

**Etiologic and diagnostic facets
of
Creutzfeldt-Jakob disease**
The effect of genes and environment

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of
Creutzfeldt-Jakob Disease**
The effect of genes and environment

**Etiologische en diagnostische aspecten
van de
ziekte van Creutzfeldt-Jakob**
Invloed van genen en omgeving

Proefschrift

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Erasmus Universiteit Rotterdam
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Para ti, mamá

Publications and manuscripts based on the studies described in this thesis

Chapter 2.1

Knight RSG, Sánchez-Juan P, Lindsey T & the 'NEUROCID' collaborative group. The 'NEUROCID' collaboration: CJD surveillance systems and results in ten countries.

Chapter 2.2

Sánchez-Juan P, Houben MPWA, Hoff JI, Jansen C, Sie MPS, van Rijn MJE, Ironside JW, Will RG, van Duijn CM, Rozemuller A. The first case of variant Creutzfeldt-Jakob disease in the Netherlands. *Journal of Neurology*. (In press)

Jansen C, Houben MPWA, Hoff JI, Sánchez-Juan P, Rozemuller, AJM, van Duijn CM. De eerste patiënt in Nederland met de nieuwe variant van de ziekte van Creutzfeldt-Jakob. *Ned Tijdschr Geneeskd* 2005;149:2949-2954.

Chapter 3.1

Sánchez-Juan P, Cousens SN, Will RG, van Duijn CM. What is the source of variant Creutzfeldt-Jakob disease outside the UK?. (Submitted)

Chapter 3.2

Sánchez-Juan P, Ward HE, de Silva R, Knight RSG, Will RG. Ophthalmic surgery and Creutzfeldt-Jakob disease. *British Journal of Ophthalmology* 2004;88:446-449.

Chapter 3.3

Sánchez-Juan P, Bishop MT, Croes EA, Knight RSG, Will RG, van Duijn CM, Manson JC. A polymorphism in the regulatory region of PRNP is associated with increased risk of sporadic Creutzfeldt-Jakob disease. (Submitted)

Chapter 3.4

Sánchez-Juan P, Bishop MT, Green A, Ladogana A, Arias-Vasquez A, Knight RSG, Pocchiari M, Will RG, van Duijn CM. No evidence for association between Tau haplotypic variants and susceptibility to Creutzfeldt-Jakob disease.

Chapter 4.1

Sánchez-Juan P, Green A, Ladogana A, Cuadrado-Corrales N, Sanchez-Valle R, Mitrová E, Stoeck K, Sklaviadis T, Kulczycki J, Hess K, Bodemer M, Slivarichová D, Saiz A, Calero M, Ingrosso L, Knight RSG, Janssens AC, van Duijn CM, Zerr I. CSF tests in the differential diagnosis of Creutzfeldt-Jakob disease. *Neurology* 2006;67:637-643.

Chapter 4.2

Sánchez-Juan P*, Sánchez-Valle R*, Green A, Ladogana A, Cuadrado-Corrales N, Mitrová E, Stoeck K, Sklaviadis T, Kulczycki J, Hess K, Krasnianski A, Equestre M, Slivarichová D, Saiz A, Calero M, Pocchiari M, Knight RSG, van Duijn CM, Zerr I. Influence of timing on CSF tests value for Creutzfeldt-Jakob disease diagnosis. *Journal of Neurology*. (In press)

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Chapter 4.3

Green A, Sánchez-Juan P, Ladogana A, Cuadrado-Corrales N, Sánchez-Valle R, Mitrová E, Stoeck K, Panagiotidis C, Sklaviadis T, Kulczycki J, Heinemann U, Hess K, Slivarichová D, Saiz A, Calero M, Mellina V, Knight RSG, van Duijn CM, Zerr I. CSF analysis in patients with sporadic CJD and other transmissible spongiform encephalopathies. *European Journal of Neurology*. (In press)

Chapter 4.4

Collins SJ*, Sánchez-Juan P*, Masters CL, Klug GM, van Duijn CM, Poleggi A, Pocchiari M, Almonti S, Cuadrado-Corrales N, de Pedro-Cuesta J, Budka H, Gelpi E, Glatzel M, Tolnay M, Hewer E, Zerr I, Heinemann U, Kretschmar HA, Jansen GH, Olsen E, Mitrová E, Alperovitch A, Brandel JP, Mackenzie J, Murray K, Will RG. Determinants of diagnostic investigation sensitivities across the clinical spectrum of sporadic Creutzfeldt-Jakob disease. *Brain* 2006;129:2278-2287.

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1

Scope of the thesis

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a unique group of fatal neurodegenerative disorders that affect both humans and animals. TSEs are characterized neuropathologically by spongiform degeneration of the gray matter, neuronal loss, astrocytosis and the accumulation of the protease resistant prion protein (PrP^{Sc}). Even though they share some similarities with other conventional neurodegenerative diseases such as Alzheimer's disease, they are unique in that they are transmissible. According to the protein-only hypothesis, PrP^{Sc} is itself the main or only component of the transmissible agent.¹

Human TSEs comprise a number of diseases with different etiology. Under this acronym we currently include: entities with unknown etiology, like sporadic Creutzfeldt-Jakob disease (sCJD), genetic syndromes associated to mutations of the human gene encoding the prion protein (PRNP) referred to as genetic CJD (gCJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI), and transmitted cases via medical procedures like iatrogenic CJD (iCJD) or via consumption of human (Kuru) or bovine infected material (variant CJD (vCJD)).

TSEs are rare disorders. The annual incidence of all human TSEs has been estimated to be around 1.67 cases per million.² However, remarkable attention has been focused on these rare diseases in the past century. This is mainly because of their unique pathogenic mechanisms and also because of their public health implications. The bovine spongiform encephalopathy (BSE) epizootic in the UK and the subsequent appearance of vCJD, as a consequence of the transmission of the BSE agent to humans through contaminated bovine products, brought these rare disorders to the centre of the world's attention. Consequently, there has been an enormous amount of publications on the subject. Indeed, if the number of publications on any disease is divided by the incidence of such disease, human prion diseases possess by far the highest 'interest factor'.

It was the BSE outbreak in the UK in the middle 1980s that motivated the establishment in 1993 of a network EURO-CJD to coordinate the epidemiological surveillance in a number of European countries with the main aim of identifying any change in incidence or characteristics of CJD that might be attributable to the BSE epizootic. The data collected since 1993 has allowed the realization of multiple studies addressing fundamental questions about key aspects of this group of diseases. This original collaboration seeded new collaborations focusing on extending CJD monitoring to other countries (NEURO-CJD) or diagnostic aspects (EPICJD).

All the studies presented in this thesis are fruit of the work of these international CJD surveillance networks. The objective of the research presented in this book is twofold: the first aim is to obtain new insights about TSE etiology and risk factors. The second aim of the thesis is to evaluate the main tests used for the diagnosis of TSEs. Both topics are analyzed under the prism of two different methodological tools, classical epidemiology in search of environmental factors, and genetic epidemiology in search of genetic determinants and gene-gene or gene-environment interactions.

Chapter 2 introduces the concept of the epidemiological CJD surveillance systems as the cornerstone of the studies part of this thesis. In chapter 2.1 we discuss in depth

the main methodological issues concerning the set up of a multinational collaborative network like NEUROCID. We present the results of the monitoring activities of the ten countries included in the NEUROCID collaboration from 1996 to 2004. In chapter 2.2 we present the description of the first case of vCID detected in the Netherlands. This patient was ascertained as part of the EUROCID monitoring of CID. The ascertainment of cases with new phenotypes of the disease is one of the main objectives of the CID surveillance systems.

In chapter 3 we present several etiological studies. Chapter 3.1 analyses the role of UK bovine exports during the 1980s or indigenous BSE cases, in the spread of vCID to other countries beyond the UK borders. In chapter 3.2 we present the results of a case control study carried out in the UK in which we studied whether ophthalmic surgery has contributed to the transmission of sporadic and variant CID. The last part of the third chapter is devoted to the study of possible genetic factors associated to risk of sporadic CID. In chapter 3.3 we performed a population based genetic association study to assess the role of a polymorphism located inside the regulatory region of PRNP. In chapter 3.4 we carried out a genetic association study aiming to assess the role of the genetic variations of Tau protein gene (MAPT) in susceptibility to vCID and sCID.

Chapter four is entirely focused on diagnostic aspects of CID. The studies included in this chapter are all international collaborative efforts that collected a huge amount of data, allowing to address important questions related to the tests commonly employed in the diagnosis of this extremely rare group of diseases. Chapter 4.1 analyses the diagnostic sensitivity and specificity of various brain derived proteins in the cerebrospinal fluid (CSF) of patients of CID and other dementias. Several biological factors that influence the sensitivity and specificity of these tests are assessed. Chapter 4.2 addresses the influence of timing on CSF tests value for CID diagnosis, and the usefulness of sequentially repeated tests. Chapter 4.3 summarizes the main findings of the routine CSF tests carried out in TSE patients. Finally, in chapter 4.4 we present a comprehensive study analyzing the main determinants of the diagnostic sensitivity of the three main tests (EEG, CSF 14-3-3 test, and MRI) performed on TSE patients. We specifically assessed the performance of those three diagnostic tests across the clinical spectrum of sporadic CID. In the last chapter the main findings of these studies and their implications are discussed together with methodological issues.

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2

**Epidemiology of Creutzfeldt-Jakob disease:
the role of international collaborative studies**

2.1

The 'NEUROCJD' collaboration: CJD surveillance systems and results in ten countries

Abstract

This paper describes the extended collaborative study group of Creutzfeldt-Jakob disease (CJD) 'NEUROCJD' and the results of their monitoring activities during a 8-years period in 10 countries with relatively small population size like Belgium, Denmark, Finland, Greece, Iceland, Ireland, Israel, Norway, Portugal, Sweden, plus the United Kingdom (UK). The overall annual mortality rate of sporadic CJD in the studied countries from 1997 to 2004 was 1 case per million inhabitants. The most common clinical presentations of sporadic CJD were the rapidly progressive dementia (74%), followed by the ataxic onset (9%). Although much less frequent than the first two, visual presentation (Heidenhein's syndrome) (4%) was also well recognized. Other clinical presentations, psychiatric (3%), sensory (2%), and extrapyramidal (0.4%) were recognized less frequently. Finally, 6% of the cases presented with a slowly progressive dementia syndrome. During the study period we ascertained 27 sCJD patients (6% of the total) younger than 50 years at onset, 51 sCJD patients (12% of the total) with disease duration superior to one year, and 69 (6%) sCJD patients with atypical clinical onset. Excluding the UK, only one case of vCJD, in the Republic of Ireland, was diagnosed during the period of observation. In conclusion, we have established CJD monitoring successfully in 9 new European countries, plus Israel, which are characterised by relatively small population size.

Introduction

Creutzfeldt-Jakob disease (CJD), a rare disease, has become the centre of intense clinical, scientific and media attention. The emergence of bovine spongiform encephalopathy (BSE) in United Kingdom (UK) cattle in the 1980s prompted the institution of national CJD surveillance in the UK, to detect any resulting change in the incidence or nature of CJD.¹ An EU-funded CJD surveillance collaborative group ('EUROCJD') began in 1993, with the following members: Austria, France, Germany, Italy, the Netherlands, Slovakia, Spain and the UK, joined in 1996 by Australia, Canada and Switzerland.² In 1996, variant CJD (vCJD, previously 'new variant CJD') was reported and it has been causally linked to BSE.^{3,4} The rapid availability of comparative data from other countries was an important factor in the early identification of variant CJD in the UK.

BSE has not been confined to the UK and past UK exports of live animals, animal products and animal feed are a potential risk to other countries. Many individuals travel widely and thus may become exposed to risks present in other countries. VCJD has been transmitted from human to human by blood and there is concern about risk from blood products and other treatments that have a potential world-wide distribution.^{5,6} VCJD is therefore a problem without national boundaries.

In 1998, a further EU-funded group ('NEUROCJD') was established with ten member countries which are characterised by relatively small population size (<12 million inhabitants): Belgium, Denmark, Finland, Greece, Iceland, Israel, Norway, Portugal and Sweden, joined in 1999 by the Republic of Ireland. In this paper we describe the design of this collaborative group, the nature of their individual surveillance systems and discuss the CJD data collected from 1997 to 2004.

