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# Proteomic Analysis of the Cerebrospinal Fluid of Patients with Creutzfeldt-Jakob Disease

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## Key Words

Cerebrospinal fluid · Creutzfeldt-Jakob disease · Proteome · Two-dimensional polyacrylamide gel electrophoresis · Surrogate markers

## Abstract

So far, only the detection of 14-3-3 proteins in cerebrospinal fluid (CSF) has been accepted as diagnostic criterion for Creutzfeldt-Jakob disease (CJD). However, this assay cannot be used for screening because of the high rate of false-positive results, whereas patients with variant CJD are often negative for 14-3-3 proteins. The aim of this study was to compare the spot patterns of CSF by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to search for a CJD-specific spot pattern. We analyzed the CSF of 28 patients [11 CJD, 9 Alzheimer's disease (AD), 8 nondemented controls (NDC)] employing 2D-PAGE which was optimized for minimal volumes of CSF (0.1 ml; 7-cm strips). All samples were run at least three times, gels were silver stained and analyzed by an analysis software and manually revised. We could consistently match 268 spots which were then compared between

all groups. By the use of 5 spots, we were able to differentiate CJD from AD or NDC with a sensitivity of 100%. CJD could also be distinguished from both groups by using a heuristic clustering algorithm of 2 spots. We conclude that this proteomic approach can differentiate CJD from other diseases and may serve as a model for other neurodegenerative diseases.

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## Introduction

Creutzfeldt-Jakob disease (CJD) is a rare and fatal neurodegenerative disease. A definite diagnosis of CJD can be made by neuropathologic or immunochemical demonstration of the pathological isoform of the prion protein (PrP<sup>Sc</sup>) in human brain tissue [1]. Up to now, the *intra vitam* diagnosis has been made according to clinical and electroencephalographic criteria [2–4]. Attempts to detect prion protein aggregates by using fluorescence-correlated spectroscopy show a sensitivity of only 20% [5]. The clinical diagnosis of CJD can be supported by

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biochemical analysis of cerebrospinal fluid (CSF). So far, only the 14-3-3 protein immunoblot has been included in the diagnostic criteria [6–9], despite the fact that other surrogate markers also have a high differential diagnostic potential and the diagnostic accuracy of 14-3-3 proteins was challenged [10–13]. Especially, this assay cannot be used for screening because of the high rate of false-positive results, whereas patients with variant CJD are often negative for 14-3-3 proteins [12, 13].

Initially, 14-3-3 proteins were described as 2 spots (P130 and P131) in 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) by Harrington in 1986, which he considered at this time to be typical for CJD and herpes encephalitis [14]. Ten years later, again his group could sequence at least 1 protein (P130) and identified it as a member of the 14-3-3 protein family [6].

Further surrogate markers were found in the CSF of CJD patients or patients with other neurological diseases applying this approach. For example, elevated ApoE levels in CJD were described [15], inter alia an isoform of  $\alpha$ -antitrypsin in Alzheimer's disease (AD) [16], granin-like neuroendocrine precursor (proSAAS) in frontotemporal dementia [17], *n-myc* oncoprotein in primary brain tumors [18] and apolipoprotein A-IV in schizophrenia [19].

So far, this 2D-PAGE approach has barely been used for routine diagnosis because of the relatively high amount of required CSF, the high time consumption and the low sensitivity in comparison to an ordinary SDS-PAGE immunoblot if only 1 protein spot is of interest.

The aim of this study was to compare the entire CSF protein pattern of CJD and AD patients and controls in a 2D-PAGE approach. For this reason, we had to optimize our 2D-PAGE setup for a microscale 2-dimensional electrophoresis (2-DE) on minigels with a minimal CSF volume and in a reasonable analysis time.

## Subjects and Methods

### Patients

We analyzed the CSF of 28 patients who were all diagnosed and followed by a specialist neurologist. Eleven patients were diagnosed as having CJD, 9 as having AD. The other 8 patients had no signs of dementia and served as nondemented control patients (NDC).

