Identification of novel risk loci and causal insights for sporadic Creutzfeldt-Jakob disease: a genome-wide association study


Summary

Human prion diseases are rare and usually rapidly fatal neurodegenerative disorders, the most common being sporadic Creutzfeldt-Jakob disease (sCJD). Variants in the PRNP gene that encodes prion protein are strong risk factors for sCJD but, although the condition has similar heritability to other neurodegenerative disorders, no other genetic risk loci have been confirmed. We aimed to discover new genetic risk factors for sCJD, and their causal mechanisms.

Methods

We did a genome-wide association study of sCJD in European ancestry populations (patients diagnosed with probable or definite sCJD identified at national CJD referral centres) with a two-stage study design using genotyping arrays and exome sequencing. Conditional, transcriptional, and histological analyses of implicated genes and proteins in brain tissues, and tests of the effects of risk variants on clinical phenotypes, were done using deep longitudinal clinical cohort data. Control data from healthy individuals were obtained from publicly available datasets matched for country.

Findings

Samples from 5208 cases were obtained between 1990 and 2014. We found 41 genome-wide significant single nucleotide polymorphisms (SNPs) and independently replicated findings at three loci associated with sCJD risk; within PRNP (rs1799990; additive model odds ratio [OR] 1·23 [95% CI 1·17–1·30], p=2·68 × 10⁻¹⁰), GAL3ST1 (rs2267161; OR 1·18 [1·12–1·25], p=1·01 × 10⁻¹⁰), and STX6 (rs3747957; OR 1·16 [1·10–1·22], p=9·74 × 10⁻⁹). Follow-up analyses showed that associations at PRNP and GAL3ST1 are likely to be caused by common variants that alter the protein sequence, whereas risk variants in STX6 are associated with increased expression of the major transcripts in disease-relevant brain regions.

Interpretation

We present, to our knowledge, the first evidence of statistically robust genetic insights associated in sporadic human prion disease that implicate intracellular trafficking and sphingolipid metabolism as molecular causal mechanisms. Risk SNPs in STX6 are shared with progressive supranuclear palsy, a neurodegenerative disease associated with misfolding of protein tau, indicating that sCJD might share the same causal mechanisms as prion-like disorders.

Funding

Medical Research Council and the UK National Institute of Health Research in part through the Biomedical Research Centre at University College London London Hospitals National Health Service Foundation Trust.

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Introduction

Prion diseases are fatal neurodegenerative conditions in humans and animals caused by the propagation of prions: atypical infectious agents comprised solely or predominately of host prion protein.¹ Prions are thought to propagate through a process of binding to normal prion protein, induction of conformational change by templating, and fission of the polymeric assembly. Prion diseases can be acquired from exposure to prions in the diet, or through medical or surgical procedures, which can result in public health crises. The cattle prion disease, bovine spongiform encephalopathy (BSE), which transmitted to mostly young British and other European adults as variant Creutzfeldt-Jakob disease (vCJD),² led to enhanced clinical diagnosis and management for all prion diseases worldwide. Inherited prion disease, caused only by mutations of the prion protein gene (PRNP), causes approximately 10–15% of the annual incidence of all prion diseases in most countries.³ The most common type of human prion disease is sporadic CJD (sCJD), a rapidly progressive dementia with a lifetime risk of approximately one in 5000, which occurs predominantly in older adults.⁴ Other than age and
Research in context

Evidence before this study
The rarity of sporadic Creutzfeldt-Jakob disease (sCJD) has been limiting in previous genome-wide association studies (GWAS) for disease risk. We searched PubMed on April 9, 2020, with the terms (“prion” OR “crauzfeldt””) AND (“genome wide association” OR “GWAS”), without language or date restrictions, and identified four relevant publications, including two directly investigating sCJD risk through genome-wide analyses. However, the sample sizes in these studies were not sufficient to identify statistically significant associations outside of the known risk at the prion protein gene (PRNP). Further studies into genetic risk factors for sCJD have primarily utilised targeted replication of putative risk variants or candidate genes to propose association.

