Supplementary Data

Supplementary Methods

Cerebrospinal fluid samples

Four centers participated in this study: (1) University Hospitals at Case Western Reserve University (CWRU), (2) National Prion Disease Pathology Surveillance Center (NPDPSC) at CWRU, (3) Department of Neurology at University of Brescia (Italy), and (4) Department of Pathophysiology and Transplantation at University of Milan, Fondazione Cà Granda, IRCSS Ospedale Maggiore Policlinico (Italy). A complete list of diagnoses and other available information is provided in Supplementary Table S1A–D below.

Animal samples

Cerebrospinal fluid (CSF) samples from control and scrapie-infected sheep and chronic wasting disease-infected deer were obtained from Justin Greenlee (USDA). The samples were collected at end-stage disease after euthanasia. Snap-frozen brain tissue harvested from wild-type mice was stored at −80°C until use. Just before use, a 10% homogenate was prepared in 1 × phosphate-buffered saline (PBS; pH 7) supplemented with 1% Triton X-100 and 2 × protease inhibitor cocktail (Roche; Cat. No. 04 693 124 001), centrifuged at $1500 \times g$ for 30 s, and a clear supernatant extracted for ferroxidase (Frx) assays. Total protein in each sample was estimated by the DC Protein Assay Kit (BioRad; Cat. No. 500-0116).

Characterization of Frx

Sensitivity to heat using Triton X-100 (detergent), sodium azide [2.5 m*M*; Sigma-Aldrich; Cat. No. S2002)], and zinc (10 μM; Sigma-Aldrich; Cat. No. Z4750) was assessed by exposing the CSF samples to 100°C for 20 min in a water bath, or pretreating with 1% Triton X-100 or sodium azide or zinc before determining Frx activity. Purified amyloid precursor protein (APP; Sigma-Aldrich; Cat. No. S9564) was used as a positive control for checking the effect of zinc.

To fractionate the CSF based on molecular mass, a single sample was divided into three parts and passed individually through membrane filters (Millipore) of 100-, 50-, and 3-kDa

Supplementary Table S1A. Available Information on Cerebrospinal Fluid Samples Analyzed in this Study A. Samples from University Hospitals at Case Western Reserve University, Ohio

Confirmed diagnosis	*Cases*	*Age*	*Sex*	*Protein (mg/dl)*	*Cell counts*	*RBC*	*Diff. cell counts*
HIV encephalopathy (EN)	1	47	M	85	1	22	NA
Altered mental status, subarachnoid hemorrhage (VS)	1	65	F	83	NA	NA	NA
Acute lymphoblastic leukemia (ND)	1	48	M	58	NA	NA	NA
Non-Hodgkin's lymphoma with CNS involvement (ND)	1	54	M	NP	NA	NA	NA
Multiple myeloma (ND)	1	73	F	NP	NA	NA	NA
Acute demyelinating encephalomyelitis (EN)	1	48	F	60	NA	NA	NA
Psuedotumor cerebri (ND)	1	39	F	43	NA	NA	NA
Non-Hodgkin's lymphoma (ND)	1	61	F	44	NA	NA	NA
Acute-onset severe headache (ND)	1	56	F	46	NA	NA	NA
Primary CNS lymphoma (ND)	1	51	M	62	NA	NA	NA
Pre-B-cell acute lymphoblastic leukemia (ND)	1	42	M	23	NA	NA	NA
Neuropathy (ND)	1	50	M	52	NA	NA	NA
Lumbar spinal stenosis (ND)	1	81	F	77	2	1	98l 2m
Vestibular migraine (ND)	1	40	F	30	8	9	45l 55m
Headache in patient with lymphoma (ND)	1	77	F	36	<1	20	100 l
ALL with intrathecal chemo (ND)	1	54	M	48	1	4	
Hydrocephalus, post-cerebellar hemorrhage (VS)	1	69	F	11	1	33	
ALL with intrathecal chemo (ND)	1	48	M	53	3	11	14n 86l
Severe headache, sinusitis *versus* viral meningitis, (ND)	1	39	M	63	<1	1	
Extrapyramidal syndrome (ND)	1	70	F	66	2	30	100m
Severe headache (ND)	1	47	F	27	<1	0	
Normal pressure hydrocephalus (ND)	1	58	M	87	<1	1	
Acute mental status change (ND)	1	67	M	106	2	112	8n 70l 21m 1e
Pneumonia with fever and headache (ND)	1	64	F	NA	4	0	20l 80m
Migraine headache (ND)	1	55	F	48	5	0	89l 11m
Normal pressure hydrocephalus (ND)	1	72	F	60	<1	32	
Suspected subacute right MCA infarct (middle cerebral artery) (VS)	1	42	F	46	NA	NA	NA
Cerebral hemorrhage (VS)	1	68	M	40	NA	NA	NA