Methods: the NEUROCJD collaborative system

The NEUROCJD collaborative group was established in 1998. Initial funding from the EU ran from 1998 to 2000, with a further grant covering the period 2001 to 2005. The member countries, with their population sizes are given in Table 1 and Table 2. The UK acted as the chair of the NEUROCJD system. There were six-monthly meetings of the members at which data were presented, problems discussed, methods harmonised and research collaborations coordinated. The collaborative project employed a Study coordinator who organized the meetings, acted as a point of contact and visited the member countries on a regular basis. The core aims of NEUROCJD were the harmonization of CJD surveillance methodologies, the collection of basic CJD data, the sharing of particular expertise and collaborative research projects. A basic data set (the 'Minimal Monitoring Data Set') was provided by each country, on a regular basis, to a central data base at Erasmus University, Rotterdam, which also acted as the data centre for the EUROCJD project. Sub-groups within NEUROCJD addressed neuropathology,

CSF testing, genetics, atypical cases and public health issues. Although methods were harmonized as far as possible, especially concerning CJD diagnostic criteria/case definitions, CSF 14-3-3 methodologies, genetic testing methodologies and the collection of common data sets, each country was responsible for establishing, designing, funding and running its own surveillance system. There were therefore differences in structure and approach between the member countries. There are various key parameters of CJD surveillance systems, including whether they are essentially based on pathological or clinical identification, whether case notification is mandatory, and whether cases are examined directly by surveillance system personnel. The essential characteristics of each member country system are given in Table 1. For example, in the UK, Belgium and Iceland, notification of cases of CJD was not mandatory during the 1997-2004 period, being officially notifiable in the remaining 8 NEUROCID countries. Mandatory notification was introduced at different times: from 1996 in Greece, Ireland, Israel and Portugal, from 1997 in Norway and Portugal, from 1998 in Sweden and from 1999 in Finland.

The information collected correspond to an 8-years monitoring period (1997-2004). All reported patients who died for any form of transmissible spongiform encephalopathy (TSE) were included in the study. Only patients fulfilling the diagnostic criteria for probable or definite disease were accepted for the data analyses.⁷ The clinical presentation of sporadic CJD cases was determined whenever possible, according to agreed definitions (Appendix 1).

Statistical analysis

Crude and age- and sex-specific mortality rates were calculated using as denominator population data from the 2004 revision of the world population prospects from the United Nations (www.unpopulation.org). Four age-interval classes were defined for the analysis (up to 14, 15 to 59, 60 to 79 and more than 80 years). Differences in mortality from sCJD among countries were assessed by means of a Standardized Mortality Ratio (SMR) based on the overall age and sex-specific mortality rates from 1997 to 2004. The 95% Confidence Intervals (CIs) were calculated assuming a Poisson distribution. Global differences of mortality among countries and among years of study were assessed by fitting a Poisson regression model, gender and age being included as covariates.

Table 1: Basic characteristics of each member country surveillance system

| Country | Nature of the surveillance system | Notifiable disease | Criteria & type of notification | Patient seen in life |
|----------|------------------------------------|--------------------|---|----------------------|
| Belgium | Epidemiology/ Neuropathology | No | All suspect cases | No |
| Denmark | Epidemiology/ Neuropathology | Since 1997 | Suspect or definite cases Causes of death register | No |
| Finland | Neuropathological/ Neurological | Since 1999 | Death certificates, referred cases, hospital records | Partial |
| Greece | Neurological/ Neuropathological | Since 1996 | Not specified | Partial |
| Iceland | Pathological/ Neurological | No | Not specified | Yes |
| Ireland | Neurological/ Neuropathological | Since 1996 | Suspect cases | Partial |
| Israel | Neuroepidemiological | Since 1999 | Hospital Autopsy Death certificates Neurogenetic labs Personal communication Family contacts | Partial |
| Norway | Epidemiological | Since 1997 | Suspect or definite cases Death certificate register Neurologists/neuropathologists | No |
| Portugal | Neuropathology/ Epidemiology | Since 1996 | All suspect cases | No |
| Sweden | Epidemiological | Since 1997 | Not specified | No |

Results: CJD data

A total of 1320 deaths from any form of TSE were reported during the observation period. This includes 1037 sporadic CJD patients (sCJD), 119 genetic CJD (gCJD) (including 9 Gerstmann-Sträussler-Scheinker syndrome cases and 1 fatal familial insomnia case), 28 iatrogenic CJD (iCJD) (all but one Irish case from the UK) and 136 variant CJD cases (vCJD) (all but one Irish case from the UK). The overall mean annual mortality rate for all TSE in the NEUROCID countries for the 8 year period (January 1997 to December 2004) was 1.3/million person-years (Table 2).

Table 2: Human prion diseases mortality rates and country surveillance parameters

| Country | Country population | All TSE | Sporadic CJD | Autopsy rates (%)* | CSF 14-3-3 testing (%)* | PRNP Mutation testing (%)* | PRNP codon 129 genotyping (%)* |
|-----------------|--------------------|---------|--------------|--------------------|-------------------------|----------------------------|--------------------------------|
| Belgium | 10,898,274 | 1.5 | 1.5 | 75.2 | 84.1 | 15.9 | 40.7 |
| Denmark | 5,659,102 | 1.2 | 1.2 | 66.0 | 50.0 | 20.0 | 24.0 |
| Finland | 5,458,960 | 1.4 | 1.3 | 88.9 | 25.9 | 22.2 | 22.2 |
| Greece | 11,520,320 | 0.7 | 0.7 | 59.3 | 89.8 | 0.0 | 0.0 |
| Iceland | 303,114 | 0.4 | 0.4 | 100.0 | 0.0 | 0.0 | 100.0 |
| Rep. of Ireland | 4,259,996 | 1.0 | 0.9 | 86.2 | 65.5 | 0.0 | 69.0 |
| Israel | 6,899,850 | 2.0 | 0.7 | 29.7 | 94.6 | 59.5 | 2.7 |
| Norway | 4,838,187 | 0.9 | 0.8 | 96.8 | 32.3 | 0.0 | 0.0 |
| Portugal | 10,892,772 | 0.8 | 0.9 | 40.6 | 67.2 | 43.8 | 45.3 |
| Sweden | 9,529,214 | 1.3 | 1.3 | 94.7 | 36.2 | 1.1 | 9.6 |
| UK | 62,293,392 | 1.4 | 1.0 | 75.9 | 59.9 | 50.8 | 66.2 |
| Total | 132,553,181 | 1.3 | 1.0 | 73.8 | 60.9 | 33.2 | 44.4 |

*Only sporadic CJD patients

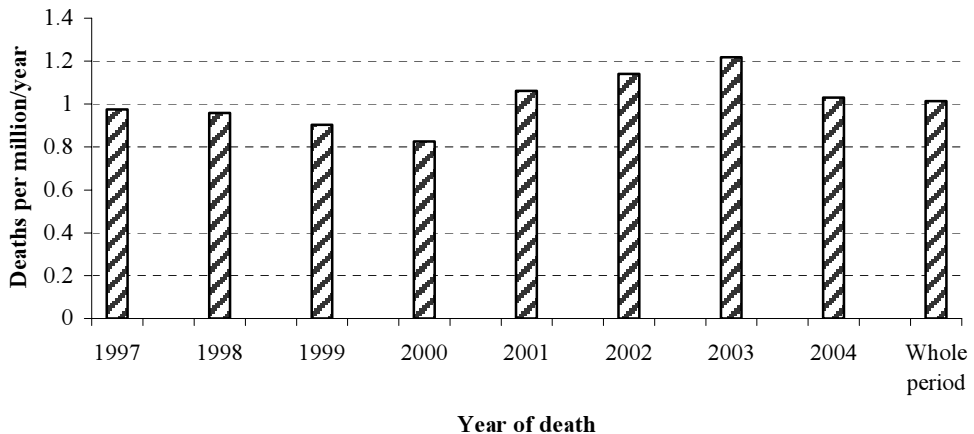


Figure 1: Annual mortality rates for sporadic CJD, by year for all countries combined over the period 1997-2004.

Sporadic CJD

The overall mean annual mortality rate of sCJD was 1.0/million person-years, the numbers of deaths and the annual mortality rates are given in Table 2, Table 3 and Figure 1. The Standardised Mortality Ratios (SMRs) for sCJD are given in Table 3. The calculation of the SMR requires demographic data that were not available in all cases and is therefore based on a total of 1007 cases. Belgium has a slightly increased SMR, with Greece and Portugal having relatively low SMRs. Because of missing data, the SMR in all three countries is likely to be an underestimate, especially for Portugal. However, a Poisson regression model suggests that there are no significant differences in the sCJD annual mortality rates between the different NEUROCJD countries over this 8 year period. The annual mortality rates for specific age categories and sex, given in Figure 2, are slightly higher in women aged more than eighty years than in men of the same age. However, as shown in Table 4, the overall sex ratio of sCJD patients is almost 50:50. The basic age, illness duration and case classification are also given in Table 4a for the sporadic and Table 4b genetic CJD cases. The median age at onset in sCJD was 66.7 years, varying from 61.5 years in Norway to 69 years in Sweden. GCJD cases presented at an earlier age than sCJD (median of 59.4 years; range from 31.7 to 81.8 years). The median disease duration was of 5 months for both sCJD and gCJD cases. The percentage of neuropathological confirmation was almost 74% for the sCJD, but these figures varied considerably ranging from almost 100% in some Scandinavian countries to only 30% in Israel. For gCJD cases this percentage was much lower (27%).

Table 3: *Standardized mortality rates and 95% confidence intervals for each country*

| Country | Total deaths from sporadic CJD | Mean annual mortality rate sporadic CJD | SMR | SMR 95% CI |
|-----------------|--------------------------------|---|------|------------|
| Belgium | 123 | 1.5 | 1.27 | 1.04-1.53 |
| Denmark | 50 | 1.2 | 1.21 | 0.90-1.59 |
| Finland | 54 | 1.3 | 1.24 | 0.93-1.63 |
| Greece | 59 | 0.7 | 0.63 | 0.48-0.81 |
| Iceland | 1 | 0.4 | 0.53 | 0.01-2.93 |
| Rep. of Ireland | 29 | 0.9 | 1.07 | 0.71-1.55 |
| Israel | 37 | 0.7 | 0.82 | 0.56-1.16 |
| Norway | 31 | 0.8 | 0.87 | 0.59-1.24 |
| Portugal | 65 | 0.9 | 0.65 | 0.49-0.84 |
| Sweden | 94 | 1.3 | 1.19 | 0.96-1.46 |
| UK | 494 | 1.0 | 1.03 | 0.95-1.13 |
| Total | 1037 | 1.0 | | |

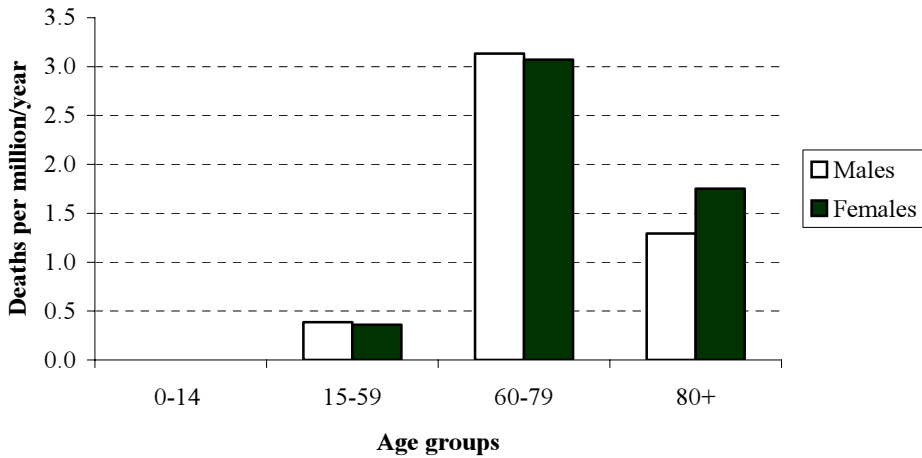


Figure 2: Age-specific mortality rates for sporadic CJD in all countries over the period 1997-2004.

