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


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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 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 the 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. We also show that highly sensitive ELISAs can be used to reliably detect PrP in CSF. Together, these findings support a monitoring strategy for the effect of a PrP-reducing drug in the CNS, and will facilitate development of prion disease therapeutics with this mechanism of action.

Significance

Human prion disease is a rapidly fatal and incurable neurodegenerative disease. Reduction of prion protein in the brain is a well-supported therapeutic hypothesis, and antisense oligonucleotides with this mechanism of action are currently in development. To facilitate clinical testing of prion protein-lowering drugs in prion disease, we show that with proper sample handling, brain prion protein levels can be monitored in cerebrospinal fluid, using existing tools, and exhibit suitable short-term stability for drug-dependent decreases to be reliably measured. Cerebrospinal fluid prion protein levels thus may usefully serve as a pharmacodynamic biomarker. This biomarker may open new paths for informative clinical trials in presymptomatic individuals who harbor high-risk mutations for genetic prion disease.
ELISA kit, the BetaPrion ELISA (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 (Discussion).

Informed by US Food and Drug Administration’s 2013 Draft Guidance on Bioanalytical Method Validation (25), we assessed the technical performance of the BetaPrion ELISA across 225 human CSF samples spanning a range of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, Sensitive, and Selectively. We assessed the assay’s precision, sensitivity, selectivity and reproducibility by analyzing 225 human CSF samples from patients with symptomatic prion disease, presymptomatic prion disease mutation carriers, patients with nonprion dementia, and patients with normal pressure hydrocephalus, as well as other nonprion controls (SI Appendix, Table S1), across 41 plates. The results broadly support the technical suitability of this assay for reliable quantification of CSF PrP (Table 1 and SI Appendix, Fig. S1).

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 (SI Appendix, Fig. S2). Comparison of the kit standard curve to a standard curve yielded systematic differences, with a mild but significant downward trend from upper left to lower right (SI Appendix, Fig. S3). Comparison of the kit standard curve to a standard curve yielded systematic differences, with a mild but significant downward trend from upper left to lower right (SI Appendix, Fig. S4 A and B and Table S1). Across all CSF samples analyzed, PrP levels varied by more than two orders of magnitude (SI Appendix, Fig. S4A), 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 (nonprion, presymptomatic 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 (SI Appendix, Fig. S4C; 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 preanalytical variability. CSF PrP was correlated with age (SI Appendix, Fig. S4D), 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 (SI Appendix, Fig. S4E), and exhibited no lumbar–thoracic gradient over serial tubes collected from the same lumbar puncture (SI Appendix, Fig. S4 F and G). After noticing that PrP levels appeared lower in smaller aliquots of the same CSF sample (SI Appendix, Fig. S5A), 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 number of transfers between polypropylene storage tubes, amount of exposure to polypyrrole pipette tips, storage aliquot size, storage temperature, and number of freeze–thaw cycles (Fig. 1A). Strikingly, increased plastic exposure in the first three conditions dramatically reduced measurable PrP in solution (Fig. 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 before aliquoting minimized PrP loss to plastic across most manipulations (Fig. 2B). 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 71% ($P = 0.37$) 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 (SI Appendix, Fig. S5), and was effective against loss to multiple plastics, but not glass (Fig. 1C). Storing CSF at room temperature for 24 h or subjecting samples to three freeze–thaw cycles had a less dramatic effect on PrP that did not appear to be affected by CHAPS (Fig. 1A and B and SI Appendix, Fig. S5 D and E).

We also investigated the relationship between measured PrP and total protein in 217 samples, using the DC total protein

### Table 1. The technical performance of the BetaPrion human PrP ELISA supports reliable quantification of PrP in human CSF