Added value of this study
Through international collaboration of sample resources, this study is, to our knowledge, the first GWAS to identify genetic variants associated with sCJD risk outside of PRNP, at genome-wide significance. Two of these variants (within STX6 and GAL3ST1) were statistically robust to replication in an independent cohort, with 5208 patients with sCJD in total included in the two-stage study design. Through statistical fine-mapping and analysis of exome sequencing and gene expression data, we propose genes that are likely to be causal, and mechanisms for both novel associations. We used patient brain samples and cell-based assays to further investigate the biological implications of these associations in relevant systems. Two further loci at PDIA4 and BMER81 were also associated with sCJD risk in gene-based tests.

Implications of all the available evidence
Identification of two novel non-PRNP loci conferring sCJD risk will provide further avenues for research, with increased evidence to support a role of modified intracellular trafficking and sphingolipid metabolism within sCJD biology, providing the potential to inform new therapeutic approaches. With the shared genetic risk of variants within STX6 and those previously identified for the tauopathy progressive supranuclear palsy, this study also supports the notion of a common so-called prion-like causal mechanism for related neurodegenerative disorders and thus potential for shared treatments.

Methods

Study design and participants
We did a GWAS using samples from patients diagnosed with probable or definite sCJD according to widely accepted criteria, which were provided by specialist or national surveillance centres in countries with populations of predominantly European ancestries (appendix pp 2, 28–32). Diagnostic criteria for probable sCJD varied over the course of sample collection for the study. Using modern diagnostic methods, including real-time quaking-induced conversion assay with CSF, a probable diagnosis refuted by post-mortem examination is extremely rare; but even more than 20 years ago, probable sCJD was a highly accurate term. Patient samples were distributed across a two-stage study design: samples were genotyped using Illumina Omniexpress arrays in the discovery stage, and additional samples were genotyped at the lead variant in each hit locus using minor groove binding probes in the replication stage. Control data from healthy individuals were obtained from publicly available datasets matched for country.
Table: Association results of discovery and replication stages

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Procedures and statistical analysis

Genotypes were imputed using the Michigan Imputation Server and standard sample and genotyping quality control measures were implemented, to generate 6 314 492 high-quality autosomal single nucleotide polymorphisms (SNPs) for subsequent analysis (appendix pp 28–32). SNPTTEST version 2.5.2 was used to perform the association test using an additive logistic regression model. Association statistics for the replication stage were generated using PLINK version 1.9 in a fixed effects meta-analysis. Each model was used to study genetic association for kuru resistance (older asymptomatic individuals who were exposed to kuru compared to patients with young onset and those born after kuru exposure). Additional exome sequencing was performed on 5019 samples using the Illumina HiSeq6000 platform.

Further gene-based analysis was performed using MAGMA version 1.06 and VEGAS2 version 2.02, and SNP heritability estimates were calculated using SumHer with standard specifications. CAVIAR and PAINTOR were utilised to generate a credible causal set for SNPs surrounding each significant locus based on linkage disequilibrium and functional annotations. eCAVIAR and eQTL colocalisation analysis was performed using 48 tissues included in the GTEx portal version 7.

Short-hairpin RNAs targeting S10X6 and Prnp were used to knockdown expression in N2aPK1/2 cells susceptible to infection with Chandler RML prions. Prion propagation was measured using the scrapie cell assay, as previously described. Expression of each proposed gene was measured by RT-qPCR in cerebellum from ten patients with sCJD and ten neurologically healthy controls. Immunohistology for syntaxin-6 and protein disulphide isomerase family A, member 4 was done on formalin-fixed paraffin-embedded frontal cortex and cerebellum of 19 patients with sCJD and 15 non-neurological disease controls.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between 1990 and 2014, we obtained 5208 sCJD samples, of which 4110 were used in the discovery stage and 1098 were used in the replication stage.

