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## Prions in the Urine of Patients with Variant Creutzfeldt–Jakob Disease

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Fabio Moda, Pierluigi Gambetti, Silvio Notari, Luis Concha-Marambio ...+10 more authors

**Institutions:** University of Texas at Austin, Case Western Reserve University, University of Los Andes, Pierre-and-Marie-Curie University ...+3 more institutions

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## ORIGINAL ARTICLE

# Prions in the Urine of Patients with Variant Creutzfeldt–Jakob Disease

Fabio Moda, Ph.D., Pierluigi Gambetti, M.D., Silvio Notari, Ph.D., Luis Concha-Marambio, B.Sc., Marcella Catania, Ph.D., Kyung-Won Park, Ph.D., Emanuela Maderna, B.Sc., Silvia Suardi, B.Sc., Stéphane Haik, M.D., Ph.D., Jean-Philippe Brandel, M.D., James Ironside, M.D., Richard Knight, M.D., Fabrizio Tagliavini, M.D., and Claudio Soto, Ph.D.

## ABSTRACT

**BACKGROUND**

From the Mitchell Center for Research in Alzheimer's Disease and Related Brain Disorders, University of Texas Medical School at Houston, Houston (F.M., L.C.-M., K.-W.P., C.S.); Foundation Carlo Besta Neurologic Institute, Milan (F.M., M.C., E.M., S.S., F.T.); National Prion Disease Pathology Surveillance Center, Case Western Reserve University, Cleveland (P.G., S.N.); Universidad de los Andes, Facultad de Medicina, Santiago, Chile (L.C.-M.); Assistance Publique–Hôpitaux de Paris, Cellule Nationale de Référence des Maladies de Creutzfeldt–Jakob, Groupe Hospitalier Pitié–Salpêtrière, INSERM Unité 1127, Université Pierre et Marie Curie–Paris 6, and Centre Nationale de la Recherche Scientifique, Unité Mixte de Recherche — all in Paris (S.H., J.-P.B.); and the National CJD Research and Surveillance Unit, Western General Hospital, University of Edinburgh, Edinburgh (J.I., R.K.). Address reprint requests to Dr. Soto at the University of Texas Medical School at Houston, 6431 Fannin St., Houston, TX 77030, or at [claudio.soto@uth.tmc.edu](mailto:claudio.soto@uth.tmc.edu).

Prions, the infectious agents responsible for transmissible spongiform encephalopathies, consist mainly of the misfolded prion protein (PrP<sup>Sc</sup>). The unique mechanism of transmission and the appearance of a variant form of Creutzfeldt–Jakob disease, which has been linked to consumption of prion-contaminated cattle meat, have raised concerns about public health. Evidence suggests that variant Creutzfeldt–Jakob disease prions circulate in body fluids from people in whom the disease is silently incubating.

**METHODS**

To investigate whether PrP<sup>Sc</sup> can be detected in the urine of patients with variant Creutzfeldt–Jakob disease, we used the protein misfolding cyclic amplification (PMCA) technique to amplify minute quantities of PrP<sup>Sc</sup>, enabling highly sensitive detection of the protein. We analyzed urine samples from several patients with various transmissible spongiform encephalopathies (variant and sporadic Creutzfeldt–Jakob disease and genetic forms of prion disease), patients with other degenerative or nondegenerative neurologic disorders, and healthy persons.

**RESULTS**

PrP<sup>Sc</sup> was detectable only in the urine of patients with variant Creutzfeldt–Jakob disease and had the typical electrophoretic profile associated with this disease. PrP<sup>Sc</sup> was detected in 13 of 14 urine samples obtained from patients with variant Creutzfeldt–Jakob disease and in none of the 224 urine samples obtained from patients with other neurologic diseases and from healthy controls, resulting in an estimated sensitivity of 92.9% (95% confidence interval [CI], 66.1 to 99.8) and a specificity of 100.0% (95% CI, 98.4 to 100.0). The PrP<sup>Sc</sup> concentration in urine calculated by means of quantitative PMCA was estimated at  $1 \times 10^{-16}$  g per milliliter, or  $3 \times 10^{-21}$  mol per milliliter, which extrapolates to approximately 40 to 100 oligomeric particles of PrP<sup>Sc</sup> per milliliter of urine.

**CONCLUSIONS**

Urine samples obtained from patients with variant Creutzfeldt–Jakob disease contained minute quantities of PrP<sup>Sc</sup>. (Funded by the National Institutes of Health and others.)

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**P**RION DISEASES ARE FATAL NEURODEGENERATIVE disorders for which no therapy or definitive noninvasive intravital diagnosis is available.<sup>1</sup> These diseases affect humans and animals. Creutzfeldt-Jakob disease in humans and scrapie, bovine spongiform encephalopathy, and chronic wasting disease in animals are the most common forms of transmissible spongiform encephalopathies. The infectious agent in transmissible spongiform encephalopathies appears to be composed exclusively of the misfolded form of the prion protein (termed PrP<sup>Sc</sup>), which self-propagates in the absence of nucleic acid.<sup>2,3</sup> PrP<sup>Sc</sup> replicates in infected persons by acting as a template for the misfolding of the cellular prion protein (PrP<sup>C</sup>). When exposed to the infectious agent, prions progressively replicate in the host body, and by the time clinical symptoms develop, large quantities of PrP<sup>Sc</sup> have accumulated in the central nervous system. Although the disease is mostly confined to the central nervous system, small quantities of PrP<sup>Sc</sup> are present in many tissues and body fluids even at early presymptomatic stages of the disease.<sup>1,4,5</sup>