NA, not available.
l, lymphocyte; m, monocyte; e, eosinophil; n, neutrophil.

Supplementary Table S1B. Samples from NPDPSC at Case Western Reserve University, Ohio

Histopathologically confirmed diagnosis	*Cases*	*Age*	*Histopathologically confirmed diagnosis*	*Case*	*Age*
CJD	2	<50	Hippocampal sclerosis (DM)	1	61
CJD	12	50–55	CNS lymphoma (DM)	1	68
CJD	19	56–60	Lymphoma (DM)	1	50
CJD	19	61–65	Baylisascaris (DM)	1	54
CJD	12	66–70	Coccidiomycosis (DM)	1	61
CJD	15	71–75	Diffuse Lewy Body disease/ frontotemporal lobar degeneration (DM)	1	60
CJD	10	76–80	Possible tauopathy (DM)	1	62
CJD	9	81–85	Leptomeningeal carcinomatosis (DM)	1	74
Dementia (undetermined cause) (DM)	4	<50	Meningoencephalitis (EN)	1	81
Dementia (undetermined cause) (DM)	7	50–55	Encephalitis (EN)	1	69
Dementia (undetermined cause) (DM)	7	56–60	Encephalitis (EN)	1	50
Dementia (undetermined cause) (DM)	9	61–65	Chronic meningoencephalitis (EN)	1	66
Dementia (undetermined cause) (DM)	8	66–70	Encephalitis (EN)	1	68
Dementia (undetermined cause) (DM)	4	71–75	Coccidioidomycosis meningoencephalitis (EN)	1	71
Dementia (undetermined cause) (DM)	5	76–80	Granulomatous meningoencephalitis (EN)	1	53
AD	1	<50	Leukoencephalopathy with axonal spheroids (DM)	1	60
AD	2	50–55	HIV encephalopathy (EN)	1	66
AD	5	56–60	Focal necrotizing leukoencephalopathy (EN)	1	69
AD	3	61–65	Prog. multifocal leukoencephalopathy (EN)	1	72
AD	8	66–70	FTD	1	68
AD	1	71–75	FTD	1	61
AD	4	76–80	FTD	1	67
AD	4	81–85	FTD	1	71
AD	3	86–90	Infract (VS)	1	57
AD	1	90–95	Cerebral vasculopathy (VS)	1	64
AD	5	NA	Angiitis with microinfarcts (VS)	1	71
Lewy body dementia (DM)	1	67	Perivenous encephalomyelitis (EN)	1	59
Cortical dysplasia (DM)	1	54	Vascular dementia (VS)	1	48
Meningeal carcinomatosis (DM)	1	55	Multi-infarct dementia (VS)	1	54
Alexander's disease (DM)	1	64	Angiitis (VS)	1	61
Leptomeningeal lymphomatosis/ leukemia (DM)	1	65	Subacute transient global ischemia (VS)	1	68
Lymphoma (DM)	1	76	Vascular dementia (VS)	1	69
Military metastases (DM)	1	64	Muti-infarct dementia (VS)	1	70
CNS lymphoma (DM)	1	71	Intravascular lymphomatosis (DM)	1	72
Glioma (DM)	1	77			

NPDPSC, National Prion Disease Pathology Surveillance Center; DM, dementias; CJD, Creutzfeldt-Jakob disease; FTD, Frontotemporal dementia; AD, Alzheimer's disease.