In the discovery stage we compared genome-wide genotype data from 4110 patients with probable or definite sCJD from countries of predominantly European ancestries with 13 569 control samples from a similar range of countries (appendix pp 2–3). Imputation using the Michigan server resulted in 6 314 492 high-quality autosomal SNPs after quality control, which were used for downstream association tests in SNPTTEST with ten population covariates. Genomic inflation (λ) was 1·026 for downstream association tests in SNPTEST with 1·27 [95% CI 1·16–1·38], p=0·040) and PDIA4

Further to the known association at PRNP on chromosome 20p13, two loci achieved genome-wide significance mapping to 1q25.3 (STX6) and 22q12.2 (GAL3ST1); figure 1; table; appendix pp 17–19. Gene-based testing with VEGAS2 additionally identified PDIA4 (p=0·040) and BMERB1 (p=0·0014), although testing with MAGMA did not support these associations (appendix pp 20–21). No significant gene sets were found. A SNP in intron 1 of the BMERB1 gene achieved borderline significance (rs6498552, odds ratio 1·27 [95% CI 1·16–1·38], p=5·75×10⁻⁸, appendix p 21). Although we acknowledge that data from multiple SNPs at a locus are needed to directly replicate gene-based test results, we selected a lead SNP from the three genome-wide significant loci as well as from PDIA4 and BMERB1 for the replication stage.

In the replication stage we generated genotype data using minor groove binding probes from 1098 patients with probable or definite sCJD, again from multiple countries of predominantly European ancestries, and compared these with genotypes from 498 016 control samples.
from the same countries (appendix pp 2–3). Association testing provided replication evidence for PRNP (rs1799990, heterozygous genotype and to a lesser extent the minor allele is protective), STX6 (rs3747957, minor allele conferred risk), and GAL3ST1 (rs2267161, minor allele was protective; table). Additionally, we explored if those loci would show an association in related prion diseases. Genotype data was generated for vCJD (acquired from exposure to BSE), iatrogenic CJD (caused by exposure to cadaveric pituitary-derived human growth hormone), or kuru (and resistance to kuru; a former epidemic of orally transmitted prion disease among people who lived in the Eastern Highlands Province of Papua New Guinea). We found no evidence for association of rs3747957 in STX6, or rs2267161 in GAL3ST1 with these phenotypes (p>0·05), implying that these loci might confer risk specific to the sporadic form of human prion disease, although all tests were underpowered because of small sample size.7,9

sCJD is known to comprise a range of different clinical and pathological phenotypes, broadly correlating with prion molecular strain types, the latter including categorisation by different proportions of three glycoforms and the apparent molecular weight of abnormal prion protein by western blotting.28 The National Prion Clinic London, UK has done longitudinal observational cohort studies of CJD involving systematic clinical assessments of patients, resulting in deep phenotype data.23,29 We tested rs1799990, rs3747957, and rs2267161 for association with age at clinical onset, clinical duration, and the slope of decline in a functional measure of disease severity, along with 27 other phenotypic variables (appendix p 4). As expected, rs1799990 in PRNP showed associations with several clinical and biomarker traits (ten associations in 30 tested hypotheses). We found no evidence for epistasis between discovered loci and genotypes at rs1799990, which is known to be a major determinant of clinical phenotype. Because association in a genomic region might not be mediated through the nearest gene, we investigated the potential mechanisms underlying associations with PRNP, STX6, and GAL3ST1. We used CAVIAR to fine-map the association signal at a locus through joint modelling of association statistics for all variants at a locus and estimation of a conditional posterior probability of causality while allowing for multiple plausibly functional SNPs.30 Around PRNP most of the SNPs identified tagged rs1799990. Unexpectedly, a cluster of SNPs located

Figure 2: Statistical fine-mapping using CAVIAR

CAVIAR utilises summary statistics and LD structure to predict the probability of each variant being causal, producing a causal set with 95% probability of containing the causal SNP, while allowing for the possibility of multiple causal SNPs. Each locus was defined as 100 variants upstream and downstream of the top SNP. Plots show causal posterior probability of each variant at PRNP (A), STX6 (B), and GAL3ST1 (C), coloured by LD (derived from 1000 Genomes Project European populations data) with the top SNP. Circles indicate variants within the 95% causal set. Triangles highlight other SNPs not predicted to be in the causal set. MB=megabases. LD= linkage disequilibrium. SNP=single nucleotide polymorphism.