Table 4a: Basic demographic and clinical characteristics of sporadic CJD patients

| Countries | Number | % Definite | % Female | Median age at onset (range) | Median duration (range) |
|-----------------|--------|------------|----------|-----------------------------|-------------------------|
| Belgium | 123 | 75.2 | 44.2 | 65.3 (31.8-88.0) | 4 (1-36) |
| Denmark | 50 | 66.0 | 52.0 | 65.9 (40.0-87.7) | 5 (1-18) |
| Finland | 54 | 88.9 | 51.9 | 67.8 (47.6-82.2) | 3 (0-44) |
| Greece | 59 | 59.3 | 52.4 | 63.0 (30.0-88.0) | 6 (1-15) |
| Iceland | 1 | 100.0 | 100.0 | | |
| Rep. of Ireland | 29 | 86.2 | 51.7 | 67.9 (49.0-82.9) | 3 (1-14) |
| Israel | 37 | 29.7 | 51.4 | 66.7 (34.5-81.7) | 4 (1-38) |
| Norway | 31 | 96.8 | 61.3 | 61.5 (53.8-85.9) | 4 (1-16) |
| Portugal | 65 | 40.6 | 56.3 | 67.3 (17.6-81.9) | 7 (1-52) |
| Sweden | 94 | 94.7 | 53.2 | 69.0 (43.0-82.0) | 4 (1-30) |
| UK | 494 | 75.9 | 50.4 | 66.8 (15.6-94.9) | 4 (0-62) |
| Overall sample | 1037 | 73.8 | 51.1 | 66.7 (15.6-94.9) | 5 (0-38) |

Table 4b: *Basic demographic and clinica characteristics of genetic CJD patients*

| Countries | Number | % Definite | % Female | Median age at onset (range) | Median duration (range) |
|------------------|---------------|-------------------|-----------------|------------------------------------|--------------------------------|
| Belgium | 3 | 66.7 | 66.7 | 51.4 (40-53.7) | 8 (2-14) |
| Denmark | | | | | |
| Finland | 4 | 75.0 | 25.0 | 41.4 (37.4-61.2) | 15.5 (3-52) |
| Greece | | | | | |
| Iceland | | | | | |
| Rep. of Ireland | 1(FFI) | 100.0 | 0.0 | | |
| Israel | 73 | 5.5 | 52.1 | 60.5 (35.0-78.0) | 5 (1-78) |
| Norway | 4 | 75.0 | 50.0 | 78.2 (68.5-81.8) | 5.5 (2-9) |
| Portugal | | | | | |
| Sweden | | | | | |
| UK | 34* | 58.8 | 55.9 | 51.7 (31.7-72.2) | 4 (0-216) |
| Overall sample | 119 | 27.5 | 52.5 | 59.4 (31.7-81.8) | 5 (0-216) |

*Included 9 GSS patients

Basic data concerning the main investigation for sCJD diagnosis (EEG, MRI scan and CSF 14-3-3 analysis) are summarized in Table 5. The most sensitive test was 14-3-3 CSF analysis, which was positive in 91.6% of the sCJD patients. The other two main tests, EEG and MRI, showed less sensitivity than CSF analysis. Periodical complexes in the EEG were present in 44.6% of the sCJD patients and basal ganglia hyperintensity in the MRI scan only in 34.0% of them. Table 5 shows PRNP codon 129 genotypic distribution of sCJD patients. We observe an excess of homozygous cases (80%) with predominance of the methionine/methionine (MM) genotype. There are some variations across countries most likely explained by the fact that the partial sample sizes are low.

Table 5: *Diagnostic investigations in sporadic CJD*

| Country | EEG no data | Positive EEG | 14-3-3 test no data | Positive 14-3-3 | MRI no data | Typical MRI | Codon 129 no data | Codon 129 | | |
|--------------------|----------------|---------------------------|---------------------------|---------------------------|----------------|---------------------------|-------------------------|---------------------------|--------------------------|--------------------------|
| | | | | | | | | MM (%) | MV (%) | VV (%) |
| Belgium | 28 | 42/95 (44.2) | 31 | 88/92 (95.6) | 81 | 29/42 (69.0) | 78 | 32/45 (71.1) | 8/45 (17.8) | 5/45 (1.1) |
| Denmark | 2 | 35/48 (72.9) | 26 | 22/24 (91.7) | 15 | 7/35 (20.0) | 38 | 9/12 (75.0) | 1/12 (8.3) | 2/12 (16.7) |
| Finland | 7 | 37/47 (78.7) | 43 | 10/11 (90.1) | 16 | 23/38 (60.5) | 46 | 6/8 (75.0) | 0/8 (0.0) | 2/8 (25.0) |
| Greece | 19 | 17/40 (42.5) | 6 | 53/53 (100.0) | 30 | 9/29 (31.0) | 59 | 0/0 | 0/0 | 0/0 |
| Iceland | 0 | 1/1 (100.0) | 1 | 0/0 | 0 | 0/1 (0.0) | 0 | 1/1 (100.0) | 0/1 (0.0) | 0/1 (0.0) |
| Rep. of Ireland | 2 | 18/27 (66.7) | 10 | 17/19 (89.5) | 8 | 3/21 (14.3) | 9 | 11/20 (55.0) | 4/20 (20.0) | 5/20 (25.0) |
| Israel | 1 | 15/36 (41.7) | 2 | 32/35 (91.4) | 21 | 1/16 (6.2) | 36 | 0/1 (0.0) | 1/1 (100.0) | 0/1 (0.0) |
| Norway | 10 | 17/21 (80.9) | 21 | 9/10 (90.0) | 31 | 0/0 | 31 | 0/0 | 0/0 | 0/0 |
| Portugal | 5 | 44/60 (73.3) | 22 | 38/43 (88.4) | 17 | 12/48 (25.0) | 40 | 16/25 (64.0) | 5/25 (20.0) | 4/25 (16.0) |
| Sweden | 43 | 29/51 (56.9) | 62 | 25/32 (78.1) | 66 | 7/28 (25.0) | 85 | 6/9 (66.7) | 1/9 (11.1) | 2/9 (22.2) |
| UK | 86 | 117/408 (28.7) | 215 | 254/279 (91.0) | 255 | 78/239 (32.6) | 167 | 195/327 (59.8) | 66/327 (10.1) | 66/327 (10.1) |
| Total | 203 | 372/834 (44.6) | 439 | 548/598 (91.6) | 540 | 169/497 (34.0) | 589 | 276/448 (61.6) | 86/448 (19.2) | 86/448 (19.2) |

The clinical presentations of cases (as defined in Appendix 1) are given in Table 6 and Figure 3. The clinical presentation of sCJD was classified in as many cases as possible and this was possible in 897 out of 1037 (86%) cases (being a little lower at 76%, 411/543, if the UK data are excluded). Difficulties included, for example, clinical notes failing to record the presence or absence of a clinical feature and absent data or insufficient detail on whether a particular feature, like cerebellar ataxia, remained in isolation for the specified period. In these cases, the clinical presentation was recorded as 'missing'. The commonest clinical presentations of sporadic CJD were the rapidly progressive dementia (74%), followed by the ataxic onset (9%). Although much less frequent than the first two, visual presentation (Heidenhein's syndrome) (4%) was also

well recognized. Other clinical presentations, psychiatric (3%), sensory (2%), and extrapyramidal (0.4%) were recognized less frequently. Finally, 6% of the cases presented with a slowly progressive dementia syndrome. The percentages of some of the clinical presentation categories varied significantly across the NEUROCID participants (Table 6). For example, rapidly progressive dementia accounted for only 38% of Greek cases and only 60% of Israeli cases, compared with 96% of cases in Norway and 74% of cases overall. The greatest variation in rapidly progressive dementia, in Greece, mostly paralleled a difference in cerebellar onset (being 38% in Greece, compared to 9% overall).

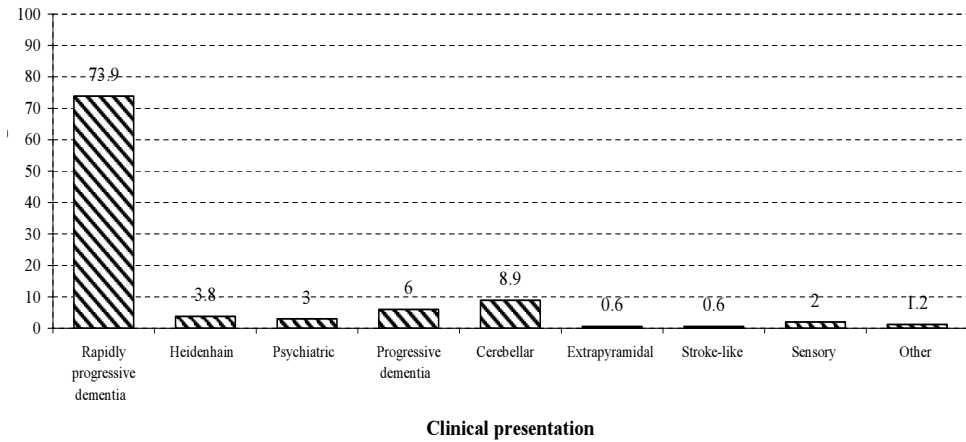


Figure 3: *Clinical presentations of sporadic CJD patients.*

Table 6: *Clinical presentation of sporadic CJD cases*

| Country | N (% of data missing) | “Data quality assessment” | Rapidly progressive dementia (%)* | Heidenhain (%) | Psychiatric (%)* | Progressive dementia (%) | Cerebellar (%)* | Extrapyramidal (%)* | Stroke-like (%)* | Sensory (%) | Other (%)* |
|-----------------|-----------------------|---------------------------|-----------------------------------|----------------|------------------|--------------------------|-----------------|---------------------|------------------|-------------|------------|
| Belgium | 90 (20.4) | yes | 56 (62.2) | 4 (4.4) | 10 (11.1) | 8 (8.9) | 12 (13.3) | 0 | 0 | 0 | 0 |
| Denmark | 44 (12.0) | yes | 35 (79.5) | 3 (6.8) | 0 | 1 (2.3) | 3 (6.8) | 1 (2.3) | 0 | 1 (2.3) | 0 |
| Finland | 46 (14.8) | yes | 32 (69.6) | 1 (2.2) | 1 (2.2) | 1 (2.2) | 6 (13.0) | 0 | 0 | 1 (2.2) | 4 (8.7) |
| Greece | 53 (10.2) | yes | 20 (37.7) | 1 (1.9) | 3 (5.7) | 7 (13.2) | 20 (37.7) | 1 (1.9) | 1 (1.9) | 0 | 0 |
| Iceland | 1 (0.0) | yes | 0 | 0 | 0 | 0 | 0 | 1 (100.0) | 0 | 0 | 0 |
| Rep. of Ireland | 23 (20.7) | yes | 17 (73.9) | 1 (4.3) | 0 | 2 (8.7) | 3 (13.0) | 0 | 0 | 0 | 0 |
| Israel | 37 (0.0) | no | 22 (59.5) | 3 (8.1) | 2 (5.4) | 4 (10.8) | 3 (8.1) | 2 (5.4) | 1 (2.7) | 0 | 0 |
| Norway | 27 (12.9) | no | 26 (96.3) | 0 | 0 | 0 | 0 | 0 | 1 (3.7) | 0 | 0 |
| Portugal | 36 (43.7) | yes | 27 (75.0) | 1 (2.8) | 1 (2.8) | 2 (5.6) | 0 | 0 | 0 | 0 | 5 (13.9) |
| Sweden | 54 (42.5) | yes | 40 (74.1) | 1 (1.9) | 0 | 1 (1.9) | 8 (14.8) | 0 | 2 (3.7) | 1 (1.9) | 1 (1.9) |
| UK | 486 (5.0) | yes | 388 (79.8) | 19 (3.9) | 10 (2.1) | 28 (5.8) | 25 (5.1) | 0 | 0 | 15 (3.1) | 1 (0.2) |
| Total | 897 (22.7) | | 663 (73.9) | 34 (3.8) | 27 (3.0) | 54 (6.0) | 80 (8.9) | 5 (0.6) | 5 (0.6) | 18 (2.0) | 11 (1.2) |

During the study period we ascertained 27 sCJD patients (6% of the total) younger than 50 years at onset, 51 sCJD patients (12% of the total) with disease duration superior to one year, and 69 (6%) sCJD patients with atypical clinical onset. Basic details and investigation results in atypical cases are given in Table 7.

Table 7: Characteristics of atypical sporadic CJD cases

| | N | Duration >1year (%) | Females (%) | Codon 129 genotype | | | Positive 14-3-3 test (%) | Typical EEG (%) | Typical MRI (%) | Pathological confirmation (%) |
|---|----|---------------------------|-----------------|--------------------|----------------|---------------|--------------------------------|--------------------|--------------------|-------------------------------------|
| | | | | MM (%) | MV (%) | VV (%) | | | | |
| Clinical onset below fifties | 27 | 6/23 (26.1) | 13/27 (48.1) | 4/8 (50.0) | 3/8 (37.5) | 1/8 (12.5) | 16/18 (88.9) | 10/23 (43.5) | 6/16 (37.5) | 21/27 (77.8) |
| Atypical presentation* | 66 | 22/53 (41.5) | 33/66 (50.0) | 15/23 (65.2) | 7/23 (30.4) | 1/23 (4.4) | 42/46 (91.3) | 27/60 (45.0) | 15/35 (42.8) | 42/65 (63.6) |
| Atypical presentation below fifties | 6 | 4/5 (80.0) | 4/6 (66.7) | 0/1 (0.0) | 1/1 (100.0) | 0/1 (0.0) | 4/4 (100.0) | 0/5 (0.0) | 1/4 (25.0) | 2/6 (33.3) |

*Clinical presentation other than rapidly progressive dementia, Heidenhaim or cerebellar onset.