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Results</th>
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<tbody>
<tr>
<td>Within-plate technical replicate reproducibility (same dilution)</td>
<td>CV = 8%</td>
</tr>
<tr>
<td>Within-plate technical replicate reproducibility (all dilutions)</td>
<td>CV = 11%</td>
</tr>
<tr>
<td>Between-plate technical replicate reproducibility</td>
<td>CV was 22% in an interplate control sample run on 17 plates on different days (SI Appendix, Supplementary Discussion).</td>
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<tr>
<td>Sensitivity</td>
<td>LLOQ is 3–5× the blank signal, depending on the plate reader used.</td>
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<tr>
<td>Selectivity</td>
<td>Nonreactive for recombinant mouse PrP, rat CSF, and cynomolgus monkey CSF (consistent with one amino acid mismatch in the reported detection antibody epitope (18)), artificial CSF, and protease-digested CSF.</td>
</tr>
<tr>
<td>Dilution linearity</td>
<td>Linear across two samples and five dilutions. See SI Appendix, Fig. S1A.</td>
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<tr>
<td>Spike recovery</td>
<td>Using AAA-quantified recombinant human PrP23-230 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 SI Appendix, Fig. S3.</td>
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<tr>
<td>Standard curve reproducibility</td>
<td>CV was &lt;10% at all six nonzero standard curve points across five replicates. See SI Appendix, Fig. S1.</td>
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CV, coefficient of variation; LLOQ, lower limit of quantification.
assay. Across all samples analyzed, a modest correlation ($r = 0.36$; Spearman rank test, $P < 1 \times 10^{-6}$) between PrP and total protein was observed (Fig. 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 of 1 mg/mL BSA increased recovery of PrP ($P < 0.05$). 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% (vol/vol) did not increase the detected PrP (Fig. 2B). Finally, as a proxy for blood contamination, we measured hemoglobin levels in 128 CSF samples and observed no correlation between CSF hemoglobin and CSF PrP (Fig. 2C). Variation in hemoglobin levels also failed to confound the test–retest reliability of CSF PrP (SI Appendix, Fig. S6). From these lines of evidence, we conclude that the PrP detected in CSF is overwhelmingly derived from the CNS.

**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 artifically 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 nonneurodegenerative disease control individuals versus all the CSF samples in our study (Fig. 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% (vol/vol) did not increase the detected PrP (Fig. 2B). Finally, as a proxy for blood contamination, we measured hemoglobin levels in 128 CSF samples and observed no correlation between CSF hemoglobin and CSF PrP (Fig. 2C). Variation in hemoglobin levels also failed to confound the test–retest reliability of CSF PrP (SI Appendix, Fig. S6). From these lines of evidence, we conclude that the PrP detected in CSF is overwhelmingly derived from the CNS.

**CSF PrP Levels in Individuals Are Stable on Short-Term Test–Retest.** 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 nonprion dementia) who had undergone two fasting morning lumbar punctures at 8- to 11-wk intervals in the context of a clinical trial (38). Lumbar punctures 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 points for a given participant was reasonably low, at 8% (Fig. 2A). Among the 217 CSF samples, total protein levels and PrP levels were modestly correlated ($r = 0.36$; $P = 6.2 \times 10^{-6}$). (A–C) Dots represent mean and line segments represent 95% confidence intervals across four to seven aliquots of the same sample, each measured in duplicate at a 1:50 dilution. (D) Dots represent mean of measurements within dynamic range, among two dilutions with 2 technical replicates each. (4) Increased polypropylene exposure substantially reduces detectable PrP. (B) Addition of 0.33% 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 on storage in glass. (D) Across 217 CSF samples, total protein levels and PrP levels were modestly correlated (Spearman’s rank correlation coefficient $r = 0.36$; $P = 6.2 \times 10^{-6}$). (A–C) Dots represent mean and line segments represent 95% confidence intervals across four to seven aliquots of the same sample, each measured in duplicate at a 1:50 dilution. (D) Dots represent mean of measurements within dynamic range, among two dilutions with two 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 preanalytical 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 SI Appendix, Fig. S8. Also, in light of subtle plate position effects (SI Appendix, Fig. S2), samples intended for comparison could be colocated 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 AAA-quantified standard curve suggests that the assay may be most useful for relative rather than absolute quantification of PrP (SI Appendix, Fig. S3B).

Our study has several limitations. First, ELISA might not detect all conformers or fragments of PrP. Although we hypothesize that the BetaPrion assay measures total PrP, we are presently developing a targeted mass spectrometry-based orthogonal method to test this hypothesis. For study of specific PrP isoforms, future ELISA development efforts could leverage additional PrP antibodies to quantify particular subsets of PrP molecules (39). Second, although we have established that CSF PrP is quantifiable in patients with genetic prion disease across a variety of mutations and has good test–retest reliability in a cohort of patients with nonprion dementia, when we embarked on the present study, we did not have access to test–retest samples from presymptomatic 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 (40). Third, the samples analyzed here were reused after collection for other research or clinical purposes, and in most cases, we cannot fully account for their sample handling history before receipt by our laboratory. Thus, our numbers

![Blood PrP contributes negligibly to the PrP detected in CSF.](image)

**Fig. 2.** Blood PrP contributes negligibly to the PrP detected in CSF. (A) PrP levels were compared by ELISA in 28 postmortem human brain samples, three blood fractions from eight 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 detectable in the red cell and buffy coat fractions only at low levels compared with PrP in CSF. (B) Spiking whole blood into CSF up to 1% by volume does not affect measured PrP. (C) Across 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 two to three aliquots of the same sample.

