference in survival is shown as a percentage extension of survival of treated animals compared to untreated controls, and p values are from a two-sided log-rank survival test.*

strain	control	treated	diff	pval
22L	180 ± 24	204 ± 6	13%	0.036
Fukuoka-1	159 ± 4	217 ± 7	37%	0.00013
ME7	160 ± 14	199 ± 10	24%	0.00065
OSU	152 ± 6	206 ± 9	36%	0.0002
RML	153 ± 4	211 ± 3	38%	0.00017
RML[ASO1]	159±4	>230	>45%	

Discussion

Here we have demonstrated the ability of PrP-lowering antisense oligonucleotides to delay prion disease in mice in a prophylactic paradigm. We show that the prophylactic efficacy of ASOs is replicable across sites, mouse strains, and dosing regimens. We show that ASO efficacy is sequence-dependent, providing evidence that the relevant mechanism of action is lowering of prion protein RNA, rather than aptameric interaction between ASOs and PrP. We demonstrate that multiple ASO chemistries are able to achieve therapeutic benefit through PrP lowering. We further show that ASO efficacy is dose responsive, generalizes across all prion strains tested, and does not appear to give rise to drug resistant prion strains.

Our ASO dose-response experiment provides, to our knowledge, the first data assessing the threshold for effect of PrP lowering, beyond the longstanding observation that heterozygous knockout mice with 50% normal PrP dramatically outlive wild-type mice following prion infection[6]. Our data suggest that the protective effect follows a full dose response curve, such that any robustly quantifiable PrP reduction should be likely to confer therapeutic benefit. This finding bears on evaluation of ASOs and other PrP-lowering therapeutics in the clinic. In combination with the observation that PrP reduction is well tolerated, it also nominates PrP as a model target for future drug modalities capable of reducing a single disease-causing protein in the brain.

Promisingly, our experiments suggest that strain specificity and drug resistance may not hinder the efficacy of ASO-mediated PrP reduction. While our experiments did not include human prion strains, our data show broad efficacy of PrP-lowering ASOs against diverse mouse-adapted prion strains, including one strain of human origin[36] and one synthetic strain[37]. These findings suggest that therapeutic strategies that aim upstream of the pathogenic conformational conversion event in prion disease may generalize across prion strains. They also support the hypothesis that PrP lowering may prove effective against all genetic subtypes of human prion disease where treatment can be initiated before symptom onset.

Our study has several limitations. We measured ASO-mediated reduction of PrP RNA by RT-PCR for each ASO and dose, and while we hypothesize that these reductions will closely predict relative reductions in PrP at the protein level, we are presently developing a rodent PrP ELISA assay to perform the corresponding protein quantification. Our study focuses on prophylactic efficacy in mice pre-treated with ASOs prior to prion infection, in order to model preventive treatment in asymptomatic individuals at risk for genetic prion disease. The efficacy of ASOs at later treatment timepoints is addressed in a companion study[30]. To limit surgical interventions, each cohort in our study received two, or at most three bolus doses of ASO. A companion study is likewise assessing chronic treatment with a longer regimen of bolus dosing[30]. Finally, the ASOs used in this study, designed against the mouse *Prnp* sequence, are preclinical tools; additional ASO development will be needed to identify ASOs against the human *PRNP* sequence that could advance to human studies.

Overall, our data support advancement of PrP-lowering ASOs toward the clinic, and provide a basis for optimism that ASO-mediated PrP-lowering may have the potential to delay the onset of genetic prion disease by extending healthy life. While prion disease trials have not traditionally focused on pre-symptomatic carriers, work is underway to identify an appropriate design for such trials (Chapter 2) and to provide supporting biomarker (Chapter 4), natural history (Chapter 5), and recruitment infrastructure. Intriguingly, while our experiments support

the hypothesis that ASO-mediated PrP-lowering can delay onset of disease, they are unable to provide a quantitative estimate of the magnitude of delay that might be achievable in humans. Beyond the truncated dosing paradigm employed, our animal models are artificially infected with prions. In pre-symptomatic human carriers of genetic prion disease mutations, it is not known when *de novo* prion formation begins. We have found in our ongoing natural history study that prion seeding activity is not detectable in the cerebrospinal fluid of the majority of pre-symptomatic carriers (Chapter 5), which may suggest that prion formation has not yet begun in these individuals. The transmission paradigm employed in the present study cannot address the impact that reduction of PrP prior to formation of the first *de novo* prions may have on the kinetics of their formation. Thus, only human studies will be able to assess the full potential of PrP-lowering ASOs to delay or prevent onset of genetic prion disease.

Methods

Study design and sites. Broadly, our goal was to assess whether ASOs could extend survival in prion-infected mice by sequence-specifically lowering PrP in the mouse brain. Scientists at Ionis Pharmaceuticals led screening and discovery of ASO molecules, as well as prioritization based on potency, based on characterization in cells and animals using RT-PCR. Two sites, the Broad Institute of MIT and Harvard (Cambridge, MA) and NIH/NIAID/Rocky Mountain Laboratories (Hamilton, MT), concurrently performed survival studies in prion animals.

ASO treatment studies were designed as controlled laboratory studies with a primary endpoint of terminal prion disease requiring euthanasia. Animals were also monitored for prion disease symptom onset, according to symptom lists detailed below. Animals that died of non-prion causes were included in calculations of all-cause mortality but excluded from analyses of prion disease symptom onset or incubation period. All-cause mortality excludes 1) animal deaths that occurred prior to any surgery (prion inoculation or ASO administration), 2) deaths

due to acute surgical complications within one day post-surgery, and 3) animals euthanized due to experimental error.

Figures 3-3C - 3-3E present data gathered at NIH/Rocky Mountain Labs. The data presented in Figure 3-2, along with all other RT-PCR data were gathered at Ionis Pharmaceuticals. For experiments shown in Figures 3-3A, 3-3F, 3-5C and Table 3-2, the first round of ASO injections was performed at Ionis Pharmaceuticals at -14 dpi and animals were then shipped to the Broad Institute, where prion inoculation, the second round of ICV injections at 76 dpi (90 days after the first injections), and subsequent monitoring and analyses were performed.

Animals. All experimental procedures involving animals were approved by Institutional Animal Care and Use Committees (Ionis IACUC Protocol P-0273, Broad Institute IACUC Protocol 0162-05-17, and NIH/RML IACUC Protocol 2015-061) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication No. 80-23). Experiments at Ionis Pharmaceuticals and the Broad Institute used C57BL/6N female mice purchased from Taconic Biosciences, Inc. (Germantown, NY). Experiments at NIH/RML used an in-house mouse strain, originally C57BL/10 mice, which has been inbred at Rocky Mountain Labs for many generations. Both mouse strains used in this study have the reference *Prnp*^{a} (MoPrP-A) haplotype[38]. Figures 3-3C - 3-3E present data from the NIH strain of C57BL/10 mice. The remainder of the animal data presented are from C57BL/6N mice.

ASO synthesis, screening and lead identification – Ionis Pharmaceuticals. ASOs were synthesized and purified as previously described[39]. After synthesis, ASOs were aseptically diluted to 100 mg/ml in PBS and frozen at -20°C. Initially, roughly 500 ASOs were designed to target the full mouse *Prnp*^{a} gene, then evaluated by electroporation into HEPA1-6 cells at 7 μM, using the HT-200 BTX Electroporator with ElectroSquare Porator (ECM830) voltage source at

135 V in 96-well electroporation plates (BTX, 2mm; Harvard Apparatus). After 24 hours, cells were harvested for RNA extraction and quantification of mouse *Prnp* mRNA by RT-PCR. The most potent ASOs advanced to a four-point dose response experiment in HEPA1-6 cells, then to screening in C57BL/6 mice. For *in vivo* screening, a single 300 µg bolus dose was delivered ICV with tissue collection at 2 weeks post dose for *Prnp* mRNA reduction.

***RT-PCR – Ionis Pharmaceuticals*.** For RNA extraction from cultured cells, lysis was performed in 300 µl of RLT buffer (Qiagen, Valencia, CA) containing 1% (v/v) 2-mercaptoethanol (BME, Sigma Aldrich). For RNA extraction from animal tissue, a 2-mm coronal section of the cortex at 1 mm posterior to injection site or a 2-mm coronal section of the thoracic spinal cord were homogenized in 500 µl of RLT buffer containing 1% (v/v) BME. RNA was isolated from 20 µL of lysate or homogenate using an RNeasy 96 Kit (Qiagen) with in-column DNA digestion with 50U of DNase I (Invitrogen, Carlsbad, CA).

RT-PCR was performed using StepOne Realtime PCR system (Applied Biosystems), as described previously[27]. The sequences of primers and probes are provided in a companion study[30]. RT-PCR results were normalized by housekeeping gene cyclophilinA/Ppia and further normalized to the level in PBS-treated mice or untreated cells.

Stereotactic intracerebroventricular (ICV) injection of ASO or PBS – Ionis Pharmaceuticals and the Broad Institute. Animals were induced with 3% isoflurane and maintained on 3% isoflurane for a surgical plane of anesthesia throughout the procedure. Animals received intraperoteneal injections of prophylactic meloxicam for pain relief. Heads were shaved and swabbed with betadine. Animals were immobilized in a stereotaxic apparatus (ASI Instruments, SAS-4100) using the 18° ear bars and tooth bar of the mouse adapter, adjusted to -8mm to keep the top of the skull level. A roughly 1-cm incision was made in the skin, and the skull was scrubbed with a sterile cotton-tipped swab to reveal the bregma. 10 µL of

saline with or without ASO (diluted from 100 mg/mL in dPBS, Gibco 14190) was drawn into a Hamilton syringe (VWR 60376-172) fitted with a 22-gauge Huber point removable needle (VWR 82010-236). The needle was dialed to the following coordinates relative to bregma: 0.3 mm anterior, 1.0 mm right, and 3.0 mm ventral (down) after the bevel of the needle disappears through the skull. 10 µL of saline was injected over the course of 10 seconds, after which the needle was kept in place for 3 minutes. The needle was removed while applying downward pressure to the skull with a cotton-tipped swab. The incision was sutured with one horizontal mattress stitch using 5-O Ethilon suture (Ethicon™ 661H). Mice recovered in heated cages.

Stereotactic intracerebroventricular (ICV) injection of ASO or PBS – NIH/Rocky Mountain Labs. Two to three month old female RML mice were anaesthetized with isoflurane and maintained on anesthesia throughout the procedure. Heads were shaved and swabbed with chlorhexidine-based surgical scrub. Mice were then positioned on a stereotaxic frame (David-Kopf Instruments). A 1-cm midline incision was made in the skin, and the drill was dialed to the following coordinates relative to bregma: 0.0 mm anterioposterior, 0.8 mm lateral (right), and 2.5 mm ventral (down). A small hole was drilled in the surface of the skull prior to placement of the 32-gauge delivery needle (World Precision Instruments). 4.5 µl of ASO containing 300 µg of ASO in PBS or PBS alone was injected into the ventricle at a rate of 1 µl/sec using an UltraMicroPump III with Micro4 pump controller (World Precision Instruments). The needle was kept in place for 1 min following injection, after which the skin incision was closed with suture. Mice recovered in heated cages and received one subcutaneous injection of 0.2 mg/kg buprenorphine for post-operative pain management.

Brain homogenate preparation – Broad Institute. Whole brains were harvested from terminally ill prion mice and frozen immediately upon sacrifice, or received frozen from collaborators. Brains were homogenized in sterile PBS at 10% weight/vol using at MiniLys beat-

beater tissue homogenizer in 7 mL tubes (program: 3 rounds, 40 seconds each, max speed) (Bertin instruments, cat. no. P000673-MLYS0-A). Homogenates being prepped for inoculation were further diluted to 1% in sterile PBS, then drawn up into a 25 mL syringe and serially extruded through progressively finer needle attachments (18G, 21G, 24G, 27G, 30G) to achieve an appropriate consistency. Injection-ready 1% brain homogenates were stored in a rubber top vial at -80°C. Prior to inoculation into animals, homogenates were irradiated at ~7.0 kGy to ensure inactivation of non-prion pathogens[40].

Intracerebral prion inoculations – Broad Institute. Animals were inoculated at age 7-10 weeks when cartilaginous skulls permit manual intracerebral inoculation directly through the skull. The dose of inoculum for all animals was 30 µL of a 1% brain homogenate of the indicated prion strain. Mice were induced with 3% isoflurane and maintained on a surgical plane of anesthesia for the procedure. Heads were swabbed with betadine. A 300 µL BD SafetyGlide Insulin 31G syringe with a 0.25" needle (BD 328449) was used to deliver 30 µL of brain homogenate freehand through the skull to a location slightly right of midline. Animals recovered in their cages.

In our first prophylactic experiment (Figures 3-3A and 3-3B), animals were monitored once per week, increasing to every other day after 120 dpi, for presence or absence of the following prion disease clinical signs routinely used in the prion literature[41]: generalized tremor, ataxia, difficulty righting from a supine position, rigidity of the tail, stare or blank look, hindlimb weakness. Animals were weighed every other day after 120 dpi. Animals were euthanized upon body condition score <2, body weight loss >20%, inability to reach food or water, severe respiratory distress, or severe neurological deficits. As Broad investigators and staff were overseeing prion-infected animals for the first time, blinding to treatment condition was not employed.

In subsequent survival experiments (Figures 3-3F - 3-3H, Figure 3-5, Table 3-2), raters blinded to treatment status performed all behavioral monitoring (including weights and nest rating.) The symptom list was updated according to experience gained in the first experiment. Under the new criteria, animals were monitored for the presence or absence of scruff, poor body condition, reduced activity, hunched posture, irregular gait/ hindlimb weakness, tremor, blank stare, and difficulty righting. Animals were monitored on the same schedule described above. Nests were rated weekly, by cage, according to criteria described below. Animals were euthanized if they met any of the following conditions: body condition score ≤2, body weight loss ≥15%, inability to reach food or water, ≥5 prion disease symptoms observed for 2 days in a row.

Intracerebral prion inoculations – NIH/Rocky Mountain Labs. Eight to twelve week old female mice were injected intracerebrally (IC) with 25 µl of 1% brain homogenate in PBS prepared aseptically from a pool of 10 brains collected from end-stage prion mice infected with the RML strain of prions. 10% brain homogenates were prepared in 0.32 M sucrose by 1) douncing 10x each with the loose and tight pestle (Wheaton glass), 2) sonication in a cuphorn sonicator (2 pulses, 1 minute each, maximum setting) and 3) centrifugation (1500xG for 5 minutes). Supernatant was aliquotted and stored at -80°C. Fresh aliquots were thawed for each set of inoculations, sonicated for 2 sonication pulses in a cuphorn sonicator at the maximum setting, then diluted to 1% in Dulbeccos medium with 2% fetal bovine serum.

For intracerebral inoculation, mice were anesthetized using saturated isofluorane vapors in a bell jar. Clinical neurological signs used to determine clinical onset included progressive deterioration of ataxia, tremors, myoclonus, weight loss, somnolence, kyphosis and poor grooming.

Nest rating system – Broad Institute. Across experiments, all four mice in a given cage were grouped into the same treatment cohort. As part of biweekly cage changes, each cage received

one cotton square nestlet (Ancare) and one unit of Enviro-dri® (Shepherd) packed paper for nest building. Once per week, allowing at least two days following cage changes for animals to remake their nests, both nest-building materials were separately rated by a blinded operator according to the following scale: 0 = unused; 1 = used/pulled apart, but flat; 2 = pulled into a three-dimensional structure.

RT-QuIC – Broad Instititute. RT-QuIC was performed according to established protocols[31]. Briefly, FPLC purified recombinant full-length mouse PrP (moPrP23-230) was added at 1 mg/mL to a reaction mix consisting of 10 mM Sodium phosphate (pH 7.0), 130 mM NaCl, 0.1 mg/ml rPrPSen, 10 µM ThT, 1 mM EDTA, and 0% SDS. 10% brain homogenates were serially diluted in 0.05% SDS/ 1x PBS/ 1X N2 media supplement (vendor). Aliquots of the reaction mix (98 µL) were loaded into each well of a black 96-well plate with a clear bottom (Nunc) and seeded with 2 µL of indicated BH dilutions, with each sample run in quadruplicate. The plate was incubated at 42°C in a BMG FLUOstar Optima plate reader with cycles of 1 min shaking (700 rpm double orbital) and 1 min rest throughout the indicated incubation time. ThT fluorescence measurements (450 +/-10 nm excitation and 480 +/-10 nm emission; bottom read) were taken every 45 min. Data sets were normalized to a percentage of the maximal fluorescence response (260,000 rfu) of the plate readers after subtraction of the baseline, as previously described[42], and plotted versus reaction time. Reactions were classified as RT-QuIC positive based previously described criteria[42].

***Data analysis and visualization*.** Data were analyzed using custom scripts in R 3.5.1.

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Chapter 4: Prion protein quantification in human cerebrospinal fluid as a tool for prion disease drug development

Publication history:

This chapter is adapted from a manuscript currently in press at the Proceedings of the National Academy of Sciences*: Vallabh SM et al. Prion protein quantification in human cerebrospinal fluid a tool for prion disease drug development.* A previous version of this manuscript is also available on bioRxiv at https://www.biorxiv.org/content/10.1101/295063v1.

Attributions:

All of the experiments in this chapter were conducted at the Broad Institute. I conceived the overall study and individual experiments in partnership with Eric Minikel. I led execution of the experiments with support from Eric Minikel. I supported data analysis and visualization, which were led by Eric Minikel. Steven E. Arnold, Henrik Zetterberg, and Stuart Schreiber provided mentorship and guidance.

Abstract

Reduction of native prion protein (PrP) levels in the brain is an attractive strategy for the treatment or prevention of human prion disease. Clinical development of any PrP-reducing therapeutic will require an appropriate pharmacodynamic biomarker: a practical and robust method for quantifying PrP, and reliably demonstrating its reduction, in the central nervous system (CNS) of a living patient. Here we evaluate the potential of enzyme-linked immunosorbent assay (ELISA)-based quantification of human PrP in human cerebrospinal fluid (CSF) to serve as a biomarker for PrP-reducing therapeutics. We show that CSF PrP is highly sensitive to plastic adsorption during handling and storage, but its loss can be minimized by addition of detergent. We find that blood contamination does not affect CSF PrP levels, and that CSF PrP and hemoglobin are uncorrelated, together suggesting that CSF PrP is CNS-derived, supporting its relevance for monitoring the tissue of interest and in keeping with high PrP abundance in brain relative to blood. In a cohort with controlled sample handling, CSF PrP exhibits good within-subject test-retest reliability (mean coefficient of variation 13% in samples collected 8-11 weeks apart), a sufficiently stable baseline to allow therapeutically meaningful reductions in brain PrP to be readily detected in CSF. Together, these findings supply a method for monitoring the effect of a PrP-reducing drug in the CNS, and will facilitate development of prion disease therapeutics with this mechanism of action.

Introduction

Prion disease — a fatal and incurable neurodegenerative disease — is caused by misfolding of the prion protein (PrP), encoded by the gene *PRNP*[1]. PrP is a well-validated drug target for prion disease: knockout animals are invulnerable to prion infection[2], heterozygous knockouts have delayed onset of disease[3], and post-natal depletion of PrP can delay or prevent prion disease[4,5]. Total knockout is tolerated in mice[6,7], cows[8], and goats[9,10], and healthy humans with one loss-of-function allele of *PRNP* have been identified[11]. Further, PrP serves as a common target uniting all subtypes of human prion disease, including Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), and Gerstmann-Straussler-Scheinker disease (GSS)[12]. Therefore, therapeutic development efforts have sought to lower PrP in the brain[13–16], and antisense oligonucleotides with this mechanism of action are currently in development[17]. Similar approaches are being explored in other neurodegenerative diseases, with promising preliminary clinical results[18,19].

