Diagnosing Sporadic Creutzfeldt-Jakob Disease by the Detection of Abnormal Prion Protein in Patient Urine

Connie Luk, PhD; Samantha Jones, BSc; Claire Thomas, BSc; Nick C. Fox, MD; Tze H. Mok, MBChB; Simon Mead, PhD; John Collinge, FRS; Graham S. Jackson, PhD

+ **Supplemental content**

IMPORTANCE Creutzfeldt-Jakob disease (CJD) is a fatal neurodegenerative disorder associated with the accumulation of infectious abnormal prion protein through a mechanism of templated misfolding. A recent report has described the detection of abnormal prion protein in the urine of patients with variant CJD (vCJD) using protein misfolding by cyclic amplification, which was apparently absent in the more common sporadic form of CJD (sCJD). A noninvasive diagnostic test could improve early diagnosis of sCJD and, by screening donations, mitigate the potential risks of prion transmission through human urine–derived pharmaceuticals. Here, we describe the adaptation of the direct detection assay, developed originally as a blood test for vCJD, for the detection of disease-associated prion protein in urine samples from patients with sCJD.

OBJECTIVE To determine the feasibility of sCJD diagnosis by adaptation of an established vCJD diagnostic blood test to urine.

DESIGN, SETTING, AND PARTICIPANTS This retrospective, cross-sectional study included anonymized urine samples from healthy nonneurological control individuals (n = 91), patients with non-prion neurodegenerative diseases (n = 34), and patients with prion disease (n = 37) of which 20 had sCJD. Urine samples obtained during the Medical Research Council PRION-1 Trial, the National Prion Monitoring Cohort Study, and/or referred to the National Prion Clinic or Dementia Research Centre at the National Hospital for Neurology and Neurosurgery in the United Kingdom.

MAIN OUTCOMES AND MEASURES Presence of sCJD infection determined by an assay that captures, enriches, and detects disease-associated prion protein isoforms.

RESULTS A total of 162 samples were analyzed, composed of 91 normal control individuals (51 male, 33 female, and 7 not recorded), 34 neurological disease control individuals (19 male and 15 female), and 37 with prion disease (22 male and 15 female). The assay's specificity for prion disease was 100% (95% CI, 97%-100%), with no false-positive reactions from 125 control individuals, including 34 from a range of neurodegenerative diseases. In contrast to a previous study, which used a different method, sensitivity to vCJD infection was low (7.7%; 95% CI, 0.2%-36%), with only 1 of 13 patients with positive test results, while sensitivity to sCJD was unexpectedly high at 40% (95% CI, 19%-64%).

CONCLUSIONS AND RELEVANCE We determined 40% of sCJD urine sample results as positive. To our knowledge, this is the first demonstration of an assay that can detect sCJD infection in urine or any target analyte outside of the central nervous system. Urine detection could allow the development of rapid, molecular diagnostics for sCJD and has implications for other neurodegenerative diseases where disease-related assemblies of misfolded proteins might also be present in urine.

Author Affiliations: MRC Prion Unit, Department of Neurodegenerative Disease, University College London Institute of Neurology, London, England (Luk, Jones, Thomas, Mead, Collinge, Jackson); Dementia Research Centre, Department of Neurodegenerative Disease, University College London Institute of Neurology, London, England (Fox); National Prion Clinic, National Hospital for Neurology and Neurosurgery, University College London Hospitals NHS Foundation Trust, London, England (Mok, Mead, Collinge).

Corresponding Author: Graham S. Jackson, PhD, MRC Prion Unit, Department of Neurodegenerative Disease, University College London Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom (g.s.jackson@prion.ucl.ac.uk).