Supplementary Table S1C. Samples from the Department of Neurology, University of Brescia, Italy

Diagnosis	*Cases*	*Age*	*Sex*
Myasthenia (ND)	1	40	F
Palsy of IV cranial nerve (ND)	1	63	F
Healthy control (ND)	1	71	M
Ataxia (ND)	1	36	M
Healthy control (ND)	1	64	F
Healthy control (ND)	1	64	F
Healthy control (ND)	1	86	M
Healthy control (ND)	1	63	F
Healthy control (ND)	1	62	M
Healthy control (ND)	1	70	F
Peripheral neuropathy (ND)	1	61	M
Peripheral neuropathy (ND)	1	53	M
Metabolic encephalopathy (DM)	1	64	M
FTD	2	55–60	M
FTD	6	61–65	M
FTD	3	66–70	M
FTD	8	55–80	F
AD	8	55–85	NA

Supplementary Table S1D. Samples from the Department of Pathophysiology and Transplantation, University of Milan, Italy

Diagnosis	*Cases*	*Age*	*Sex*	*Protein (mg/dl)*	*Glucose (mg/dl)*	*WBC/mm^3*
Ipostenia right hand (ND)	1	79	F	29	48	0.4
Psych (ND)	1	67	F	29	63	2.2
Deficiency VI NC (ND)	1	70	F	51	48	3.2
Radiculopathy (ND)	1	59	F	25	73	1.2
Idiopathic epilepsy (ND)	1	60	F	26	53	1.9
Acute lymphatic leukemia (ND)	1	NA	F	NA	NA	NA
Hydrocephalus (ND)	1	87	M	26	60	0.7
Epilepsy (ND)	1	73	M	NA	NA	NA
Memory loss (negative neurol exam) (ND)	1	NA	F	27	43	1.2
Psych (ND)	1	77	F	24	37	1.6
Psych (ND)	1	NA	M	34.4	54	0.5
Healthy control (ND)	1	NA	F	24	45	1.1
Psych (ND)	1	NA	M	46	46	2.8
Healthy control (ND)	1	NA	M	60	60	1
Healthy control (ND)	1	60	M	28	81	0.2
Psych (ND)	1	NA	F	23	59	0.5
Psych (ND)	1	58	F	27	56	2
MCI improved over time (2-year follow-up) (ND)	1	78	M	36	47	1.6
AD	1	NA	F	40	60	6.7
AD	1	NA	F	34	67	1
AD	1	NA	F	NA	NA	NA
AD	1	77	F	29	43	0.7
AD	1	NA	F	38	54	<1
AD	1	NA	F	NA	NA	NA
AD	1	79	F	48	60	<1
AD	1	NA	M	53	50	2
AD	1	NA	F	NA	NA	NA
AD	1	66	M	39	68	2
AD	1	64	F	27	64	3
AD	1	67	F	42	56	15
AD	1	77	M	NA	NA	NA
AD	1	82	F	28	53	1
AD	1	62	F	25	61	1
AD	1	NA	M	44	64	0.6
AD	1	NA	M	48	NA	NA
FTD	1	NA	M	35	60	1.6
FTD	1	63	F	29	71	0.8
FTD	1	NA	F	NA	54	0.8
FTD	1	NA	M	47	NA	NA
FTD	1	NA	F	46	183	1
FTD	1	63	F	NA	49	2.5
FTD	1	NA	M	57	NA	NA
FTD	1	58	F	42	56	1.2
Parkinson's disease (DM)	1	NA	M	37.5	61	28
Parkinson's disease (DM)	1	NA	M	33	57	1
Parkinson's disease (DM)	1	NA	F	22	50	0.8
Parkinson's disease (DM)	1	NA	M	36	43	1.5
Progressive aphasia *versus* semantic dementia (DM)	1	NA	F	NA	44	1.4
Lewy body dementia (DM)	1	NA	M	67	NA	NA
Lewy body dementia (DM)	1	NA	M	70	60	0.9
Corticobasal degeneration (DM)	1	NA	F	36	80	1.6
Progressive supranuclear palsy (DM)	1	NA	F	24	67	12

MCI, mild cognitive impairment.