According to the clinical criteria, all suspected cases of CJD were classified and received at least one cranial computed tomography or magnetic resonance imaging before clinical staging to exclude ischemic stroke, hemorrhage, or space-occupying lesions as a cause of the illness. All patients in this study were classified according to the WHO [7] as having 'probable' CJD. Four of these

patients with CJD were later neuropathologically verified as CJD cases.

Diagnosis of AD was made according to the established NINCDS-ADRDA criteria [20].

The NDC group comprised 8 patients with functional gait disturbance ( $n = 2$ ), depression ( $n = 2$ ), left abducens paresis, recurrent confusion by chronic urinary infection, lumboschialgia, vertigo, and blurred pain syndrome of the limbs. These patients had no sign of acute or chronic inflammation or intrathecal immune response in their CSF. No patient of this group had signs of cognitive decline.

### Procedures

**Sample Preparation.** Lumbar CSF was taken in approximately 5-ml portions by the neurologist and sent to our CSF lab. After the analysis of the routine parameter, the CSF samples were stored at  $-80^{\circ}\text{C}$  until preparation was performed. All samples have been taken in the same way. Prior to 2-DE, to each aliquot of 600  $\mu\text{l}$  of native CSF 1,200  $\mu\text{l}$  ice-cold acetone was added and proteins were precipitated overnight at  $-20^{\circ}\text{C}$  and pelleted by centrifugation (10 min at 14,000  $g$ ). The supernatant was discarded and the pellet air dried and afterwards resolved in 150  $\mu\text{l}$  lysis buffer [9.5 M urea, 2% (w/v) CHAPS, 0.8% Pharmalyte pH 3–10 and 1% (w/v) DTT]. The solution was vortexed for 15 min in an ultrasound water bath. Twenty-five microliters of the 150  $\mu\text{l}$  dissolved proteins in lysis buffer were later used for each gel. This volume corresponds to 100  $\mu\text{l}$  of native CSF. Up to 4 gels were made from each patient, which corresponds to 400  $\mu\text{l}$  of native CSF.

**Isoelectric Focusing.** For isoelectric focusing, commercially available linear immobilized pH gradient (IPG) strips were used (pH 4–7, 7 cm, Amersham Pharmacia Biosciences). The IPG was performed according to Gorg et al. [21] using the Multiphor-System. The IPG gel strips were rehydrated overnight at room temperature in a solution containing 8 M urea, 0.5% (w/w) CHAPS, 15 mM DTT and 0.2% (w/v) Pharmalyte (pH 3–10). The rehydrated strips were loaded with the CSF proteins precipitated from 100  $\mu\text{l}$ , solubilized in 25  $\mu\text{l}$  rehydration buffer and focused for 8,075 Vh.

**Equilibration.** After focusing, single strips were equilibrated twice for 10 min in 5 ml of equilibration buffer [6 M urea, 2% (w/v) SDS, 0.72 M bistris, 0.32 M bicine, 30% (v/v) glycerol]. In the first equilibration step 50 mg dithiothreitol and in the second step 240 mg iodoacetamide were added.

**Two-Dimensional Polyacrylamide Gel Electrophoresis.** The second-dimension separation was performed according to Laemmli using the BioRad system with SDS polyacrylamide gels consisting of 12% (w/v) total acrylamide monomer. The focused gel strips were laid onto the top of the polymerized SDS gels and covered by a low-melting agarose buffer [1% (w/v) low-melting agarose, 0.25% SDS, 0.36 M bistris, 0.16 M bicine, a trace of bromophenol blue] which had previously been heated to melting temperature. After polymerization, gels were run in pairs at 24 mA/gel at constant current and at room temperature.

**Gel Staining and Analysis.** Proteins were detected by silver staining according to Rabilloud [22]. Stained gels were scanned and the image files were imported to the analysis software Image master 3.1 (Amersham Biosciences).

**Table 1.** Main characteristics of investigated patient groups and CSF samples

	Gender (male/female)	Age years	14-3-3 positive	Tau protein pg/ml	S-100B ng/ml
CJD (n = 9)	4/5	68 (53–83)	9/9	7,478 (1,071–19,208)	9.3 (n = 7) (2.1–14.9)
AD (n = 9)	1/8	64 (52–80)	1/8	759 (359–1,840)	2.06 (n = 4) (1.54–2.74)
NDC (n = 8)	2/6	62 (57–78)	n.a.	n.a.	n.a.