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See Online for appendix

For more on SNP heritability see http://doi.org/s3ummer
For more on CAVIAR see http://genetics.cs.ucla.edu/caviar
Previously studies have reported that variants at the PRNP locus might confer an increased risk for sCJD, independently of rs1799990.20,21 To further delineate the genetic architecture of the PRNP risk locus, we first performed an association analysis under a heterozygous model, which is more appropriate for the known mechanism, and confirmed rs1799990 as the lead SNP (p=1·0·01×10⁻¹³; appendix p 22). In a conditional analysis, adjusting for heterozygosity at rs1799990, the lead SNP was rs6139515 (p=8·98×10⁻⁴). This SNP, which is in low linkage disequilibrium with rs1799990 (r²=0·04), is correlated with PRNP transcript levels in tibial nerve in human brain (frequency of 0·018), and TC (frequency of 0·315). We have found no evidence of an association driven by the rs55647628-T allele using a haplotype-based test (appendix p 13). Furthermore, analyses of 501 CJD samples by exome sequencing did not identify additional rare variants in GAL3ST1 or STX6.

Expression of STX6, GAL3ST1, PDIA4, and BMERBI mRNA was slightly reduced in bulk analysis of post-mortem cerebellar brain tissue from patients with sCJD, but only to a similar extent as genes that have been suggested as good comparators (appendix p 24). Immunohistology of frontal cortex (in 19 patients with sCJD) showed that syntaxin-6 expression is restricted to neurons of different sizes, although other cell types, probably astrocytes or oligodendrocytes, were less consistently stained. In the cerebellum, syntaxin-6 staining was observed in Purkinje cells and in large neurons of the dentate nucleus, and a fine granular staining was seen in the molecular layer (appendix p 25). In all neuron populations of cerebellum and forebrain, for more on PAINTOR see https://hpc.nih.gov/apps/PAINTOR.html

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![Figure 2: Colocalisation of GWAS results at STX6 locus with expression quantitative trait loci](image-url)

Plot of -log₁₀ of p values from the GWAS analysis at the STX6 locus (black) and the expression quantitative trait locus association analysis from the GTEx dataset (red) for: STX6 expression in the caudate (A), STX6 expression in the hypothalamus (C), and the putamen (B), STX6 locus association analysis from the GTEx dataset (red) for: STX6 expression in the hypothalamus (C), and the putamen (B). Peaks correspond to the colocalisation posterior probability in the expression quantitative trait locus and GWAS CAVIAR analysis, with a higher degree of colocalisation with increasing colocalisation posterior probability (appendix p 12).

GWAS=genome-wide association study. Mb=megabases.
the staining pattern was fine granular, and was located in the cytoplasm, but did not extend into the processes. The staining pattern was compatible with the predicted target, the Golgi apparatus. The pattern for both syntaxin-6 and PDIA4 was indistinguishable between patients with CJD and controls (appendix p 26).

Based on GTEx data, we hypothesised that increased expression of STX6 in deep brain nuclei increases risk of prion disease. To test whether this might be conferred through facilitating prion propagation in mammalian neuronal cells, we depleted prion-susceptible mouse neuroblastoma-derived cells (N2aPK1/2) of STX6 expression using RNA interference. Using the automated scrapie cell assay we measured the impact of STX6 knockdown on prion propagation using Prnp knockdown cells, known to inhibit prion propagation in this assay, as positive controls. Figure 4 shows that STX6 depletion, unlike Prnp depletion, does not consistently reduce the ability of N2aPK1/2 cells to propagate RML prions.