Supplementary Table S2. Performance of 14-3-3 in Discriminating CJD from DM

Biomarker	*14-3-3*	*14-3-3 & t-tau*
Area under ROC	0.57	0.71
95% CI	0.50–0.64	0.61–0.80
Sensitivity	93.7	91.3
95% CI	85.2–97.6	81.4–96.4
Specificity	21.1	50.0
95% CI	10.1–37.8	32.2–67.8
Positive LR	1.2	1.8
95% CI	1.0–1.4	1.3–2.6
Negative LR	0.3	0.2
95% CI	0.1–0.9	0.1–0.4
AIC	1.25	1.08
Accuracy	70.1	78.2

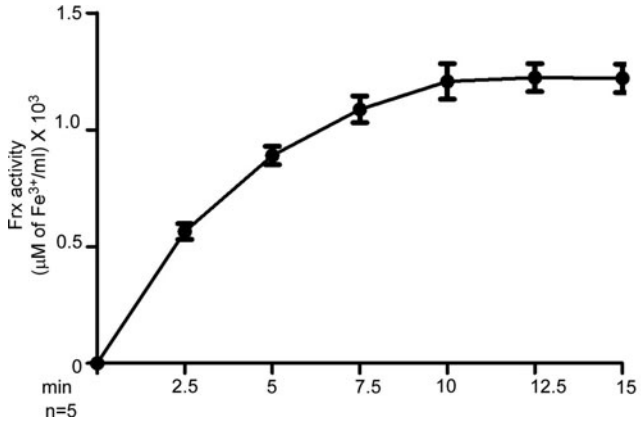
CJD ($n = 94$) *versus* all-DM ($n = 75$).
CI, confidence interval; ROC, receiver-operating characteristic; LR, likelihood ratios; AIC, Aikake Information Criterion.

cut-off, or each sample was passed sequentially through the filters. The fractions were reconstituted to the original volume before estimating Frx activity. For proteinase-K (PK) treatment, the samples were exposed to 50 μg/ml of PK for 1 h at 37°C, and residual Frx activity was estimated. Frx activity in the mouse brains was estimated in 10 μl of 10% brain homogenate prepared in 1 × PBS (pH 7), 1% Triton X-100, and 2 × protease inhibitor cocktail, using ceruloplasmin (Cp) as a positive control and homogenization buffer as a blank. The activity was normalized to the protein concentration.

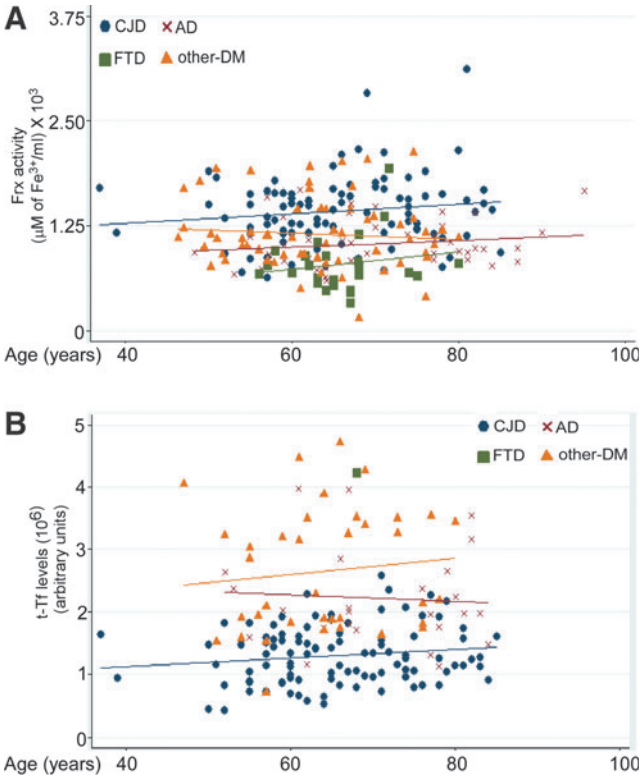
All assays were performed in triplicate, and data are presented as mean ± standard error of the mean, or as median with 5%–95% confidence interval (CI) using GraphPad Prism Software Version 5. One-way ANOVA, followed by Bonferroni's multiple comparison test, was used to compare the values between groups.