P rion diseases are lethal, transmissible neurodegenerative conditions of humans and animals caused by misfolding and aggregation of the prion protein (PrP).[1,2] It is the transmissibility of disease that is a defining feature of the group and has resulted in several high-profile epidemics of acquired prion disease, including, for example, kuru in humans, bovine spongiform encephalopathy in cattle, and its human counterpart variant Creutzfeldt-Jakob disease (vCJD).[3] Prion disease may also occur as sporadic CJD (sCJD) without any obvious exposure to an infectious source, the clinical syndrome being rapidly progressive with a median survival of around 4 months.[4] Central to the pathogenesis of prion disease is the autocatalytic, templated remodeling of the host's normal cellular PrP, leading to accumulation of abnormal PrP isoforms typified by insoluble aggregates often termed PrP^{Sc}.[5-8] It is increasingly recognized that more common neurodegenerative diseases, such as Alzheimer disease, also involve similar seeding processes and worldwide efforts are now being made to establish the precise role of prionlike mechanisms in a range of human diseases.[9-12]

As a consequence of widespread exposure to bovine spongiform encephalopathy prions, it is thought that as many as 1 in 2000 of the UK population may be prion carriers.[13] Significant public health concerns are associated with the transmissibility of prion diseases, and the potential prevalence of vCJD infection has required widespread precautionary measures to limit iatrogenic transmission. The risks of iatrogenic transmission from individuals with sCJD have long been established, with the primary sources being contaminated neurosurgical instruments and devices[14] and cadaveric donor-derived materials such as dura mater and pituitary growth hormone.[15] The incidence of sCJD is around 2 persons per million per year, which typically occurs with increasing incidence from late middle age onwards and has a uniform sex and geographical distribution. The lifetime risk for sCJD is around 1 in 5000 in the United Kingdom. Unfortunately, approximately half of patients are only diagnosed as having the disease at a clinically advanced state.[16]

The presence of PrP^{Sc} and other abnormal isoforms of PrP is a specific and potentially sensitive diagnostic indicator for prion disease and methods for their detection have been developed that can be applied to a range of tissues and fluids.[17-21] The impetus for the development of diagnostic methods has been the emergence of vCJD, which is characterized by a widespread peripheral pathology[22] and the infection of blood[23,24] and urine.[25] Despite the development of an assay for the diagnosis of vCJD using blood[21,26] and the reporting of vCJD infection being detectable in urine,[25] the application of such techniques to sCJD has been confounded by the restriction of significant pathology to the central nervous system. Coincident with the reporting of detection in the urine of patients with vCJD was the failure to detect any signs of infection in the samples of patients with sCJD using the same technique.[25] However, the method used has never been convincingly demonstrated to detect sCJD and notably no positive control individuals were included in this study. While the application of amyloid seeding assays to cerebrospinal fluid (CSF) have proved effective for the diagnosis of sCJD, the invasive nature of this

Key Points

Question Is abnormal prion protein present in the urine of patients with sporadic Creutzfeldt-Jakob disease?

Findings In this cross-sectional study, 40% of patients with sporadic Creutzfeldt-Jakob disease excreted abnormal prion protein in their urine, an indication absent in healthy and neurological disease control individuals.

Meaning Sporadic Creutzfeldt-Jakob disease can be diagnosed using urine-based testing and the screening of urine used for the production of pharmaceuticals may be possible.

procedure has limited their application to patients with a high index of suspicion of prion disease.[27]

A noninvasive premortem biochemical test for sCJD diagnosis could significantly reduce the extended time currently taken to establish a firm diagnosis, which can compose most of a patient's remaining life[16] and reduce the threshold for testing in patients with relatively early, nonspecific clinical features. Here we report the application to urine testing of an adapted blood assay, originally developed for vCJD.[21] Strikingly, the assay was able to detect sCJD infection in almost half of the patient samples, the first demonstration of an assay capable of diagnosing sCJD from urine or any peripheral analyte.

Methods

Source of Urine Samples

This study was approved by the local research ethics committees of the University College London Institute of Neurology and the National Hospital for Neurology and Neurosurgery. Patient and control urine samples were obtained with written informed consent from patients enrolled in the Medical Research Council PRION-1 Trial,[28] National Prion Monitoring Cohort Study, and/or referred to the National Prion Clinic or Dementia Research Centre at the National Hospital for Neurology and Neurosurgery. Diagnoses of CJD were made according to established criteria.[27,29] All samples were collected using standard urinalysis preservatives (boric acid, sodium formate, and sodium borate) and stored frozen at −70°C in multiple aliquots of 2 mL and thawed before use. Samples were analyzed blind to the operators and only decoded after analysis and determination of positive or negative status.