**Discussion**

We report, to our knowledge, the first GWAS in a human prion disease powered to detect alleles with the modest effect sizes typical of complex diseases. We identified new risk factors for sCJD, including variants which appear to have pleiotropic effects in neurodegenerative diseases. Further to the known effects at PRNP codon 129, we report two independently replicated loci and evidence to support the conclusion that risk variants modify the primary sequence of the encoded protein (GAL3ST1) or increase expression in brain tissues (STX6). Although a multitude of potential binding partners for prion protein and mechanisms for the modification of prion infection have been proposed, GWAS discoveries have great value because risk variants identified are implicitly causal in the human disease. Therapeutic targets underpinned by genetic evidence have better chances of successful drug development, further encouraging research into the mechanisms that underpin these signals.

Risk variants in sCJD might act at different disease stages: increasing the chance of the spontaneous generation of prion proteins or increasing the susceptibility to prion infection. GWAS discoveries have great value because risk variants identified are implicitly causal in the human disease. Therapeutic targets underpinned by genetic evidence have better chances of successful drug development, further encouraging research into the mechanisms that underpin these signals.
of prions, reducing prion clearance, enabling prion propagation throughout brain tissue, or modifying the downstream toxic effects of prion propagation on brain cells. We did not find any evidence of a role for risk variants in the modification of clinical or pathological disease phenotypes, or in modified expression of risk genes at the end stage of the disease, but it is too early to draw confident conclusions in this respect. Altering the expression of Stx6 in a cellular model of prion infection did not modify the susceptibility of mouse cells to infection or the accumulation of abnormal forms of prion protein. Our functional data therefore point to a role early in the disease process, perhaps in altering the risk of spontaneous prion formation in the brain, but studies in other models are warranted.

STX6 encodes syntaxin-6, an eight exon, 255 amino-acid protein that localises to the trans-Golgi network, and recycling and early endosomes. Syntaxin-6 is thought to form part of the t-SNARE complex involved in the decision of a target membrane to accept the fusion of a vesicle. 11 The intracellular location of abnormal prion protein in prion-infected cells involves the plasma membrane where conversion is primarily thought to occur, 29 as well as early and recycling endosomes, late endosomes, and the perinuclear region. 42 Other studies implicate the endocytic-recycling compartment or multivesicular bodies as sites of generation of prions, and dysregulation of trafficking genes by sCJD. 11,13 Intracellular trafficking has also been implicated in the degradation of prions. 12 The modification of trafficking of normal or abnormal prion protein by syntaxin-6 might be a focus for future investigation. 16

There has been considerable recent discussion about the extent to which neurodegenerative diseases associated with the accumulation of misfolded proteins or peptides are similar to prion diseases in their pathogenesis. 7 This concept provokes the suggestion that prion diseases and prion-like disorders might share genetic risk factors. Progressive supranuclear palsy is an uncommon neurodegenerative cognitive and movement disorder associated with the accumulation of abnormal forms of microtubule-associated protein tau with four repeats. 30,31 Variants in STX6 are in a haplotype with SNPs previously identified as associated with progressive supranuclear palsy, with shared risk alleles (appendix p 14). 14 Pleiotropic effects at this locus shared between prion diseases and tauopathy lend support to the concept of prion-like disorders and indicate the possibility of genetically inspired interventions across multiple neurodegenerative disorders.

GAL3ST1 encodes galactose-3-O-sulfotransferase 1, a 423 amino-acid protein that localises to the Golgi network in oligodendrocytes, and is the sole enzyme responsible for the sulfation of membrane sphingolipids to form sulfatides—a major brain lipid and component of the myelin sheath. 42 Degradation of sulfatides is catalysed by ARSA in the lysosome; recessive defects in this enzyme cause metachromatic leukodystrophy: a lysosomal storage disorder associated with profound central and peripheral demyelination. 42 Knockout of Gal3st1 in mice results in a neurological phenotype associated with abnormal myelin maintenance with age, histological abnormalities at the paranodal junctions, and abnormal diffusion tensor imaging. 43 Furthermore, in a GWAS of UK Biobank participants, rs2267161 in GAL3ST1 was significantly associated with multiple changes in white matter microstructure measured using brain diffusion tensor imaging. 44 Sphingolipid metabolism and myelin maintenance have both been previously implicated in prion protein function and prion diseases. 45,46 Multiple genes in the sphingolipid metabolic pathways are dysregulated early in the pathogenesis of mouse prion diseases, a finding consistent between inbred mouse lines and prion strains. 47 Knockout of prion protein in mice, or naturally in goats, results in a demyelinating neuropathy, which in goats is associated with abnormal sphingolipid metabolism. 48-50