It is widely accepted that bovine spongiform encephalopathy has been transmitted to humans, generating a new disease termed variant Creutzfeldt-Jakob disease.<sup>6-8</sup> Although the number of patients with this disease is fairly small (228 cases reported as of this writing), it is unclear how many people may carry infectious material due to a preclinical or subclinical condition. A recent retrospective study of archived surgically resected appendixes in the United Kingdom estimated that the prevalence of asymptomatic variant Creutzfeldt-Jakob disease infection in the U.K. population was approximately 1 case per 2000 persons; this suggests that approximately 30,000 people in the United Kingdom might be carriers of potentially infectious variant Creutzfeldt-Jakob disease prions.<sup>9</sup> The potential existence of a large number of carriers raises the possibility of horizontal transmission of variant Creutzfeldt-Jakob disease from human to human, a course that has already led to hundreds of deaths in other human transmissible spongiform encephalopathies.<sup>10</sup> Studies in animal models have shown that bovine spongiform encephalopathy infection often results in subclinical or carrier states, which on secondary transmission can produce the complete disease

in a much more efficient way.<sup>11-13</sup> This scenario is a concern, since cases of variant Creutzfeldt-Jakob disease have been linked to transfusion of blood donated by infected persons at the preclinical stage of the disease.<sup>14-16</sup>

Noninvasive tests that can detect prions in samples of body fluids with high sensitivity and specificity could be useful for estimating the prevalence of variant Creutzfeldt-Jakob disease prion infection, reducing the risk of iatrogenic transmission, and facilitating prophylactic treatment of the disease. One of the most promising techniques for high-sensitivity detection of PrP<sup>Sc</sup> is the protein misfolding cyclic amplification (PMCA) assay, which mimics in vitro the prion replication process occurring during the pathogenesis of prion disease.<sup>17</sup> PMCA is a cyclic process that can detect the equivalent of a single oligomeric particle of PrP<sup>Sc</sup>.<sup>18</sup> This high level of sensitivity has permitted detection of PrP<sup>Sc</sup> in the blood and urine of experimentally infected animals at both the symptomatic<sup>19,20</sup> and presymptomatic<sup>21</sup> stages of the disease. PMCA has been successfully used to detect PrP<sup>Sc</sup> in affected species, including humans.<sup>22</sup> We investigated whether PrP<sup>Sc</sup> is present in the urine of patients with variant Creutzfeldt-Jakob disease and whether it can be detected by means of PMCA. Urine testing is the least invasive type of sampling for detection of a disease marker, and urine has been shown to carry small quantities of infectious PrP<sup>Sc</sup> in experimental animal models.<sup>20,23-26</sup>

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## METHODS

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### PATIENTS AND STUDY OVERSIGHT

We analyzed urine samples obtained from 68 patients with sporadic Creutzfeldt-Jakob disease and 14 patients with variant Creutzfeldt-Jakob disease (clinical information about these patients is provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org), as well as 4 patients with genetic prion diseases, 50 patients with other neurodegenerative disorders (including Alzheimer's disease, Parkinson's disease, frontotemporal dementia, motor neuron disease, and progressive supranuclear palsy), 50 patients with nondegenerative neurologic disorders (including cerebrovascular disease, multiple sclerosis, epilepsy, brain tumors, autoimmune encephalitis, meningitis,

myeloradiculopathy, and peripheral neuropathy), and 52 healthy persons. The diagnosis of human transmissible spongiform encephalopathy in all patients (except for 1 patient with variant Creutzfeldt-Jakob disease) was pathologically confirmed on autopsy or by means of biopsy of brain tissue, lymphoid tissue, or both. The diagnosis of the other neurologic diseases was established by means of clinical examination, as well as imaging and laboratory tests, when available. All patients and controls included in the study were homozygous for methionine at codon 129 of the gene encoding prion protein (*PRNP*) (*PRNP* genotyping is described in the Supplementary Appendix). Urine was collected at various times during the symptomatic period of the diseases and kept frozen at  $-80^{\circ}\text{C}$  until analysis.

The methods of urine collection and genetic analysis were approved by the institutional review boards at the authors' study centers, and all study participants provided written informed consent. The sponsors had no role in the study design, data collection and analysis, preparation of the manuscript, or decision to submit it for publication.

#### URINE PROCESSING

To attempt direct detection of  $\text{PrP}^{\text{Sc}}$  by means of Western blotting, 1 ml of each urine sample was concentrated by means of centrifugation at high speed ( $100,000\times g$  for 1 hour at  $4^{\circ}\text{C}$ ). The supernatant was discarded, and pellets were washed with phosphate-buffered saline. After washing, pellets were resuspended in  $10\ \mu\text{l}$  of phosphate-buffered saline. Samples were subjected to proteinase K digestion and Western blotting, as described below.

For PMCA, urine samples were processed as shown in Figure 1. Briefly, 1 ml of each sample was subjected to an initial low-speed centrifugation ( $5000\times g$  for 20 minutes at  $4^{\circ}\text{C}$ ) to precipitate debris. The supernatant and pellet fractions were both used for  $\text{PrP}^{\text{Sc}}$  detection after concentration by means of centrifugation, as described in Figure 1. For the studies with 10 ml of urine samples, a simplified procedure was performed in which samples were subjected to a single centrifugation at  $100,000\times g$  for 1 hour at  $4^{\circ}\text{C}$ . In this way, the materials associated with both debris and supernatant were included in the sample. The pellet was resuspended directly in  $100\ \mu\text{l}$  of PMCA substrate.