Clinical trials of PrP-lowering therapies will be enhanced by early determination of whether PrP is indeed being lowered effectively at a tolerated dose. The brain is the target tissue for any prion disease therapeutic, but is difficult to monitor directly. Cerebrospinal fluid (CSF) is produced by the choroid plexus of the ventricles, flows in and around the spinal cord and is in intimate contact with interstitial fluid of brain parenchyma. CSF more closely reflects the biochemistry of the brain than blood or any other accessible tissue, and is obtainable through a minimally invasive lumbar puncture (LP). PrP levels in CSF range from tens to hundreds of ng/mL, within the range of standard protein detection assays. Multiple groups have reported successful detection of PrP in human CSF using ELISA assays, including the one currently commercially available human PrP ELISA kit, the BetaPrion® ELISA assay[20–24] (Analytik Jena, Leipzig, Germany). The assay is best described as measuring total PrP, which is the variable of interest for PrP-lowering therapeutics (see Discussion).

Informed by FDA's 2013 Draft Guidance on Bioanalytical Method Validation[25] we assessed the technical performance of the BetaPrion® ELISA assay across *N*=225 human CSF samples spanning a range of diagnoses. We then used this assay to investigate the biological suitability of CSF PrP as a pharmacodynamic biomarker for PrP-reducing therapeutics.

Results

The BetaPrion® Human PrP ELISA quantifies total CSF PrP reproducibly, precisely, sensitively, and selectively

We assessed the assay's precision, sensitivity, selectivity and reproducibility by analyzing *N*=225 human CSF samples from symptomatic prion disease patients, pre-symptomatic prion disease mutation carriers, non-prion dementia patients, and normal pressure hydrocephalus (NPH) patients as well as other non-prion controls (Supplemental Table S-1) across 41 plates. The results broadly support the technical suitability of this assay for reliable quantification of CSF PrP (Table 4-1 and Supplemental Figure S-1).

Table 4-1. The technical performance of the BetaPrion® human PrP ELISA assay supports reliable quantification of PrP in human CSF. *Abbreviations: coefficient of variation (CV); lower limit of quantification (LLOQ); amino acid analysis (AAA).*

Experiment	Results
Within-plate technical replicate reproducibility (same dilution)	CV = 8%
Within-plate technical replicate reproducibility (all dilutions)	CV = 11%
Between-plate technical replicate reproducibility	CV = 22% in an interplate control sample run on 17 plates on different days (see Supplementary Discussion).
Sensitivity	LLOQ is 3-5× the blank signal, depending on the platereader used.
Selectivity	Non-reactive for recombinant mouse PrP, rat CSF and cynomolgus monkey CSF (consistent with one amino acid mismatch in the reported detection antibody epitope[23]), artificial CSF and protease-digested CSF.
Dilution linearity	Linear across two samples and five dilutions. See Figure S-1A.
Spike recovery	Using AAA-quantified recombinant HuPrP23-230 as a standard, spike recovery of recombinant PrP in CSF was 90% across five concentrations. Titration of a high PrP CSF sample into a low PrP sample resulted in linear recovery. See Supplemental Figure S-3.
Standard curve reproducibility	CV < 10% at all six non-zero standard curve points, across five replicates. See Supplemental Figure S-1.

In assessing within-plate variability we discerned plate position effects for control samples, with a mild but significant downward trend from upper left to lower right (Supplemental Figure S-2). Comparison of the kit standard curve to a standard curve made from recombinant human prion protein quantified by amino acid analysis (AAA) yielded systematic differences, with implications for kit use for absolute versus relative quantification of PrP (Supplemental Figure S-3B; see Discussion).

Standardized storage and handling are essential to reliable quantification of CSF PrP

PrP was measurable by ELISA in all *N*=225 CSF samples analyzed, including in CSF from individuals with 13 different genetic prion disease mutations (Supplemental Figure S-4A-B,

Supplemental Table S-1). Across all CSF samples analyzed, PrP levels varied by over two orders of magnitude (Supplemental Figure S-4A), ranging from 1.9 to 594 ng/mL. PrP was reduced in individuals with symptomatic prion disease, as previously reported[20,21,23,24,26]. Within matched cohorts containing individuals with prion disease, however, diagnostic category (non-prion, pre-symptomatic genetic, symptomatic genetic, and sporadic prion disease) explained only a minority of variance in CSF PrP level (adjusted R^2 = 0.23, $P < 1 \times 10^{-7}$, linear regression). After excluding individuals with symptomatic prion disease, PrP still differed significantly between the various cohorts included in our study, and within-cohort variation was also dramatic (Supplemental Figure S-4C; mean ~20-fold difference between highest and lowest sample within a cohort). These observations led us to search for other factors that might contribute to either biological or pre-analytical variability. CSF PrP was correlated with age (Supplemental Figure S-4D), but among our samples age is confounded with cohort, diagnosis, and likely many unobserved variables, making it unclear whether this correlation is biologically meaningful. CSF PrP did not differ according to sex (Supplemental Figure S-4E), and exhibited no lumbar-thoracic gradient over serial tubes collected from the same LP (Supplemental Figure S-4F-G). After noticing that PrP levels appeared lower in smaller aliquots of the same CSF sample (Supplemental Figure S-5A), we hypothesized that differences in sample handling might be one major source of variability in observed CSF PrP levels.

It is known that other neurodegenerative disease-associated amyloidogenic proteins have a high affinity for plastics[27–29], but PrP's stability under different handling conditions has not previously been systematically investigated. To assess PrP's susceptibility to differential CSF sample handling, we subjected aliquots of a single CSF sample to variations in 1) number of transfers between polypropylene storage tubes, 2) amount of exposure to polypropylene pipette tips, 3) storage aliquot size, 4) storage temperature, and 5) number of freeze-thaw cycles (Figure 4-1A). Strikingly, increased plastic exposure in the first three conditions dramatically reduced measurable PrP in solution (Figure 4-1A). To promote PrP solubility in our samples, we

experimented with adding small amounts of 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), a common zwitterionic surfactant known to enhance protein solubility in multiple contexts[30–32]. Addition of 0.03% CHAPS prior to aliquotting minimized PrP loss to plastic across most manipulations (Figure 4-1B). For instance, transferring a CSF sample to a new microcentrifuge tube three times eliminated at least 73% of detectable PrP ($P < 1 \times 10^{-6}$, two-sided t test) without CHAPS, but only 7.1% ($P = 0.37$) of PrP was lost in the presence of 0.03% CHAPS. Addition of CHAPS also increased total PrP recovery, presumably by preventing loss to the single polypropylene tube and tips used for plating samples (Supplemental Figure S-5), and was effective against loss to multiple plastics but not glass (Figure 4-1C). Storing CSF at room temperature for 24 hours or subjecting samples to three freeze-thaw cycles had a less dramatic impact on PrP that did not appear to be affected by CHAPS (Figure 4-1A-B and Supplemental Figures S-5D and S-5E).

We also investigated the relationship between measured PrP and total protein in N=217 samples, using the DC total protein assay. Across all samples analyzed, a modest correlation ($r = 0.36$, Spearman rank test, $P < 1 \times 10^{-7}$) between PrP and total protein was observed (Figure 4-1D), which may reflect either a biological phenomenon, or simply the ability of higher ambient protein levels to serve a blocking function that partially offsets PrP loss by adsorption. In support of the latter interpretation, addition to CSF of 1 mg/mL bovine serum albumin increased recovery of PrP (Supplemental Figure S-5F), though it was less effective than CHAPS at preventing loss due to transfers.

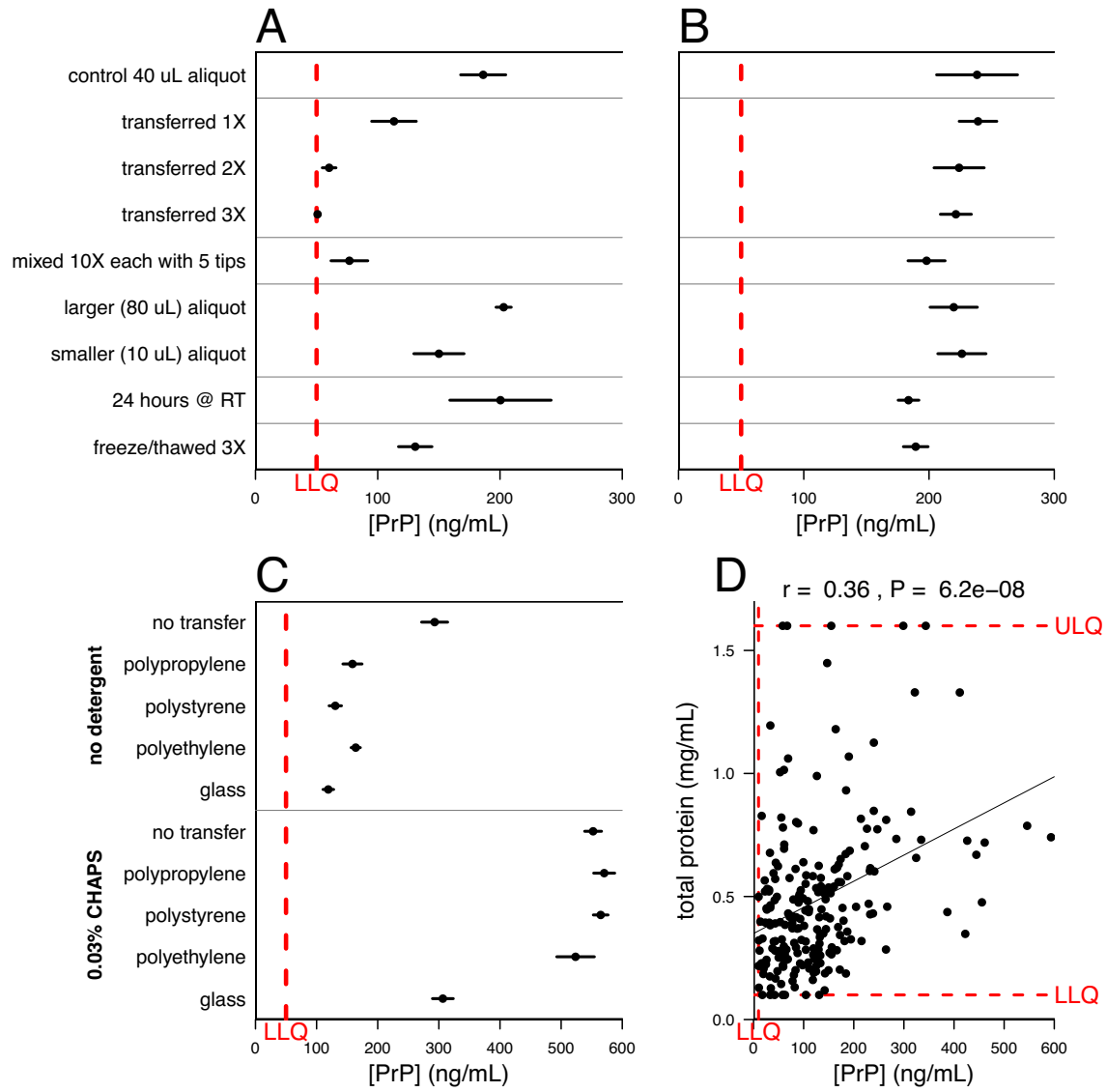

Figure 4-1. Storage and handling can dramatically reduce the amount of PrP detected in CSF samples unless appropriate measures are taken. *In A-C, dots represent mean and line segments represent 95% confidence intervals across 4 to 7 aliquots of the same sample, each measured in duplicate at a 1:50 dilution. In D, dots represent mean of measurements within dynamic range, among 2 dilutions with 2 technical replicates each. A. Increased polypropylene exposure substantially reduces detectable PrP. B. Addition of 0.03% CHAPS detergent to samples increases PrP recovery and consistently mitigates PrP loss to plastic. C. Addition of CHAPS (bottom) increases total PrP recovery and shows similar rescue across plastics, but substantial PrP loss is still observed upon storage in glass. D. Across 217 CSF samples, total protein levels and PrP levels were modestly correlated (Spearman's rank correlation coefficient = 0.36,* $P=6.2\times10^{-8}$*). In A-C, dots represent mean and line segments represent 95% confidence intervals across 4 to 7 aliquots of the same sample, each measured in duplicate at a 1:50 dilution. In D, dots represent mean of measurements within dynamic range, among 2 dilutions with 2 technical replicates each.*

PrP in CSF is CNS-derived and unlikely to be confounded by blood contamination

CSF PrP is an informative tool in prion disease only insofar as it is a faithful proxy for PrP levels in the CNS, the relevant target for any future therapeutic. CSF proteins derive from two major sources, CNS and blood, with proportional contribution driven by relative tissue abundance of a given protein[33,34]. Blood proteins may enter CSF either through passive diffusion as CSF flows along the spinal cord[35], or artifactually if blood from a traumatic lumbar puncture contaminates the collected CSF. To assess the contribution of blood-derived PrP to overall CSF PrP, we compared PrP levels across brain samples and red blood cell, buffy coat and plasma fractions of blood from non-neurodegenerative disease control individuals, versus all of the CSF samples in our study (Figure 4-2A). Among blood fractions, PrP was most consistently detected in buffy coat, in keeping with reports that blood PrP emanates chiefly from platelets[36,37]; we also detected PrP above the lower limit of quantification in some red cell samples, but never in plasma. As the average PrP concentration in all three blood fractions was still well below that in brain and was lower than that in 96% of CSF samples analyzed, the risk of confounding signal from blood-derived PrP appears negligible. Consistent with this conclusion, spiking whole blood into CSF at up to 1% (v/v) did not increase the detected PrP (Figure 4-2B). Finally, as a proxy for blood contamination we measured hemoglobin levels in *N*=128 CSF samples and observed no correlation between CSF hemoglobin and CSF PrP (Figure 4-2C). Variation in hemoglobin levels also failed to confound the test-retest reliability of CSF PrP (Supplemental Figure S-6). From these lines of evidence, we conclude that the PrP detected in CSF is overwhelmingly derived from the CNS.

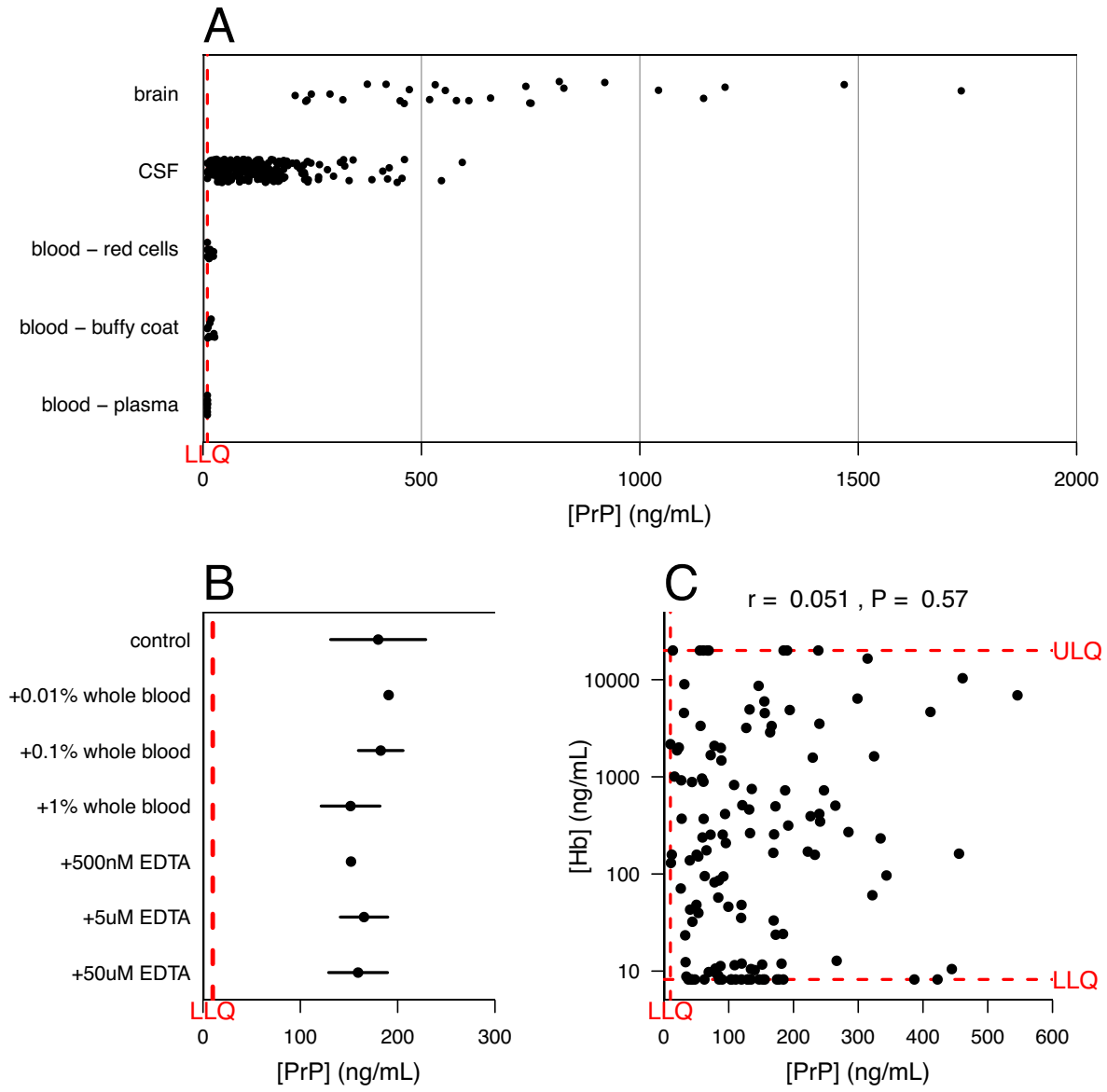

Figure 4-2. Blood PrP contributes negligibly to the PrP detected in CSF. *A. PrP levels were compared by ELISA in N=28 postmortem human brain samples, three blood fractions from N=8 individuals each, and all N=225 CSF samples analyzed in the present study. PrP is abundant in a range of human brain regions, undetectable in human plasma, and is detectable in the red cell and buffy coat fractions only at low levels compared to PrP in CSF. B. Spiking whole blood into CSF up to 1% by volume does not impact measured PrP. C. Across N=128 CSF samples spanning multiple cohorts and diagnostic categories, hemoglobin and PrP levels in CSF are uncorrelated. In A and C, dots represent mean of measurements within dynamic range, among 2 technical replicates per dilution. In A-C, dots represent mean and line segments represent 95% confidence intervals across 2 to 3 aliquots of the same sample.*

CSF PrP levels in individuals are stable on short-term test-retest

In order for CSF PrP levels to serve as a meaningful biomarker, they must be stable enough in one individual over time that a drug-dependent reduction could be reliably detected. We quantified PrP in pairs of CSF samples collected from nine individuals — placebo-treated controls with non-prion dementia — who had undergone two fasting morning lumbar punctures at 8-11 week intervals in the context of a clinical trial[38]. LPs were performed according to a standardized protocol by a single investigator, and samples were subsequently processed uniformly. Under these highly controlled conditions, the mean CV between timepoints for a given participant was reasonably low at 13% (Figure 4-3). Higher CVs of 33% - 41% were observed in three other cohorts where sample handling appears to have been less uniform (Supplementary Discussion and Supplemental Figure S-7), consistent with PrP's susceptibility to pre-analytical perturbations (Figure 4-1).