Values are listed as means and range in parentheses. n.a. = Not ascertained.

**Table 2.** Detection and percentage of spot presence in gels and patients

	Spot 87	Spot 100	Spot 162	Spot 213	Spot 230
CJD					
Gels (n = 28)	28 (100)	25 (89.3)	28 (100)	25 (89.3)	27 (96.4)
Patients (n = 9)	9 (100)	6 (66.7)	9 (100)	7 (77.8)	8 (88.9)
AD					
Gels (n = 28)	24 (93)	4 (15.4)	10 (38.5)	3 (11.5)	4 (15.4)
Patients (n = 9)	9 (100)	3 (33.3)	6 (66.7)	1 (11.1)	2 (22.2)
NDC					
Gels (n = 28)	0 (0)	2 (8.7)	2 (8.7)	2 (8.7)	1 (4.4)
Patients (n = 9)	0 (0)	2 (25)	1 (12.5)	2 (25)	1 (12.5)

Figures in parentheses indicate percentages.

## Results

One hundred and nine gels were analyzed from 28 patient samples. Some of the gels had to be excluded due to differences in staining intensities. To reduce the individual gel-to-gel differences, a minimum of 3 gels was prepared per sample. After excluding unusable gels and samples with less than 3 gels, 77 gels out of 109 and 26 patients out of 28 were left. Of these 26 patients, 9 were suffering from CJD and 9 from AD. Samples of 8 patients were used as NDC. All data concerning the CSF samples are shown in table 1.

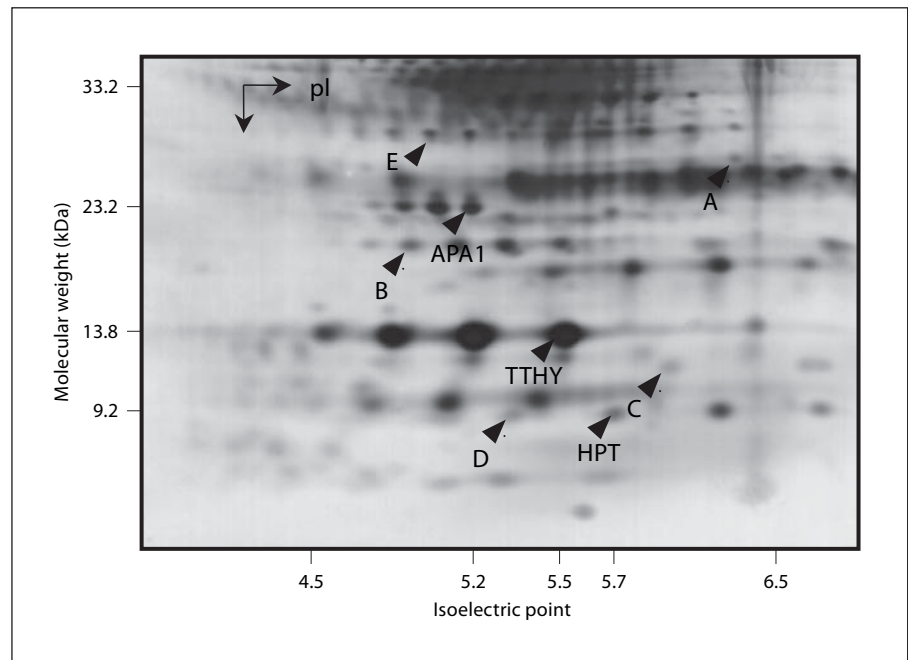
An area was selected in which protein spots can be detected easily as isolated instead of blurred spots. This area includes spots of a molecular weight between 29 and 3 kDa, as well as an isoelectric point between 4 and 7.

All spots in this area were detected by the software (Image Master, Amersham Biosciences) and manually revised. More than 350 spots were found and 268 spots were matched to a reference gel. As a quantitative analysis of silver-stained gel is in discussion, analyses were based on the presence of spots in the gels.