PDIA4 and BMRB1 loci, identified in the discovery stage by gene-based analysis, were not replicated at their lead SNPs; however, the replication sample was necessarily limited by the rarity of the disease, and the lead SNPs had a lower allele frequency than at other risk loci. Further attempts to replicate are justified as gene-based test results are driven by multiple SNPs at each locus.

In conclusion, we present the first evidence of statistically robust genetic associations in sporadic human prion disease that implicate intracellular trafficking and sphingolipid metabolism as molecular causal mechanisms. Future work might further test the hypotheses derived from these discoveries in prion disease model systems, and examine the effects of genome-wide genetic variation on clinical, pathological, and molecular phenotypes in sporadic and inherited prion diseases.

Contributors
EJ, HH, EV, and JU did the main data collection and analysis. AD, HS, TC, PN, LQ, JW, JL, ZJ, SBr, PJ, AN, THM, and PdH contributed specific sections of data collection and analysis. SCs, CS, SS, GGK, MDG, AG, KS, HB, AA, HK, Sjvi, CAI-V, CMvdD, BS, EG, PPL, MC, OC, PS-J, AS, FM-T, EB-A, SH, J-LJ, J-PB, PAm, J-CL, PP, AB-S, Sca, AP, AL, MP, SA, GM, RK, SZ, IZ, SBo, MBC, GHJ, KJ, JB, PG, JS, and BA contributed to sample collection and phenotyping. JC and SM had overall supervision of the study and obtained funding. SM and EJ drafted the text and figures for the manuscript. All authors contributed to editing of the text and figures for the manuscript.

Declaration of interests
HB reports grants from Federal Office for Health, Swiss Government, during the conduct of the study. SH reports grants from Santé Publique France, during the conduct of the study; grants from LFBI Biomedicaments, Institut de Recherche Servier, and MedDay Pharmaceuticals, outside the submitted work; BA reports grants from Centers for Disease Control and Prevention, during the conduct of the study. KA reports grants from Ono Pharmaceuticals, outside the submitted work. SM reports grants from Medical Research Council and National Institute of Health Research’s Biomedical Research Centre at University College London Hospitals NHS Foundation Trust, during the conduct of the study. GGK reports personal fees from Biogen, outside the submitted work. BA reports research grants from Genescreen, outside the submitted work. SM reports grants from Medical Research Council and National Institute of Health Research’s Biomedical Research Centre at University College London Hospitals NHS Foundation Trust, during the conduct of the study. SL reports personal fees from LFB during the conduct of the study. SH reports grants from Santé Publique France, during the conduct of the study; grants from LFBI Biomedicaments, Institut de Recherche Servier, and MedDay Pharmaceuticals, outside the submitted work; and has a patent method for treating prion diseases (PCT/EP2019/070457) pending. PAm reports personal fees from Fondation Alzheimer, and personal fees and other from Genescreen, outside the submitted work. AJ reports grants from National Institutes of Health, outside the submitted work; and has a patent method for treating prion diseases (PCT/EP2019/070457) pending. PdH reports personal fees from Biogen, outside the submitted work. JC reports grants from Medical Research Council and National Institute of Health Research’s Biomedical Research Centre at University College London Hospitals NHS Foundation Trust, during the conduct of
the study; and is a director and shareholder of D-Gen: an academic spinout in the field of prion disease diagnostics, decontamination, and therapeutics. MP reports personal fees from Ferrer Pharmaceuticals and the Collection of National Chemical Compounds and Screening Centre, and non-financial support from Fondazione Cellule Staminali, outside the submitted work. MDG reports grants from National Institutes of Health and National Institute on Aging (R01 AG031089, R56 AG055619, and R01AG066256) and Alliance Biosecure, personal fees and other from Quest Diagnostics, and other from The Michael J Hoozer Family Fund, during the conduct of the study, and personal fees from Blode Therapeutics and Bioscience Biopharma, and other from Grand Rounds, outside the submitted work; has consulted for 3D Communications, Adept Field Consulting, Advanced Medical, Best Doctors, Second Opinion, Gerson Lehrman Group, Guidepoint Global, InThought Consulting, Market Plus, Trinity Partners, Biohaven Pharmaceuticals, Quest Diagnostics, and various medical-legal consulting firms; has received speaking honoraria for various medical centre lectures and from Oakstone publishing; has received past research support from Alliance Biosecure, CureFSP, the Tau Consortium, and Quest Diagnostics; and serves on the board of directors for San Francisco Bay Area Physicians for Social Responsibility and on the editorial board of Dementia & Neuropsychologia. All other authors declare no competing interests.