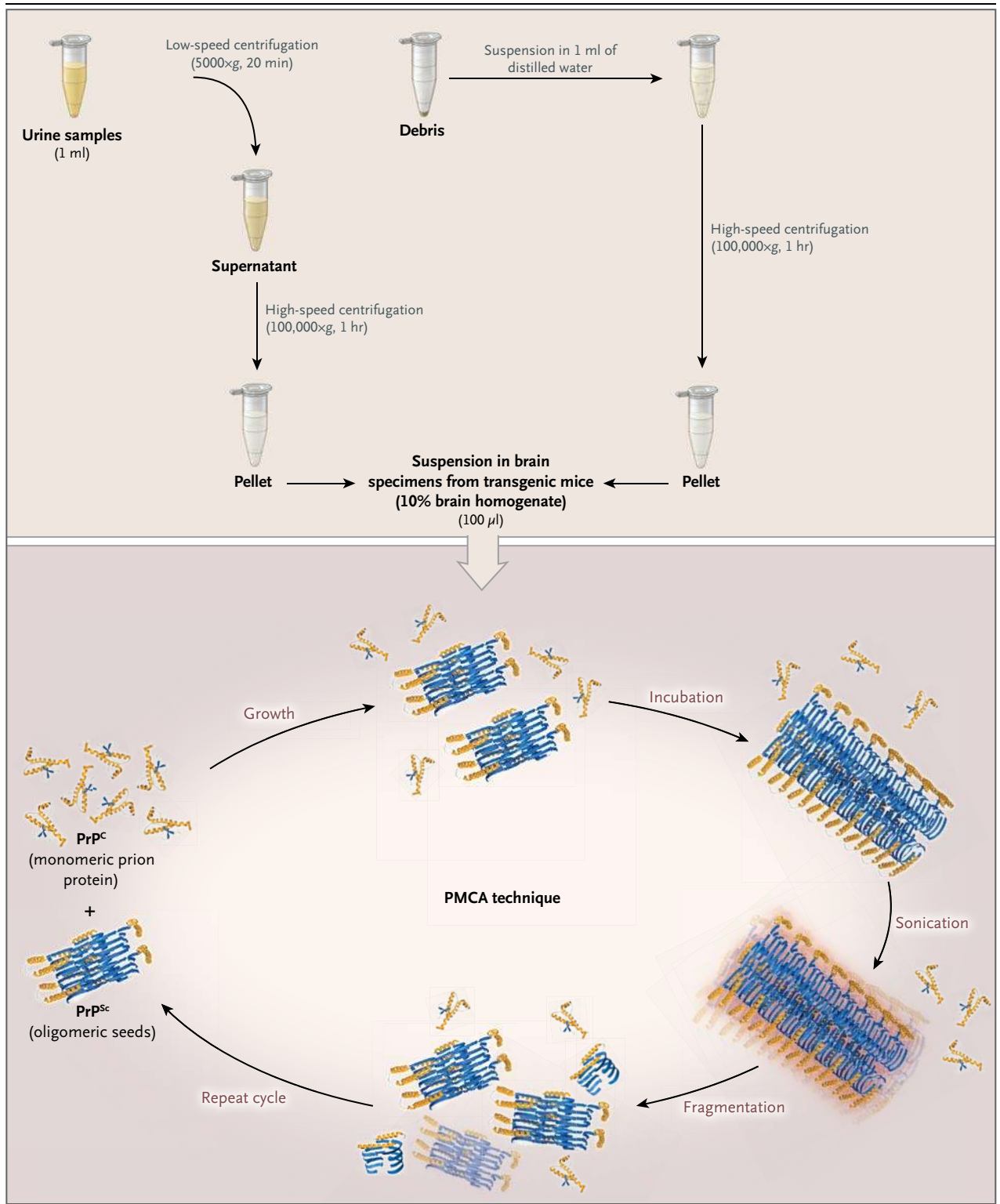
#### Figure 1 (facing page). Urine Processing and Protein Misfolding Cyclic Amplification (PMCA) Procedure.

One milliliter of urine was first centrifuged at low speed to separate debris. The pellet fraction containing cellular debris was resuspended in 1 ml of water and subjected to high-speed centrifugation ( $100,000\times g$ ) to precipitate misfolded prion protein ( $\text{PrP}^{\text{Sc}}$ ). The pellet was resuspended directly in brain homogenate from transgenic mice expressing human prion protein and used as a substrate for PMCA. The supernatant fraction from the low-speed centrifugation was centrifuged at high speed to pellet  $\text{PrP}^{\text{Sc}}$  and remove urine compounds that interfere with PMCA. The pellet was resuspended in brain homogenate from transgenic mice and used for PMCA amplification. The PMCA procedure comprises cycles of amplification in which incubation of oligomeric seeds with the monomeric prion protein to allow growth of the polymers is followed by fragmentation of the aggregates by means of sonication to multiply the number of nuclei for prion replication. After 96 PMCA cycles, the cellular prion protein ( $\text{PrP}^{\text{C}}$ ) substrate is refreshed, and samples are subjected to a new round of PMCA cycling.

#### PMCA PROCEDURE

PMCA was performed as previously described.<sup>22</sup> As a substrate, we used brain specimens obtained from transgenic mice expressing human prion protein with methionine homozygosity at *PRNP* codon 129. Brains were harvested after perfusion, and 10% homogenate was prepared in conversion buffer (phosphate-buffered saline containing 150 mM sodium chloride and 1% Triton X-100) with the addition of protease inhibitors. To increase the efficiency of PMCA, 6 mM EDTA and 0.05% digitonin were added to the brain homogenate.

Urine samples, processed as described above, were mixed with 10% brain homogenate (from transgenic mice expressing human prion protein) in tubes containing three polytetrafluoroethylene (Teflon) beads and subjected to 96 cycles of PMCA with the use of a microsonicator (model Q700, Qsonica). Each cycle consisted of 29 minutes 20 seconds of incubation at 37 to  $40^{\circ}\text{C}$ , followed by a 40-second pulse of sonication set at a potency of 270 to 280 W. After one round of 96 PMCA cycles, an aliquot of the amplified material was diluted by a factor of 10 into fresh brain homogenate from transgenic mice and an additional round of PMCA cycles was performed. To avoid sample cross-contamination between rounds, thorough decontamination of instruments and equipment was performed with the



use of 2 N sodium hydroxide or 4 M guanidine isothiocyanate.

#### PROTEINASE K DIGESTION AND WESTERN BLOT ANALYSIS

Samples were treated with proteinase K (100  $\mu$ g per milliliter) for 1 hour at 37°C with shaking. The reaction was stopped with the use of lithium dodecyl sulfate sample buffer (NuPAGE, Invitrogen), and samples were developed by means of Western blotting as previously described.<sup>22</sup>

## RESULTS

#### PrP<sup>Sc</sup> DETECTION IN URINE BY WESTERN BLOTTING AND PMCA

In a first attempt to detect PrP<sup>Sc</sup> in urine, samples obtained from patients with various forms of transmissible spongiform encephalopathies, as well as from persons with other neurodegenerative diseases and healthy controls, were concentrated and analyzed by means of Western blotting after proteinase K digestion. The results showed that even after concentration by a factor of 100, no protease-resistance prion protein signal was detectable in any of the samples analyzed (Fig. S1A in the Supplementary Appendix). To further attempt detection of PrP<sup>Sc</sup> in human urine, we used the PMCA technique. First, we optimized the method, using samples of urine from healthy persons that were supplemented with various dilutions of variant Creutzfeldt–Jakob disease brain homogenate, ranging from 10<sup>-5</sup> to 10<sup>-12</sup>. After one and two rounds of 96 PMCA cycles, we could detect human PrP<sup>Sc</sup> at a brain homogenate dilution of 10<sup>-10</sup> and 10<sup>-12</sup>, respectively (Fig. 2A). No PrP<sup>Sc</sup> was detected even after seven rounds of PMCA in urine samples that were supplemented with 10% healthy brain homogenate (Fig. S1B in the Supplementary Appendix).