Genetic CJD

Genetic cases are very rare. They were identified in Belgium, Finland, Norway, Israel and the UK, accounting for 9% of all TSE cases. Diagnosis was based, whenever possible, on PRNP genetic analysis; a clear family history was not present in all cases. The only underlying mutations identified, in countries other than the UK, were E200K (n=74) and D178N (n=6) (Table 8).

Table 8: Frequency of PRNP genetic mutations by country

| Country | E200K | D178N | Family history | Total | % of all TSE cases | Mortality rate* |
|-----------------|-------|-------|----------------|-------|-----------------------|--------------------|
| Belgium | 1 | 0 | 2 | 3 | 2.4 | 0.04 |
| Denmark | 0 | 0 | 0 | 0 | 0.0 | 0.00 |
| Finland | 1 | 3 | 0 | 4 | 6.9 | 0.10 |
| Greece | 0 | 0 | 0 | 0 | 0.0 | 0.00 |
| Iceland | 0 | 0 | 0 | 0 | 0.0 | 0.00 |
| Rep. of Ireland | 0 | 1 | 0 | 1 | 3.5 | 0.03 |
| Israel | 70 | 2 | 1 | 73 | 66.4 | 1.36 |
| Norway | 2 | 0 | 2 | 4 | 11.4 | 0.11 |
| Portugal | 0 | 0 | 0 | 0 | 0.0 | 0.00 |
| Sweden | 0 | 0 | 0 | 0 | 0.0 | 0.00 |
| UK | 9 | 0 | 16 | 34 | 3.7 | 0.05 |
| Total | 83 | 6 | 21 | 119 | 9.0 | 0.11 |

*Deaths per million inhabitants/year

Iatrogenic CJD

In countries other than the UK, there was only one iatrogenic case identified in the 8 year period, in the Republic of Ireland. This was due to cadaveric-derived human growth hormone, in a male, aged 37 years at onset, presenting with cerebellar ataxia and dying in 2001, with neuropathological confirmation of CJD.

Variant CJD

In the NEUROCID zone one variant case was identified in the period 1997-2004, in the Republic of Ireland. The individual was female, PRNP codon 129-MM, dying in 1999 after an illness duration of 7 months. Because this individual had spent a significant period of time living in the UK during the relevant UK dietary risk period, it was considered that she had actually contracted the illness while in the UK. In the UK 135 deaths of vCJD were registered during the study period.

Discussion

The surveillance of CJD poses certain problems that are particular (although not unique) to this disease. Firstly, CJD is rare with even the commonest form (sCJD) having an annual mortality rate of only around 1/million/ year. Therefore, it can be difficult to maintain awareness and interest in the disease.^{2,8} However, the identification of variant CJD stimulated intense general and medical interest in prion diseases. Secondly, there are no validated simple, non-invasive, pre-mortem diagnostic tests. Cases should be definitively detected at autopsy, but not all suspect cases have autopsies, reflecting different cultural and medical practices. Case ascertainment based solely on autopsy confirmation is likely to be incomplete. Cerebral biopsy is invasive, requires particular precautions, and is not usually necessary on strictly clinical grounds.

Clinical diagnostic criteria have been established and validated, especially for sporadic and variant forms of CJD, but their appropriate and reliable application requires the involvement of suitably experienced clinicians.⁷ The necessary clinical experience or laboratory techniques (such as CSF 14-3-3 analysis) may be difficult to obtain in a country with a relatively small population and, hence, a small number of cases. However, collaborative systems, such as NEUROCID, are designed to help with such problems, by sharing of expertise and common provision of laboratory tests.

It should be emphasized that the reliable and complete identification of cases of vCJD requires the identification of all types of CJD. The differential diagnosis may differ across populations. In the UK, the most important differential diagnosis of vCJD is sCJD, while in the remaining countries is other non-CJD dementias. Atypical cases of sCJD may affect uncharacteristically young individuals, have prolonged duration and present with atypical symptoms, all of which may suggest the possibility of vCJD.⁹

NEUROCID is based on each country's individual surveillance systems. The available funding and facilities varies from country to country with different geographical, cultural or medical system factors dictating the need for different surveillance approaches. Methods applicable in a densely populated, geographically

small country, with a relatively small neuroscience community, like the UK, may not be ideal in a country with a larger geographical area, a smaller population and a different neurological service structure. Three main medical specialities are usually involved in CJD surveillance: epidemiologists/public health doctors, clinical neurologists and neuropathologists. Some involvement of all of these disciplines is essential at some point of case evaluation, but the primary responsibility for the surveillance system varies from country to country (see Table 1). For example, in the UK, the primary point of notification is to clinical neurologists, an attempt is made to see all individual cases in life, and neuropathology is an integral aspect of the system. In Belgium, the primary method of case ascertainment is through epidemiology systems with neuropathology. Clinical neurologists are involved, but individual cases are not seen in life by the surveillance system. There are three broad methods of clinical data collection. Firstly, the CJD surveillance system (CJDSS) may obtain them from routine clinical case records. Secondly, they may request special forms to be completed by clinicians, relatives and others. Finally, a CJDSS clinician may personally assess the suspect case, including a direct interview with the relatives. General clinical experience and anecdotal data strongly suggest that the last method is likely to yield the most complete, detailed and reliable information. However, this is a relatively expensive method; involving the employment of a suitably trained clinician and travel over a potentially wide area. In five of the NEUROCJD countries, cases are not seen in life by the system and only one country (the UK) has a definitive policy of visiting all suspect cases when possible. While visiting cases should increase the quality of the diagnosis and clinically diagnosed patients, may encourage referral of atypical cases and allows the collection of more detailed data, it may not be vital for simple case ascertainment (as the relative uniformity of data in Table 2 and Table 3 suggest).

Notification & Sporadic CJD Mortality Rates

The actual notification criteria and routes vary from all clinically suspect cases, through neuropathologically confirmed cases, to death certificate registers, with some countries employing multiple methods (Table 1). In some countries, the criterion is simply 'all suspect cases' without further definition. There are, of course, potential difficulties in trying to precisely define notification criteria. Any very general clinical criteria such as 'atypical dementias' or 'neuro-psychiatric disease in the young' may lead to unmanageable numbers of referrals. If the primary notifiers are clinical neurologists, it should be possible to base notification criteria on sensible clinical judgment. On the other hand, very specific criteria, based on previous experience, may overlook atypical cases or those without autopsy. Unless all cases undergo cerebral biopsy and/or autopsy, notification based purely on neuropathological criteria will, necessarily, overlook cases. The available autopsy rates are given in Table 2. Case identification based on death certification may lead to detection of cases not notified in life, but is likely to be unreliable. Notification based on investigation reports and results has obvious problems in the absence of a definitive diagnostic test (this issue is discussed further below). For

a rare disease like CJD, especially if one wishes to detect atypical cases, multiple, overlapping methods of case ascertainment are advisable.

The overall annual mortality rate of sCJD was 1.0 per million for the 11 countries during 1997-2004. There was some variation over time (Figure 1), but this would be expected especially in countries with relatively small populations and, after fitting a Poisson regression model, the overall differences in mortality across the study years were not statistically significant ($p=0.42$). There was variation between countries (Table 3) and there was one country (Belgium) and two countries (Greece and Portugal) with high and low SMRs respectively. However, the Poisson regression analysis did not suggest any significant difference in the annual mortality rates of the 11 countries.

In this collaborative study, there is no clear indication that one form of notification system is inherently superior to another. The lack of mandatory formal notification is not associated with relatively lower incidence figures. Indeed, in Belgium, the annual mortality rate (AMR) and SMR are relatively high despite voluntary notification and both Greece and Portugal, with mandatory notification, have relatively low AMRs and SMRs. In countries that introduced formal notification during the 1997-2004 period, there is no evidence of a subsequent rise or fall in numbers of cases identified.

The age-specific mortality rates for males and females are given in Figure 2. The overall profile is generally similar to that reported in other countries, with the highest rates in the 60-79 age band and a fall in the later age band.^{8,10} The significance of this age profile is uncertain. In particular, it is unclear if there is under ascertainment in the elderly. However, whatever the explanation, it appears to affect all countries, both within, and without, NEUROCID in a similar way. The sex ratio is almost 50:50, comparable to data from other countries, although some previous reports have shown a slight female preponderance.^{2,8} The latest data from EUROCID, which is monitoring CJD in a larger population, is around 50:50 (personal communication Sánchez-Juan).

Clinical Presentation of sCJD

As discussed previously, the clinical data collection policy is not homogeneous in all NEUROCID countries. This fact resulted in incomplete data sets on clinical data for many countries and also may account for some of the variation in findings between countries.

The clinical presentation categories were defined after discussion at the early meetings of the NEUROCID group and reflected the known clinical profile of sCJD and unusual features that might cause diagnostic difficulty, including with respect to differentiating from vCJD (Table 6). In general, the categories were based on identifying the typical 'rapidly progressive dementia' cases and those characterized by initial specific features (that typically, later, progress into more general brain involvement). The final definitions of these categories inevitably included some relatively arbitrary elements (for example, the required duration of pure cerebellar features in the definition of a 'cerebellar onset' case). However, they were based on the considerable previous clinical experience of the study participants. The complete definitions are given in Appendix 1.

A few points should be emphasized. Firstly, the category of 'rapidly progressive dementia' covers the typical presentation of sCJD and, while dementia is a vital component, other neurological features are commonly associated and, in retrospect, a better term might have been 'rapidly progressive encephalopathy'. Secondly, while cerebellar features are very common in the early phase of sCJD, the 'cerebellar' category was specifically defined as only those cases with a progressive pure cerebellar syndrome, without other features, for a period of at least 2 weeks. Thirdly, visual symptoms and cortical blindness are relatively common features in the course of sCJD, but 'Heidenhain's' presentation was specifically defined as only those cases with progressive pure visual features for at least 2 weeks. Finally, the 'progressive dementia' category was defined as those cases with a relatively slowly progressive cognitive impairment (without other specific neurological features) that might therefore suggest dementia diagnoses other than CJD.

The overall most common presentations were, unsurprisingly, a rapidly progressive dementia and a cerebellar onset, accounting overall for 74% and 9% of sporadic cases, respectively. Cerebellar onset (which has been termed the Brownell-Oppenheimer form of sCJD) is well recognized and can pose initial diagnostic difficulties as there a number of causes of a progressive isolated cerebellar syndrome.^{11,12} The precise frequency of this presentation has not been clearly established. Cerebellar features are relatively common initial or early accompaniments of the typical rapidly progressive encephalopathy so characteristic of sCJD, but, in this study, we wanted to specifically delineate the pure cerebellar onset cases.¹³ Heidenhain's syndrome, another well recognized form of sCJD, is rare but the true frequency has been uncertain; it accounted for only 4% of cases in NEUROCID.¹⁴ Visual symptoms and eventual cortical blindness are not uncommon in typical sCJD, but we concentrated on those cases with a progressive pure visual problem which can pose diagnostic problems and may lead to initial referral to ophthalmology services. Presentations with psychiatric or sensory features, that might cause differential diagnostic difficulty with vCJD, accounted overall for only 3% and 2% of presentations respectively. Progressive dementia cases (as defined here), with their relatively slow clinical course and the absence of early non-dementia features may be particularly difficult to differentiate from other more common diseases. Rather surprisingly, as we expected them to be exceptionally rare, they represented 6% of all cases. However, this amounts to only 54 such cases in a total population of around 133 million, over an 8 year period. Extra-pyramidal and stroke-like presentations were indeed very rare (each being only 0.6% of cases). Stroke-like presentation was reported in 5.6% of 532 cases of definite and probable sporadic CJD in the UK but, this was not confirmed in our cases, prospectively applying an agreed definition¹⁵ (Appendix 1).

The significance of different presenting modes of sCJD is uncertain. Of course, if sCJD results from a spontaneous somatic PRNP mutation or spontaneous PrP protein misfolding, then it is reasonable to expect a variety of presentations reflecting random anatomical distributions of the original event (although this would not, in itself, explain why certain specific presentations are commoner than others). Following Parchi and

colleagues, the clinical features of sCJD can be correlated, to some extent, with the associated PRNP codon 129 polymorphism and the PrP protein type.¹⁶ However, this does not explain the clinical variations and we do not have sufficiently complete data (especially with respect to PrP type) in NEUROCID to classify the cases in this manner. Different presentations could conceivably reflect different causes and two recent studies have reported that surgical procedures are a risk factor for sCJD, suggesting that perhaps at least some sCJD cases might be iatrogenically acquired.^{17,18} Therefore, the observed differences in presenting patterns between countries could be of interest.