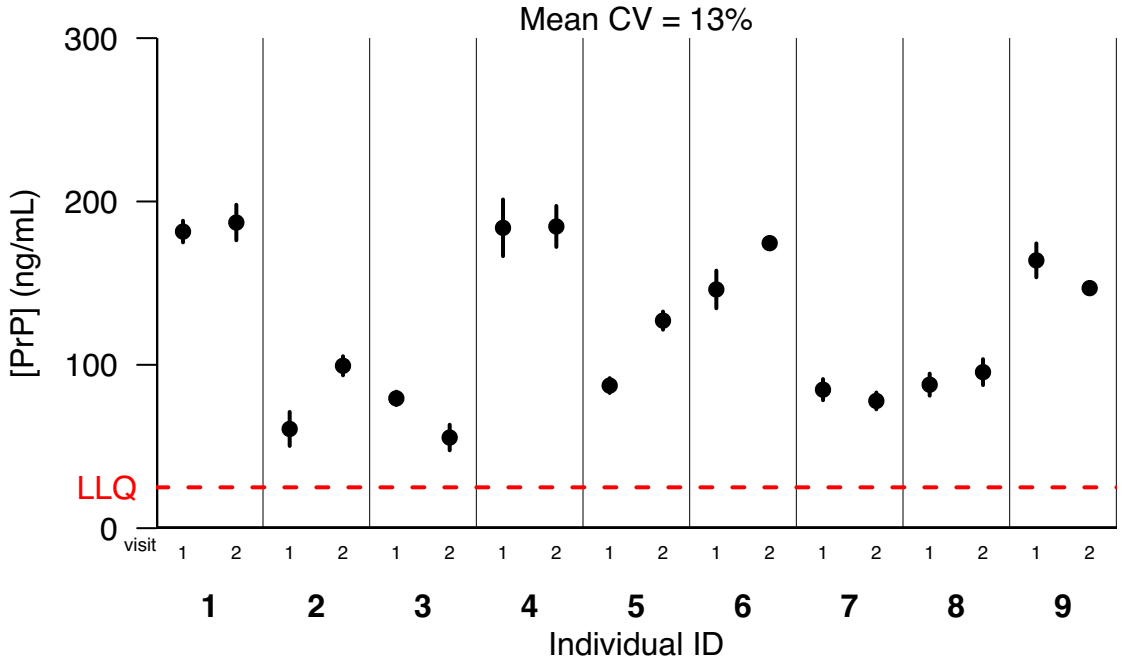

Figure 4-3. Test-retest stability of CSF PrP. *Uniformly processed CSF samples were provided from a past clinical trial, from placebo-treated individuals with mild, non-prion cognitive impairment. Fasting morning lumbar punctures were performed by one investigator on nine individuals then repeated at an interval of 8-11 weeks. Dots represent means, and line*

Figure 4-3 (Continued) *segments 95% confidence intervals, of measurements within dynamic range among 2 dilutions with 2 technical replicates each.*

Discussion

Our data support the use of CSF PrP quantification as a pharmacodynamic biomarker for clinical trials of PrP-lowering therapeutics. CSF PrP is CNS-derived, rather than blood-derived, so it should respond to PrP lowering in the brain. With appropriate protocols, it can be measured reproducibly and with favorable test-retest reliability.

Our experiments suggest best practices for sample handling and assay use. CSF PrP is sensitive to pre-analytical factors, but the addition of 0.03% CHAPS detergent mitigates the most dramatic such factor by minimizing PrP loss to plastic. A recommended CSF collection and processing protocol is detailed in Supplemental Figure S-8. Also, in light of subtle plate position effects (Supplemental Figure S-2), samples intended for comparison be co-located on the ELISA plate, and/or plate position should be adjusted for using standard curves or control samples. Our comparison of the kit standard curve to an amino acid analysis (AAA)-quantified standard curve suggests that the assay may be most useful for relative rather than absolute quantification of PrP (Supplemental Figure S-3B).

As ELISA depends on the presence and conformational availability of two epitopes, it cannot be taken for granted that ELISA detects all conformers or fragments of PrP. To this end, we have recently developed a targeted mass spectrometry-based assay that serves as an orthogonal method for CSF PrP quantification. Experiments on this platform support the hypothesis that the BetaPrion assay measures total PrP, and have confirmed key findings of this study, including the correlation between PrP and total protein levels in CSF, and reduced PrP levels in the CSF of symptomatic prion disease patients[39].

Our study has several limitations. First, while we have established that CSF PrP is quantifiable in genetic prion disease patients across a variety of mutations and has good test-

retest reliability in a cohort of patients with non-prion dementia, when we embarked on the present study we did not have access to test-retest samples from pre-symptomatic genetic prion disease mutation carriers. To address this shortfall, in summer 2017 we launched a longitudinal clinical research study at Massachusetts General Hospital through which we are collecting serial CSF from *PRNP* mutation carriers and controls (Chapter 5). Third, the samples analyzed here were re-used after collection for other research or clinical purposes, and in most cases we cannot fully account for their sample handling history prior to receipt by our lab. Thus, our numbers may exaggerate the inter-individual variation in CSF PrP in the population.

Our data reproduce the previously reported phenomenon that PrP levels in CSF are reduced by approximately half in symptomatic prion disease patients[21,23,24] (Supplemental Figure S-4). Multiple plausible biological mechanisms could explain these findings: incorporation of PrP into insoluble plaques[40], internalization of misfolded PrP in the endosomal-lysosomal pathway[41], and post-translational downregulation of PrP as a function of disease[42]. It is therefore possible that an intrinsic reduction in CSF PrP in the course of symptomatic disease could confound the use of PrP as a biomarker for the activity of PrP-lowering drug tested in a symptomatic population. Although it is important to be aware of this potential limitation, symptomatic patients are not the population most in need of such a biomarker. The rapid progression of prion disease has enabled symptomatic trials to use cognitive or survival endpoints[43–45], and future trials may be further benefit from the use of real-time quaking induced conversion (RT-QuIC) to detect misfolded prions in symptomatic patient CSF[46–48].

Instead, this biomarker may have its greatest utility in pre-symptomatic individuals carrying high-risk genetic prion disease mutations. As trials in symptomatic neurodegenerative disease patients continue to fail or prove uninterpretable, it is increasingly recognized that therapeutic efforts must aim further upstream in the disease process[49]. Though identifiable by genetic testing, genetic prion disease mutation carriers appear healthy up to the stark precipice of symptom onset, creating a compelling case for prevention. Because following pre-

symptomatic individuals to a clinical endpoint appears infeasible[50], lowering CSF PrP has been proposed as a surrogate endpoint to enable pre-symptomatic trials of agents such as the antisense oligonucleotides currently in development (Chapter 2). In this context, CSF PrP may have a near-term opportunity to serve, not just as a pharmacodynamic biomarker, but as a pivotal readout that enables a rational therapeutic to be tested for its ability to extend healthy life, thus honoring the opportunity provided by predictive genetic testing.

Methods

***Cerebrospinal fluid samples*.** De-identified human CSF samples were provided by multiple clinical collaborators and include some previously published samples[38,51]. Samples were shipped on dry ice and stored at -80°C. Prior to use, samples were thawed on ice and centrifuged at 2,000 × g at 4°C. Ninety percent of the volume was pipetted into a new tube to separate supernatant from cellular or other debris, aliquotted into new polypropylene storage tubes and refrozen at -80°C. For indicated samples, 0.03% CHAPS detergent by volume (final concentration, from a 3% CHAPS stock) was pre-loaded into the supernatant receiving tube prior to the post-centrifugation transfer, then mixed into the sample by gentle pipetting prior to aliquotting.

Quantification of human PrP in CSF, brain tissue and blood using the BetaPrion® human PrP ELISA kit. Across experiments, PrP was quantified using the BetaPrion® human PrP ELISA kit (Analytik Jena, cat no. 847-0104000104) according to the manufacturer's instructions. This sandwich ELISA is configured in 96-well format and relies on an apparently conformational human PrP (HuPrP) capture antibody and a horseradish peroxidase (HRP)-conjugated primary detection antibody to HuPrP residues 151-180[23]. In brief, samples were diluted into blocking buffer (5% BSA and 0.05% Tween-20 in PBS, filtered prior to use) at concentrations ranging

from 1:100 to neat depending on the anticipated PrP content of the sample type. All samples were plated in duplicate. Lyophilized standards and kit reagents were diluted fresh for same-day use, with the exception of wash buffer and blocking buffer, excess of which were stored at 4°C for reuse within 4 weeks. The assay format comprises twelve modular 8-well strips which enabled partial plates to be run in some cases. Following all add and incubation steps the absorption per well was read in either a SpectraMax or FluoStar Optima plate reader at 450 nm with 620 nm absorbance also monitored as baseline. Data was exported as a text file and analyzed in R.

Unknown CSF samples were run at two dilutions each (typically 1:10 and 1:50). Only one out of 225 CSF samples analyzed fell below the range of the assay's lower limit of detection (1 ng/mL final) at a 1:10 dilution, and was re-run neat, yielding a result of 1.9 ng/mL. Except where noted, samples were run in technical duplicate at two dilutions, and error bars represent 95% confidence intervals around the mean.

Human brain samples were obtained from the Massachusetts Alzheimer's Disease Research Center (ADRC; *N*=26 samples from 5 different control individuals without neurodegenerative disease, with post-mortem intervals of 23-72 hours, representing diverse cortical and subcortical regions) and from the National Prion Disease Pathology Surveillance Center (*N*=2 samples of frontal cortex from non-prion controls) homogenized in PBS with 0.03% CHAPS at 10% weight/vol in 7mL tubes (Precellys no. KT039611307.7) using a MiniLys tissue homogenizer (Bertin no. EQ06404-200-RD000.0) for 3 cycles of 40 seconds at maximum speed. The resulting 10% brain homogenates were diluted 1:10 and 1:100 in blocking buffer for ELISA.

Human blood fractions were obtained from Zen-Bio (3 fractions – red blood cell, buffy coat, and plasma – from 8 individuals each), 0.03% CHAPS was added, and samples were then mixed either by pipetting up and down or by homogenization in a MiniLys using the same protocol described above. Blood fractions were diluted 1:10 in blocking buffer for ELISA.

Negative controls. Rat and cynomolgous monkey CSF (BioReclamation IVT; two samples each from two separate animals) and artificial CSF (Tocris no. 3525) were aliquotted and stored at -80°C. For protease-digested CSF, two CSF samples with 0.03% CHAPS (measured to contain 273 and 643 ng/mL PrP undigested) were digested with 5 µg/mL Proteinase K (WW Grainger Co. cat. no. 5000186667) at 37C for 1 hour, after which the digestion was halted with 4 mM PefaBloc (Sigma Aldrich cat. no. 11429868001) immediately prior to use in ELISA.

Recombinant prion protein purification. For spike-in experiments and attempted detection of mouse recombinant PrP, in-house purified recombinant full-length human prion protein and mouse prion protein were purified from *E. coli* using established vectors (a generous gift from Byron Caughey's laboratory at NIH Rocky Mountain Labs) according to established methods[52,53]. Protein concentration was determined by 280 nm absorbance on a NanoDrop, and by amino acid analysis (AAA) performed in duplicate (New England Peptide) after the addition of 0.03% CHAPS.

Storage and handling experiments. For all storage and handling experiments, each condition was run in parallel on four identical aliquots made from one original CSF sample, and each aliquot was plated in duplicate. For all transfer experiments, 40 µL CSF aliquots were thawed on ice, then the full volume was transferred to a new 500 µL storage tube the indicated number of times and allowed to sit for a minimum of fifteen minutes in each tube. Where not otherwise indicated, tubes were polypropylene, and sample aliquots were 40 µL.

Total protein assay. The DC total protein assay (Bio-Rad cat. no. 5000111) was used according to the manufacturer's instructions to measure total protein across 217 CSF samples (all samples in this study except for the N=8 lumbar-thoracic gradient samples, Supplemental Figures S-4F and S-4G). This assay, similar in principle to a Lowry assay, combines the protein

with an alkaline copper tartrate solution and Folin reagent[54]. The protein reacts with copper in the alkaline medium, then reduces the Folin reagent to yield species with a characteristic blue color in proportion to abundance of key amino acids including tyrosine and tryptophan.

Whole blood spike-in. Human whole blood (Zen-Bio) was spiked into parallel aliquots of a single CSF sample containing baseline mid-range PrP at 1%, 0.1%, or 0.01% per volume. EDTA spike-ins were performed in parallel to control for EDTA preservative carried in the blood sample. Samples were refrozen following spike-in then re-thawed for use to ensure lysis of cellular fractions prior to PrP quantification.

Bethyl Laboratories Human Hemoglobin ELISA. Hemoglobin was quantified in 128 human CSF samples using the Human Hemoglobin ELISA kit (Bethyl Laboratories no. E88-134), according to the manufacturer's instructions. Samples for this analysis spanned diagnostic categories including normal pressure hydrocephalus, non-prion dementia, symptomatic genetic and symptomatic sporadic prion disease. Samples were diluted 1:10 and 1:100 for most experiments, an in some cases 1:20 and 1:100. All samples were plated in duplicate.

Blinding procedures. Assay operators were blinded to diagnosis for prion disease CSF cohorts. For test-retest cohorts, assay operators were blinded to test-retest pairing for Metformin trial samples and MIND Tissue Bank samples; pairing was known but collection order unknown for UCSF samples; pairing and order were known for Sapropterin trial samples.

Statistics, data, and source code availability. All statistical analyses were conducted, and figures generated, using custom scripts in R 3.1.2. Raw data from platereaders, associated metadata, and source code sufficient to reproduce the analyses reported herein are publicly available at: https://github.com/ericminikel/csf_prp_quantification/

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Chapter 5: Preliminary findings from the Massachusetts General Hospital genetic prion disease biomarker study

Publication history:

This chapter is adapted from a manuscript in preparation.

Attributions:

This chapter includes study components performed at the following two sites, in partnership with a number of collaborators:

1. The Broad Institute. Sonia Vallabh, Eric Minikel, Brendan Blumenstiel (Associate Director, Genomics R&D), Anna Koutoulas (Project Manager, Genomics Platform).
2. Massachusetts General Hospital. Steven E. Arnold (PI), Alison McManus (nurse practitioner), Victoria Williams (neuropsychologist), Becky Carlyle, (lab operations manager), Jessica Gerber (program manager), Holly Duddy (registered nurse), Chloe Nobuhara (technician), David Urick (technician), Chase Wennick (clinical research coordinator).

I conceived the overall study together with Steven Arnold and Eric Minikel and I collaborated closely on design and interpretation of study components across sites. The Massachusetts General Hospital team led all clinical aspects of this study including recruitment, enrollment, participant visits, participant assessments, and sample collection and processing. At the Broad site, I designed and executed biofluid assays, and collaborated with Eric Minikel on data analysis and visualization. Eric Minikel, Brendan Bluemstiel, and Anna Koutoulas led genotyping, including development and implementation of the targeted capture genotyping assay.

Abstract

Prion disease is a rapidly progressive dementia that can arise through a known set of genetic mutations in the prion protein gene (*PRNP*). Though prion disease is currently universally fatal, genetically targeted therapies to reduce prion protein (PrP) levels in the brain are under development. Pre-symptomatic carriers of genetic prion disease mutations may be well positioned to benefit from such therapies, and here we report the first results from an ongoing natural history study aiming to facilitate meaningful trials in this population. We show that within-subject test-retest PrP levels in cerebrospinal fluid (CSF) are comparably stable in mutation carriers and non-carrier controls, both over short (2-4 month) and longer (10-18 month) terms, supporting the use of CSF PrP levels as a biomarker in pre-symptomatic trials of PrP-lowering therapeutics. Carriers have normal levels of two CSF markers of neuronal damage, total tau and neurofilament light chain (NfL), arguing against a years- or decades-long biochemically detectable prodromal phase of genetic prion disease. Out of fifteen pre-

symptomatic carriers, we report one carrier with detectable CSF prion seeding activity, as measured by the real-time quaking induced conversion (RT-QuIC) assay. This observation suggests that RT-QuIC may offer the earliest detectable fluid marker of genetic prion disease. However, as RT-QuIC cannot distinguish the majority of carriers from non-carrier controls, this marker likely cannot be used as a basis for either recruitment or assessment of efficacy in pre-symptomatic trials. Overall, our results suggest that genetic prion disease carriers are healthy for most of their lives, and support a primary prevention model for pre-symptomatic trials. They also confirm that CSF PrP may serve as a useful biomarker in such trials.

Introduction

Prion disease is a uniquely rapid neurodegenerative disease of humans and other mammals. The hallmark feature of human prion disease is an aggressive symptomatic course that is usually fatal within less than six months of the first symptom[1–3]. Due to its rarity, prion disease is not typically diagnosed until most of the short disease course has elapsed[2], by which time patients are suffering from advanced dementia. Most cases of prion disease arise spontaneously, and in such cases, termed “sporadic,” there are presently no prospects for identifying individuals at risk before the onset of symptoms. However, roughly 15% of cases are genetic, arising from known, dominant, highly penetrant protein-altering variants in *PRNP*[4,5]. Depending on the *PRNP* mutation, such cases may be clinically termed genetic Creutzfeldt-Jakob Disease (gCJD), fatal familial insomnia (FFI), or Gerstmann-Straussler-Scheinker syndrome (GSS)[4]. In such cases predictive genetic testing creates an opportunity for early identification, which in turn could enable early therapeutic intervention to delay or prevent disease onset, with the goal of preserving meaningful quality of life.

All previous reported clinical trials in prion disease have recruited symptomatic patients, who are referred to trial centers by diagnosing neurologists[6–8]. In order to facilitate future clinical trials in presymptomatic mutation carriers, it will be critical to organize and characterize healthy

individuals carrying a range of genetic prion disease associated mutations, alongside non-carrier controls. In the present study, we report findings to date from a genetic prion disease carrier-control cohort representing multiple pathogenic *PRNP* mutations that we are assembling and following longitudinally.

Therapeutics to reduce prion protein (PrP) in the brain are under development for the treatment of prion disease (Chapter 3). Data to date indicate that PrP levels in cerebrospinal fluid (CSF) provide a reasonably accessible proxy for PrP levels in the brain (Chapter 4), and will be a useful tool for drug dosing, monitoring, and potentially approval. However, the stability of this biomarker has yet to be characterized in genetic prion disease mutation carriers. Multiple groups have observed that CSF PrP is decreased in symptomatic disease[9–11]. While this decline appears to progress over the symptomatic course[11], it is not clear when it begins relative to onset. CSF PrP levels have been reported to be lower in scrapie-infected sheep than in control sheep during the pre-clinical incubation period following transmission and immediately preceding onset of this horizontally acquired prion strain[12]. However, the implications for human genetic prion disease, which lacks a clear corollary of the post-transmission incubation period, are not clear. We therefore set out to assess within-subject test-retest stability of CSF PrP over short term and longer-term intervals.