Spots of interest were defined as present (intense staining intensities) in samples of CJD and less present (intensities near background or not visible) in samples without CJD (<10%). The conditions were determined as follows: first, the lower limit for presence in CJD samples was 85% and the upper limit for presence in non-CJD samples was 20%. The second determination was to differentiate demented (CJD, AD) and nondemented patients by a lower limit of 90% in the group of CJD and AD and an upper limit of 10% in the group of NDC.

The first requirement was fulfilled by 3 spots, whereas only 1 spot could be found to fulfil the second condition. A further spot was present in 38% of AD gels, which was also present in 100% of CJD gels, but could rarely be seen in NDC gels (9%). In total, we observed 5 spots that appear to be relevant and differentially expressed in gels of patients with CJD, AD or NDC (table 2). These spots were isolated locally in the gel and are easy to distinguish from other spots. The spots were demonstrated both in the total map of spots (fig. 1) and in a zoomed area of interest (fig. 2).

**Fig. 1.** Silver-stained 2-DE gel of CJD CSF. Proteins are separated by size and charge. Spots of interest are marked by arrowheads and letters and depicted in figure 2 (spot A corresponds with spot 100, spot B with spot 162, spot C with spot 213, spot D with spot 230, spot E with spot 87). pI = Isoelectric point; APA1 = apolipoprotein A1; TTHY = transthyretin; HPT = haptoglobin.



Known proteins were used to extrapolate the molecular weight and isoelectric point of spots of interest. These known proteins were isoforms of haptoglobin [isoelectric point 5.7, molecular weight 9,270 Da; serial number of SWISS-2DPAGE (<http://www.expasy.org/cgi-bin/ch2d-search-sn>): 2D-000CH8], transthyretin (isoelectric point 5.49, molecular weight 13,800 Da; serial number 2D-000CFA) and apolipoprotein A1 (isoelectric point 5.22, molecular weight 23,149 Da; serial number 2D-000CBM). Based on these data, spots of interest were calculated by rule of three (table 3).

#### Heuristic Clustering

Based on the detection of spot 87 and spot 213, we selected the patients according to a heuristic clustering (fig. 3). First, the gels were split up into groups dependent on the detection of spot 87. If they were positive for spot 87, the samples were clustered into two distinct branches by detection of spot 213. After the first splitting, all non-demented patients and 2 cases with AD were segregated to one branch. On the other branch, we found all cases with CJD and 23 samples with AD. After the next segregation by spot 213, 25 CJD samples and 3 AD samples were clustered to one branch and 3 CJD samples and 21 AD samples to the other. The AD samples that were clustered after the second splitting to the branch of CJD samples were only samples from 1 patient. Further CSF anal-

ysis of this patient revealed a similar marker pattern to that found in CJD patients (positive 14-3-3 SDS/immunoblot and highly elevated tau protein level). All other patients with AD could be excluded by clustering the samples.