#### PrP<sup>Sc</sup> DETECTION IN URINE OF PATIENTS WITH VARIANT CREUTZFELDT–JAKOB DISEASE

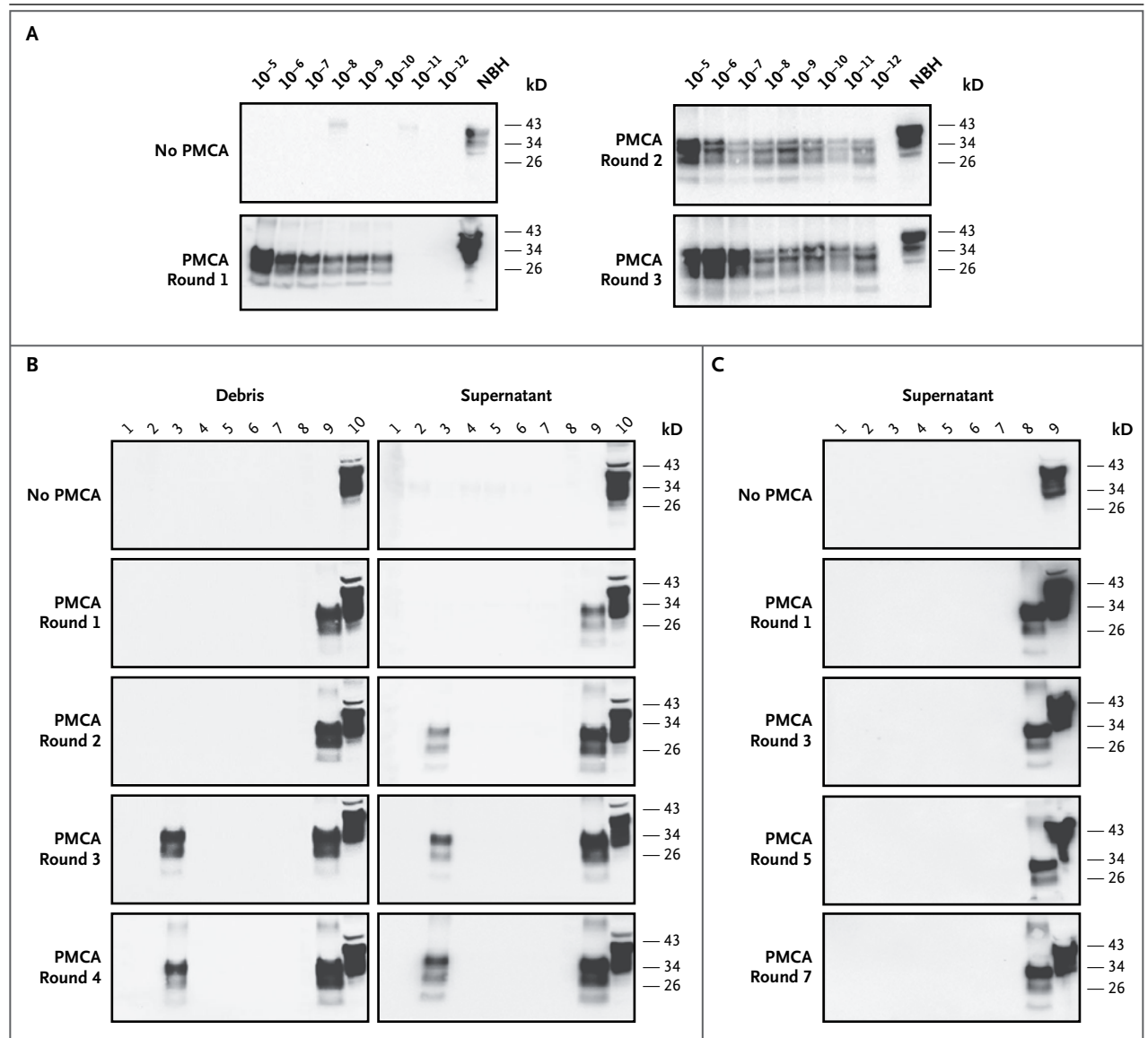
Next, we used PMCA to detect PrP<sup>Sc</sup> in urine samples obtained from 238 persons (Table 1), including 68 patients with sporadic Creutzfeldt–Jakob disease, 14 patients with variant Creutzfeldt–Jakob disease (Table S1 in the Supplementary Appendix), 4 patients with genetic forms of transmissible spongiform encephalopathies (3 patients with the Gerstmann–Sträussler–Scheinker syndrome and 1 with familial Creutzfeldt–Jakob disease), 50 patients with other neurodegenerative

diseases, 50 patients with nondegenerative neurologic diseases, and 52 healthy persons. All patients and healthy controls were homozygous for methionine at PRNP codon 129.

Figure 2B shows results of some of the samples analyzed. Among all samples tested in a blinded manner, only the one obtained from a patient with variant Creutzfeldt–Jakob disease (lane 3 in Fig. 2B) was positive for PrP<sup>Sc</sup>, besides the positive control sample (lane 9 in Fig. 2B) consisting of urine from a healthy control that was supplemented with a 10<sup>-5</sup> dilution of variant Creutzfeldt–Jakob disease brain homogenate. In the urine sample obtained from the patient with variant Creutzfeldt–Jakob disease, PrP<sup>Sc</sup> was detectable both in the debris and in the supernatant fraction after precipitation of the debris (Fig. 2B). No signal was detected in any of the urine samples obtained from patients with other forms of human transmissible spongiform encephalopathies (i.e., sporadic Creutzfeldt–Jakob disease, familial Creutzfeldt–Jakob disease, and the Gerstmann–Sträussler–Scheinker syndrome), patients with other degenerative or nondegenerative neurologic disorders, and healthy controls, even after seven rounds of PMCA (Fig. 2B and 2C).