To date, pathological biomarkers consistently measurable before symptoms have not been identified in genetic prion disease mutation carriers. Previous studies have employed serial FDG-PET imaging[13], MRI imaging[14], and tests of sensory thresholds and lower limb reflexes[15] to characterize pre-symptomatic mutation carriers, but have largely found changes to coincide with or follow symptom onset, with suggestive prodromal changes reported only roughly one year before onset in a few individuals. If a consistent indicator of prodromal disease could be identified, such a marker could inform design and stratification of pre-symptomatic trials. On the other hand, if no prodrome is systematically identifiable in the cohort of healthy mutation carriers presently available for research and trials, this may highlight a substantial

difference between prion disease and more slowly progressive neurodegenerative diseases[16–19], again with implications for trial design.

As imaging techniques, though useful in the diagnosis of sporadic CJD[20], appear to offer limited sensitivity to some genetic prion disease subtypes even in the clinical stage of disease[21], we chose instead to focus on fluid biomarkers. Three such biomarkers – total tau protein (t-tau), neurofilament light chain protein (NfL), and RT-QuIC seeding activity – were chosen for tracking in carrier CSF based on their well-established elevation in symptomatic prion disease[9,22–30]. These markers have not been systematically prospectively assessed in pre-symptomatic carriers. One study reported nominally elevated CSF NfL in a single pre-symptomatic P102L carrier, but at levels still overlapping the range seen in controls[28]. Meanwhile two studies have seen single individuals convert from RT-QuIC negative to positive. A P102L carrier was reported to be RT-QuIC negative two years before onset, but positive at symptom onset[28], while one E200K carrier was reported to be negative two months after symptom onset, but positive four months after onset[23].

Results

Three groups of individuals were recruited to the study: 1) confirmed mutation carriers of *PRNP* variants, 2) untested individuals at 50% risk of carrying a *PRNP* variant, usually established through confirmed mutation status of a first-degree relative; and 3) non-carrier controls. Mutation carriers and at-risk individuals were only eligible if determined by pre-screening to be pre-symptomatic and able to give consent to participate. Between July 2017 and time of analysis, 33 individuals completed at least one study visit. For all participants, genotyping was performed by targeted capture and short-read sequencing, for research purposes only, to determine *PRNP* mutation status. Results are summarized in Table 5-1. Of the 33 initial participants, at time of writing 25 have completed one follow up visit 2-4 months

after the initial study visit, and three have completed a third study visit 10-18 months after their initial study visit.

Demographics of participants to date are summarized in Table 5-1. At each study visit, in addition to undergoing a blood draw and lumbar puncture, participants completed an array of tests and questionnaires assessing their cognitive, neuropsychological, and motor abilities and daily functioning. In support of their pre-symptomatic status, carriers perform comparably to controls on these assessments (Supplemental Table S-2). Of note, all participants scored 20/20 points at each visit on the prion disease-specific MRC functional rating scale.

Table 5-1: Demographic overview of study participants. *Participant number, age, sex,* PRNP *genotype, and total number of study visits at time of analysis.*

		***PRNP* mutation carriers**	**Non-carrier controls**
N		22	11
Age at first visit		42.4±15.5	42.7±12.8
Sex	Male	6	4
	Female	16	7
***PRNP* genotype**	Wild type	0	11
	E200K	8	0
	D178N	7	0
	P102L	3	0
	Other	4	0
Number of completed study visits	1 visit	3	2
	2 visits	17	8
	3 visits	2	1

Within subject test-retest stability of CSF PrP

To assess the short-term within-subject stability of CSF PrP levels in genetic prion disease mutation carriers and controls, we quantified PrP levels in CSF samples given by study participants at 2-4 month intervals using an ELISA assay that we have previously shown appropriate for this purpose (Chapter 4). Following collection, CSF used for this analysis was handled uniformly according to an established protocol that includes addition of 0.03% CHAPS detergent to maintain PrP solubility (Chapter 4). CSF PrP levels were reasonably stable over

this interval and similar between carriers (mean CV = 8.2%) and controls (mean CV = 6.7%) (Figure 5-1A). While all reasonable efforts were made to standardize CSF collection, in some cases clinical variations were noted, including use of drip collection rather than aspiration and aberrantly low sample yields. On average, the six individuals whose CSF was handled differently between the two visits showed greater, though still reasonable, variation in CSF PrP levels (mean CV = 14.9%) compared to all other participants (mean CV = 5.7%).

At the time of analysis, three individuals had completed a third study visit 10-18 months after their initial study visit, enabling a preliminary longer-term analysis of CSF PrP test-retest stability. Across two mutation carriers and one control, CSF PrP levels were again steady with a mean CV of 4.4% across all three visits (Figure 5-1B). A comparison with test-retest CSF PrP levels in samples shared from other retrospective cohorts lacking uniform sample handling suggests that our sample collection and processing protocol is controlling the pre-analytical variability we previously reported (Chapter 4). Across all participants, CSF PrP was modestly correlated with CSF total protein ($P = 0.0067$, $r = 0.38$, two-sided Spearman's correlation), as we have previously observed in CSF samples from other cohorts (Chapter 4). This correlation could be driven by biological factors, or may reflect a blocking function whereby higher total protein content insulates PrP from adsorption to plastic during collection and handling.

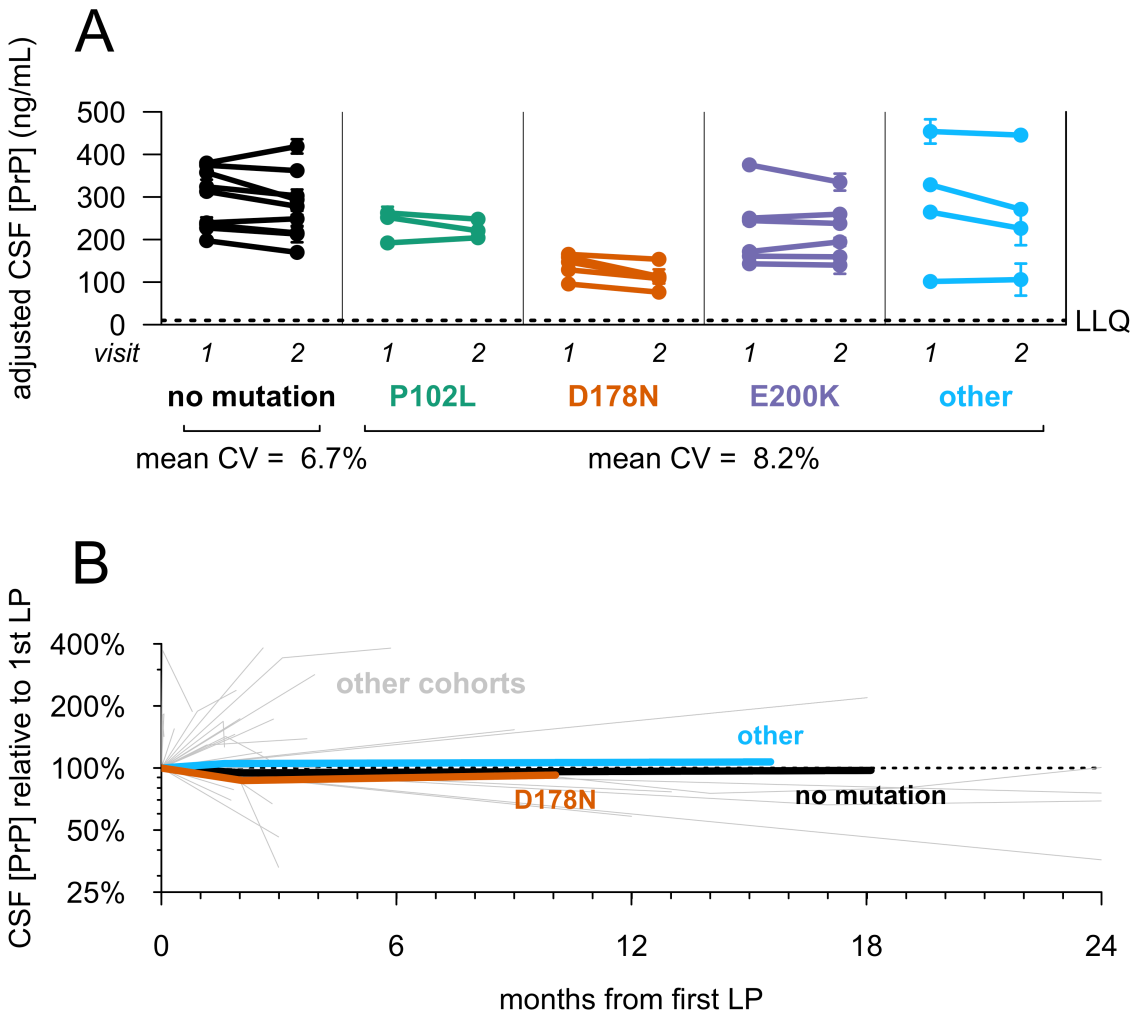

Figure 5-1: Test-retest stability of CSF PrP. *Uniformly processed CSF samples were collected from lumbar punctures performed by one of two investigators. CSF PrP levels were quantified using the BetaPrion human ELISA assay, with samples from the same individual co-located on the same plate. The operator was blinded as to carrier/control status. Dots represent means, and line segments 95% confidence intervals, of measurements within dynamic range with 2 technical replicates each. A. 21 individuals gave two CSF samples at an interval of 2-4 months. While test-retest samples were co-located on the same ELISA plate for PrP measurement, multiple plates were needed to measure samples for all subjects, therefore CSF PrP levels have been normalized to an interplate (IPC) control sample, such that the IPC control PrP value for each plate matches the first in the series. B) Three participants with the noted genotypes gave three CSF samples at the following intervals: initial visit, 2-4 month follow-up visit, 10-18 month follow-up visit. For each subject, PrP levels for all visits have been normalized to levels at the first visit, such that the first LP is defined as 100%. Grey lines show PrP test-retest stability for CSF samples shared from other cohorts. Note that data from other cohorts is reproduced from Supplemental Figure S-7.*

Pathological biomarkers in participant CSF

To assess the presence of neuronal damage markers in carrier and control CSF, we measured total tau and NfL levels in participant CSF by ELISA. For both t-tau and NfL, levels in carrier and control CSF were similar ($p = 0.049$ and $p = 0.82$ respectively, 2-sided Kolmogorov-Smirnov test), with a weak trend toward higher t-tau in non-carrier controls (mean 261±73 pg/mL) than in mutation carriers (241±102 pg/mL). All samples fell within or below the normal ranges reported by other studies using the same ELISA assays[9,25,26]. By contrast, as expected, both proteins were highly elevated in positive control CSF from symptomatic prion disease patients ($p = 7.2 \times 10^{-8}$ for t-tau; $p = 1.7 \times 10^{-14}$ for NfL, 2-sided Kolmogorov-Smirnov test) (Figures 5-2A and 5-2B). To assess potential changes over time in markers of neuronal damage, we measured total t-tau and NfL in the longer-term serial CSF samples available for three longitudinal participants spanning 10-18 months. Across all three visits, levels of both proteins remained low with no change over time seen in either carriers or control ($p=0.17$ for t-tau; $p = 0.45$ for NfL, linear regression) (Figures 5-2C and 5-2D).

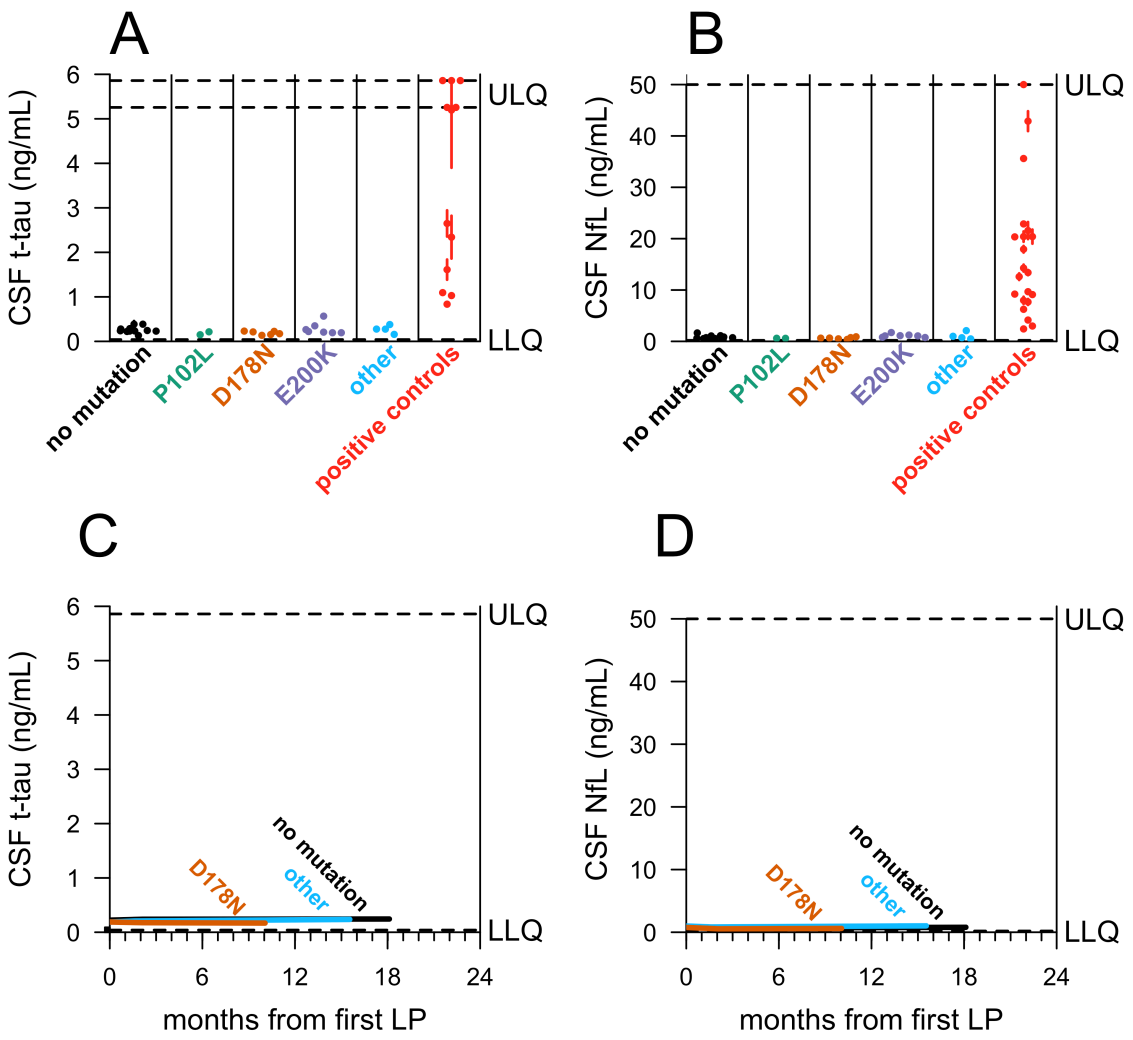

Figure 5-2: Markers of neuronal damage in carrier and control CSF. *CSF* ***A)*** *Total tau (t-tau) and* ***B)*** *neurofilament light (NfL) were measured for 22 participants who have made at least one study visit, for whom genotypes were available at time of analysis, and for whom appropriate CSF aliquots were available. For each participant included, samples were taken from the most recent visit at time of analysis. We used the Innotest hTau Ag ELISA kit (Fujirebio) and NF-light RUO ELISA (Tecan). Both assays included positive control CSF samples from post-mortem confirmed clinical cases of both sporadic and genetic prion disease (shown in red). The operator was blinded as to mutation status. Dots represent means, and line segments 95% confidence intervals, of measurements within dynamic range with 2 technical replicates each. The two ULQ lines for t-tau represent the distinct upper limits of quantification for two different kit lots.* ***C and D)*** *For participants who had completed three visits, both total tau and NfL were measured by ELISA for all three visits to assess longitudinal dynamics. As in Figure 5-1B, CSF from the following three timepoints is represented for each participant: initial visit, 2-4 month follow-up visit, 10-18 month follow-up visit. Dots represent means, and line segments 95% confidence intervals, of measurements within dynamic range with 2 technical replicates each. Serial samples were co-located on the same ELISA plate.*

To assay prion seeding activity in participant CSF samples, we leveraged the real-time quaking induced conversion (RT-QuIC) assay, following a published protocol optimized for CSF-seeded reactions[22]. The RT-QuIC assay recapitulates prion-seeded templated misfolding in a well, and provides a fluorescent readout of aggregation kinetics by leveraging amyloid-binding dye thioflavin T. Second-generation conditions for CSF-seeded reactions have achieved 100% specificity as well as high, if somewhat subtype-dependent, sensitivity for symptomatic prion disease in other cohorts[22,27,31]. According to previously published criteria, positive reactions were defined as those where at least 2/4 replicates reached a predetermined fluorescence threshold within 24 hours of initiation of the assay[22]. Under these conditions, positive controls including CSF from both sporadic and genetic post-mortem confirmed prion disease cases were identified as positive with 88% sensitivity, comparable to reported results[22,27,31] (Figure 5-3A). Non-carrier control samples were negative (Figure 5-3B), as were 14/15 carrier samples (Figures 5-3C - 5-3F). One E200K sample gave a positive result (Figure 5-3E) and is discussed in greater detail below.