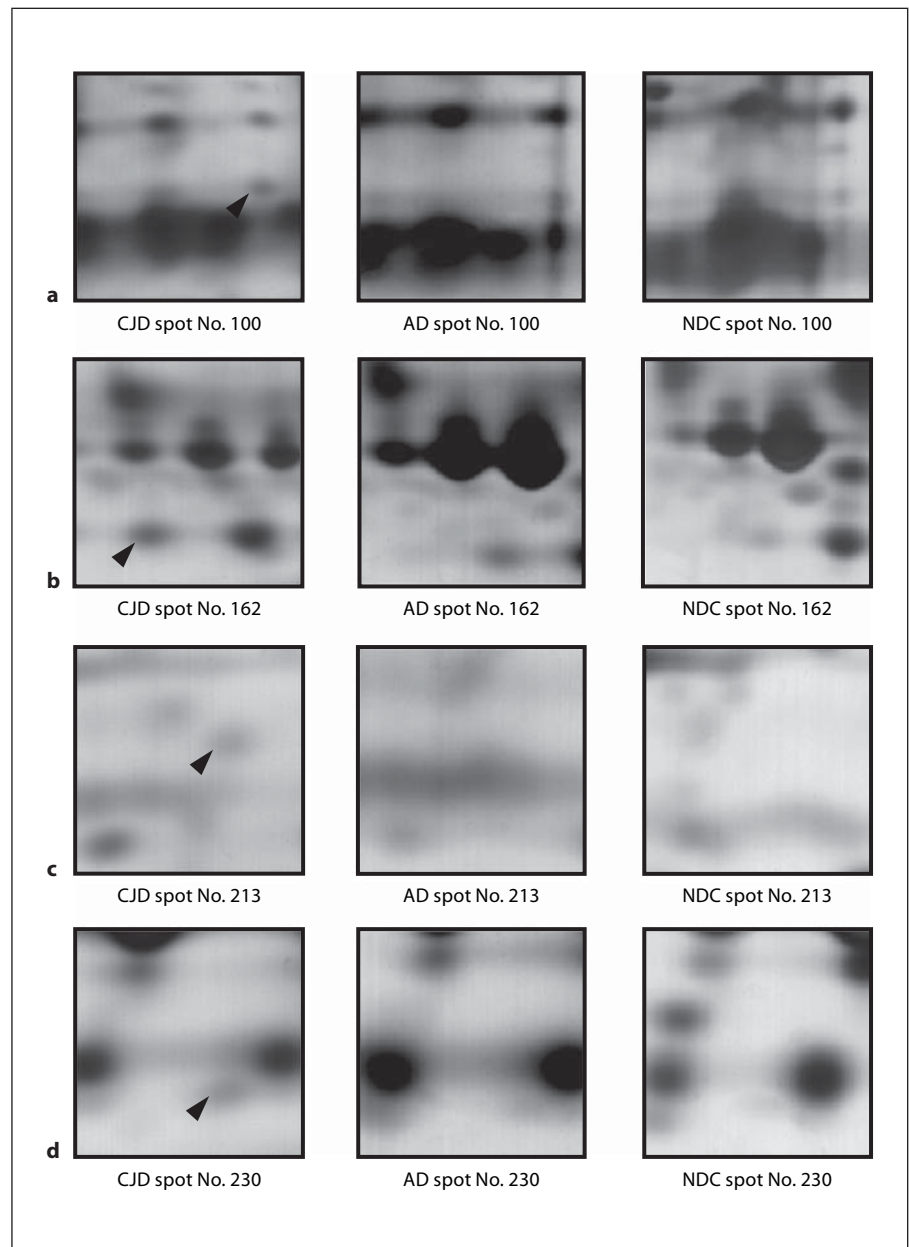
Only in 3 samples of CJD patients could spot 213 not be detected.

#### Single and Combined Diagnostic Sensitivity and Specificity

All spots were analyzed for sensitivity and specificity. The highest sensitivity was obtained for spots 87 and 162 for CJD, whereas the best specificity was obtained for spots 213 and 230. By a combined analysis of these spots we obtained higher diagnostic values. The two best combinations are shown in table 4.

#### Discussion

The differential diagnosis of dementias is usually based upon clinical criteria, while neurochemical data are rarely included as diagnostic algorithms. In the case of sporadic CJD, the measurement of 14-3-3 proteins in CSF was included in the diagnostic criteria by the WHO [6, 7, 14, 23, 24]. However, there is still a need for other markers as so far no surrogate marker has been shown to



**Fig. 2.** Region of silver-stained 2-DE from each group demonstrated in one exemplary sample.

**Table 3.** Molecular weights and isoelectric points of spots found

	Isoelectric point	Molecular weight, kDa
Spot 87	5.33	25.2
Spot 100	6.61	23.7
Spot 162	5.25	19
Spot 213	6.31	11.9
Spot 230	5.66	9.3

support the diagnosis sufficiently. Especially patients with variant CJD mainly have a negative 14-3-3 immunoblot [12, 25]. Apart from the diagnostic approach, it can be doubted that the 14-3-3 proteins play an important pathophysiological role in transmissible spongiform encephalopathies [26].