Testing Procedure

Samples were tested using a modification of a method previously described.[21] Briefly, 400 µL of each sample was diluted 1:1 into 400-µL buffer (200mM Tris; 4% weight/volume bovine serum albumin; 4% weight/volume 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2 tablets complete protease inhibitors [Roche]; and 80 units of Benzonase [grade II, Merck]) containing 23 mg of Capture Matrix (CM) (sub–45-µm stainless steel particles; Goodfellow) and incubated overnight. The CM was isolated using a magnetic rack, supernatant discarded, and washed repeatedly with 1 mL of

phosphate-buffered saline (PBS) plus 0.05% volume/volume Tween-20 (PBST). After the final wash, all liquid was removed and the CM heated at 110°C. To each tube, 50 μL of biotinylated primary antibody (ICSM18; D-Gen Ltd) prepared at 1 μg/mL in PBS plus 1% volume/volume Tween-20 (PBST*) was added and incubated at 37°C for 1 hour. Samples were washed repeatedly with 1-mL PBST, isolating CM each time. Each sample was then incubated with High Sensitivity NeutrAvidin-HRP (Pierce) prepared at a 1:100 000 dilution in PBST* at 37°C for 45 minutes. Samples were again washed repeatedly with 1-mL PBST, isolating CM each time. To each sample, 60 μL of SuperSignal enzyme-linked immunosorbent assay Femto chemiluminescent substrate (Pierce) was added and CM was evenly distributed into 3 replicate wells of a black 96-well plate (Greiner). Each plate included a set of 6 quality-control samples (5 negative control normal urine samples and 1 positive control sample). An additional 80 μL of chemiluminescent substrate was added to each well immediately prior to the plate scan. The total luminescent output over a fixed integration time was measured for each well using a M1000 plate reader (Tecan).

Positivity Criteria and Data Analysis

Samples were scored reactive if the mean chemiluminescence from 3 replicate wells exceeded an on-plate cutoff threshold of the mean plus 5 SDs of 5 negative control normal urine samples. Thus, samples with a ratio relative to cutoff greater than 1 were scored reactive. All samples were tested twice with those that were repeat reactive (ie, reactive in both analyses considered positive for prion disease). Nonreactive and single-reactive samples were considered negative.

Conductivity Measurements

To control for the possibility of dehydration in patients with prion disease producing higher urine direct detection assay (DDA) signals, an approximation to the concentration levels of urine samples was determined by conductivity measurement. Conductivity is known to correlate with urine osmolality and specific gravity[30,31] and was determined using a low-volume conductivity meter (Horiba Laqua Twin B-771). Calibration of the conductivity meter was performed using a 1.41-mS/cm standard solution and the conductivity of urine samples determined by taking the mean of 3 independent measurements from 3 separate aliquots of 100 μL of urine sample, washing the electrode with double-distilled water between readings.

Results

DDA Analysis of Urine From Patients With Prion Disease

A panel of urine samples obtained from a total of 37 patients with confirmed prion disease and 125 control individuals, from both normal individuals and patients with neurological diseases other than prion disease, were analyzed using an adaptation of a previously reported DDA blood test for vCJD infection. The samples were tested in groups of 12, with a quality-control panel on each plate consisting of 5 negative normal control samples and 1 positive control sample. Each sample was tested in triplicate wells across 2 independent runs. An arbitrary cutoff was determined from the mean plus 5 SDs of the panel of 5 normal samples in the quality-control panel. A ratio for the mean chemiluminescence of each sample to the plate cutoff was calculated for each sample and, if greater than 1, a sample was classified as reactive. Only samples that were repeat reactive in both test runs were scored as positive. Of the 162 blinded samples tested, 15 and 22 samples were scored as reactive in the first and second test runs, respectively, with 10 samples scored repeat reactive across the 2 test runs (**Figure 1**). All of the 10 positive samples were from patients with prion disease (1 from a case of vCJD, 1 from a growth hormone-related case of iatrogenic CJD, and 8 from patients with sCJD). A summary of the major clinical findings in all 37 cases of prion disease is provided in the eTable in the Supplement.