Similar results were obtained with the rest of the samples analyzed (Fig. 3A). Indeed, a positive signal for PrP<sup>Sc</sup> was detected in both the supernatant and debris fraction for 13 of the 14 urine samples obtained from patients with variant Creutzfeldt–Jakob disease (Table 1). No signal was detected in any of the other samples analyzed, indicating that, with this technique, PrP<sup>Sc</sup> is detectable exclusively in the urine of patients with variant Creutzfeldt–Jakob disease. PMCA enables PrP<sup>Sc</sup> detection in patients with variant Creutzfeldt–Jakob disease with an estimated sensitivity of 93% and an estimated specificity of 100%, and the positive and negative predictive values are close to 100% (Table 2). To investigate whether larger samples of urine from different control groups might be positive, we performed an experiment using samples of 10 ml of urine obtained from 10 patients with sporadic Creutzfeldt–Jakob disease, 10 patients with other neurodegenerative diseases, and 10 healthy controls. Samples were processed as described above, and all material obtained from the 10 ml of urine was used for the PMCA assays. As shown in Figure 3B, no signal was detected in any of these samples.

After proteinase K digestion, the electropho-



**Figure 2. PrP<sup>Sc</sup> Detection in Urine by Means of PMCA.**

As shown in Panel A, to optimize PrP<sup>Sc</sup> detection by means of PMCA, 1 ml of urine from healthy persons was supplemented with samples of variant Creutzfeldt-Jakob disease brain homogenate at distinct dilutions ( $10^{-5}$  to  $10^{-12}$ ). After processing by means of high-speed centrifugation, samples were subjected to various rounds of 96 PMCA cycles in the presence of 10% brain homogenate from transgenic mice expressing human prion protein, which was used as a substrate for PMCA. The PrP<sup>Sc</sup> signal was assessed by means of Western blot analysis after proteinase K digestion. NBH refers to the normal (healthy) brain homogenate. As shown in Panel B, urine samples obtained from patients with sporadic Creutzfeldt-Jakob disease MM1 (methionine homozygote, prion protein type 1) (lanes 1, 2, and 4), variant Creutzfeldt-Jakob disease (lane 3), and healthy controls (lanes 5, 6, and 7) were processed. Fractions corresponding to debris and supernatant were collected for each sample and subjected to serial rounds of 96 PMCA cycles. As a positive control, a urine specimen obtained from a healthy person (lane 9) was supplemented with a  $10^{-5}$  dilution of variant Creutzfeldt-Jakob disease brain homogenate. Lane 8 shows the molecular-weight marker, and lane 10 shows the normal brain homogenate, which was not treated with proteinase K (used as an electrophoretic migration marker). The result with the urine sample from one patient with variant Creutzfeldt-Jakob disease was representative of the 13 samples with positive test results. As shown in Panel C, urine samples obtained from patients who had other neurodegenerative disorders, including Alzheimer's disease (lane 1), frontotemporal dementia (lanes 2 and 4), Parkinson's disease (lane 3), and progressive supranuclear palsy (lane 5) were subjected to seven serial rounds of PMCA. The results for rounds 1, 3, 5, and 7 are shown, as well as the results with no amplification. Lane 6 shows a urine sample from a healthy control, lane 7 shows the molecular-weight marker, and lane 9 shows the normal brain homogenate not treated by proteinase K. Lane 8 shows samples of urine from a healthy control supplemented with a  $10^{-5}$  dilution of variant Creutzfeldt-Jakob disease brain homogenate. All samples were treated with proteinase K, except the NBH used as a PrP<sup>Sc</sup> migration control.



**Table 1. Urine Samples That Were Positive for PrP<sup>Sc</sup> Detection by PMCA, According to Study Group.\***

Group	Urine Samples in Which PrP <sup>Sc</sup> Was Detected
	no./total no.
Patients with variant Creutzfeldt–Jakob disease	13/14
Patients with sporadic Creutzfeldt–Jakob disease	0/68
Patients with genetic prion disease (familial Creutzfeldt–Jakob disease or Gerstmann–Sträussler–Scheinker syndrome)	0/4
Patients with other degenerative neurologic diseases	0/50
Patients with nondegenerative neurologic diseases	0/50
Healthy controls	0/52

\* PMCA denotes protein misfolding cyclic amplification, and PrP<sup>Sc</sup> misfolded prion protein.