The presentation definitions were agreed by all participants, discussed regularly at collaborative group meetings and their application reviewed, on a case by case basis, by two collaborators (PSJ & RK) prior to the final results analysis. However, it is difficult to be absolutely certain that these variations are definite, especially given the variations in source material (such as case note review versus direct examination). Further study would be necessary to confirm these variations.

In 10 cases (1.3% of all sCJD cases from the 11 countries, including the UK), the clinical presentation was classified as 'other'. This group included the following presenting features: an isolated progressive hemiparesis, focal seizures, and isolated dysphasia. These represent very exceptional modes of presentation of sCJD.

Atypical sCJD

'Atypical sCJD' cases were considered according to three criteria: age at onset <50 years (27 cases), illness duration >1 year (51 cases), and 'atypical clinical presentation', being defined as any presentation other than rapidly progressive dementia, cerebellar or Heidenhein's, (66cases) (Table 7). Onset of sCJD below the age of 50 is a relatively rare phenomenon, our figure of 27 out of 439 (6%) with available information (UK data excluded) being in line with previously reported figures and the latest EUROCID comparative figure is 5%^{2,8} (personal communication Sánchez-Juan).

The median duration of illness in this study was 5 months and only 51 out of 418 (12.2%) with available information (UK data excluded) had illness durations of greater than 1 year, broadly in line with other reports.^{9,19}

Of the 66 that were 'atypical' by virtue of their clinical presentation, illness duration data were available on 53 and, of these 53, 22 had durations of >1 year. If the 'atypical' cases presenting with progressive dementia are excluded, then only 10 of them had durations >1 year. Data on age at onset were available on 56 and, of these 56, only 6 were <50 years at onset. There were 6 cases in total who were both <50 years at onset and had illness durations of >1 year; representing 6 such cases out of a total population of around 133 million over 7 years.

It is obviously important to have neuropathological confirmation particularly in clinically atypical cases. This was achieved in 78% of those with onset under 50 years and in 64% of those with atypical clinical presentations; all the cases met the agreed criteria for 'probable sCJD'.⁷

Males and females were represented equally in the 'atypical' cases, although females were apparently over-represented in those cases with both an atypical presentation and young age at onset, there were only 6 such cases in total. The distribution of PRNP codon 129 genotypes in cases with illness onset below 50 years, showed a relative reduction of MM and a relative increase in MV (compared with the usual distribution for sCJD). This is in line with previous reports.⁹ However, the numbers here are too small for statistical significance ($p=0.49$), with only 8 of the <50 years onset group being genotyped. The investigation results in this group are discussed below.

Genetic CJD

Overall, genetic cases accounted for 9% of all TSE cases, which is a slightly lower figure than that generally reported.²⁰ There was a striking variation between the member countries. Five countries reported no cases, in 3 countries genetic cases represented from 2.4% to 3.7% of all cases (Belgium, Republic of Ireland, UK), in Finland the figure was 6.9%, in Norway 11.4% and in Israel, 66.4% (Table 8). Israel was already recognized to have a high incidence of genetic cases.²¹ Although the differences among countries were highly significant ($p<0.001$) there may represent the presence of founder mutations. PRNP genotyping is required for the absolute distinction of gCJD from sCJD as a positive family history is not always present in genetic cases.²⁰ This can be performed on a simple blood sample and it also allows for the PRNP codon 129 genotype to be determined, which may be important in the characterization of atypical cases, and for research purposes. Genetic analysis is available in 6 countries. In other countries, samples have been analyzed through the collaborative project, where necessary and possible.

Variant CJD

One important aim of the NEUROCID collaboration was the identification of any cases of vCJD in the 10 non-UK countries. It is, of course, impossible to state with absolute confidence that no cases of vCJD were missed in the 1997-2004 period. However, with the individual surveillance systems operating, the collaborative mechanisms of NEUROCID and the discussion of any atypical cases, there is a high degree of confidence that vCJD did not occur in any of the 11 countries outside of the UK and Ireland. The single case in Ireland is detailed in the results section above.

This analysis relates to the 1997-2004 period, but in 2005, a case of vCJD was identified in Portugal; the Portuguese surveillance system and the NEUROCID collaboration successfully identifying the case. A further three cases have been identified in Ireland since 2004; one case considered as contracting the illness while in the UK, but two cases being regarded as truly intrinsic to Ireland.

Investigations

The EEG has been an important part of the investigation of suspect CJD cases, mainly because a characteristic periodic pattern is seen in the majority, but not all, of sporadic

cases. This finding has been incorporated into the internationally accepted clinical diagnostic criteria.^{7,22,23} In practice, the EEG has become significantly less important in case classification since the incorporation of the CSF 14-3-3 test into the diagnostic criteria. In our study, the EEG showed the characteristic changes in around 45% of all sporadic cases (Table 5) which is broadly comparable with figures from other studies. In a study of 29 cases from Germany, with repeated EEGs being undertaken, periodic activity was found in 67%.²² In a further study from a grouping of countries involving over 2000 cases, the EEG showed such changes in 58.4%.²³ There are variations between the collaborating countries from 30% to 81% of cases with typical EEGs (ignoring Iceland, with only one case in total). This almost certainly reflects different testing policies, repeat EEGs being undertaken with different frequencies in different countries, partly according to how much reliance is placed on EEG rather than CSF 14-3-3. The EEG is generally less useful in atypical CJD so it is notable that characteristic changes were seen in about the same percentage of our 'atypical' cases, at 44% (Table 7) (although in none of the 5 cases with both atypical presentation and young age of onset).^{16,23}

CSF 14-3-3 has been found to be sensitive and specific for sCJD (provided testing is undertaken in a carefully selected context).^{23,24} 14-3-3 testing has been established in most of the NEUROCJD countries. For small population countries, with relatively few suspect cases, it is possible for samples to be sent to laboratories elsewhere in particularly important cases. 14-3-3 testing has potential technical difficulties and it is important that any laboratory providing such a service should have sufficient numbers of samples to gain and maintain experience. Laboratories therefore tend to be established on a national basis (as is true for the NEUROCJD countries). Exchange of samples and comparison of experience is important to maintain standards. The NEUROCJD collaboration has allowed and encouraged these practices. Referral of cases for 14-3-3 testing is, of course, another means of case ascertainment. Within NEUROCJD, close collaboration between the laboratory service and the surveillance system has proved invaluable. 14-3-3 was positive in 92% of our cases (Table 5), overall which is in line with previous reports.²⁴ There was some statistically significant variation between countries ($p=0.04$), with the lowest figure being 78% in Sweden. Interestingly, the 14-3-3 test was similarly useful in our 'atypical' cases, with around 90% showing positive results (Table 7).

A vital role of the **MRI** in the investigation of suspect cases is in the exclusion of other possible diagnoses. In sCJD, certain relatively specific features have been described, but the MRI has not yet been incorporated into the standard diagnostic criteria.^{25,26} In variant CJD, however, very characteristic changes have been found in around 85% of cases.²⁷ The changes may be relatively difficult to reliably identify and requires an update of the radiologist. An international collaboration allows for more experienced countries to review suspect scans in situations of uncertainty. Overall, 34% of our sCJD MRI scans showed characteristic changes, although there were highly significant variations

($p < 0.001$) between countries, from 6-69% of cases (Table 5). These variations in sensitivities will reflect the use of different MRI sequences, differential policies concerning repeat studies in individual cases and the increased understanding over time of the changes characteristic of sCJD.

In this article we summarize the design and characteristics of the extended collaborative study group of CJD 'NEUROCID', and the results of their monitoring activities during an 8-years period. Relevant methodological issues and the main problems encountered are discussed. We describe our experience and results focusing on the clinical presentation of sCJD and the atypical subtypes. VCJD cases in Ireland and in Portugal were successfully detected by their national surveillance systems.

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Appendix: classification of presenting symptoms

The following is an attempt to classify the different modes of presentation. Since it may not be possible to accurately classify a particular case, if the case does not clearly fit one of the specified categories then the code 8 'missing' value should be used.

Code 0: rapidly progressive dementia

The majority of cases will probably be in this category. The precise presenting symptom will vary from case to case, but the picture is an encephalopathic illness with dementia (and other neurological features), progressing rapidly over weeks to a few months, with no individual cognitive or physical deficit being present alone for more than two weeks.

Code 1: slowly progressive dementia

These cases present with a slowly progressive dementia, developing over months to years, without any other significant neurological features for the first six months.

Code 2: cortical blindness (Heidenhain)

These cases present with impairment of visual acuity and/or field, progressing onto cortical blindness, without other significant clinical deficit for the first two weeks of illness. The visual symptoms might include visual loss, visual inattention, visual illusions and visual hallucinations. It is essential that the symptoms progress to cortical blindness. Cases with other onsets that progress to include cortical blindness are not included in this category.

Code 3: psychiatric onset

These cases present with psychiatric symptoms such as depression, anxiety, paranoia, and delusions, without the presence of other features for a period of at least four weeks. Non-specific malaise or apathy do not count unless accompanied by some of the above symptoms. Visual or auditory hallucinations alone do not count, but may accompany the above features.

It may be difficult to distinguish between the early features of dementia and a more specifically psychiatric onset. Behavioural change straightforwardly due to a developing dementia is not included in this category. The essential characteristic of this presentation is that the patients present with a disturbance that suggests a psychiatric disturbance rather than an obvious dementia and that specifically neurological features are absent.

Code 4: cerebellar onset

The presentation is with a progressive cerebellar syndrome without other significant features, for at least two weeks.

Code 5: extrapyramidal onset

The presentation is with an extrapyramidal syndrome involving Parkinsonian features with or without chorea, athetosis or dystonia, but without other significant features for at least two weeks.

Code 6: stroke-like onset

The presentation is abrupt enough for a diagnosis of stroke to be entertained in the initial stages.

Code 7: other

None of the above described presentations is applicable.

Code 8: missing

There is no clear clinical information available or the information does not allow a definite classification according to the above criteria.

2.2

The first case of variant Creutzfeldt-Jakob disease in the Netherlands

Abstract

As of July 2006, 191 cases of variant Creutzfeldt-Jakob (vCJD) disease have been identified. We report the first case of vCJD in the Netherlands. The patient was a young Dutch woman who had never traveled to the United Kingdom (UK). The brain MRI scan played a fundamental role in the clinical diagnosis. After death the diagnosis was confirmed by neuropathological examination and western blot analysis of the brain. The clinical characteristics of the patient, MRI scan images, neuropathological study, PrP^{Sc} western blot profile and PRNP M129V genotype, were all indistinguishable from that reported in UK vCJD patients. The identification of this patient had important public health implications for the country.

Introduction

In 1996, a new variant of Creutzfeldt-Jakob disease (vCJD) linked to the cattle bovine spongiform encephalopathy (BSE) epidemic was described in the United Kingdom (UK).^{1,2} Up until now, 191 cases have been reported worldwide. Most of these cases have occurred in the UK (n=159), but in recent years vCJD has been identified in a number of European countries with indigenous outbreaks of BSE, including 18 cases in France, four in Ireland, two in the Netherlands and single cases in Portugal, Italy and Spain. However, a growing number of cases of vCJD have also been identified in countries outside Europe including Japan (n=1), the United States (n=2) and Canada (n=1) and also in Saudi Arabia (n=1), in which BSE has not been identified. A number of the non-UK cases, including two Irish, the Canadian, the two US cases, and possibly the Japanese case, may well have been infected during periods of residence in the UK, but the majority of cases (n=25) occurring outside the UK, like the two cases happened in the Netherlands, had never visited the UK.³ In this study we describe the first patient identified with vCJD in the Netherlands.⁴

Case report

The patient is a 26-year-old woman with previous medical history of two abortions in 1996 and in 1998 and psychiatric history of anxiety and a phobic disorder since 1999. However, one year previous to the vCJD disease onset, she had completely recovered from her psychiatric problems and was able to function normally. She worked in a catering and food-processing business for 6 years, from 1998 to the disease onset. She consumed all types of meat frequently, including raw meat, and particularly processed meat products. She had no history of neurosurgical procedures, hormonal treatments, or tissue or organ grafts, and she had never received or donated blood. She never traveled to the UK. She had no family history of dementia or any other neurodegenerative disorder.