There were no clear associations with any aspects of the clinical history, disease progression, or investigations. The National Prion Clinic has developed a functionally oriented rating scale to measure CJD disease progression termed the MRC Prion Disease Rating Scale.[4] The mean MRC Scale score was 4 of 20 for positive cases and 5 for negative cases ($P = .57$; t test). The patient with least disease progression of sCJD had an MRC Scale score of 10 of 20. However, at present, most sCJD cases in the United Kingdom are diagnosed at advanced clinical stages for a variety of reasons; we had no samples from any patient with sCJD at earlier disease stages. All genotypes at polymorphic codon 129 of the prion protein gene, an important susceptibility factor and disease modifier,[32] were represented in both DDA positive and negative cases. Typical investigation findings, including periodic sharp wave complexes on electroencephalography, abnormal signal on diffusion-weighted brain magnetic resonance imaging in the cortex and basal ganglia, and abnormal 14-3-3 protein levels in CSF were found in an expected proportion of DDA positive and negative cases (eTable in the Supplement).

Based on the criteria of a sample being repeat reactive in 2 independent runs, 10 of 37 (27%) of the prion disease urine samples were identified in the absence of any false-positive reactions. However, considering sCJD in isolation, sensitivity of detection was 8 of 20 samples (40%; 95% CI, 19%-64%), with 100% specificity (95% CI, 97%-100%).

Comparison of the mean DDA results expressed as ratios relative to cutoffs for the 4 types of prion disease analyzed confirms significantly elevated abnormal PrP levels in both sCJD and iatrogenic CJD with respect to control ($P < .001$), but with levels in both vCJD and inherited prion disease failing to achieve statistical significance, possibly owing to the low numbers of samples analyzed (**Figure 2**).

Conductivity of Urine Samples As a Measure of Concentration

Conductivity, a simplified measure of osmolality that correlates with urine concentration,[30,31] was measured in approximately a third of the samples (n = 55), where sufficient volume was available. No correlation was observed between urine concentration and DDA signals, confirming patient hydration was not the cause of elevated abnormal PrP signals in urine (**Figure 3**).