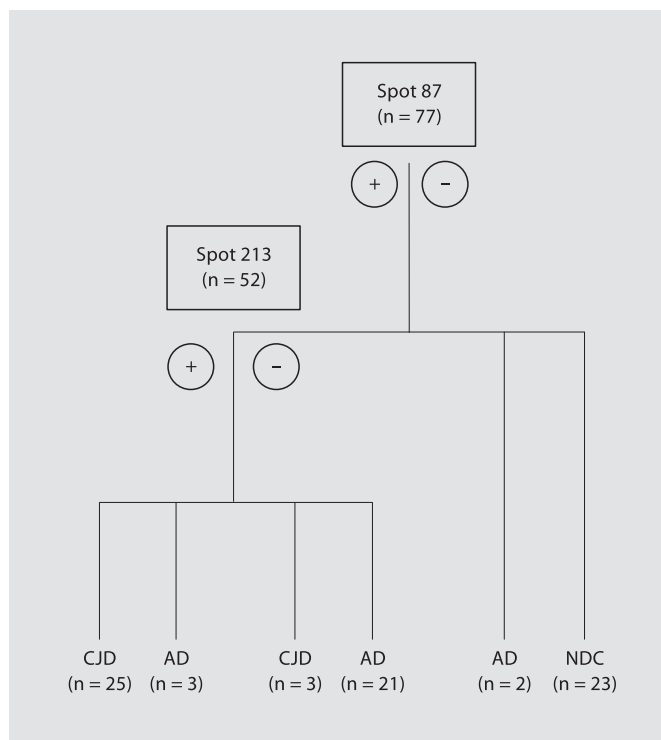
Our aim was to investigate if a diagnosis can be made by a disease-specific spot pattern. This approach is now mainly in use for chip-based techniques but is rarely used in a 2D-PAGE approach [27]. As CSF volume is often lim-

**Table 4.** Values for sensitivity (sen) and specificity (spe) of CSF spots for clinical diagnosis of CJD

Spot	tp, n	tn, n	fp, n	fn, n	sen	spe
87	28	25	24	0	1	0.54
100	25	43	6	3	0.93	0.81
162	28	37	12	0	1	0.7
213	25	44	5	3	0.93	0.83
230	27	44	5	1	0.98	0.84
87 and 230	27	45	4	1	0.98	0.87
162 and 230	27	45	4	1	0.98	0.87

tp = True positive; tn = true negative; fp = false positive; fn = false negative.

**Fig. 3.** Heuristic clustering of CJD versus AD and versus NDC using detection of spot 87 followed by spot 213 by 2D-PAGE.



ited, we had optimized the protocol to analyze a minimal volume of CSF. In earlier investigations, it was necessary to investigate sample volumes between 250  $\mu$ l and 2 ml of CSF [15, 28]. Here we used a volume of only 100  $\mu$ l CSF. We concentrated our analysis on an area between 29 kDa and 3 kDa and an isoelectric point between 4 and 7 because proteins with higher molecular weights were not clearly separated in this setup with 7-cm strips. Although this is a comparably small area, we were able to detect a minimum of 268 spots for subsequent analysis. A further advantage of these small gels is the minimum of time consumption and cost for an analysis.

We found 5 protein spots constantly expressed only in the CSF of patients suffering from CJD. These spots do not match in molecular weight and isoelectric point with the currently used markers (14-3-3, tau protein, S-100B, NSE). Spot 213 may represent H-FABP, which was currently identified in the CSF of CJD patients [29]. Additionally, none of these spots matched with earlier described spots which were found in a similar study [15]. We were not able to validate the spots which were shown in this study. This might be due to the small patient number which had been investigated in this former study (n = 3) [15].

Either by heuristic clustering or by pattern analysis we were able to obtain similar values for sensitivity and specificity, as it was described for currently used biomarkers [12, 30–32]. Certainly, it remains to be seen if these high levels of sensitivity and specificity persist when a larger number of patients is investigated. As these markers do not correspond to the aforementioned CJD biomarkers, it will be of special interest to investigate the potential of the shown spot pattern in the differential diagnosis of variant CJD, as the currently known markers were often false negative.

On the basis of our results, we cannot conclude that these spots are involved in the pathophysiology of CJD. In further studies with larger amounts of CSF, these spots should be attempted to be identified, so that the pathophysiological function can be investigated. However, this does not limit the use of our spot pattern for the diagnosis of CJD.

In the future, it has to be the goal to generate reference gels of special diseases, so that a diagnosis can be made by pattern analysis as it has already been described for AD [33] and schizophrenia [19].

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