Data sharing
Summary statistics are available through the GWAS catalog at National Human Genome Research Institute-European Bioinformatics Institute via study accession number GCST90001389. Further data are available upon request to the corresponding author.

Acknowledgments
We thank Richard Newton (University College London Institute of Prion Diseases) for support with images and University College London Genomics who did the array processing for CJD samples. This study makes use of data generated by the Wellcome Trust Case-Control Consortium. A full list of the investigators who contributed to the generation of the data is available from the Wellcome Trust Case-Control Consortium. Funding for the project was provided by the Wellcome Trust and Medical Research Council. We thank patients, their families and carers, UK neurologists and other referring physicians, co-workers at the National Prion Clinic, and our colleagues at the National Creutzfeldt-Jakob Disease Research and Surveillance Unit, Edinburgh, UK. We thank the present and previous directors and the staff of the Papua New Guinea Institute of Medical Research, especially the kuru project field team, and the communities of the kuru-affected region, for their generous support. We gratefully acknowledge the help of the late Carleton Gajdusek, the late Joseph Gibbs, and their associates from the former Laboratory of Central Nervous System Studies of the National Institutes of Health, Bethesda, MD, USA for archiving and sharing old kuru samples. The kuru studies were initially funded by a Wellcome Trust Principal Research Fellowship in the Clinical Sciences to JC, and since 2001, all other aspects of the work were funded by the Medical Research Council. Several authors at University College London and University College London Hospitals receive funding from the Department of Health’s National Institute of Health Research Biomedical Research Centres funding scheme. Some of this work was supported by the Department of Health and funded National Prion Monitoring Cohort study. SCo receives a National Health and Medical Research Council Practitioner Fellowship (APP105784). THM is supported by a Fellowship award from Alzheimer’s Society, UK (341 [AS-CTF-16b-007]) and Creutzfeldt-Jakob Disease Support Network UK Research Support Grants. Many other national funders were involved in international Creutzfeldt-Jakob disease surveillance and sample collection. Funding for the collection of Polish samples for study was partially provided by the EU Joint Programme Neurodegenerative Disease Research and Medical University of Lodz. DrWe thank for Social Responsibility and on the editorial board of Dementia & Neuropsychologia. All other authors declare no competing interests.