Figure 1. Assaying a Blind Panel of 162 Urine Samples

A Run 1

B Run 2
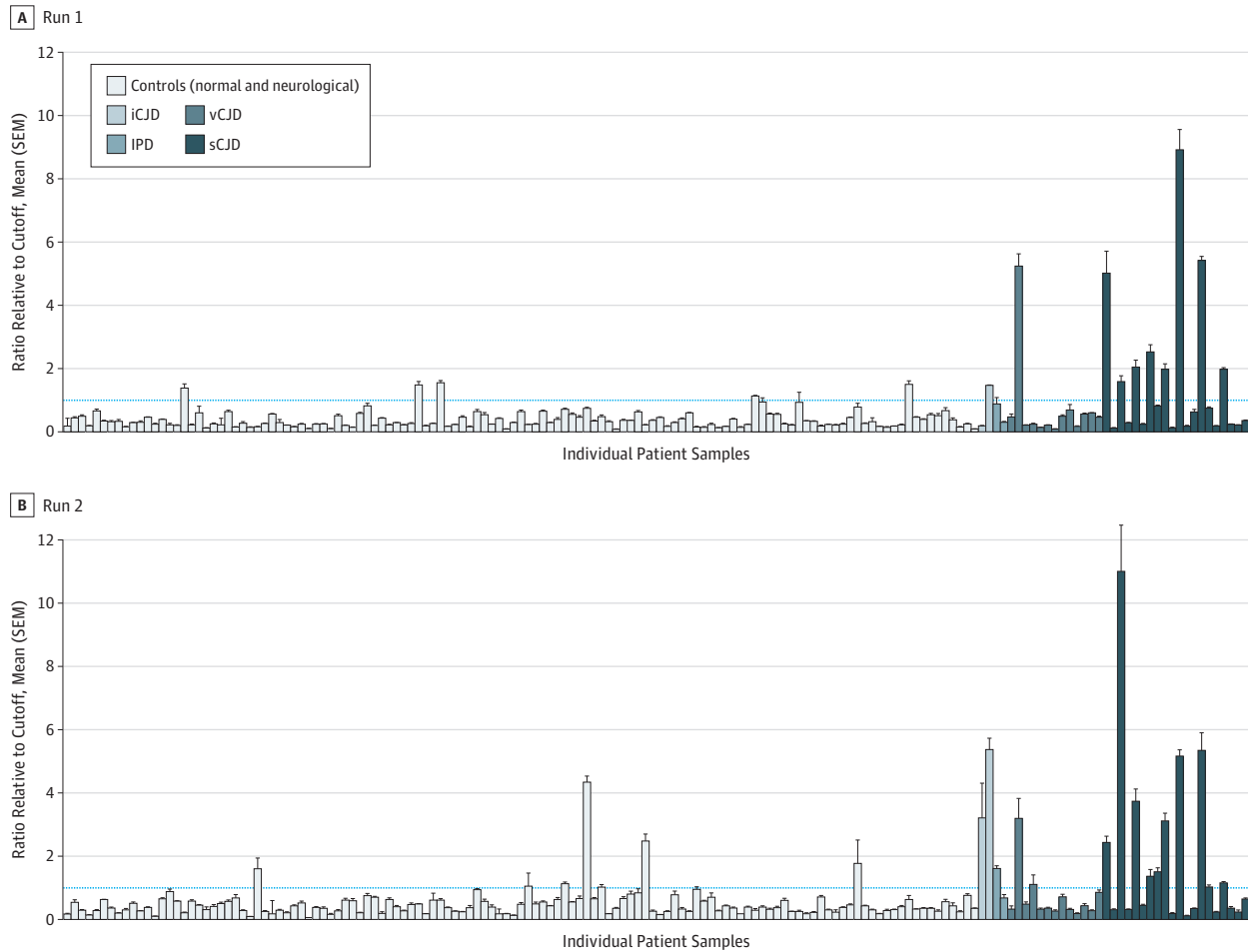
Testing was performed in 2 independent assays on a blind panel of 162 urine samples from patients with diagnoses of sporadic Creutzfeldt-Jakob disease (sCJD; n = 20), variant CJD (vCJD; n = 13), inherited prion disease (IPD; n = 2), and growth hormone–related iatrogenic CJD (iCJD; n = 2) and healthy normal and neurological disease control individuals (n = 91 and n = 34, respectively). Neurological disease control individuals comprised those with Alzheimer disease (n = 21), Huntington disease (n = 3), frontotemporal dementia (n = 3), familial Alzheimer disease (n = 2), and early-onset dementia (n = 1) and referrals to the Nation Prion Clinic determined as non-prion disease (n = 4). Runs 1 and 2 represent data from the individual assay runs of blind samples. Data are shown as the chemiluminescent signal ratio relative to a cutoff determined from the mean +5 x SD of normal control individuals (see the Methods section).

Discussion

Blood is a commonly used analyte for a range of diagnostic and prognostic tests, being readily accessible with minimal risk in almost all patients. We have previously reported the development and validation of a blood test for vCJD,[21,26] which despite excellent performance characteristics against vCJD was unable to detect infection in the blood of patients with sCJD. An accurate diagnosis of sCJD in patients with advanced symptomatic disease can usually be achieved[16] without recourse to molecular diagnostics but the ability to diagnose disease in patients much earlier would have obvious benefits, particular with respect to early entry into therapeutic trials.