From November 2003, the patient developed new psychiatric symptoms, particularly anxiety and aggressive behavior. In spring 2004, the family also noticed some forgetfulness. A few months later, the psychiatric symptoms worsened. She suffered from panic attacks, became very dependent and apathetic, and showed regressive behavior. She also had visual hallucinations in which she saw animals. In July 2004, two weeks after a dental extraction, she started complaining of severe facial pain in her upper jaw. Her dentist examined her but no organic cause was found. At that point she was seen by a psychiatrist who suspected that she was suffering from anxiety and a conversion disorder.

In September 2004, the patient developed involuntary movements of her right foot, with dystonic eversion-inversion posturing and occasionally some jerking movements. She also developed paraesthesiae in the lower limbs and gait difficulties, especially

when walking in the dark. In November, the family also noticed dysarthria and problems in the motor coordination of the upper limbs. She had several episodes of blurring of vision. During this period, she was twice hospitalized in a psychiatric ward because of increasing anxiety, regressive behavior and facial pain.

From January to March 2005 the involuntary movements worsened significantly, accompanied by generalized stiffness, now involving all extremities. In March the patient was again hospitalized in a psychiatric ward with the clinical diagnosis of a conversion disorder, and treatment with Haloperidol was started. Two days later she suffered a tonic-clonic seizure followed by hyperthermia that required admission to an intensive care department. No cause was found for the persistent high temperature, and the neuroleptic medication was withdrawn. The patient was treated for malignant neuroleptic syndrome without success. The neurological state of the patient worsened rapidly.

On neurological examination she was mute, opened her eyes to verbal stimuli but there was no visual fixation. She had variable extrapyramidal rigidity in the extremities with dystonic movements of the hands and feet, and she had a positive Babinski sign in her right foot. Routine laboratory examinations only revealed mild increases of the aspartate aminotransferase (ASAT) and creatine phosphokinase (CPK), which later normalized. The cerebrospinal fluid was normal, and 14-3-3 protein was not detectable. Tau protein determination in CSF was performed after the referral of the patient to the CJD registry, and it showed abnormally high levels (1314 ng/l). The electroencephalogram showed generalized slowing, more evident in the left hemisphere, but without any periodic complexes. Brain MRI showed symmetrical hyperintensities in the pulvinar and the dorsomedial nuclei of the thalami on T₂- and fluid-attenuated inversion recovery (FLAIR)-weighted images (Figure 1). Genotyping of the prion protein gene (PRNP) identified no mutation and homozygosity for methionine at codon 129. Following the current WHO criteria, the patient was diagnosed in life with probable vCJD based on the clinical features, laboratory findings, brain MRI images and PRNP genotyping.⁵ In May 2005 the patient died, and the diagnosis was confirmed by post mortem neuropathological examination of the brain, which showed typical features of vCJD, including florid plaques (Figure 2). Western blot analysis of unfixed brain tissue showed a characteristic type 2B PrP^{Sc} isoform (Figure 3).

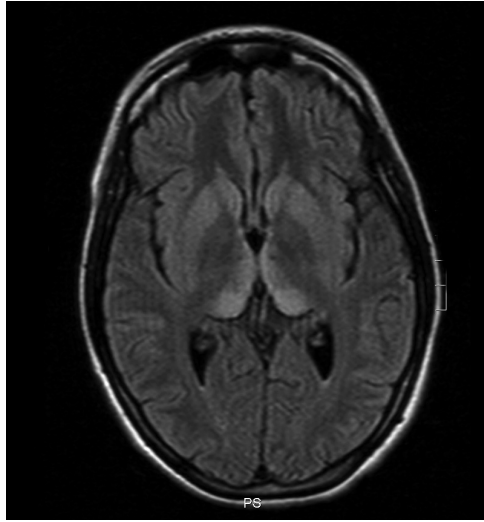


Figure 1: FLAIR-weighted images of the brain MRI study, showing bilateral symmetrical regions of hyperintensity in the pulvinar and the dorsomedial nuclei of the thalami ('hockey stick sign').

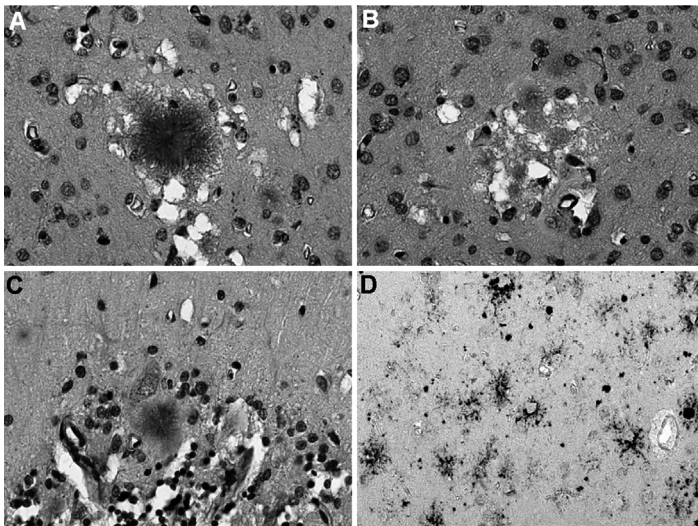


Figure 2: Neuropathology of variant CJD:

- A. A florid plaque surrounded by small areas of spongiform change in the occipital cortex (Luxol-PAS stain).
- B. Multiple small cluster plaques in the occipital cortex (Luxol-PAS stain).
- C. A kuru-type plaque in the cerebellar cortex (Luxol-PAS stain).
- D. Immunohistochemistry for prion protein (using monoclonal antibody 3F4) shows strong staining of plaques in the occipital cortex, along with amorphous and pericellular deposits.

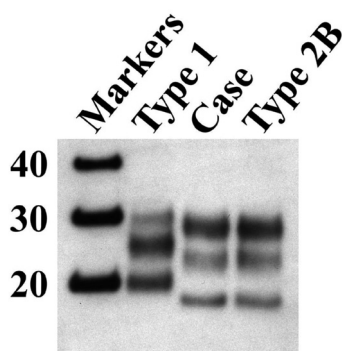


Figure 3: Western blot analysis of protease-resistant prion protein in this patient (Case) shows an identical pattern of glycosylation and size of the unglycosylated fragment (lowest band ~ 19Kd) to a control case of vCJD (Type 2B), which is markedly different from a case of sporadic CJD (Type 1). (Reproduced with permission of Prof. Dr. A.J.P.M. Overbeke editor of *NTVG*).

Discussion

Non-UK cases of vCJD may potentially have been infected during a period of residence in the UK in the 1980s and early 1990s (for example the US and Canadian cases). Our patient had never traveled to the UK, but may have been exposed through either indigenous BSE infected animals or BSE infected exports from the UK to the Netherlands during the 1980s and early 1990s. Thus far, all non-UK cases of vCJD who had never previously visited the UK show disease characteristics analogous to the UK cases. The clinical features, PRNP codon 129 genotype, neuropathological findings and western blot PrP^{Sc} isoform of our patient are indistinguishable from the United Kingdom vCJD cases, pointing towards a common etiological agent.

The Netherlands CJD surveillance system is part the EURO-CJD group, a EU-funded network established in 1993 with the aim of monitoring the disease incidence and detecting new vCJD cases.⁶ In the Netherlands, only 80 BSE cases have been reported.⁷ The first case of variant CJD diagnosed in the Netherlands highlights the importance of continuing multinational surveillance of human prion diseases, even in countries with a low BSE incidence, as a matter of fact, during the preparation of this manuscript a second probable vCJD case has been ascertained in our country.⁸

The early detection of vCJD patients is crucial as there are major public health implications, including of tracing of blood products from patients who were blood donors and tracing of surgical instruments, in this case dental instruments. Despite of a long disease course (18 months) the diagnosis in this patient was particularly difficult due to the past psychiatric history. The brain MRI findings played a crucial role in the diagnosis by the treating neurologist (J.I.H.). This triggered the notification to the CJD registry in Rotterdam. This case report underscores the value of the MRI in the diagnosis

of vCJD and the need for an increased awareness of radiologists and neurologists of the MRI findings in vCJD, including in countries with a low incidence of BSE.

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3

**Aetiological aspects of
Creutzfeldt-Jakob disease**

3.1

What is the source of variant Creutzfeldt-Jakob disease outside the UK?

Abstract

A growing number of cases of variant Creutzfeldt-Jakob disease (vCJD) in countries outside the United Kingdom (UK) are being identified. The correlation between the number of vCJD cases and that of indigenous bovine spongiform encephalopathy (BSE) is low, raising the question whether the spread of vCJD is rather due to import products from the UK. We have analysed the occurrence of vCJD cases outside the UK in relation to the level of imported bovines and bovine products from the UK from 1980 to 1990. Our studies suggest that the numbers of live bovine imported from the UK correlates to the occurrence of vCJD cases outside the UK as good if not better than that with indigenous BSE cases. An important lesson from a public health perspective is that imports of livestock and bovine products from the UK have contributed substantially to a global spread of vCJD.

Introduction

In 1996 a new variant of Creutzfeldt-Jakob disease (vCJD) was described in the United Kingdom (UK).¹ By August 24th 2006, 196 cases had been reported worldwide, with the majority (83%) (n=162) occurring in the UK. Laboratory and epidemiological studies have yielded convincing evidence for a causal link between vCJD and the bovine spongiform encephalopathy (BSE) epizootic in cattle^{2,3} with the likely route of primary human infection through dietary exposure to bovine tissues containing high levels of infectivity.³

In recent years vCJD has been identified in a number European countries with indigenous outbreaks of BSE, including 20 cases in France, four in Ireland, two in the Netherlands and single cases in Portugal, Italy and Spain. However, a growing number of cases of vCJD have also been identified in countries outside Europe with a minimal incidence of BSE including Japan, the United States and Canada and also in Saudi Arabia, in which BSE has not been identified.⁴ A number of the non-UK cases, notably two Irish, one Canadian and two US cases, and possibly the Japanese case, may have been infected during periods of residence in the UK, but the majority of cases (28 out of 34) occurring outside the UK had never visited the UK. On the other hand, although vCJD cases have occurred in countries with very low incidence of BSE, no cases have been reported in countries with higher incidence of BSE such as Switzerland (460 reported cases of BSE) and Germany (395 cases).⁵ Thus, there is a rather poor correlation between the incidence of indigenous BSE and the incidence of vCJD in some countries, raising questions as to the source of infection in the cases outside the UK. Were they exposed to infectivity derived from indigenous cases of BSE or to infected bovine material imported from the UK? An analysis of infection risk in France suggests that the most likely source of vCJD in that country is the import of infected material from the UK,⁶ while in the Republic of Ireland the transmission of BSE to humans was estimated to be equally likely from indigenous BSE or from UK imports.⁷ Given the apparently weak association between the occurrence of indigenous BSE and vCJD in some countries, we studied the occurrence of vCJD cases outside the UK in relation to the level of imported bovines and bovine products from the UK from 1980 to 1990.

Methods

An EU surveillance network, established in 1993, assures prospective surveillance for CJD using standard methods⁸ in 18 European countries and the USA, Canada, Israel and Australia. By 24th August 2006, 32 cases of vCJD had been identified in these countries (excluding the UK) (Table 1). A further two cases had been identified in countries outside the Surveillance network (in Japan and Saudi Arabia). Cases in Canada (n=1), the USA (n=2), Ireland (n=2), and possibly Japan (n=1) were considered as likely to have been infected during their period of residence in the UK.

Table 1: *Worldwide vCJD cases up to August 2006*

| Country of residence at disease onset | N |
|--|----------|
| UK | 162 |
| France | 20 |
| Rep. of Ireland | 2+2* |
| Italy | 1 |
| USA | 2* |
| Canada | 1* |
| Saudi Arabia | 1 |
| Japan | 1* |
| Netherlands | 2 |
| Portugal | 1 |
| Spain | 1 |

*Most likely exposed to BSE in the UK.