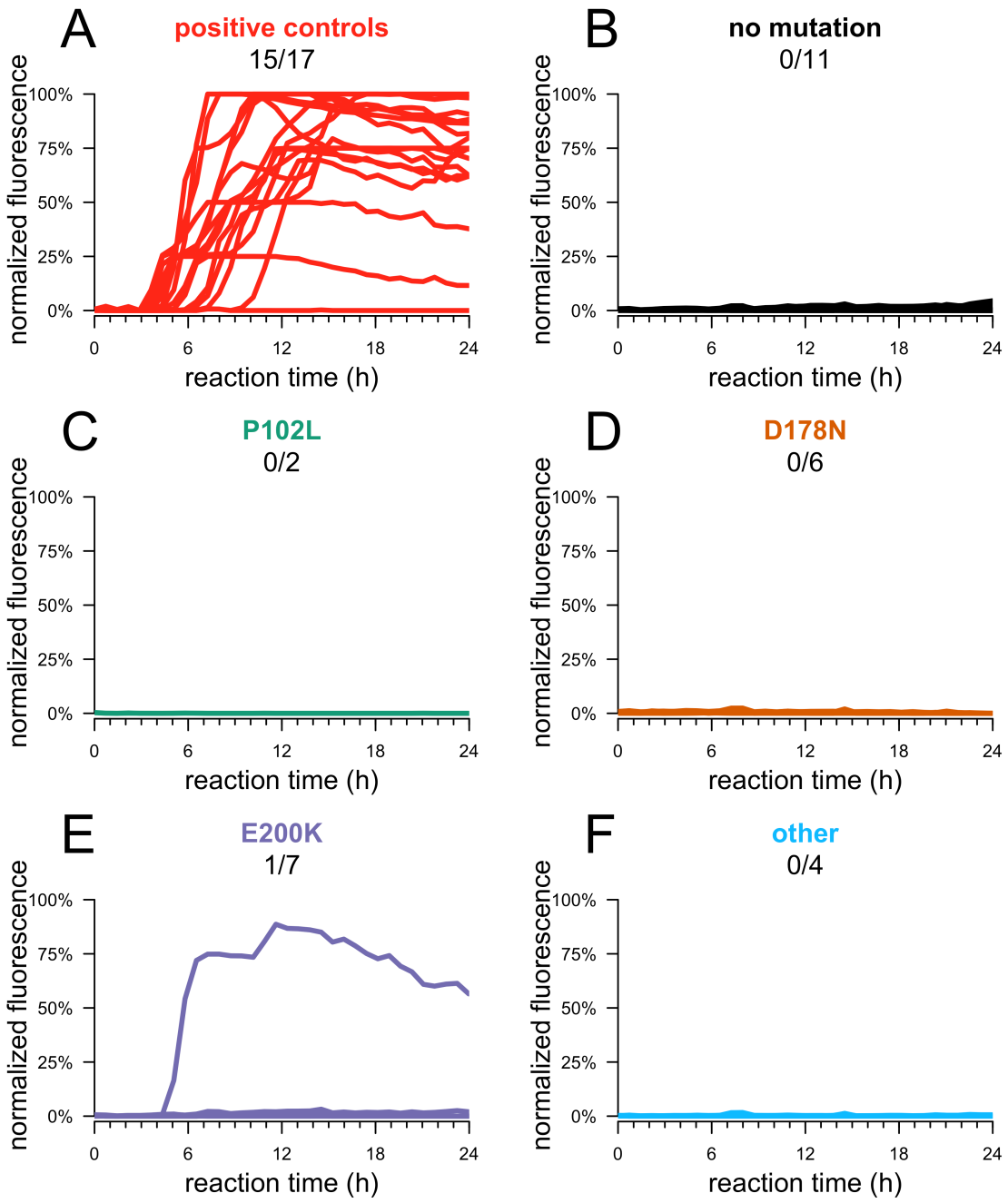

Figure 5-3: RT-QuIC seeding activity in carrier and control CSF. *RT-QuIC was performed on CSF from 22 participants who have made at least one study visit, for whom genotypes were available at time of analysis, and for whom appropriate CSF aliquots were available. For each participant, samples were taken from the most recent visit at time of analysis. RT-QuIC was performed following established second-generation protocols for second-generation CSF RT-QuIC. Reactions were seeded with 20 uL CSF from N=17 post-mortem confirmed prion disease cases or N = 30 MGH study participants, with each reaction run in quadruplicate. Kinetic curves – normalized thioflavin T (ThT) fluorescence (y axis) vs. time in hours (x axis) – are shown for each replicate. The operator was blinded to sample mutation status.*

RT-QuIC positive pre-symptomatic carrier

One participant, a carrier of the E200K mutation older than the average age of onset for this mutation, showed RT-QuIC seeding activity upon analysis of CSF from their second visit. We subsequently performed RT-QuIC, t-tau and NfL analysis on CSF from both visits 1 and 2 for this individual. For both visits, 4/4 replicates were positive for RT-QuIC seeding activity, while total tau and NfL remained in the normal range (Table 5-2). Over the two month interval between visits 1 and 2, no striking changes were seen in the cognitive, psychiatric, motor or daily living assessments (Table 5-2 and Supplemental Table S-3). This participant's score on the Montreal Cognitive Assessment declined nominally between visits 1 and 2, to just below the cutoff of 26 typically used to bound the normal range[32], but it is not clear that this difference is meaningful. This individual remains asymptomatic roughly one year after their second visit. Notably, this participant's CSF PrP levels were stable between visits 1 and 2.

Table 5-2: Comparison of visits for one RT-QuIC-positive study participant. *This participants first and second study visits were separated by two months. RT-QuIC replicates were designated as positive based on criteria described above, in methods, and elsewhere[22]. T-tau, NfL, and PrP were measured by ELISA as described in Figures 5-1 and 5-2.*

		Visit 1	Visit 2
Assessment scores	MRC prion disease rating scale	20	20
	Montreal Cognitive Assessment	27	25
	NIH Toolbox Cognitive Battery composite	109	104
Fluid biomarkers	CSF t-tau (ng/mL)	0.57	0.60
	CSF NfL (ng/mL)	1.27	1.48
	RT-QuIC (positive replicates)	4/4	4/4
	CSF PrP (ng/mL)	380	394

Lumbar puncture tolerability

As future therapeutic interventions for prion disease, such as antisense oligonucleotides, may be delivered intrathecally, we sought to gather feedback from study participants on the clinical experience of undergoing a lumbar puncture for research. Following each lumbar puncture, participants completed a brief survey in which they were asked to rate their anxiety

prior to the LP, as well as their post-procedure anxiety at the prospect of a future LP, by appropriately marking a Likert-type scale. For participants experiencing their first LP, self-reported anxiety declined from mean 51.2% to mean 26.1% (P = 0.0017 by a 2-sided Student's t test) following the procedure (Figure 5-4).

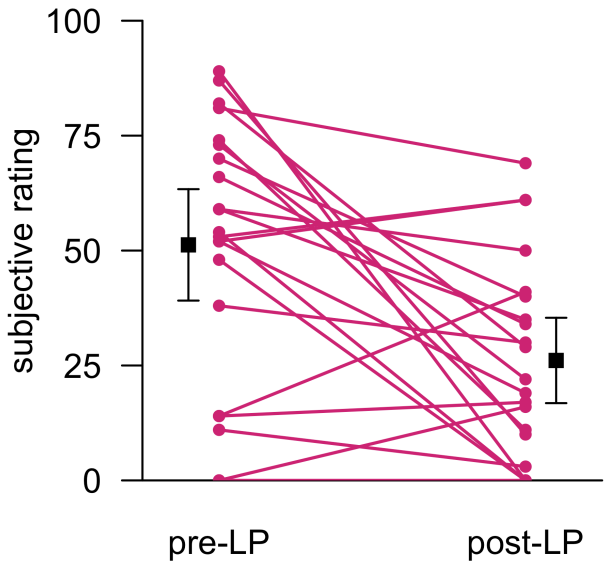

Figure 5-4: Pre- and post-procedure anxiety in participants experiencing their first lumbar puncture. *Following the lumbar puncture procedure, participants were asked to quantitatively rank both their anxiety before the procedure and their post-procedure anxiety when contemplating a future LP, using a Likert-type scale. Responses are normalized to the length of the scale and shown only for first study visits, for individuals who had not undergone a previous LP.*

Discussion

Above, we describe interim results from an ongoing longitudinal clinical study characterizing genetic prion disease mutation carriers and mutation-negative controls. This is the first report that we know of to prospectively characterize fluid biomarkers in healthy individuals carrying genetic prion disease predisposing mutations.

Our analysis of test-retest CSF PrP indicates that CSF PrP levels are reasonably stable over short and intermediate terms in both carriers and controls. For samples with no noted handling aberrations, we observed a mean CV of less than 6% over 2-4 months intervals. This

level of biotemporal stability is comparable to that reported for core CSF biomarkers including amyloid beta (Aβ) 1-38, Aβ 1-40, t-tau, and NfL over a similar term[33]. Analysis of a small number of longer-term follow-up samples suggest that CSF PrP levels may be equally stable over up to a year or more, with a mean CV of less than 5%. Notably, CSF PrP variability in this cohort appears lower than in our previous analysis using samples from placebo-treated individuals with mild cognitive impairment enrolled in a clinical trial (mean CV = 13%), a finding that likely reflects greater control of pre-analytical factors afforded by our CSF processing protocol.

PrP levels in the CSF of asymptomatic D178N mutation carriers have been reported to be lower than in individuals with other *PRNP* mutations or no mutation[11], a finding replicated here. This difference has been interpreted by some to represent a prodromal disease process underway[11]. However, other reports suggest that preferential degradation of D178N mutant PrP may occur constitutively, independent of initiation of the disease process[34–37], leading to lower baseline PrP levels in carriers of this mutation. Our data support the latter interpretation. Relatively lower CSF PrP levels in D178N carriers appear to be stable over short (Figure 5-1A) and longer terms (Figure 5-1B), suggesting that this level is constant rather than a dynamic function of approaching symptom onset, which to date has not been observed for any study participant. Notably, CSF PrP levels were stable even in one E200K mutation carrier with RT-QuIC seeding activity (Table 5-2). Thus, the decline in CSF PrP levels seen in symptomatic disease likely emerges later in the disease process, and should not confound CSF PrP stability in carriers with no observable pathology.

Broadly, these findings suggest that CSF PrP levels are stable enough in any one individual, regardless of *PRNP* mutation, to informatively report on a PrP-lowering therapeutic such as a PrP-lowering antisense oligonucleotide in the central nervous system, over time frames likely to be of relevance to dose-finding and biomarker-based trials. In a Phase I/II trial of the Huntingtin-lowering ASO HTT Rx, mutant Huntingtin protein was reduced by a mean of 40% in CSF in the two highest dose cohorts[38,39]; our data suggest that if a similar reduction in PrP

levels could be achieved, measurement of CSF PrP could reliably facilitate its detection. The lumbar puncture tolerability data we were able to collect suggests that intrathecal delivery of a drug will not be a barrier to treatment among pre-symptomatic carriers of *PRNP* mutations. While our study is biased toward highly motivated carriers willing to participate in research, this same bias will likely apply to trial recruitment, vouching for the relevance of these data.

Our analysis of CSF pathological biomarkers found most carriers to be indistinguishable from non-carrier controls. Previous reports have identified anecdotal prodromal signatures in one individual no more than roughly a year in advance of symptomatic onset[13,14,28]. We replicate this finding for RT-QuIC by showing that prion seeding activity is detectable in 1/15 pre-symptomatic carriers, and appears to pre-date symptom onset by a minimum of one year. This finding suggests that for individuals harboring the E200K mutation, RT-QuIC seeding activity may be the first detectable pathological change. However, 3/4 E200K carriers and 11/11 carriers of other mutations were negative by RT-QuIC, suggesting that this signal does not represent a long nor consistent carrier prodrome.

Our study has several limitations. Our analyses to date provide only short-term and cross-sectional findings with the exception of a small number of samples from longer-term follow up visits. Moving forward, participants will be seen at annual intervals where feasible, with an eye to enhancing the longitudinal analysis of CSF PrP and enabling longitudinal tracking of pathological biomarkers. In the present analyses of CSF and NfL, ELISA sensitivity limited our analysis of neuronal damage markers to CSF, but we will next expand to measuring these markers in plasma using the single-molecule array (Simoa) platform[28,29]. We chose to perform RT-QuIC analysis according to the best validated protocol for human CSF, and achieved 88% sensitivity to detect positive controls and 100% specificity with regard to known non-carrier controls. However, alternate RT-QuIC protocols are available using human[23] or bank vole[40] recombinant PrP substrate, and offer additional routes for further testing of participant samples.

To date, our study suggests that carriers of *PRNP* variants are healthy, with no sign of prion pathology, for the majority of their lives. Put differently, these data do not support analogies between the disease state of the average carrier and the clinically silent incubation phase of prion disease observed in animal models. It remains possible that a fluid biomarker that reliably presages symptom onset further in advance will emerge from further study, and we will continue to expand our cohort as well as to conduct longitudinal follow-up. However, our present findings reflect where the field will stand as therapeutics presently in development approach clinical trials, and suggest a major practical difference between prion disease and more slowly progressive dementias. Clinical trials in other neurodegenerative diseases have at times relied on a secondary prevention model, using presence of biomarkers of sub-clinical pathology as indication of disease imminence, and as gating criteria for enrollment[41,42]. Our cohort represents the pre-symptomatic *PRNP* mutation carriers who are available in the near term to participate in research and in trials, with the implication that such an approach is not supported in this context. Pre-symptomatic trials in genetic prion disease may therefore be better served by a primary prevention model based on genetic risk.

Methods

Protocol approvals and consents. This study was approved by the Partners Institutional Review Board in April 2017 (protocol #2017P000214). All participants provided written informed consent at the time of study enrollment. This study did not provide predictive genetic testing for genetic prion disease. If at-risk participants did not know their own genetic status, the clinical team was blinded to their status. The research team performed *PRNP* genotyping on de-identified samples for research purposes only.

Assessments of cognitive, neuropsychiatric, motor and daily functioning.

- The **Medical Research Council (MRC) prion disease rating scale**[3] used to assess whether participants were experiencing intermediate to advanced symptoms of prion disease. This 20-point scale broadly assesses activities of daily living compromised by advancing disease, such as cognitive function, speech, mobility, personal care, feeding, and continence. People in reasonable health should score 20 points, with deductions reflecting inability to independently perform basic daily tasks.
- The **Montreal Cognitive Assessment (MoCA)**[32] was used as a screen for mild cognitive impairment. This 10-minute test uses simple tasks to assess short-term memory, visuospatial abilities, executive function, attention, concentration, working memory, language, and orientation to time and place. Points are assigned per task out of a possible thirty total.
- **The NIH Toolbox Cognition Battery**[43] was used to test episodic memory, executive function and attention, working memory and processing speed. The global composite score reflects the following 8 subtests from the toolbox, administered via iPad:
    - The **Dimensional Card Sort Test (DCCS)** was used to test cognitive flexibility using sorting bivalent test pictures; participants are required to rapidly change tasks by sorting on different dimensions. Scoring is based on a combination of accuracy and reaction time.
    - The **Picture Sequence Memory test** was used to test episodic memory by requiring patients to recall the order of a series of pictures. Scoring is based on the number of picture pairs in the sequence that are correctly placed adjacent to one another.
    - The **Flanker Inhibitory Control and Attention test** was used to measure attention and inhibitory control by requiring the participant to focus on and report

on one stimulus while inhibiting attention to others. Scoring is based on a combination of accuracy and reaction time.

- The **List Sorting Working Memory** was used to test working memory by requiring participants to sequence different stimuli presented both visually and orally. Scoring is based on the number of items correctly sequenced in ascendingly difficult conditions.
- The **Picture Vocabulary test** was used to test receptive vocabulary using an adaptive format in which participants were required to match photographs to an audio recording of a word. Scoring is based on the number of correct answers.
- The **Oral Reading Recognition** test was used to measure reading ability. Participants are asked to read a series of words, pronouncing them accurately as possible. Each word is scored as right or wrong by the administrator.
- The **Pattern Comparison Processing Speed Test** was used to measure processing speed by through rapid determination of whether two images are the same or different. Scoring is based on the number of correct answers in a 90-second period.
- The **Rey auditory verbal learning test (RAVLT)** was used to test immediate recall by asking participants to recall a list of fifteen unrelated words in three learning trials. In addition to its conventional use, the test was reintroduced 20-30 minutes before the end of the NIH battery to test delayed memory following intervening tasks. Scoring is based on the total number of words correctly recalled.

- The **Delis-Kaplan Executive Functioning System (D-KEFS)**[44] is a set of nine tests used to assess executive function. We used two tests from this battery. 1) The **color-word interference test (CWIT)**, based on the Stroop color interference test[45], stages ascendingly difficult reading tasks to test processing speed, verbal inhibition, and

cognitive flexibility. Scoring is based on completion time for each task. 2) The D-KEFS **verbal fluency test (VFT)** measures the subjects' ability to generate words swiftly in three conditions: a phonemic format (letter fluency), from overlearned concepts (category fluency), and while simultaneously shifting between overlearned concepts (category switching). Scoring is based on the number of correct responses per category.

- The **trailmaking test (TMT**), part of the Halstead-Reitan Neuropsychological Battery[46], measures processing/graphomotor speed (Part A) and mental flexibility within the executive function domain (Part B) through a timed visual motor sequencing task. Scoring is based on completion time for each condition.
- The **grooved pegboard test**[47] measures fine motor manipulative dexterity of both the dominant and non-dominant hand. Scoring is based on time time completion for each hand.
- The **digital clock drawing test (dCDT)** is a variant of the traditional clock drawing test, which measures cognitive function through subjective clinical rating of a subject's ability to draw a standard clock face showing a specified time using pen and paper. In this variant, the participant draws with a digitized ballpoint pen that collects spatial and temporal data on its use, allowing many features of the behavior to be quantified and compiled into a composite task score[48].
- **Questionnaires.** The following standard instruments, all previously described, were administered to collect self-reported information on participants' sleep, motor, mental health and cognitive function in the context of their daily lives: 1) Everyday Cognition ("ECog")[49,50], 2) the Athens Insomnia Scale[51], 3) the Epworth Sleepiness Scale[52], 4) the Beck Anxiety Inventory[53], 5) the Beck Depression Inventory[54], and 6) the Motor Aspects of Experiences of Daily Living assessment[55].

Blood processing and genotyping. Blood was collected in EDTA tubes, then allowed to clot for 30 mins, before centrifugation at 1000 rpm and aliquoting into 0.5 mL aliquots. Samples were codified for analysis and genotyping; genotypes are used for research purposes only. Whole blood samples were submitted to the Broad Genomics platform for DNA extraction. The *PRNP* region was enriched using a custom targeted capture probe set (Twist Biosciences) and subjected to short-read sequencing for single nucleotide variant detection. OPRI detection was performed by Genewiz using a protocol provided by Piero Parchi and Anna i) Stella — after amplification with primers GCAGTCATTATGGCGAACCTTGGCTG (forward) and TGCATGTTTTCACGATAGTAACGG (reverse), PCR products were sized on a 2% agarose gel.

Lumbar puncture and CSF processing. The lumbar puncture (LP) for CSF collection was performed using a standardized protocol with a 24G atraumatic Sprotte needle by either Dr. Steven Arnold, M.D or Dr. Alison McManus, D.N.P. The time of day for LP was kept consistent across subjects and 20 mL CSF was collected per subject where possible. Following collection, CSF was handled uniformly according to an established protocol designed to minimize PrP loss to plastic through measures including i) highly controlled and minimized plastic exposure, ii) uniform storage in aliquots no smaller than 40 µL, and iii) addition of 0.03% CHAPS detergent to a subset of CSF to maintain PrP solubility (Chapter 4). Samples were then frozen and banked at the Broad Institute where they are stored at -80°C until analysis. CSF aliquots containing 0.03% CHAPS were used for PrP quantification by ELISA; neat CSF aliquots with no additive were used for t-tau ELISA, NfL ELISA, and RT-QuIC. Because some LPs were anomalous or unsuccessful, for some participants CHAPS CSF, neat CSF, or both were not available. These individuals were excluded from the corresponding analyses. All analyses were performed by researchers blinded to participant identity and carrier/control status.

Post-LP survey. Following each LP, participants completed a brief survey that we designed to assess the experience, either on paper or via iPad. They were asked whether they had previously had an LP, and if so, how many. Participants were then asked to mark an X on a 14-cm Likert-type scale[56] to indicate 1) their level of anxiety before the LP procedure, and 2) their current feelings at the prospect of a future LP. In both cases, the response was marked on a continuous spectrum bounded by the two extremes of "Not anxious at all" and "Extremely anxious." Responses were normalized to the full length of the scale.

PrP ELISA. PrP levels were quantified at the Broad Institute using the BetaPrion Human ELISA assay that we have previously shown suitable for this purpose (Chapter 4), according to the manufacturer's instructions and as described elsewhere (Chapter 4). All samples were diluted 1:50 in blocking buffer (0.05% Tween, 5% BSA, 1x PBS) and assayed in duplicate, with samples from the same individual co-located on the same plate to facilitate comparison. Following termination of the colorimetric development reaction, absorbance per well was measured at 450 nm as well as at 620 nm for background subtraction using a FLUOStar Optima absorbance plate reader, then fit to an internal standard curve to generate PrP concentrations in ng/mL.