The ability of disease-associated abnormal PrP to catalyze the conversion of recombinant PrP into amyloid conformations has been exploited in the development of amyloid-

seeding assays capable of detecting the presence of prion disease infection in a variety of tissues and fluids.[18,19,33] However, the detection of sCJD prion infection has been limited to the tissues of the central nervous system and CSF. In one study, analysis of CSF obtained from patients with sCJD using amyloid seeding indicated the detection limit is sufficient for excellent discrimination of affected individuals from control individuals, with a sensitivity of around 90%.[34] However, the requirement for CSF limits the application of the assay, as the collection of such samples is an invasive procedure that is generally performed only when significant neurological symptoms and signs are evident.

An alternative to blood as a peripheral analyte, and one that can be readily obtained for routine diagnostic use, is urine. Our study has shown for the first time, to our knowledge, that it is feasible to identify sCJD using a urine sample by capture of disease-associated PrP on a stainless steel matrix and detection
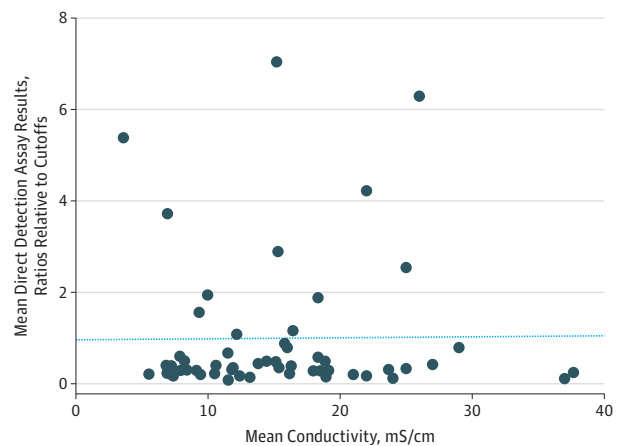
Figure 2. Discrimination of Various Forms of Prion Disease From Normal and Neurological Control Individuals
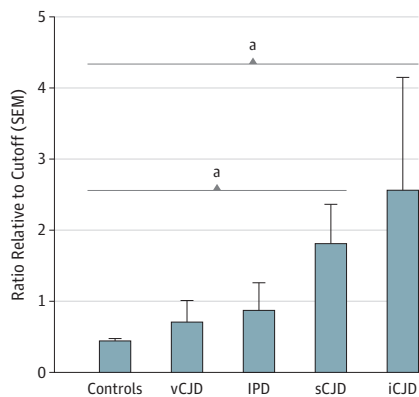
Data shown are the group mean ratios relative to cutoffs for both assay runs combined for the groups of samples indicated; 91 normal control individuals and 34 neurological disease control individuals (for details see Figure 1 legend), 13 with variant Creutzfeldt-Jakob disease (vCJD), 20 with sporadic Creutzfeldt-Jakob disease (sCJD), 2 with inherited prion disease (IPD), and 2 with iatrogenic inherited prion disease (iCJD). Error bars represent the SEM. The 2-tailed P values for comparison of control samples with prion disease cohorts support a significant difference for both sCJD and iCJD.

a $P < .001$.

Figure 3. Correlation of Urine Sample Direct Detection Assay Values and Conductivity

The conductivities of 55 urine samples were quantified as milliSiemens per cm and plotted vs the mean of the previously determined ratios relative to cutoff values on the y-axis. A Pearson correlation coefficient was calculated as 0.058 for the association between the 2 values, which was not significantly different from 0 (P = .68; 2-tailed t test). The blue line superimposed on the data is the best fit of a linear regression, highlighting the lack of correlation with a gradient of 0.002.