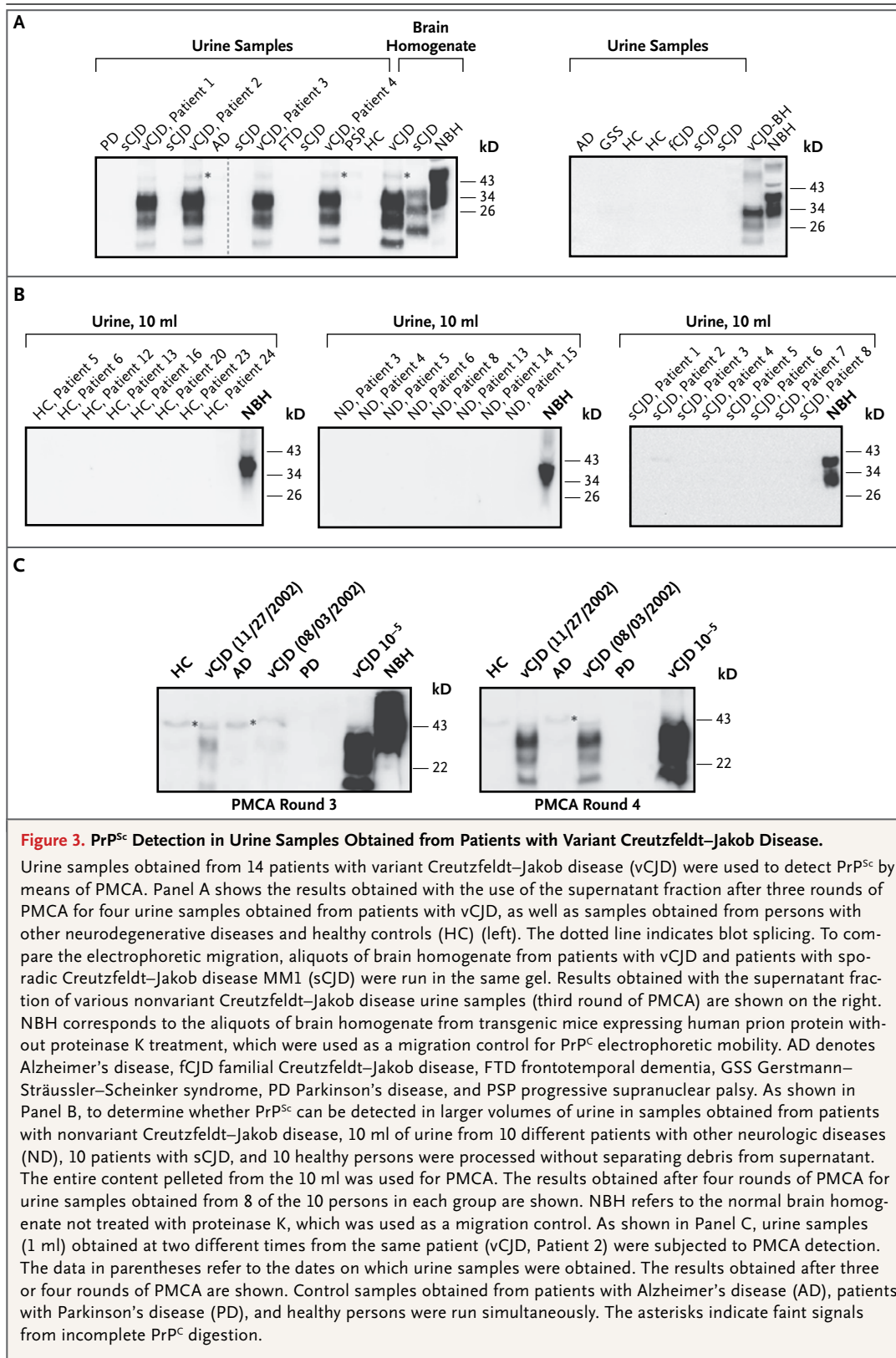
retic characteristics of PrP<sup>Sc</sup> in urine were the same in all the patients with variant Creutzfeldt–Jakob disease, regardless of their country of origin, and corresponded to the typical electrophoretic features of variant Creutzfeldt–Jakob disease (i.e., a type 2B mobility with predominance of the diglycosylated isoform) (Fig. 3A). All tests were repeated twice, and we never detected a false positive result in any of the 224 samples obtained either from patients with other diseases or healthy controls. To study whether PrP<sup>Sc</sup> is detectable in the urine of patients with variant Creutzfeldt–Jakob disease at different times during the clinical phase of the disease, urine obtained at two distinct times from a single patient with variant Creutzfeldt–Jakob disease (vCJD, Patient 2 in Fig. 3A, and in Table S1 in the Supplementary Appendix) was analyzed by means of PMCA. As shown in Figure 3C, the 2 samples from this patient, collected 117 days apart, were both positive for PrP<sup>Sc</sup>, with similar characteristics. However, the sample obtained later in the course of the clinical disease appeared to have a larger quantity of PrP<sup>Sc</sup>, since it required one less PMCA round for detection (Fig. 3C).

#### CONCENTRATION OF PrP<sup>Sc</sup> IN URINE FROM PATIENTS WITH VARIANT CREUTZFELDT–JAKOB DISEASE

To estimate the concentration of PrP<sup>Sc</sup> in urine from patients with variant Creutzfeldt–Jakob disease, we used the quantitative PMCA procedure, as previously described.<sup>27</sup> First, we estimated the concentration of PrP<sup>Sc</sup> in the variant Creutzfeldt–Jakob disease brain homogenate used to calibrate PMCA. The estimation was based on a com-

parison of the PrP<sup>Sc</sup> signal by means of Western blot analysis with a known concentration of recombinant prion protein (Fig. S2A in the Supplementary Appendix). Using this procedure, we calculated that the PrP<sup>Sc</sup> concentration in the brain of this patient was approximately 100  $\mu$ g per gram.

Thereafter, we supplemented urine from a healthy control with different concentrations of this brain homogenate and used these samples for PrP<sup>Sc</sup> detection (Fig. S2B in the Supplementary Appendix). One round of PMCA enabled us to detect approximately  $10^{-12}$  to  $10^{-13}$  g of PrP<sup>Sc</sup> per milliliter in urine. Two rounds of PMCA permitted detection of  $10^{-15}$  to  $10^{-16}$  g per milliliter, and three or more rounds of PMCA consistently detected  $10^{-16}$  g per milliliter of PrP<sup>Sc</sup> (Fig. S2B in the Supplementary Appendix), which is the last detectable quantity. Of the 13 urine samples obtained from patients with variant Creutzfeldt–Jakob disease in which PrP<sup>Sc</sup> was detectable by means of PMCA, 1 was positive in the first round, 6 were positive in the second round, 3 in the third round, and 3 in the fourth round. Therefore, on average, PrP<sup>Sc</sup> was detectable in urine in approximately 2.5 rounds of PMCA. Accordingly, our estimation is that the urine of patients with variant Creutzfeldt–Jakob disease contains a PrP<sup>Sc</sup> concentration of approximately  $1 \times 10^{-16}$  g per milliliter, or  $3 \times 10^{-21}$  mol per milliliter (assuming the molecular weight of the monomer). This finding extrapolates to a concentration of approximately 2000 molecules of prion protein monomer per milliliter of urine. Since most estimations for the average size of the infectious PrP<sup>Sc</sup> particle indicate that it contains between