![Test–retest stability of CSF PrP.](image)

**Fig. 3.** Test–retest stability of CSF PrP. Uniformly processed CSF samples were provided from a past clinical trial, from placebo-treated individuals with mild, nonprion cognitive impairment. Fasting morning lumbar punctures were performed by one investigator on nine individuals and then repeated at an interval of 8–11 wk. Dots represent means, and line segments 95% confidence intervals, of measurements within dynamic range among two dilutions with two technical replicates each.
may exaggerate the interindividual variation in CSF PrP in the population.

PrP levels in CSF as measured by ELISA are reduced by approximately half in patients with symptomatic prion disease (21, 23, 24), a phenomenon reproduced here (SI Appendix, Fig. S4). Multiple plausible biological mechanisms could explain these findings: incorporation of PrP into insoluble plaques (41), internalization of misfolded PrP in the endosomal–lysosomal pathway (42), and posttranslational down-regulation of PrP as a function of disease (43). 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 (44–46), and future trials may be further benefit from the use of real-time quaking induced conversion to detect misfolded prion “seeds” in symptomatic patient CSF (47–49).

Instead, this biomarker may have its greatest utility in presymptomatic 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 (50). Although 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 presymptomatic individuals to a clinical endpoint appears infeasible (51), low-cost, rapid, and easily administered biomarkers that could confer the potential for early intervention are much needed. However, a few key findings have raised caution in the enthusiasm of using PrP as a suitable biomarker in the earlier stages of prion disease:

- PrP is expressed at near basal levels in the brain of individuals without neurodegenerative disease, with postmortem intervals of 23–72 h, representing diverse cortical and subcortical regions and from the National Prion Disease Pathology Surveillance Center (n = 2 samples of frontal cortex from nonprion controls) homogenized in PBS with 0.03% CHAPS at 10% weight/vol in 7 mL tubes (Precellys no. KT039611307.7), using a Minilys tissue homogenizer (Bertin no. EQ60400-200-R0000.0) for three cycles of 40 s at maximum speed. The resulting 10% brain homogenates were diluted 1:10 and 1:100 in blocking buffer for ELISA.

- Human brain fractions were obtained from the Massachusetts Alzheimer’s Disease Research Center (n = 26 samples from 5 different control individuals without neurodegenerative disease, with postmortem intervals of 23–72 h, representing diverse cortical and subcortical regions) and from the National Prion Disease Pathology Surveillance Center (n = 2 samples of frontal cortex from nonprion controls) homogenized in PBS with 0.03% CHAPS at 10% weight/vol in 7 mL tubes (Precellys no. KT039611307.7), using a Minilys tissue homogenizer (Bertin no. EQ60400-200-R0000.0) for three cycles of 40 s at maximum speed. The resulting 10% brain homogenates were diluted 1:10 and 1:100 in blocking buffer for ELISA.

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**Methods**

**CSF Samples.** De-identified human CSF samples were provided by multiple clinical collaborators and included some previously published samples (38, 52). Samples were shipped on dry ice and stored at −80 °C. Before 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, aliquoted 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-added to the supernatant receiving tube before the postcentrifugation transfer, and then mixed into the sample by gentle pipetting before aliquoting.

**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 capture antibody and a horse-radish peroxidase-conjugated primary detection antibody to human PrP residues 151–180 (23). In brief, samples were diluted into blocking buffer (5% BSA and 0.05% Tween-20 in PBS, filtered before use) at concentrations ranging from 1:100 to neat, depending on the anticipated PrP content of the sample type. All aliquots of a single CSF sample containing baseline midrange 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 after spike-in and then rethawed for use to ensure lysis of cellular fractions before PrP quantification.

**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; SI Appendix, Fig. S4 F and G). This assay, similar in principle to a Lowry assay, combines the protein with an alkaline copper tartrate solution and Folin reagent (55). 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 midrange 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 after spike-in and then rethawed for use to ensure lysis of cellular fractions before 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, nonprion dementia, and symptomatic genetic and symptomatic sporadic prion disease. Samples were diluted 1:10 and 1:100 for most experiments, and 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 MassGeneral Institute for Neurodegenerative Disease Tissue Bank samples; pairing was known but collection order unknown for University of California, San Francisco, samples; pairing and order were known for sarpoterin 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 plate readers, associated metadata, and source code sufficient to reproduce the analyses reported here are publicly available at: https://github.com/erciminkelks/dprp_quantification.

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