using anti-PrP monoclonal antibodies. While the sensitivity of diagnosis is relatively low, at 40%, the high mean signals obtained in the assay suggest this could be improved considerably by pretreatment or processing of large volumes of urine prior to assay. It is likely the current diagnostic sensitivity could be significantly enhanced by improvements in the analytic sensitivity of the assay but it is unclear whether this could approach 100%, as there is likely to be variability in the presence and concentration of abnormal PrP in the urine of individual patients. The high mean signals observed in the 2 samples from cases of growth hormone–related iatrogenic CJD suggest such an assay may also have significant sensitivity to cases of secondary CJD infection, although this will require the analysis of larger numbers of samples. The detection of abnormal misfolded PrP in the urine of patients with CJD is somewhat unexpected and intriguingly suggests other neurodegenerative diseases associated with protein misfolding may have diagnostic urine signatures. An unparalleled advantage of urine as an analyte is the easy availability of large sample volumes for research, which will facilitate the development of protocols for the isolation and characterization of the specific form of abnormal PrP present, leading to improved diagnostic sensitivity.

The origin of disease-associated PrP in the urine of patients with sCJD is unknown, but it is clear that urine contains cellular PrP, largely in a truncated form that carries only a partial glycosylphosphatidylinositol anchor lacking the associated lipid moiety[35,36] and, therefore, there is the potential for prion replication in situ as well as accumulation from the glomerular filtration of blood. Although urine is normally considered to lack detectable protein in the absence of kidney disease, in fact, normal kidney function results in trace pro-

tein concentrations of between 50 and 200 μg mL^{-1}, which is sufficient to result in the accumulation of abnormal PrP derived from blood. It is notable that although abnormal PrP is present in urine, it is not readily detectable in blood. A plausible explanation is the enrichment of abnormal PrP by the action of filtration in the kidney to concentrations that are detectable, but this could also be explained by active prion replication in the kidney or urinary tract. Certainly all the requirements for prion amplification are present: normal cellular PrP, disease-associated abnormal PrP, and a slightly denaturing environment provided by urea. However, it is important to note that transmission studies have indicated the potential prion titers in native urine are low (<0.38 infectious units/mL^{-1})[37] or absent, and there are no reported cases of CJD resulting from sexual transmission, with the evidence from experimental models indicating that this is unlikely to occur.[38,39] The complex milieu of blood contributes to greater difficulties in specifically detecting disease-related signals whereas urine is a relatively "clean" analyte, with low background levels of protein, which may be sufficient to account for the apparent discrepancy alone.

Prion protein has previously been identified in commercially available injectable urine-derived gonadotropin products that are used to treat infertility in women[40] and undescended testes in boys[41] and are available for illicit use in body building and as performance-enhancing drugs.[42] This fact, coupled with the detection of abnormal PrP in the urine of patients with vCJD,[25] has raised questions about the safety of such products should the purification methods result in the copurification of prions as is known to have been the case for human cadaveric pituitary-derived hormones.[15] Typically, urine is sourced from postmenopausal women with a median age of

older than 50 years, an age cohort where sCJD can be expected, although exclusion criteria exist for donations, analogous to those in place for donating blood. Despite transmission studies indicating the potential prion titers in native urine are low,[37] it is conceivable that commercial purification of hormone products could concomitantly enrich for prion infectivity. Our finding, that the urine of patients with sCJD contains abnormal PrP is consistent with concerns that urine-derived fertility products could contain infectious prions as sCJD remains the most common form of prion disease and has a uniform worldwide incidence.

Our findings are in marked contrast to those recently reported using the technique of protein misfolding by cyclic amplification, where a high sensitivity for the detection of vCJD infection in urine was observed and yet none of the 68 patient samples from sCJD tested positive.[25] A critical limitation to the interpretation of this finding is that the assay method (protein misfolding by cyclic amplification) has never been convincingly demonstrated to detect sCJD and indeed no positive controls were included in this study. Parallel CJD strain-dependent differences are observed with other assay methods, some of which are attributable to differences in peripheral pathogenesis[21,22,43,44] but some of which remain unexplained.[45]

Conclusions

The detection of disease-related PrPs in the urine of patients with sCJD is the first demonstration that sCJD can be diagnosed from urine of any peripheral analyte by biochemical means. This offers the possibility of improved early diagnosis of sCJD and the potential precautionary screening of human urine–derived pharmaceuticals.

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