**Figure 3. PrP<sup>Sc</sup> Detection in Urine Samples Obtained from Patients with Variant Creutzfeldt–Jakob Disease.**

Urine samples obtained from 14 patients with variant Creutzfeldt–Jakob disease (vCJD) were used to detect PrP<sup>Sc</sup> by means of PMCA. Panel A shows the results obtained with the use of the supernatant fraction after three rounds of PMCA for four urine samples obtained from patients with vCJD, as well as samples obtained from persons with other neurodegenerative diseases and healthy controls (HC) (left). The dotted line indicates blot splicing. To compare the electrophoretic migration, aliquots of brain homogenate from patients with vCJD and patients with sporadic Creutzfeldt–Jakob disease MM1 (sCJD) were run in the same gel. Results obtained with the supernatant fraction of various nonvariant Creutzfeldt–Jakob disease urine samples (third round of PMCA) are shown on the right. NBH corresponds to the aliquots of brain homogenate from transgenic mice expressing human prion protein without proteinase K treatment, which were used as a migration control for PrP<sup>C</sup> electrophoretic mobility. AD denotes Alzheimer’s disease, fCJD familial Creutzfeldt–Jakob disease, FTD frontotemporal dementia, GSS Gerstmann–Sträussler–Scheinker syndrome, PD Parkinson’s disease, and PSP progressive supranuclear palsy. As shown in Panel B, to determine whether PrP<sup>Sc</sup> can be detected in larger volumes of urine in samples obtained from patients with nonvariant Creutzfeldt–Jakob disease, 10 ml of urine from 10 different patients with other neurologic diseases (ND), 10 patients with sCJD, and 10 healthy persons were processed without separating debris from supernatant. The entire content pelleted from the 10 ml was used for PMCA. The results obtained after four rounds of PMCA for urine samples obtained from 8 of the 10 persons in each group are shown. NBH refers to the normal brain homogenate not treated with proteinase K, which was used as a migration control. As shown in Panel C, urine samples (1 ml) obtained at two different times from the same patient (vCJD, Patient 2) were subjected to PMCA detection. The data in parentheses refer to the dates on which urine samples were obtained. The results obtained after three or four rounds of PMCA are shown. Control samples obtained from patients with Alzheimer’s disease (AD), patients with Parkinson’s disease (PD), and healthy persons were run simultaneously. The asterisks indicate faint signals from incomplete PrP<sup>C</sup> digestion.

**Table 2. Estimated Sensitivity, Specificity, and Predictive Value of PMCA for PrP<sup>Sc</sup> Detection in Urine from Patients with Variant Creutzfeldt–Jakob Disease.\***

Groups	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
	% (95% CI)		%	
Variant Creutzfeldt–Jakob disease vs. other transmissible spongiform encephalopathies	92.9 (66.1–99.8)	100 (95–100)	100	98.3
Variant Creutzfeldt–Jakob disease vs. other neurologic diseases	92.9 (66.1–99.8)	100 (96.4–100)	100	99.0
Variant Creutzfeldt–Jakob disease vs. all†	92.9 (66.1–99.8)	100 (98.4–100)	100	99.6

\* Estimates of sensitivity and specificity, with 95% confidence intervals (CI), and predictive values were calculated with the use of MedCalc software according to the following formulas: sensitivity = [true positive results ÷ (true positive results + false negative results)] × 100; specificity = [true negative results ÷ (false positive results + true negative results)] × 100; positive predictive value = [true positive results ÷ (true positive results + false positive results)] × 100; and negative predictive value = [true negative results ÷ (true negative results + false negative results)] × 100.

† All refers to all samples that were not obtained from patients with variant Creutzfeldt–Jakob disease (i.e., patients with sporadic Creutzfeldt–Jakob disease, familial transmissible spongiform encephalopathies, or other neurologic diseases [including other degenerative neurologic diseases and nondegenerative neurologic disorders] and healthy controls).

20 and 50 monomers of prion protein,<sup>28,29</sup> we conclude that 1 ml of urine obtained from a patient with variant Creutzfeldt–Jakob disease has between 40 and 100 oligomer equivalents of PrP<sup>Sc</sup>.

## DISCUSSION

We found that small quantities of PrP<sup>Sc</sup> are excreted into the urine of most patients with variant Creutzfeldt–Jakob disease. The one patient (Patient 8 in Table S1 in the Supplementary Appendix) with a urine sample that was found to be negative had a disease duration of 114 months and was receiving treatment with intracerebroventricular administration of pentosan polysulfate, an agent that has been studied for the treatment of variant Creutzfeldt–Jakob disease.<sup>30</sup> Another patient with variant Creutzfeldt–Jakob disease (Patient 10), whose urine was positive for PrP<sup>Sc</sup>, was receiving similar treatment; however, this patient had disease with a duration of 16 months (Table S1 in the Supplementary Appendix).

PrP<sup>Sc</sup> was detectable only after extensive amplification by means of PMCA, suggesting that the concentration of the infectious protein in urine is small. Estimations by means of quantitative PMCA suggest that 1 ml of urine obtained from a patient with variant Creutzfeldt–Jakob disease contains as little as 40 to 100 oligomeric PrP<sup>Sc</sup> particles. The results also indicate

that urinary PrP<sup>Sc</sup> has the ability to self-propagate in vitro by inducing the misfolding of normal human PrP<sup>C</sup>. PrP<sup>Sc</sup> was not detectable in any of the urine samples obtained from patients who had other human transmissible spongiform encephalopathies, suggesting that it is an exclusive feature of variant Creutzfeldt–Jakob disease. This result is not entirely surprising, considering that variant Creutzfeldt–Jakob disease is acquired by infection (probably by oral ingestion), and studies have shown more extensive peripheral involvement in variant Creutzfeldt–Jakob disease than in the sporadic or inherited forms of transmissible spongiform encephalopathies.<sup>5,31,32</sup> Indeed, PrP<sup>Sc</sup> is readily detectable by means of immunohistochemical or biochemical studies in lymph nodes, tonsil, spleen, portions of the intestinal tract, kidney, liver, pancreas, and skeletal muscle from patients with variant Creutzfeldt–Jakob disease.<sup>5,32–34</sup>

In conclusion, the results of our study show that PrP<sup>Sc</sup> can be detected in the urine of patients with variant Creutzfeldt–Jakob disease with high sensitivity and specificity. Further studies are needed to determine whether the detection of PrP<sup>Sc</sup> in urine can be used diagnostically.