Data on indigenous BSE cases detected by both passive and active surveillance were obtained from the World Organization for Animal Health web page (http://www.oie.int/eng/info/en_esbmonde.htm).⁵ The number of live cattle and the tonnage of carcass meat exported from the UK were derived from UK Custom and Excise Data.⁹

We have included in our analysis all countries covered by the EU surveillance network. As the exposure was expressed as a total amount rather than per capita, we performed the analysis based on number of vCJD cases rather than rates. We plotted the incidence of vCJD (number of cases) by country against (1) the number of cases of indigenous BSE, (2) the numbers of live bovines imported from the UK from 1980 to 1990 and (3) the tonnage of carcass meat imported from the UK during the same period of time. In the analysis we included only the non-UK vCJD cases that are thought likely to have been infected outside the UK. A factor likely to be important for BSE exposure is the temporal distribution of UK exports. It has been estimated that the number of BSE infected cattle entering the human food supply peaked in the UK around 1989,⁷ although with respect to exports peak exposure may have been later, around 1992-1993. For example, although Germany imported the second greatest tonnage of carcass meat globally, 85% of these imports were before 1988. In contrast, 70% of the live stock

imports to the Netherlands were between 1987 and 1990. To account for this factor we weighted the number of live bovines and the carcass meat tonnage imported each year by the size of the UK BSE epizootic that year (reported clinical cases). Non-parametric Spearman's rank correlation coefficients (r_s) were calculated to evaluate whether there was evidence of a correlation between exposure and outcome.

In order to assess the independent effects of each of the exposure sources we fitted a Poisson regression model with number of cases of indigenous BSE, numbers of live bovines imported from the UK, and the tonnage of carcass meat imported from the UK, the last two variables with the weighting described above. We included in the Poisson analysis all EU network countries.

Results

Figure 1 shows a scatter plot of the number of cases of indigenous BSE in non-UK countries and the number of non-UK vCJD cases per country. There is strong evidence of a correlation between these two variables ($r_s=0.7$; 95% CI 0.37 to 0.87; $p=0.001$) in the countries belonging to the EU network (Table 2). However, when we include in our analysis Japan and Saudi Arabia as well, the only two countries outside the EU network where vCJD cases have been detected, the correlation coefficient drops substantially ($r_s=0.55$; 95% CI 0.17 to 0.79; $p=0.008$) (Table 2). Figure 2 shows a scatter plot of live bovine imports from the UK and non-UK vCJD cases by country. The correlation between these two variables is high in EU network countries ($r_s=0.73$; 95% CI=0.42 to 0.89; $p<0.001$). Including Japan and Saudi Arabia results in a slightly lower estimate ($r_s=0.65$; 95% CI=0.31 to 0.84; $p=0.001$) (Table 2). Figure 3 shows a scatter plot of the tonnage of carcass meat imports from the UK and the number of non-UK vCJD cases by country. The correlation between these two variables is also high in EU network countries ($r_s=0.65$; 95% CI=0.29 to 0.85; $p=0.002$). When we include Japan and Saudi Arabia in the analysis, we obtain very similar results ($r_s=0.67$; 95% CI=0.35 to 0.85; $p=0.001$) (Table 2).

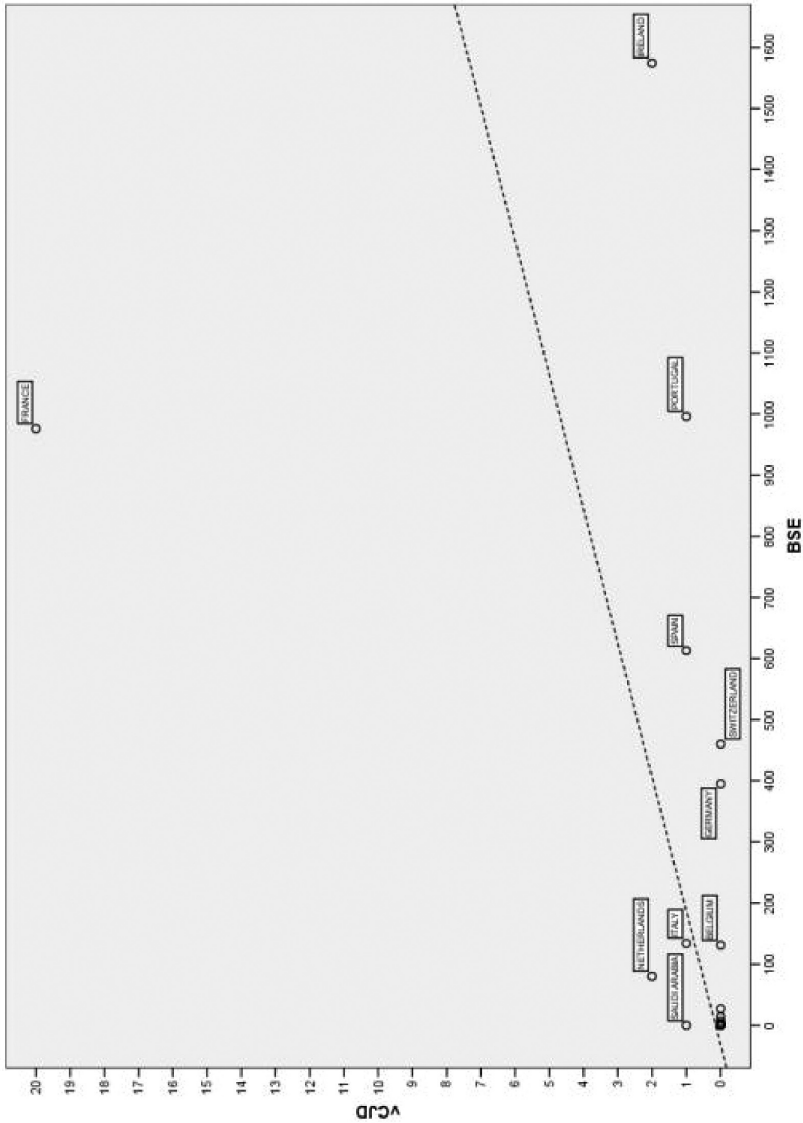


Figure 1: Scatter plot of the number of cases of indigenous BSE in non-UK countries and the number of non-UK vCJD cases per country.

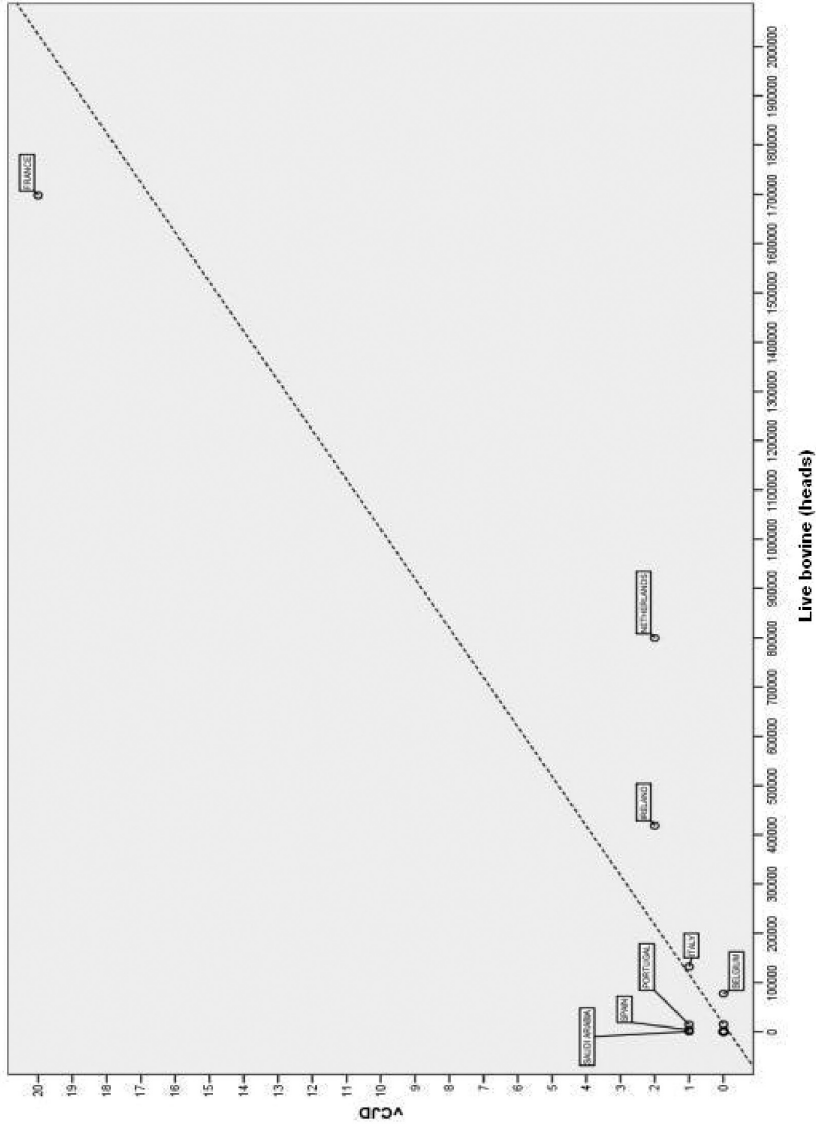


Figure 2: Scatter plot of live bovine imports from the UK (1980 to 1990) and the number of non-UK vCJD cases per country.

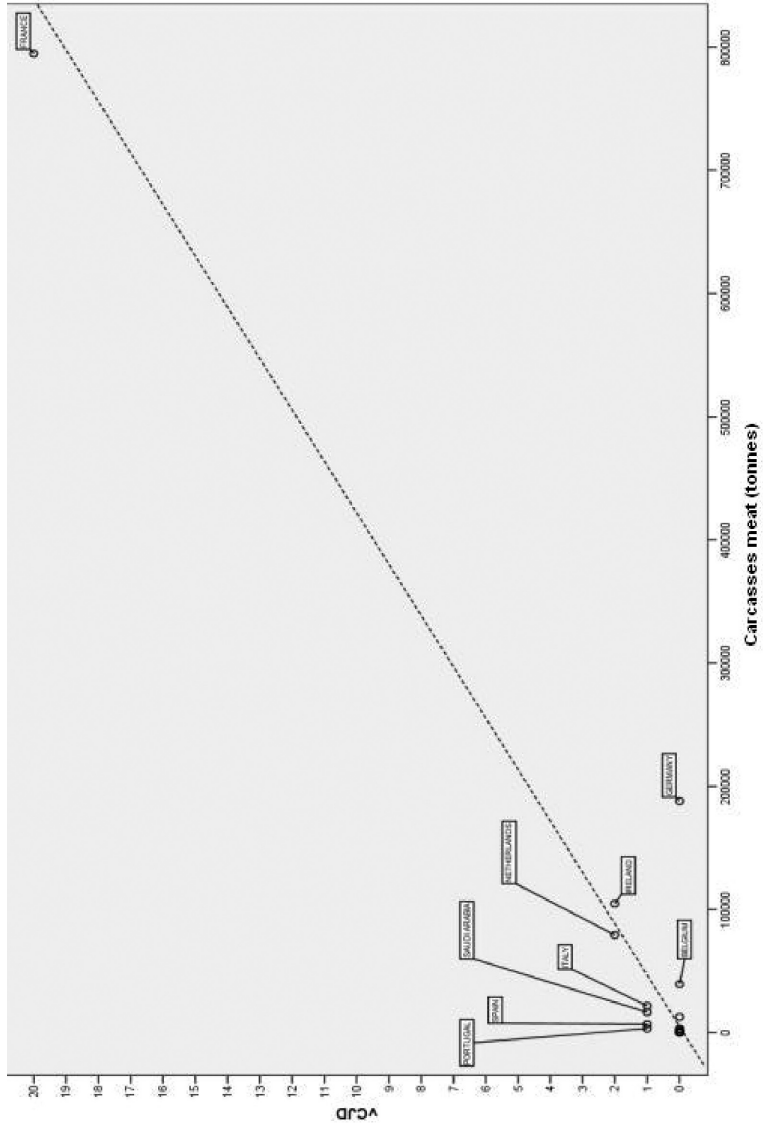


Figure 3: Scatter plot of the tonnage of carcass meat imports from the UK (1980 to 1990) and the number of non-UK vCJD cases per country of bovine carcasses meat (tonnes) imported from the UK from 1978 to 1999.

Table 2: Results of non-parametric correlation analyses between number of vCJD cases and the three studied exposure sources

| Exposure | Countries and vCJD cases included in analysis | | | |
|------------------------------------|--|--|--|---|
| | All EU network countries, including cases likely to have been infected in UK | All EU network countries plus Japan and SA, excluding cases likely to have been infected in UK | All EU network countries plus Japan and SA, including cases likely to have been infected in UK | All countries except France, including cases likely to have been infected in UK |
| Indigenous BSE cases | $r_s=0.70$ (0.37-0.87) p=0.001 | $r_s=0.60$ (0.21-0.82) p=0.005 | $r_s=0.55$ (0.17-0.79) p=0.008 | $r_s=0.48$ (0.06-0.75) p=0.03 |
| Live bovines imported from the UK* | $r_s=0.73$ (0.42-0.89) p<0.001 | $r_s=0.63$ (0.26-0.84) p=0.003 | $r_s=0.65$ (0.31-0.84) p=0.001 | $r_s=0.60$ (0.23-0.81) p=0.005 |
| Carcase meat imported from the UK* | $r_s=0.65$ (0.29-0.85) p=0.002 | $r_s=0.53$ (0.11-0.79) p=0.02 | $r_s=0.67$ (0.35-0.85) p=0.001 | $r_s=0.60$ (0.23-0.81) p=0.004 |
| | | | | $r_s=0.44$ (0.01-0.73) p=0.05 |
| | | | | $r_s=0.44$ (0.01-0.73) p=0.05 |
| | | | | $r_s=0.43$ (-0.00-0.73) p=0.05 |

* Weighted by the temporal distribution of the export in relation to the size of the BSE epizootic in the UK.