Total tau. CSF total tau (t-tau) was measured using the Innotest hTau Ag ELISA kit (Fujirebio) according to the manufacturer's instructions. Study samples were diluted 1:4; positive control symptomatic prion disease samples were diluted 1:10. All samples were assayed in duplicate with samples from the same individual co-located on the same plate to facilitate comparison. Following termination of the colorimetric development reaction, absorbance per well was measured at 450 nm as well as at 620 nm for background subtraction using a FLUOStar Optima absorbance plate reader, then fit to an internal standard curve.

NfL. CSF neurofilament light (NfL) was measured using the NF-light RUO ELISA (Tecan) according the manufacturer's instructions. Study samples were diluted 1:2; positive control symptomatic prion disease samples were diluted 1:5. All samples were assayed in duplicate with samples from the same individual co-located on the same plate to facilitate comparison. Following termination of the colorimetric development reaction, absorbance per well was measured at 450 nm as well as at 620 nm for background subtraction using a FLUOStar Optima absorbance plate reader, then fit to an internal standard curve.

RT-QuIC. The real-time quaking induced conversion (RT-QuIC) assay was performed according to an established RT-QuIC protocol for detection of prion seeds in CSF[57] that is widely used for diagnosis of symptomatic prion disease patients. Briefly, truncated recombinant Syrian hamster prion protein (SHaPrP 90-230) was purified from E. coli according to established protocols, then frozen at -80°C following determination of concentration by NanoDrop. On the day of use, PrP was thawed and centrifuged at 5,000 x g for 5 minutes at 4C in a PALL 100 kDa filter tube. 80 uL of reaction mix and 20 uL of CSF were combined in each well of a black 96-well plate with a clear bottom (Nunc) with final concentrations as follows: 300 mM NaCl, 10 mM phosphate buffer, 1 mM EDTA, 10 uM thioflavin T, 0.002% SDS, 1 mg/mL SHaPrP. All samples were loaded in quadruplicate with each plate containing negative control CSF (healthy mutation-negative individuals) and positive control CSF (symptomatic prion disease patients). After sealing (Nalgene Nunc International sealer), plates were incubated in a BMG FLUOstar Optima plate reader at 55°C for 40h with continuous cycles of 60 s shaking (700 rpm, double-orbital) and 60 s rest, and ThT fluorescence measurements every 45 min (excitation 450 nm, emission 480 nm, bottom read.) Following termination of the experiment, fluorescence readings were merged per well to generate kinetic curves, and the threshold for a positive well was set as the mean value of all negative wells plus 10 standard deviations. A sample was considered overall positive if at least two of four replicates crossed this threshold.

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Chapter 6: Conclusion

Next steps for prion disease

In this thesis, we have outlined a path to the prevention of genetic prion disease, and provided evidence that PrP-lowering antisense oligonucleotides may prove the first therapeutic to successfully walk this path. While much work remains, we are pursuing next steps of ASO development in close collaboration with pharmaceutical, regulatory, academic, and clinical partners. It is our hope that the data presented in this thesis will support the launch of a ASO trial in pre-symptomatic genetic prion disease mutation carriers, and that this modality will ultimately prove capable of extending healthy life in those at risk for prion disease.

While ASOs provide a central pivot and immediate motivation for our efforts, much of the knowledge gained and infrastructure assembled here can be repurposed towards future prion disease drug development efforts. First, we have robustly validated PrP lowering as a therapeutic hypothesis in prion disease, providing a pharmacological proof of concept that dovetails with longstanding genetic data. Second, we have established a regulatory and biomarker framework centered on the therapeutic hypothesis of PrP-lowering that could be considered for advancement of any drug with this mechanism of action. Third, we have built a cohort of pre-symptomatic carriers of genetic prion disease mutations that with whom we hope to continue to work moving forward. Future efforts can benefit from this investment in both characterization and teambuilding.

Antisense oligonucleotides offer a strong starting point for PrP-lowering drugs, owing in part to their deliverability to the whole brain, which will be essential to combat prion disease. While most potent in the cortex, intrathecally delivered ASO impact target levels in subcortical regions of the nonhuman primate[1,2] and human brain[3] to a degree that our work suggests would be meaningful in prion disease. With that said, there is room for improvement. Future PrP-

lowering drugs may aim to complement the pattern of ASO distribution with more potent knockdown in subcortical brain structures. Several potential therapeutic modalities may rely on viral vectors for delivery, and while it is not clear that brain-wide distribution of such vectors is currently achievable, recent advances[4,5] lend cause for optimism in the medium term. Groups developing new modalities for brain-wide reduction of a single disease-causing protein may fruitfully turn to prion disease as a unique test case where animal survival can provide a reliable, quantitative, and biologically meaningful readout of drug activity.

Beyond prion disease

Genetic prion disease has several unique features. The rapidness of the symptomatic course, variability in age of onset, and rareness of the indication render trials with a clinical endpoint particularly challenging. At the same time, the genetic and mechanistic clarity of the disease create three powerful and related opportunities: we can identify healthy carriers at high risk, design plausible near-term targeted therapies, and meaningfully test those therapies in healthy carriers. For all of these reasons, prion disease may be well positioned to pioneer a new model of genetically informed prevention, where all aspects of treatment trials, from recruitment to drug target to readout of efficacy, pivot on the genetic cause of disease.

With that said, for all its extremity, prion disease may differ more in degree than in kind from other neurodegenerative diseases. Tools and vocabulary from the prion field are propagating, prion-like themselves, into adjacent literatures, as increasing attention is paid to conformational templating of other disease-associated proteins in the brain[6–8]. Leveraging these mechanistic parallels, RT-QuIC is now coming online as a diagnostic for a spectrum of disorders[9]. Distinct molecular strains of other disease-associated proteins have been described[10–13], and we should be attentive to the possibility that the corresponding heterogeneity in molecular pathology may, as in prion disease, confound certain therapeutic approaches[14].

Indeed, many of the challenges that we have outlined for prion disease therapeutic development – multiple or unknown pathogenic species, diversity in clinical presentation, variable rates of progression, barriers to early diagnosis – resonate across the field.

Prion disease has the asset of clear monogenic cause, illuminating one path forward through the minefield of heterogeneity. The gift of an unequivocal genetic target lays out a clear therapeutic hypothesis and enables every step of its realization, from genetic validation in animals and humans, to development of targeted therapies, genetically informed biomarkers, and rationally selected trial cohorts. For other diseases in which causation is comparably clear, the infrastructure we have assembled may offer one relevant model, especially as genetically targeted platform-based therapies achieve greater track records and modularity. In such cases, leveraging simplicity towards the nearest-term feasible therapeutic is likely to require a relinquishing of mysteries – an acceptance that many open biological questions will remain, just as they will with prion disease, on the day when we test an effective treatment in humans for the first time.

For diseases with more complex or unsolved genetic and molecular bases, the insights to enable upstream intervention may not yet be in place. But it is clear that scientists across sectors, including at the FDA, are keen to see developments in this direction[15,16]. Mounting disappointments in neurodegenerative clinical trails are driving increased recognition that preservation of healthy brains may be more than just a distant, aspirational future use for drugs initially developed against active disease. In some cases, leveraging a genetically informed head start against brain disease may be our only viable plan of attack.

Concluding thoughts: prevention

The case for primary prevention of neurodegeneration – for carefully informed, rigorously assessed therapeutic intervention upstream of any shadow of disease – can be made in terms

of scientific feasibility. It can be made in terms of logistics. But the deeper call to action is larger than the sum of these details, and more human. For all of us there exist a small number of human brains that represent the most important assemblages of molecules on Earth. It is when we picture these few brains, in all their vulnerability – their irreplaceable, idiosyncratic networks, about which we ultimately know so little – that the mandate for primary prevention emerges most clearly. Human ambition will drive ongoing efforts to model, simulate, rewire, reverse engineer, build and rebuild the brain, and surely these efforts will enrich and surprise us. But our strongest foot forward against the ravages of dementia may lie in embracing a humbler role – not that of supervisor, architect, or engineer, but that of guardian.

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Appendix

Supplement to Chapter 4: Prion protein quantification in human cerebrospinal fluid as a tool for prion disease drug development

Supplementary Discussion

Technical parameters of the BetaPrion® ELISA kit.

As noted in Table 4-1, for one sample included as an inter-plate control on 17 different plates, we observed an inter-plate CV of 22%. The 17 plates included in our analysis include plates from three different manufacturer lots, run by two different operators (SV and EVM), read on two different platereaders (Fluostar Optima and Spectramax), all of which factors may contribute to the variability we observed.

On an intra-plate basis, we also observed slightly higher variability when including dilutions than when only comparing replicates at a single dilution (CV=11% vs. 8%). Most samples were analyzed at two dilutions, 1:10 and 1:50, with two replicates each. In many cases, one dilution or the other fell outside the assay's dynamic range, but among *N*=87 samples for which both the 1:10 and 1:50 dilutions had both replicates fall within the dynamic range of the assay (1 to 20 ng/mL final), the PrP level indicated by the 1:10 dilution was on average 3.5% higher than the 1:50 dilution.

Plate position effects

To assess whether plate position affects apparent PrP levels in ELISA, we ran two whole ELISA plates loaded with technical replicates of the same CSF sample (v1209 with 0.03% CHAPS). One plate was loaded with a single channel pipette taking 29 minutes (Supplemental Figure S-2A and S-2B) and the other was loaded with a multichannel pipette taking 11 minutes (Supplemental Figure S-2C and S-2D). A visually subtle, yet significant (*P* = 1.5e-14, linear regression), decline in apparent PrP level is seen across the plate. For instance, in Supplemental Figure S-2A, the ten replicates loaded last (wells G9-H6) are on average 22%

lower than the ten replicates loaded first (wells A11-B8). Adjustment based on the standard curves abolishes this slope, and reduces the CV among technical replicates (Supplemental Figure S-2B and S-2D).

Spike recovery experiments

While we ultimately achieved 90.5% recovery of recombinant human PrP spiked into CSF, this successful outcome was preceded by a number of experiments that usefully illuminate constraints of working with both the BetaPrion® ELISA assay and CSF PrP as an analyte. In our first experiment, recombinant full-length human PrP with concentration orthogonally established by amino acid analysis (AAA) was spiked into two CSF samples previously established to have high and low baseline PrP. Compared to the expected recovery, the recombinant protein gave a much higher signal than expected, with 392-451%, over-recovery (Supplemental Figure S-3A). This surprising finding suggested to us that the concentration of PrP in kit standards may be lower in practice than the stated concentration. To test this hypothesis, we directly compared the kit standard curve to a matched standard curve prepared with our recombinant PrP. This experiment confirmed that kit standards appeared lower than AAA-quantified PrP standards by a factor of roughly 4 (Supplemental Figure S-3B). We conclude that kit standards, while technically reproducible, may most usefully inform relative rather than absolute quantification of PrP.

We next attempted to assess spike recovery in an internally consistent system by comparing recombinant PrP spiked into CSF to a recombinant PrP standard curve. We diluted recombinant PrP in CSF, then serially diluted into additional CSF to create a five-point series. The series of samples was re-frozen and measured by ELISA the next day. Under these intensive handling conditions, we observed only ~50% recovery even though the samples contained 0.03% CHAPS (Supplemental Figure S-3C). We hypothesized that the CHAPS additive, while helpful, could not fully protect against the high levels of plastic exposure involved

in serial dilution of CSF. To test this hypothesis, we redid the experiment in C with special attention to protecting PrP from plastic adsorption. Recombinant PrP was diluted in blocking buffer to prepare a series of solutions at 100x the desired final concentrations of points in the spike series. These samples were then added to CSF aliquots at a 1:100 concentration, and used in a same-day ELISA experiment. With this level of attention to plastic exposure and the elimination of an additional freeze-thaw cycle relative to the standard curve, PrP was preserved near expected levels with 90.5% recovery observed (Supplemental Figure S-3D).

Finally, to assess recovery from a different angle, we titrated a high-PrP CSF sample into a low-PrP CSF sample at varying ratios, again ensuring minimal and consistent CSF handling. Under these conditions, we observed linear and proportional recovery of PrP (Supplemental Figure S-3E). These experiments provide additional evidence that the quality of PrP measurement afforded by the BetaPrion® ELISA assay is dependent on appropriate sample processing.

CSF aliquot size and PrP loss

We observed that when working with experimental aliquots of CSF, lower volume aliquots appeared to have consistently lower PrP levels (Supplemental Figure S-5A). This effect is likely due to increased exposure of the sample to plastic due to the higher surface area to volume ratio in the polypropylene storage tube. This explanation would be consistent with observed PrP loss across multiple regimens of plastic exposure (see Figure 4-1). Notably, while aliquot size profoundly impacts PrP recovery from small (< 100 µL) aliquots, it does not appear to impact PrP levels in substantially larger CSF volumes. When comparing 1, 3 and 5 mL draws of a pooled CSF sample into identical 5 mL syringes, we did not see a difference in measured PrP (Supplemental Figure S-5B). The cylindrical shape of the syringe could also contribute to this finding, as the surface-area-to-volume ratio difference between different syringe volumes is less dramatic than that for very small sub-aliquots. These data have clinical implications: while

downstream sub-aliquotting and storage can impact PrP levels, different syringe volumes during LPs performed with gentle aspiration will not greatly influence PrP recovery.

Handling of test-retest samples

We analyzed within-subject test-retest reliability of CSF PrP in four cohorts (Supplemental Figure S-7). Here is what we know about the handling history of these samples:

- Metformin trial placebo controls (Steven Arnold). Mean CV = 13% (Figure 4-3 and Supplemental Figure S-7A). *N*=18 samples comprise 2 lumbar punctures from each of 9 placebo-treated individuals from a randomized trial of metformin in individuals with mild cognitive impairment due to either Alzheimer disease or suspected non-amyloid pathology (SNAP). Test-retest interval ranged from 8 to 11 weeks. Lumbar punctures were performed fasting between 8:00a and 10:00a. CSF samples were handled according to a uniform protocol by the same staff, aliquotted into 0.5 mL aliquots within 1 hour of collection and then frozen on dry ice before storage at -80°C. The aliquots we received, approximately 1.75 years after the last sample was collected, were all 0.25 mL, indicating another round of freeze/thaw and aliquotting had occurred in the interim, but all samples were received in identical tubes with identical labeling.
- Sapropterin dihydrychloride trial participants (Kathryn Swoboda). Mean CV = 33% (Supplemental Figure S-7B). *N*=28 samples comprise 3 lumbar punctures from 8 individuals and 2 lumbar punctures from 2 individuals, all with Segawa syndrome (biallelic *GCH1* loss-of-function), enrolled in a trial monitoring effects of sapropterin dihydrochloride on CSF biomarkers. Test-retest interval ranged from 5 to 25 weeks. Lumbar punctures were performed at various times of day. Details of sample handling history are not known, but the aliquots we received were of various sizes (range: 150 µL to 1.3 mL) and were stored in different types of tubes (screw cap and flip top) with varied

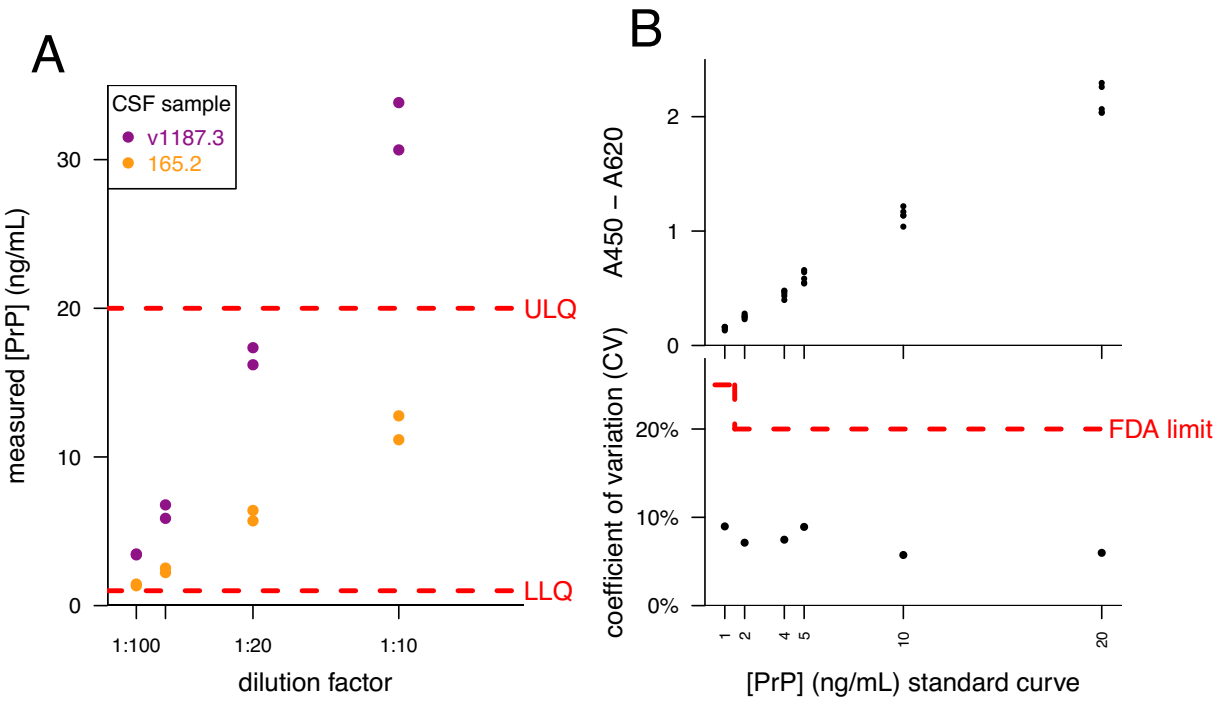
labeling (electronically generated and hand-written), suggesting a diverse sample handling history.