The views expressed in this article are those of the authors and not necessarily those of the U.K. Department of Health.

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## REFERENCES

1. Aguzzi A, Calella AM. Prions: protein aggregation and infectious diseases. *Physiol Rev* 2009;89:1105-52.
2. Prusiner SB. Prions. *Proc Natl Acad Sci U S A* 1998;95:13363-83.
3. Soto C. Prion hypothesis: the end of the controversy? *Trends Biochem Sci* 2011;36:151-8.
4. *Idem*. Diagnosing prion diseases: needs, challenges and hopes. *Nat Rev Microbiol* 2004;2:809-19.
5. Wadsworth JD, Joiner S, Hill AF, et al. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet* 2001;358:171-80.
6. Collinge J. Variant Creutzfeldt-Jakob disease. *Lancet* 1999;354:317-23.
7. Bruce ME, Will RG, Ironside JW, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* 1997;389:498-501.
8. Scott MR, Will R, Ironside J, et al. Compelling transgenic evidence for transmission of bovine spongiform encephalopathy prions to humans. *Proc Natl Acad Sci U S A* 1999;96:15137-42.
9. Gill ON, Spencer Y, Richard-Loendt A, et al. Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey. *BMJ* 2013;347:f5675.
10. Brown P, Preece M, Brandel JP, Sato T, McShane L, Zerr I. Iatrogenic Creutzfeldt-Jakob disease at the millennium. *Neurology* 2000;55:1075-81.
11. Hill AF, Collinge J. Subclinical prion infection in humans and animals. *Br Med Bull* 2003;66:161-70.
12. Bishop MT, Hart P, Aitchison L, et al. Predicting susceptibility and incubation time of human-to-human transmission of vCJD. *Lancet Neurol* 2006;5:393-8.
13. Lasmézas CI, Fournier JG, Nouvel V, et al. Adaptation of the bovine spongiform encephalopathy agent to primates and comparison with Creutzfeldt-Jakob disease: implications for human health. *Proc Natl Acad Sci U S A* 2001;98:4142-7.
14. Llewelyn CA, Hewitt PE, Knight RS, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet* 2004;363:417-21.
15. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet* 2004;364:527-9.
16. Wroe SJ, Pal S, Siddique D, et al. Clinical presentation and pre-mortem diagnosis of variant Creutzfeldt-Jakob disease associated with blood transfusion: a case report. *Lancet* 2006;368:2061-7.
17. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* 2001;411:810-3.
18. Saá P, Castilla J, Soto C. Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. *J Biol Chem* 2006;281:35245-52.
19. Castilla J, Saá P, Soto C. Detection of prions in blood. *Nat Med* 2005;11:982-5.
20. Gonzalez-Romero D, Barria MA, Leon P, Morales R, Soto C. Detection of infectious prions in urine. *FEBS Lett* 2008;582:3161-6.
21. Saá P, Castilla J, Soto C. Presymptomatic detection of prions in blood. *Science* 2006;313:92-4.
22. Morales R, Duran-Aniotz C, Diaz-Espinoza R, Camacho MV, Soto C. Protein misfolding cyclic amplification of infectious prions. *Nat Protoc* 2012;7:1397-409.
23. Haley NJ, Mathiason CK, Carver S, Zabel M, Telling GC, Hoover EA. Detection of chronic wasting disease prions in salivary, urinary, and intestinal tissues of deer: potential mechanisms of prion shedding and transmission. *J Virol* 2011;85:6309-18.
24. Murayama Y, Yoshioka M, Okada H, Takata M, Yokoyama T, Mohri S. Urinary excretion and blood level of prions in scrapie-infected hamsters. *J Gen Virol* 2007;88:2890-8.
25. Seeger H, Heikenwalder M, Zeller N, et al. Coincident scrapie infection and nephritis lead to urinary prion excretion. *Science* 2005;310:324-6.
26. Gregori L, Kovacs GG, Alexeeva I, Budka H, Rohrer RG. Excretion of transmissible spongiform encephalopathy infectivity in urine. *Emerg Infect Dis* 2008;14:1406-12.
27. Chen B, Morales R, Barria MA, Soto C. Estimating prion concentration in fluids and tissues by quantitative PMCA. *Nat Methods* 2010;7:519-20.
28. Silveira JR, Raymond GJ, Hughson AG, et al. The most infectious prion protein particles. *Nature* 2005;437:257-61.
29. Tixador P, Herzog L, Reine F, et al. The physical relationship between infectivity and prion protein aggregates is strain-dependent. *PLoS Pathog* 2010;6(4):e1000859.
30. Newman PK, Todd NV, Scoones D, et al. Postmortem findings in a case of variant Creutzfeldt-Jakob disease treated with intraventricular pentosan polysulfate. *J Neurol Neurosurg Psychiatry* 2014 February 19 (Epub ahead of print).
31. Brandner S, Whitfield J, Boone K, et al. Central and peripheral pathology of kuru: pathological analysis of a recent case and comparison with other forms of human prion disease. *Philos Trans R Soc Lond B Biol Sci* 2008;363:3755-63.
32. Head MW, Ritchie D, Smith N, et al. Peripheral tissue involvement in sporadic, iatrogenic, and variant Creutzfeldt-Jakob disease: an immunohistochemical, quantitative, and biochemical study. *Am J Pathol* 2004;164:143-53.
33. Peden AH, Ritchie DL, Head MW, Ironside JW. Detection and localization of PrPSc in the skeletal muscle of patients with variant, iatrogenic, and sporadic forms of Creutzfeldt-Jakob disease. *Am J Pathol* 2006;168:927-35.
34. Notari S, Moleres FJ, Hunter SB, et al. Multiorgan detection and characterization of protease-resistant prion protein in a case of variant CJD examined in the United States. *PLoS One* 2010;5(1):e8765.

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