Copper oxidase assay

The copper oxidase assay was performed as described by Djoko *et al.* (20). This assay is based on a quantitative decrease in absorbance at 562 nm when Cu^+ in the substrate $[Cu^I(Bca)_2]^{3-}$ is oxidized to Cu^{2+} by the copper oxidase activity of the test sample.



SUPPLEMENTARY FIG. S1. Estimation of Frx activity of normal CSF samples over a period 15 min based on the method of Duce *et al.* (22). CSF, cerebrospinal fluid; Frx, ferroxidase.

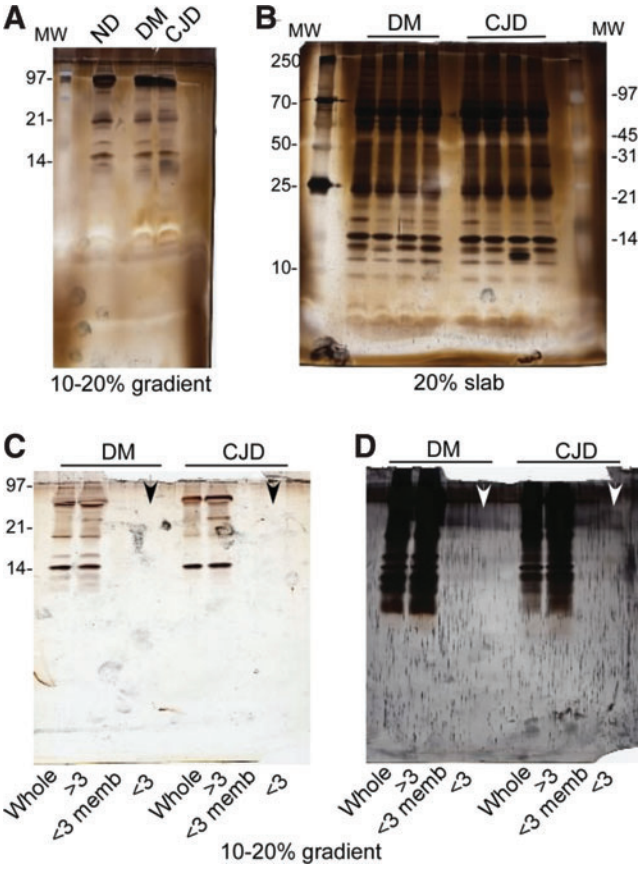


SUPPLEMENTARY FIG. S2. Scatter plot of Frx and t-Tf *versus* age for the four disease groups. (A, B) Regression lines fitted to each group displayed nonsignificant slopes, indicating an insignificant effect of patient age on Frx activity and t-Tf levels. Tf, transferrin.