- MIND external lumbar drains (MGH MIND Tissue Bank). Mean CV = 40% (Supplemental Figure S-7C). *N*=18 samples comprise 3 days of external lumbar drains from 4 patients and 2 days of lumbar drains from 3 patients, with a test-retest interval ranging from 1 day to 4 months. These individuals were being evaluated at MGH for normal pressure hydrocephalus (*N*=7), *C. dificile* infection (*N*=1), or *Herpes simplex* infection (*N*=1). CSFs from these in-patient lumbar drains had contact with diverse plastics for varying amounts of time before freezing. In general, the samples passed through a pressure-measuring burette made of cellulose acetate propionate (CAP) before draining into a polyvinyl chloride (PVC) bag. CSF was later collected from the bag and frozen in either polystyrene (PS) or polypropylene (PP) tubes. Aliquots we received were of two different sizes: 0.5 mL and 4.0 mL.
- Pre-symptomatic and symptomatic PRNP mutation carriers (Michael Geschwind). Mean CV=34% in each (Supplemental Figures S-7D and S-7E). Samples were collected between 2009 and 2017 at two sites (UCSF Parnassus NIH GCRC/CTSI and subsequently on the UCSF Mission Bay Neuroscience Clinical Research Unit) with multiple different physicians performing lumbar punctures according to a uniform protocol. Test-retest interval ranged from 2 months to 6 years. Samples were collected at various times of day and kept under refrigeration for variable amounts of time, ranging from a few hours to overnight, before being sent to UCSF CoreLabs. Samples collected prior to September 2016 were frozen immediately upon receipt at CoreLabs, and were later thawed and aliquotted in the first half of 2017. Beginning September 2016 CoreLabs aliquotted the samples upon receipt using polypropylene pipette tips (Rainin RT-L1000F) into 0.5 mL cryovials (Fisher 02-681-333) prior to first freeze. The sub-aliquots that we received were in identical tubes with uniform labels, and were all labeled

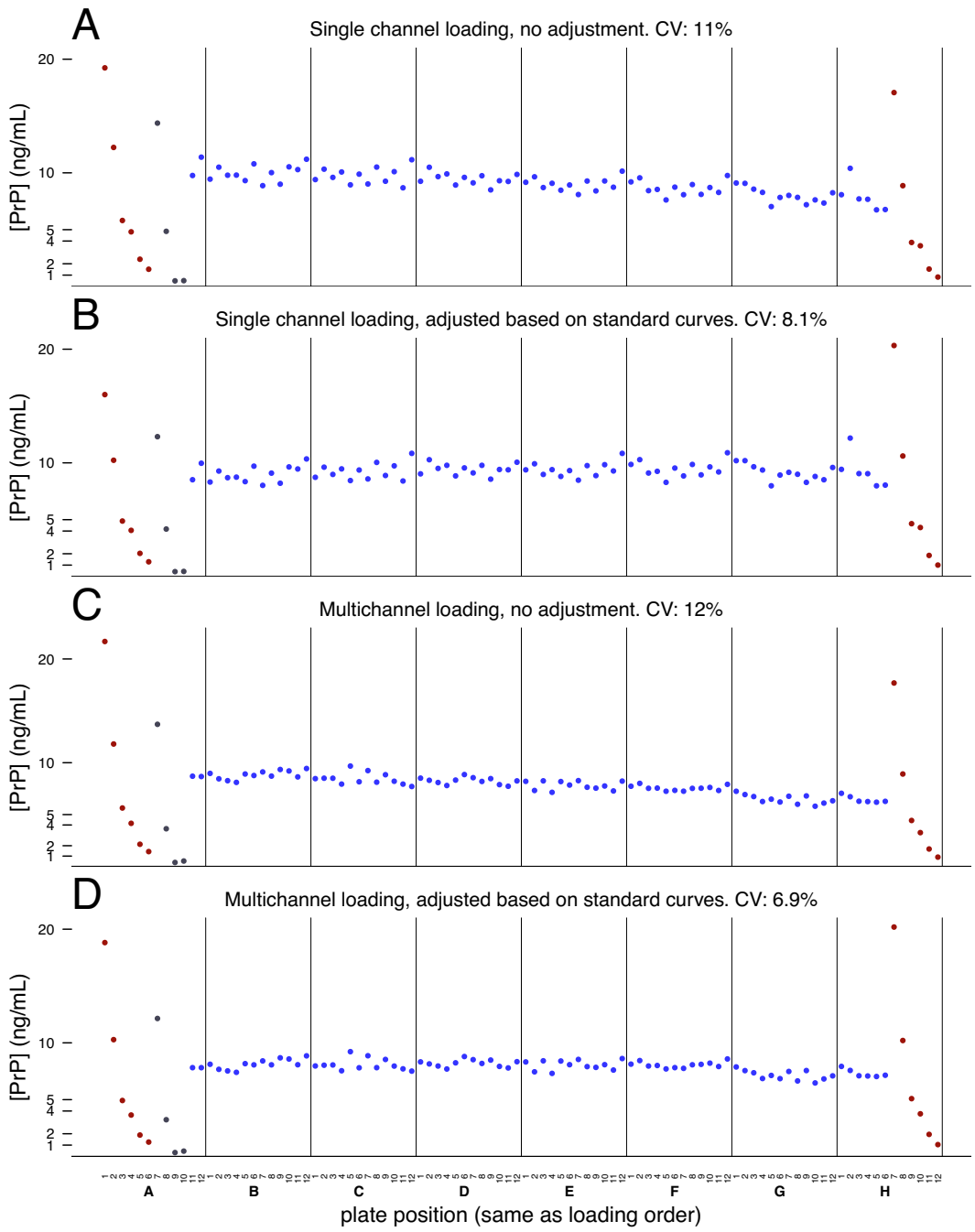
as being 250 µL, however, we found that the actual recoverable volume in each tube varied, with some as low as 100 µL; all data reported here are from aliquots with at least 140 µL.

Supplemental Table S-1: CSF samples analyzed. Abbreviations: normal pressure hydrocephalus (NPH); mild cognitive impairment with suspected non-amyloid pathology (MCI-SNAP).

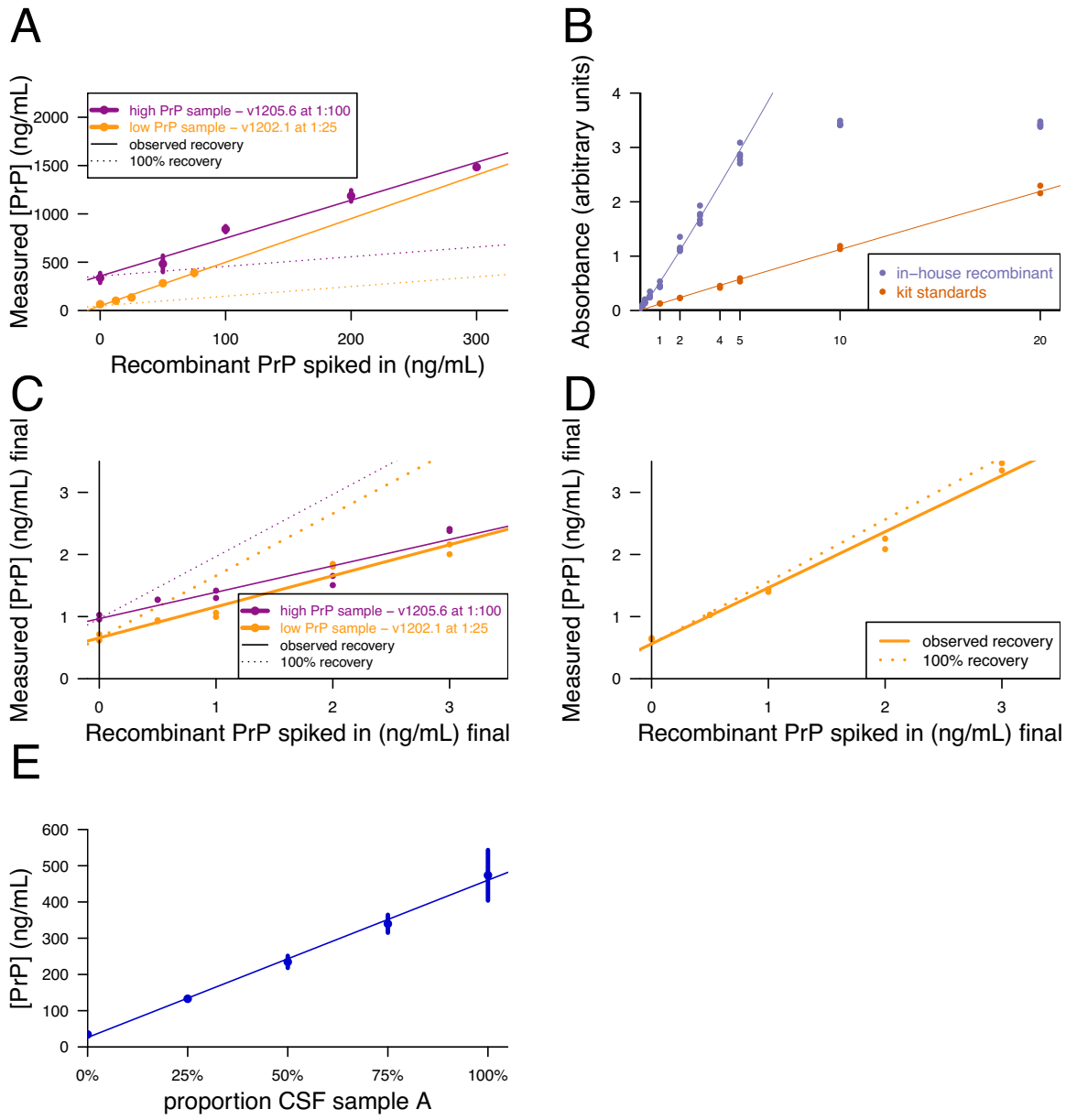
Cohort (Collaborator)	***N***	**Diagnosis**	**Description**
Metformin trial (Steven Arnold)	18	Alzheimer disease and MCI-SNAP	Placebo-treated controls from a randomized trial monitoring effects of metformin on CSF biomarkers(33). 8-11 week test-retest. Samples were handled uniformly (see Supplementary Discussion) and were centrifuged prior to freezing.
MGH MIND Tissue Bank	27	NPH, *C. dificile*, herpes simplex	Large volume assay development samples from NPH patients (*N*=9), test-retest lumbar drains (*N*=18), and lumbar-thoracic gradient samples (*N*=8). Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Sapropterin trial (Kathryn J Swoboda)	28	Segawa syndrome (*GCH1* loss of function)	Patients who received sapropterin dihydrochloride in a trial monitoring effects on CSF biomarkers (*N*=10 individuals). 5-25 week test-retest. Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Bologna prion referrals (Piero Parchi)	34	Symptomatic prion and non-prion dementias	Dementia patients referred to the CJD Reference Center at University of Bologna due to suspected prion disease. Samples are autopsy-confirmed positive or negative for prion disease. Prion samples include sporadic and genetic (E200K, *N*=5). Prior to arriving at Dr. Parchi's lab from referring physicians, samples were variably centrifuged or not, and variably shipped frozen, cold, or at room temperature. Samples not marked as previously centrifuged were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
Göttingen prion referrals (Inga Zerr)	29	Symptomatic prion and non-prion dementias	Dementia patients referred to the CJD Reference Center at University of Göttingen due to suspected prion disease. Samples are autopsy-confirmed positive or negative for prion disease. Prion samples include sporadic and genetic (D178N, *N*=2; E200K, *N*=2; V210I *N*=2). Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab. These samples were received after the data in Figure 4-1 were generated, so we added 0.03% CHAPS prior to sub-aliquotting and ELISA.
Cognitive impairment (Henrik Zetterberg)	20	Cognitive impairment	Patients with undiagnosed cognitive impairment and normal levels of CSF tau, phospho-tau, and amyloid beta. Samples were centrifuged for 10 minutes at 2,000xG after receipt in our lab.
UCSF (Michael Geschwind)	61	Symptomatic and pre-symptomatic genetic prion disease	Participants with *PRNP* mutations in the Early Diagnosis of Human Prion Disease study at UCSF(47). The cohort includes *N*=61 samples from *N*=40 distinct individuals (28 pre-symptomatic and 12 symptomatic), with 1 to 5 samples per person collected at intervals ranging from 2 months to 6 years. Mutations represented include P102L (*N*=4 individuals), D178N (*N*=6), E200K (*N*=16), and ten other mutations (details omitted to protect patient privacy), including five with literature evidence for high penetrance and five without (see companion paper by Minikel et al). These samples were received after the data in Figure 4-1 were generated, so we added 0.03% CHAPS prior to sub-aliquotting and ELISA. Samples were never centrifuged.
TOTAL	**225**		



Supplemental Figure S-1: The BetaPrion® Human PrP ELISA kit quantifies PrP in a technically reproducible and sensitive manner. *A) Consistent dilution linearity was observed within the assay's stated dynamic range of 1 – 20 ng/mL PrP, providing reassurance that this technique can be used to compare PrP levels across samples even when these levels differ by one log. Purple and yellow dots represent two different samples measured in duplicate at each of four dilutions. B) Five replicates of the kit's internal six-point standard curve, reconstituted from lyophilized standards, were run in parallel on one plate. Across the dynamic range of the assay, the coefficient of variation falls below 10% for all points and well below the 20% FDA recommended limit in standard variability for ligand-binding assays.*



Supplemental Figure S-2: Plate position effects. *Computed PrP levels for standard curves (red), kit controls (gray), or the CSF sample (blue) in two whole plates loaded with technical replicates of the same CSF sample (NPH sample v1209 with 0.03% CHAPS) using either a single channel pipette (A-B) or a multichannel pipette (C-D). Displayed are the unadjusted PrP values (A and C) or the PrP values after adjustment based on the difference between the standard curves at the beginning and end of the plate (B and D). See Supplementary Discussion for further interpretation.*

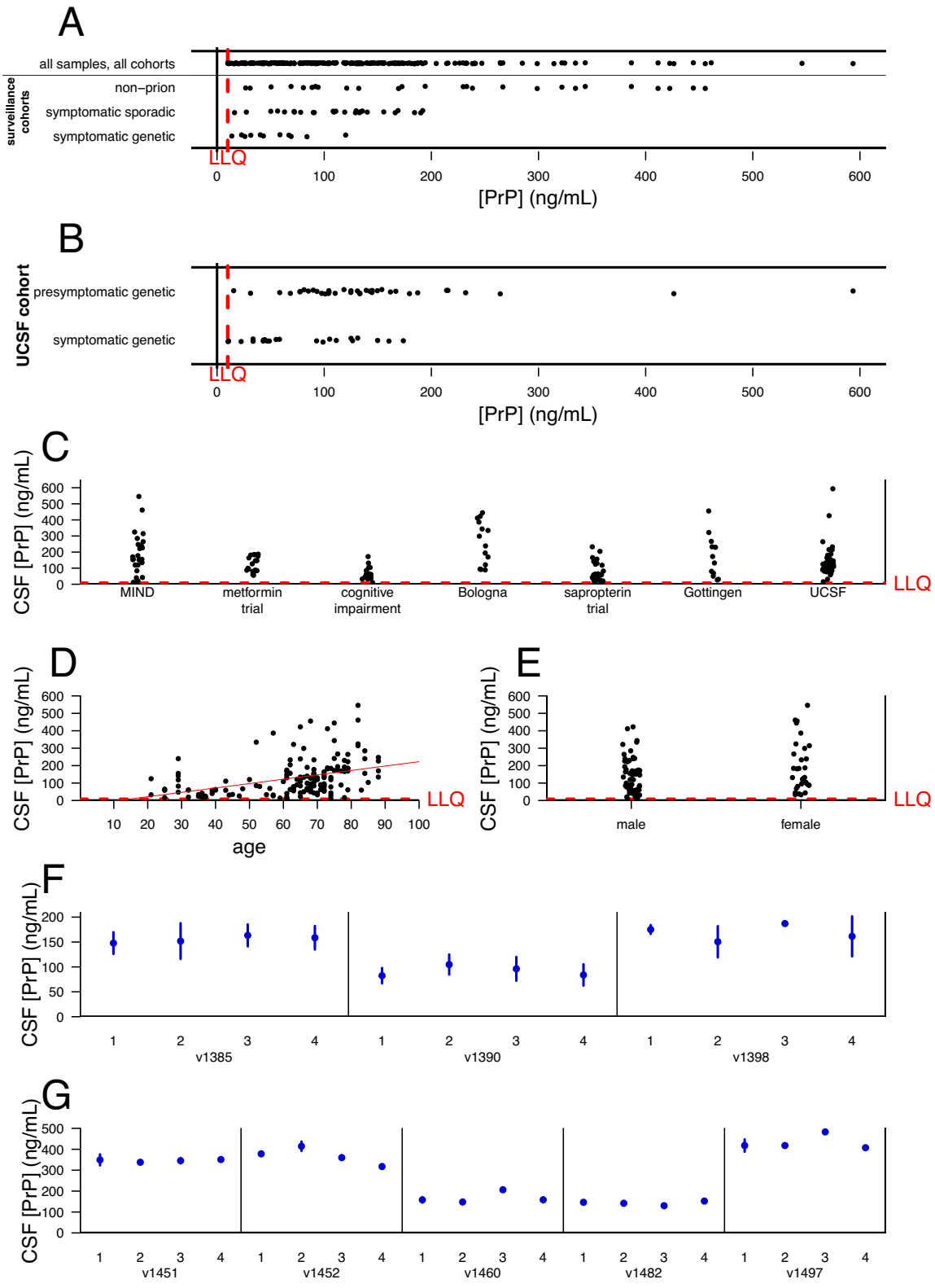


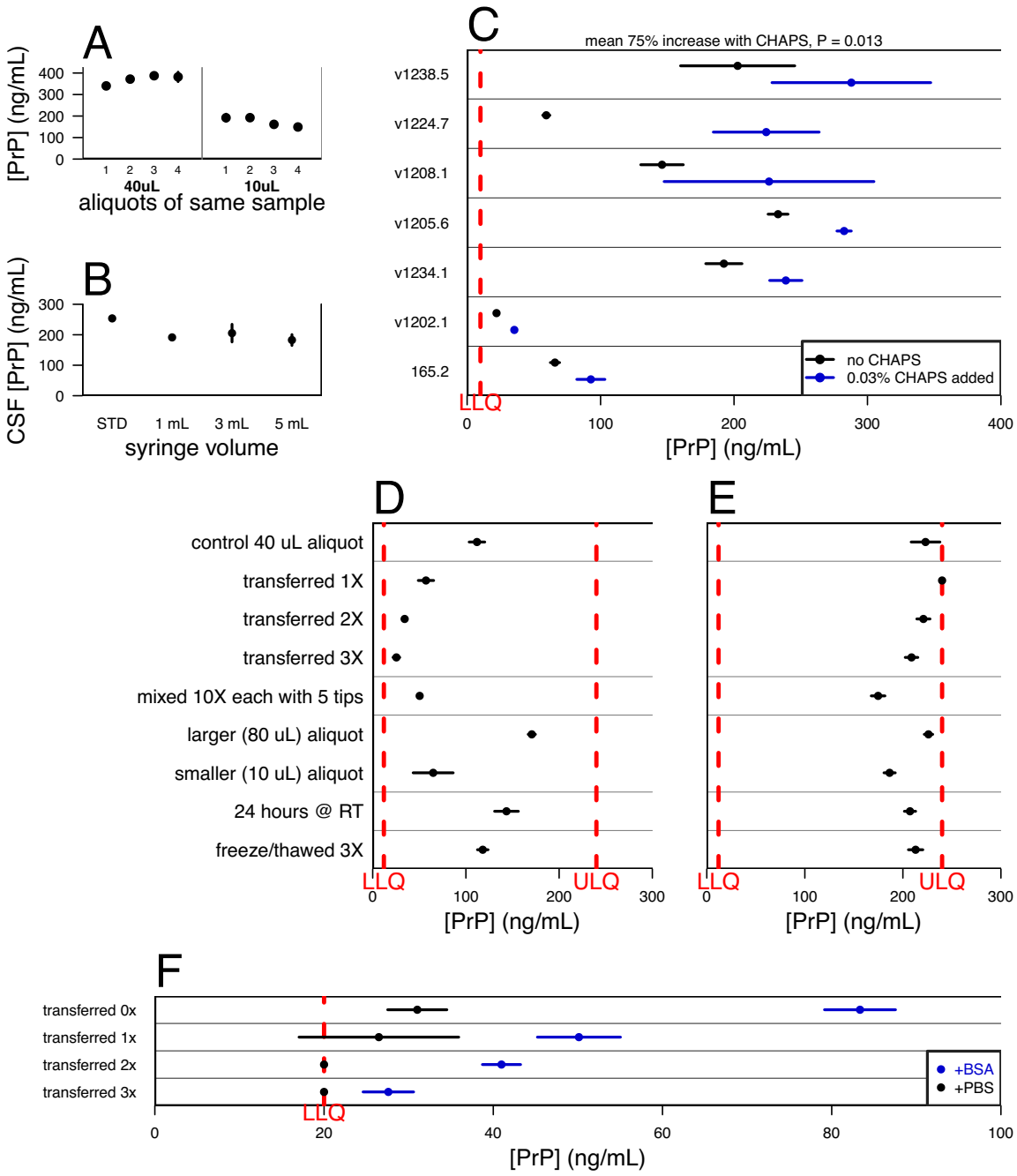
Supplemental Figure S-3: Spike recovery experiments. *A) In-house produced full-length recombinant human prion protein, quantified by amino acid analysis (AAA) was spiked into two CSF samples previously established to have high and low baseline PrP. Recombinant PrP was over-recovered by 392-451% (meaning that measured concentrations were ~4x the expected concentrations) when compared to kit standards. B) A recombinant standard curve was prepared from AAA-quantified recombinant huPrP to match the nominal concentrations of each of the six points on the BetaPrion® kit standard curve. Direct comparisons of the two series by ELISA showed the recombinant curve to be contain roughly 4x greater PrP at each point. C) Recombinant huPrP was measured according to a recombinant PrP standard curve. Recombinant PrP was diluted in CSF, then serially diluted into additional CSF to create a five-point series. The series of samples was re-frozen and measured by ELISA the next day. Under these conditions we observed 50.0% and 42.5% recovery for two different samples. D) The experiment in C was redone with the following modifications. Recombinant PrP was diluted directly in the initial aliquot tube with blocking buffer (5% BSA and 0.05% Tween-20 in PBS,*