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We thank Richard Newton (University College London Institute of Prion Diseases) for support with images and University College London Genomics who did the array processing for CJD samples. This study makes use of data generated by the Wellcome Trust Case-Control Consortium. A full list of the investigators who contributed to the generation of the data is available from the Wellcome Trust Case-Control Consortium. Funding for the project was provided by the Wellcome Trust and Medical Research Council. We thank patients, their families and carers, UK neurologists and other referring physicians, co-workers at the National Prion Clinic, and our colleagues at the National Creutzfeldt-Jakob Disease Research and Surveillance Unit, Edinburgh, UK. We thank the present and previous directors and the staff of the Papua New Guinea Institute of Medical Research, especially the kuru project field team, and the communities of the kuru-affected region, for their generous support. We gratefully acknowledge the help of the late Carleton Gajdusek, the late Joseph Gibbs, and their associates from the former Laboratory of Central Nervous System Studies of the National Institutes of Health, Bethesda, MD, USA for archiving and sharing old kuru samples. The kuru studies were initially funded by a Wellcome Trust Principal Research Fellowship in the Clinical Sciences to JC, and since 2001, all other aspects of the work were funded by the Medical Research Council. Several authors at University College London and University College London Hospitals receive funding from the Department of Health’s National Institute of Health Research Biomedical Research Centres funding scheme. Some of this work was supported by the Department of Health and funded National Prion Monitoring Cohort study. SCo receives a National Health and Medical Research Council Practitioner Fellowship (APP105784). THM is supported by a Fellowship award from Alzheimer’s Society, UK (341 [AS-CTF-16b-007]) and Creutzfeldt-Jakob Disease Support Network UK Research Support Grants. Many other national funders were involved in international Creutzfeldt-Jakob disease surveillance and sample collection. Funding for the collection of Polish samples for study was partially provided by the EU Joint Programme Neurodegenerative Disease Research and Medical University of Lodz. We thank Dr Maria Styczynska from Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, for kindly providing control DNA samples for the Polish cohort. The Italian National Surveillance of Creutzfeldt-Jakob Disease and Related Disorders is partially supported by the Ministero della Salute, Italy. The German National Reference Centre for Transmissible Spongiform Encephalopathies is funded by grants from the Robert Koch Institute. The Dutch National Prion Disease Registry is funded by the National Institute for Public Health and the Environment (Ministry for Health, Welfare, and Sports) and is conducted under the leadership of the National Coordination Infection Control (Landelijke Coördinatie Infectieziektebestrijding [LCI Landelijke coördinatie infectieziektebestrijding]). PSJ was supported by Instituto de Salud Carlos III (Fondo de Investigación Sanitaria, PI16/01652) Acción Estratégica en Salud integrated in the Spanish National I+D+i Plan and financed by Instituto de Salud Carlos III Subdirección General de Evaluacion and the Fondo Europeo de Desarrollo Regional (FEDER “Una Manera de Hacer Europa”). We thank Inés Santistube and the Valdecilla Biobank (PTT/0015/0019), integrated in the Spanish Biobank Network, for their support and collaboration in sample collection and management. The study on Italian controls was supported by the Ministero dell’Istruzione, dell’università e della Ricerca MIUR project “Dipartimenti di Eccellenza 2018–2022” (DISD18000410001) to the Department of Medical Sciences, University of Torino, Torino, Italy (GM) and the Associazione Italiana per la Ricerca sul Cancro (JG 2018 Id.21390 to GM). The Three-City Study was performed as part of a collaboration between the Institut National de la Santé et de la Recherche Médicale (Inserm), the Victor Segalen Bordeaux II University, and Sanofi-Synthélabo; the Fondation pour la Recherche Médicale funded the preparation and initiation of the study. The Three-City Study was also funded by the Caisse Nationale Maladie des Travailleurs Salariés, Direction Générale de la Santé, MGEN, Institut de la Longévité, Agence Française de Sécurité Sanitaire des Produits de Santé, the Aquitaine and Bourgogne Regional Councils, and Agence Nationale de la Recherche, and ANR supported the COGINUT and COVADIS projects, Fondation de France, and the joint French Ministry of Research INSERM Cohortes et collections de données biologiques programme. Lille Génépol received an unconditional grant from Eisai. The Three-City biological bank was developed and maintained by the laboratory for genomic analysis LAG-BRC Institut Pasteur de Lille. This work was also funded by the Pasteur Institut de Lille, the Lille Métropole Communauté Urbaine, the Haut-de-France council, the European Community (FEDER), and the French Government’s LABEL DISTALZ programme (development of innovative strategies for a transdisciplinary approach to Alzheimer’s disease). The French National Surveillance Network for Creutzfeldt-Jakob Disease is supported by Santé Publique France. MDG would like to thank Megan Casey for her assistance with sample collection and management.

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