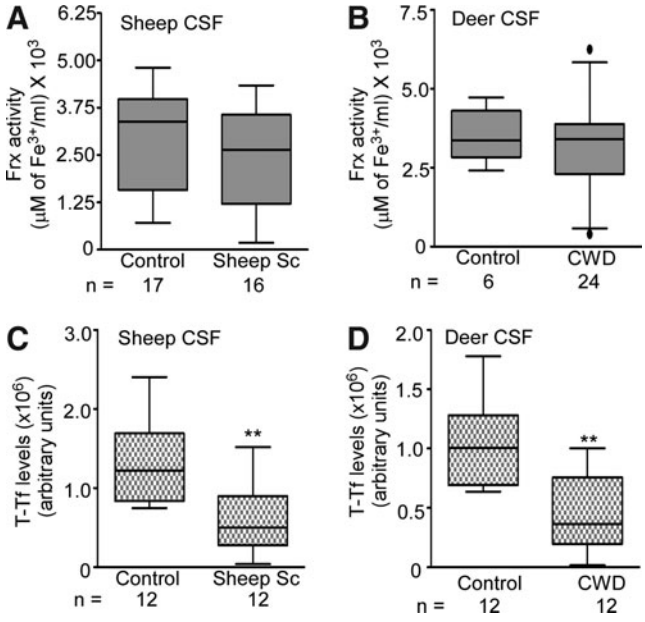
Briefly, 150 μl of the reaction mix with 2 μl substrate complex (see below) and 148 μl 50 m*M* Bis–tris buffer (pH 7) (containing sodium dithionite also known as sodium hydrosulfite to a final concentration of 1 m*M*) were added to a microtiter plate, and the absorbance at 562 nm was measured. Subsequently, 50 μl of CSF was added, and the decrease in absorbance was measured every 2 min for 30 min in the Bio-Tek Synergy 4 plate reader. H_2O_2 at a final concentration of 1% was used as a positive control. Reaction lacking the substrate complex was taken as a negative control. (The substrate complex $[Cu^I(Bca)_2]^{3-}$ contained 40 μM of $CuSO_4$ [Sigma-Aldrich; Cat. No. C1297] and 100 μM of Na_2Bca [Sigma-Aldrich; Cat. No. D8284] and was prepared by mixing the stock solutions of $CuSO_4$ and Na_2Bca in a 1:2.5 molar ratio). The stock solutions were prepared in 50 m*M* Bis–tris buffer (pH 7) containing 1 m*M* Sodium dithionite (Sigma-Aldrich; Cat. No. 157953).

Western blotting

An equal volume of CSF from each case, regardless of the protein content or cell count, was resolved by denaturing SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and probed with antibodies specific to transferrin (Tf; Genetex, Inc.; Cat. No. GTX 21223), Cp (Dako; Cat. No. 2016-10), and APP (Millipore; Cat. No.



SUPPLEMENTARY FIG. S3. Protein profile of CSF samples from different disease groups. (A, C) and **(B)** are silver-stained 10%–20% gradient and 20% slab SDS-PAGE gels, respectively, showing the protein profile in unfractionated CSF samples from ND, DM, and CJD **(A, B)** and fractionated DM and CJD samples **(C). (D)** Overdeveloped version of **(C)**. No bands are detected in the <3-kDa fraction (black and white arrowheads). CJD, Creutzfeldt-Jakob disease; DM, dementias; ND, non-DM.



SUPPLEMENTARY FIG. S4. Scrapie infection does not alter Frx activity, but decreases the t-Tf levels in the CSF of infected cases. (A, B) Frx activity in postmortem CSF from scrapie-infected sheep and CWD-infected deer does not differ from matched controls. **(C, D)** Tf levels are significantly decreased in the CSF from scrapie-infected sheep and CWD-infected deer. $n = 12$, $^{**}p < 0.01$. CWD, chronic wasting disease.

MAB348), either sequentially or on separate membranes. Appropriate HRP-conjugated secondary antibody followed by ECL Plus (GE Healthcare; Cat. No. RPN 2132) was used to detect the reactive bands. To reduce variability due to multiple-sample analysis, known quantities of purified Tf, Cp, and APP were included in each gel, and strips of the PVDF membranes corresponding to a specific protein were exposed to an X-ray film simultaneously. The immunoreactive bands were quantified with UN-SCAN-IT software (version 6.1; Silk Scientific, Inc.) using three exposures from a single membrane showing an exponential increase in the band intensity. The groups were compared by one-way ANOVA, followed by Bonferroni's multiple-comparison test.

Silver staining

An equal volume of unfractionated and fractionated CSF samples from dementias (DM) and Creutzfeldt-Jakob disease cases was resolved on a 10%–20% gradient or 20% polyacrylamide gels and stained using Silver Stain Plus (BioRad; Cat. No. 161-0499), according to the manufacturer's instructions. Briefly, the gels were fixed in the fixative enhancer solution for 20 min, washed twice for 10 min each, and exposed to a solution containing silver complex, reduction moderator, and an image development reagent. The reaction was stopped with 5% acetic acid when the protein bands reached the desired intensity.