Supplemental Figure S-3 (Continued) *filtered prior to use). It was further diluted in blocking buffer to prepare a series of solutions at 100x the desired final concentrations of points in the spike series. These samples were then added to CSF aliquots at a 1:100 concentration. These samples were then diluted in blocking buffer to their final plating concentration and measured in a same-day ELISA experiment. Under these conditions we observed 90.2% recovery. E) A high-PrP CSF sample (sample A) was titrated into a low-PrP CSF sample at varying ratios, with minimal CSF handling. We observed linear recovery of PrP. See Supplementary Discussion for further interpretation.*

Supplemental Figure S-4: Candidate explanations for variability in CSF PrP levels. *A) Within cohorts of individuals referred with a possible diagnosis of prion disease (Göttingen and Bologna cohorts), PrP levels are lower in individuals with prion disease than in individuals with other diagnoses. PrP levels in sporadic prion disease CSF average 42% of non-prion samples (P = 0.0001, Kolmogorov-Smirnov test) and in genetic prion disease CSF average 19% of non-prion samples (P = 2.6e-6, Kolmogorov-Smirnov test). B) Among individuals with a PRNP mutation (UCSF cohort), PrP levels in symptomatic individuals average 53% of those in pre-symptomatic individuals (P = .001, Kolmogorov-Smirnov test). C) CSF PrP levels vary dramatically between different cohorts in our study, even after excluding individuals with symptomatic prion disease (P = 1.1e-8, Type I ANOVA). D) CSF PrP is positively correlated with age (r = 0.47, P = 1.9e-9, Spearman rank test), although among our samples age is confounded with cohort, diagnosis, and likely with other unobserved variables, so it is unclear whether this correlation is biologically meaningful. For example, consider symptomatic prion disease patients in the two prion surveillance cohorts (Bologna and Göttingen). Symptomatic genetic patients were on average younger than symptomatic sporadic patients (mean 55 vs. 68 years old, P = 0.001, Kolmogorov-Smirnov test), and controlling for genetic vs. sporadic diagnosis eliminated any trend towards correlation between age and CSF PrP (linear regression, P = 0.37 with diagnosis as covariate, P = 0.04 without). E) Excluding individuals with symptomatic prion disease, CSF PrP does not differ between men and women (P = 0.31, Kolmogorov-Smirnov test). F) CSF PrP exhibits no lumbar-thoracic gradient within ~30 mL intrathecal CSF drips. From each of three individuals with normal pressure hydrocephalus, 29-32 mL of intrathecal CSF was collected via drip in 4 polystyrene tubes of 7-8 mL each, with "1" being the first tube and "4" being the final tube. Because CSF from further up the spinal column is expected to drain downward as CSF is removed, "1" represents the most lumbar CSF while "4" is the most thoracic. PrP exhibits no trend across tubes (P = 0.81, linear regression). Error bars show technical replicates performed in duplicate. G) CSF PrP likewise exhibits no lumbar-thoracic gradient when ~20 mL of CSF is drawn using gentle aspiration with a 24G Sprotte needle. Approximately 5 mL of CSF was drawn in each of four syringes; again, "1" is the most lumbar and "4" is the most thoracic. These samples included individuals diagnosed with Alzheimer's disease, Parkinson's disease, and undiagnosed individuals. PrP exhibits no trend across syringes (P = 0.93, linear regression). Error bars show technical replicates performed in duplicate.*

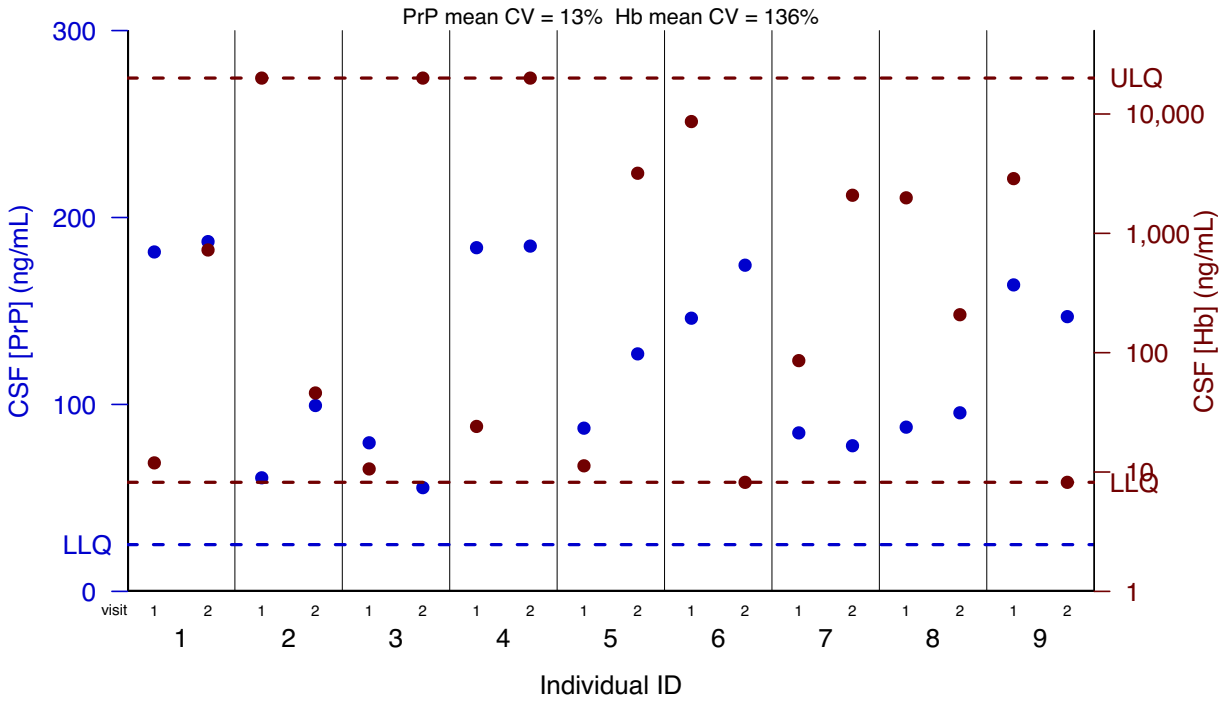
Supplemental Figure S-4 (Continued)



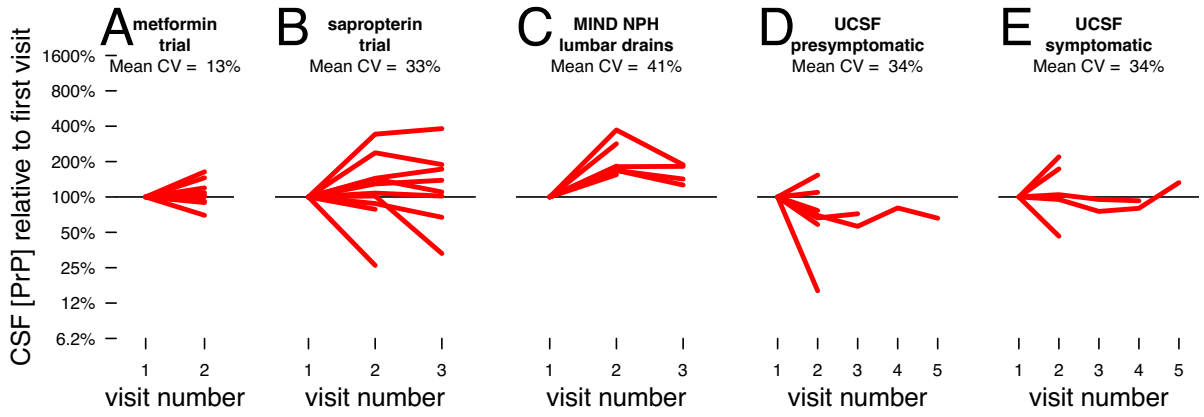


Supplemental Figure S-5: Additional evidence for loss of PrP to plastic adsorption. A) *Differently sized aliquots of sample v1187 appear to have different PrP levels. Each dot is the mean, and line segment the 95% confidence intervals, of two technical replicates on the same plate. These samples did not contain CHAPS. B) A pooled CSF standard (STD) was warmed to 37°C and various volumes (1 mL, 3 mL, or 5 mL) were drawn into identical 5 mL syringes using a 24G Sprotte needle and allowed to sit for 15 minutes before ejection into tubes, centrifugation, and aliquotting. Samples were handled identically except for the volume drawn into the syringe. See Supplementary Discussion. C) After aliquotting and freeze/thaw, CSF samples were diluted*

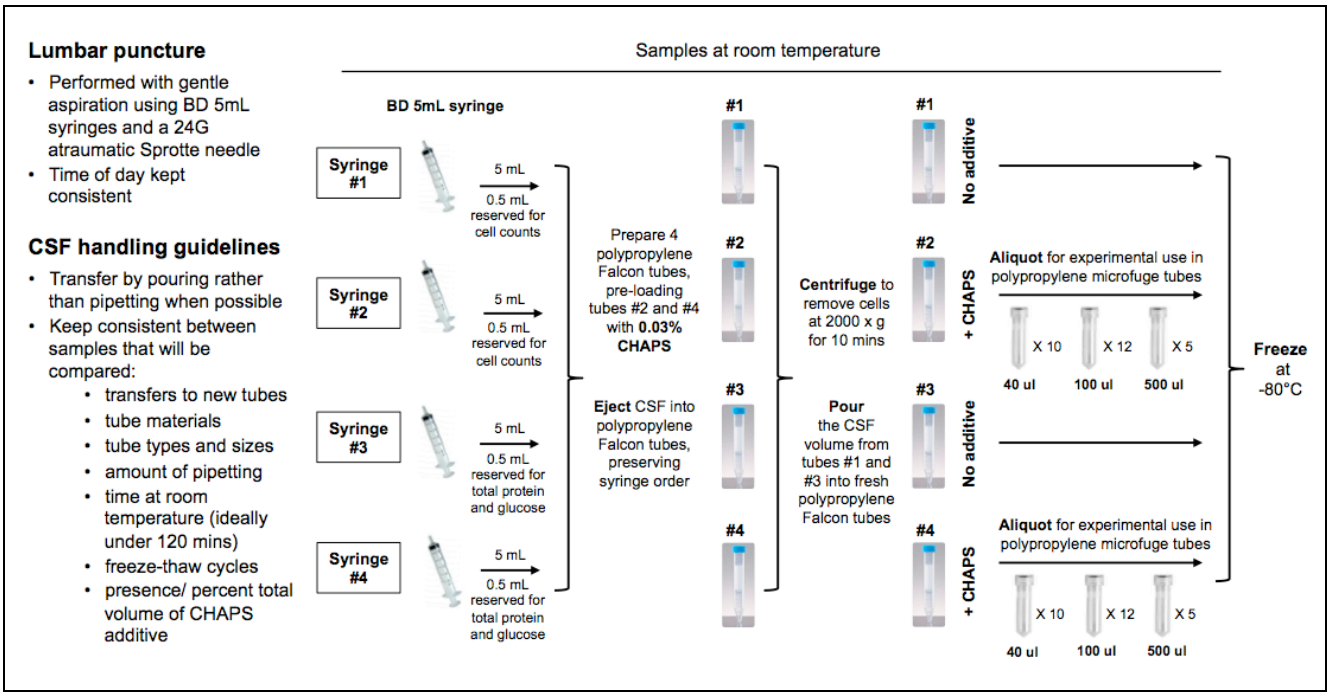
Supplemental Figure S-5 (Continued) *into blocking buffer neat (black) or after addition of a final concentration of 0.03% CHAPS to the original storage tube (blue). Addition of CHAPS resulted in a 75% increase in apparent PrP level. See Supplementary Discussion. D and E) Replication of the findings from Figure 4-1A and 4-1B. The data in Figure 4-1 were generated using CSF samples from two different individuals; to rule out the possibility that some other inter-individual difference, rather than CHAPS, explained the difference in plastic loss, we repeated the experiment but with a single CSF sample divided into two halves which were then aliquotted without (D) or with (E) 0.03% CHAPS, subjected to the same battery of perturbations and plated at the same dilution. Because CHAPS increases overall PrP recovery, some replicates in (E) are at the upper limit of quantification; nevertheless, the results recapitulate Figure 4-1. F) 1 mg/mL (final concentration) BSA (blue), or PBS as a control (black), were added to CSF sample 165.2, which had an initial total protein level at the low end of the distribution of our samples (measured at 0.22 mg/mL with PBS), bringing it up to a total protein level at the high end of our samples (measured at 1.15 mg/mL after BSA spike-in). BSA or PBS were added after centrifugation but prior to aliquotting at 40 uL and re-freezing. 4 tubes of each sample were subsequently thawed and diluted into blocking buffer for analysis. Total recovery of PrP is increased in the BSA-spiked samples, analogous to panel B, although BSA is less effective at mitigating loss upon further transfer between tubes (compare to Figure 4-2A).*



Supplemental Figure S-6: Hemoglobin in test-retest samples. *Overlaid are PrP levels (blue, same data as shown in Figure 4-3) and hemoglobin levels (red) in test-retest samples. PrP exhibited good test-retest reliability (mean CV=13%) despite dramatic variation in hemoglobin (mean CV=136%), providing further evidence that blood contamination does not influence CSF PrP level.*



Supplemental Figure S-7. Test-retest reliability of CSF PrP in additional cohorts. *Test-retest CSF PrP levels in A) metformin trial participants (Arnold) over 8-11 weeks, with mean CV=13% (same data from Figure 4-3 but plotted normalized to the PrP level at the first visit); B) sapropterin dihydrochloride trial participants (Swoboda) over 5-25 weeks, with mean CV=33%, C) NPH lumbar drains (MGH MIND Tissue Bank) over 1 day to 4 months, with mean CV=40%, D) pre-symptomatic and E) symptomatic PRNP mutation carriers (Geschwind) over 2 months to 6 years, each with mean CV=34%. The repeated 34% is not an error: the mean CVs in (D) and (E) happen to be the same (34.28% and 34.25%). See Supplementary Discussion for details on sample handling in these cohorts.*



Supplemental Figure S-8: Protocol for collection of CSF for PrP measurement. *We have incorporated our findings into the above protocol, which we are using to collect test-retest CSF for the purposes of PrP measurement in our ongoing clinical study.*

Supplement to Chapter 5: Preliminary findings from the Massachusetts General Hospital genetic prion disease biomarker study

Supplemental Table S-2: Measures of cognitive, psychiatric, motor and daily functioning in all MGH study participants. *Scores are averaged across group (carrier vs. non-carrier) at first completed study visit. P values are from two-sided Kolmogorov-Smirnov tests. Bonferroni corrected p values account for a multiple testing burden of N=19 for all measures shown. Raw scores are provided according to the methods standard for each test, with the exception of the digital clock drawing test, where digitally recorded features were compiled into a composite score as previously described*[47]*.*

		PRNP **mutation carriers**	**Non-carrier controls**	**P value (raw)**	**P value (Bonferroni corrected)**
Assessment scores	Montreal Cognitive Assessment	27.7±1.6	28.5±1.7	0.2	1
	NIH Toolbox Cognitive Battery composite	104.5±11.7	118.5±13.1	0.0048	0.09
	Digital clock-drawing test	77.6±18.5	78.9±18.4	0.58	1
	Trailmaking test, Part A	21.7±7.6	20.8±8.4	0.11	1
	Trailmaking test, Part B	58.5±32.0	44.2±11.1	0.036	0.68
	Color-word interference test, inhibition time	52.3±13.6	46.7±8.4	0.45	1
	Color-word interference test, switching time	59.6±15.6	53.3±10.5	0.19	1
	Letter fluency	39.9±13.2	49.8±10.3	0.076	1
	Category fluency	42.8±11.4	45.8±11.7	0.39	1
	Grooved pegboard test, dominant hand	70.5±15.6	61.8±10.1	0.054	1
	Grooved pegboard test, non-dominant hand	79.9±22.0	67.7±14.8	0.025	0.47
	Rey auditory verbal learning test with delay	9.0±3.5	10.4±2.9	0.42	1
Questionnaire scores	MRC prion disease rating scale	20.0±0.0	20.0±0.0	1	1
	Epworth Sleepiness Scale	6.3±3.7	4.8±3.6	0.66	1
	Athens Insomnia Scale	5.9±4.4	5.1±5.7	0.54	1
	Motor Aspects of Experience	1.0±1.5	0.2±0.6	0.28	1
	Beck Anxiety Inventory	7.4±7.2	4.0±5.1	0.088	1
	Beck Depression Inventory	6.8±6.2	5.2±6.6	0.51	1
	Everyday Cognition	12.5±1.3	12.2±0.6	1	1

Supplemental Table S-3: Additional measures of cognitive, psychiatric, motor and daily functioning for one RT-QuIC positive MGH study participant. *Visits were separated by two months. Raw scores are provided according to the methods standard for each test, with the exception of the digital clock drawing test, where digitally recorded features were compiled into a composite score as previously described*[47]*.*

		Visit 1	Visit 2
Assessment scores	Digital clock drawing test	51	56
	Trailmaking test, Part A	40	51
	Trailmaking test, Part B	178	120
	Color-word interference test, inhibition time	63	70
	Color-word interference test, switching time	86	73
	Letter fluency	43	37
	Category fluency	38	36
	Grooved pegboard test, dominant hand	122	131
	Grooved pegboard test, non-dominant hand	157	155
	Rey auditory verbal learning test with delay	6	6
Questionnaire scores	Epworth Sleepiness Scale	3	2
	Athens Insomnia Scale	7	1
	Motor Aspects of Experience	1	0
	Beck Anxiety Inventory	9	5
	Beck Depression Inventory	4	6
	Everyday Cognition	12	12