We evaluated whether our findings were dependent on the data from France, which has the largest number of non-UK cases. We repeated the analyses excluding France: all three correlation coefficients remained statistically significant (Table 2). We also repeated the analysis including the 6 cases detected outside the UK but thought to have been infected in the UK. The inclusion of these cases resulted in a reduction in all three correlation coefficients (Table 2). To analyse the independent effect of each of the three exposure sources we fitted a Poisson regression model including all EU network countries. When all three variables were included in the model, there was some evidence that both the number of live bovine imported from UK ($p=0.04$) and the number of indigenous BSE cases ($p=0.001$) were associated with the number of vCJD cases, but no evidence of an association with imports of carcass meat from the UK. When we included in the model Japan and Saudi Arabia, the association between number of vCJD cases and the same two variables (live bovine ($p=0.05$); indigenous BSE ($p=0.02$)) remained of statistical significance.

Discussion

The data suggest that live bovine imports from the UK from 1980 to 1990 correlate with the numbers of vCJD cases in countries outside the UK. Specifically, the correlation between vCJD cases and live bovine imports may be as good if not better than correlation with indigenous BSE cases, suggesting that live bovine imports from the UK may have been an important source of exposure in at least some of the countries where vCJD have been detected. These results are consistent with an analysis of French data suggesting that UK bovine imports were likely to have been a more important source of infectivity than indigenous BSE.⁶ Thus, a proportion of cases observed to date outside the UK may have been infected by imports from the UK rather than by exposure to indigenous BSE.

The inclusion in the analysis of the 6 non-UK cases thought to be infected in the UK reduced all three correlation coefficients, as one would expect if the supposition that they were infected in the UK is correct.

These findings come with a number of important caveats attached. First, they are based on small numbers of vCJD cases. Even a small number of additional non-UK cases in future could alter the findings substantially. Second, the analyses of imports are based on UK Customs and Excise data, and not all of which been validated by importing countries. Even if these data are reasonably accurate, the actual level of BSE infectivity entering the human food chain in importing countries cannot be estimated as there are many important unknown variables such as the age distribution of imported live bovines, the age at slaughter of these animals, the culinary habits in each country and the possibility that some of the UK imports may have been re-exported to other countries. Third, a proportion of indigenous BSE-infected cattle will have gone undetected until the introduction throughout the EU of the active abattoir testing program for BSE in 2000/2001; and even now cattle in the early stages of infection are unlikely to be detected.

It is also of interest that none of the 162 UK cases of vCJD identified up to August 2006 were born after 1989, the year in which the specified bovine offal ban was introduced to minimize human exposure to BSE, while two of the 34 non-UK vCJD cases have dates of birth after 1989. Measures equivalent to the UK SBO ban were not introduced in many continental European countries until 2000.

Despite these caveats, our results support that, globally, imports from the UK may have been an important source of infection, and for some countries they even may have been the main source. If this is so, our findings have a number of implications: (1) past UK exports, and in particular export of live bovines, may be the major determinant of the current incidence of vCJD outside the UK. (2) The greatest volume of these exports was to France, the Netherlands and Ireland. (3) Outside the UK, exposure to BSE via imports from the UK ceased in 1996, while exposure to indigenous BSE is likely to have continued at some level until the measures introduced in 2000.

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3.2

Ophthalmic surgery and Creutzfeldt-Jakob disease

Abstract

This paper reviews the data on ophthalmic surgery in sCJD and vCJD from the archives of the National CJD Surveillance Unit from 1990-2002, including information on both sporadic and variant Creutzfeldt-Jakob disease (CJD). We did not find evidence of any association between past history of ophthalmic surgery and risk of either sporadic or variant CJD. One interesting finding in our study is that sporadic CJD patients with early visual symptoms are often referred at first to ophthalmologists, and not infrequently they are diagnosed with cataracts and often treated. The information included in our study is of potential interest for ophthalmologists and public health.

Introduction

The occurrence of variant Creutzfeldt-Jakob Disease (vCJD) and the probable causal link with bovine spongiform encephalopathy in cattle have increased interest in the search for possible environmental sources of sporadic CJD (sCJD). Iatrogenic CJD is rare. Up to the year 2000 there had been 267 cases reported worldwide: 3 cases secondary to human corneal grafting (one confirmed, one probable and one possible case), 114 related to human dura mater grafts, 139 related to human growth hormone treatment, 4 related to human pituitary gonadotrophin therapy and 7 linked to neurosurgical procedures or stereotactic EEG electrodes.¹

Because of the marked resistance of the infectious agent of CJD to conventional sterilisation techniques, there is concern about the possibility of transmission of infection via surgical instruments in contact with infected tissue, especially in neurosurgery or ophthalmic surgery. Other forms of surgery have also been putatively linked to iatrogenic CJD with epidemiological studies showing that a history of past surgical interventions may be a significant risk factor for sCJD.^{2,3} In vCJD the presence of prion protein (PrP) immunostaining in systemic lymphoreticular tissues suggests that the risk from contaminated surgical instruments may be greater than in sCJD, in which these tissues do not stain for PrP.

In addition to the iatrogenic cases of CJD related to corneal grafts, there is experimental evidence of infectivity in eye tissues in CJD patients. The infectious form of PrP, PrP^{Sc}, has been found in the retina of sCJD and vCJD cases with comparable levels to those found in brain.⁴ Intracerebral inoculation of pooled CJD eye tissue in nonhuman primates transmitted the disease,⁵ as did inoculation of infected cornea in the anterior chamber of uninfected guinea pigs.⁶ Although, to our knowledge there have not been documented cases of CJD secondary to ophthalmic surgery other than corneal transplantation, there is a possibility that ophthalmic surgery might be a risk procedure for the accidental iatrogenic transmission of CJD. Because the levels of infectivity in prion diseases increase to high levels in the brain, and probably the eye, late in the incubation period, cases of CJD undergoing eye surgery during the clinical illness are most likely to represent the greatest risk of contaminating surgical instruments.

Recently it has been shown that the experimental transmission of metallic surface-bound prions is highly efficient.⁷ Steel wires in contact with the brain of pre-symptomatic mice needed only 5 minutes to acquire an infectious load equivalent to the injection of a 1% homogenate of brain. Infected wires were inserted transiently into the brains of indicator mice and only 30 minutes of exposure was sufficient to result in infection. The same wires remained infective when reintroduced into another set of indicator mice.

The evidence from laboratory studies underlines the importance of precautions to minimise the risks of iatrogenic transmission of CJD, but an important question is whether the concerns raised by experimental work translate into an actual risk in the clinical setting. This paper reviews the data on ophthalmic surgery in sCJD and vCJD

from the archives of the National CJD Surveillance Unit from 1990-2002, including information on both sCJD and vCJD.

Methods

We have analysed the past surgical history of sCJD and vCJD cases with specific reference to ophthalmic surgery. Cases of CJD were identified in the current prospective UK national surveillance project (1990-October 2002) by direct notification or from death certificates (see reference 8 for detailed methodology) and were classified as definite, probable or possible cases of sCJD or vCJD according to published diagnostic criteria. Only definite or probable cases were included in this analysis. All cases with a history of ophthalmic surgery were identified from the database, which has a specific code for this type of surgery. Information on past ophthalmic surgery was obtained from relatives, general practitioner records and/or copies of case notes. Case files were examined to identify the type of surgery, date of surgery and hospital in which the surgery had taken place. In cases in which the surgery was carried out after the onset of clinical symptoms of CJD detailed information on the clinical course was extracted.

The frequency of a history of eye surgery in sCJD and vCJD was compared with data on the frequency of past eye surgery in age and sex-matched control groups. During the period of the study the case-control study has evolved. Between 1990-1998 a single hospital control was obtained for the sCJD cases and from 1999-2002 a single community based control was identified. From 1996-2002 a single hospital control was obtained for vCJD cases and since 1998 attempts have been made to obtain 4 community based controls per case of vCJD. Because of the limited numbers of controls in sCJD and the infrequency of past eye surgery this study reports on unmatched comparisons of the frequency of past eye surgery.

Results

58 cases of sCJD (11%) out of 510 with information available had a history of intraocular surgery, with an average of 1.34 interventions per patient. The types of operation in the total of 81 cases having undergone any form of ocular surgery are listed in Table 1 and the years of operation in Figure 1. Eight patients with vCJD (6%) out of 125 with information available had a history of eye surgery and all were squint corrections in childhood, with the exception of one case with a history of surgery for retinal detachment carried out 15 months before the development of symptoms. Ten cases of sCJD underwent eye surgery during the prodromal (within 3 months of onset) or early symptomatic phase of the disease, the majority cataract operations. Four out of these 10 patients had the Heidenhain variant of sCJD, with visual onset and early development of cortical blindness (Table 2). Below are three illustrative case reports.

Table 1: *Types of operation*

| | Cases (n=78) | Hospital Controls (n=39) | Community Controls (n=20) |
|---------------------------|-------------------------|-------------------------------------|--|
| Intraocular surgery* | 55 (70%) | 29 (74%) | 17 (85%) |
| Extraocular surgery | 17 (22%) | 6 (16%) | 3 (15%) |
| Laser Therapy | 4 (5%) | 2 (5%) | - |
| Information not available | 2 (3%) | 2 (5%) | - |

*Cataract, Trauma and Glaucoma

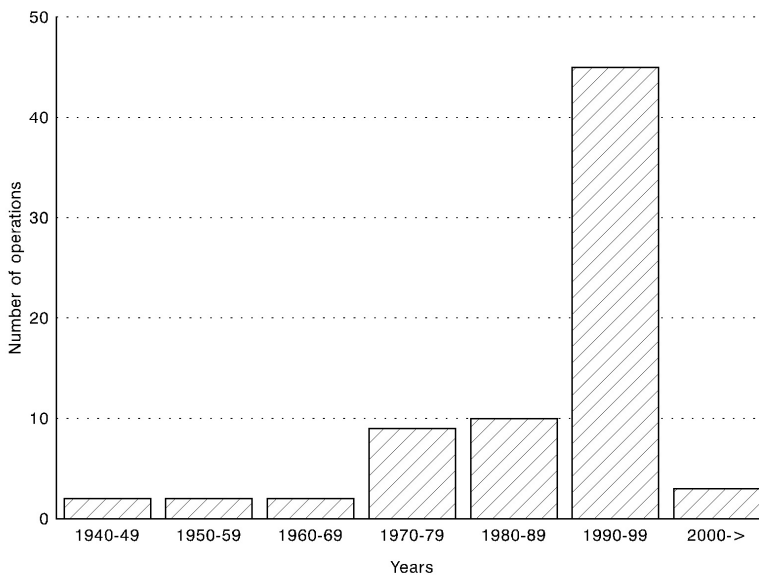
**Figure 1:** *Year of eye surgery in sporadic CJD.*

Table 2: Sporadic CJD patients with ophthalmic surgery after clinical onset

| | Date of Surgery | Intervention | Heidenhain Variant | Diagnosis |
|---------|-----------------|-----------------------|--------------------|-----------|
| Case 1 | 1992 | Cataract | | Definite |
| Case 2 | 1992 | Laser Therapy | * | Definite |
| Case 3 | 1992 | Cataract | * | Definite |
| Case 4 | 1993 | Cataract | | Definite |
| Case 5 | 1996 | Cataract | | Definite |
| Case 6 | 1998 | Dacryocystorhinostomy | | Definite |
| Case 7 | 1998 | Cataract | | Definite |
| Case 8 | 1999 | Cataract | * | Definite |
| Case 9 | 1999 | Laser Therapy | * | Definite |
| Case 10 | 2000 | Cataract | | Definite |

Case 3

A 78 year-old woman presented with deteriorating vision. Two years previously she had undergone a right cataract extraction, and her recent visual symptoms were attributed to maturation of the untreated left eye cataract. This was operated on without improvement in vision. Post-operatively her general condition deteriorated rapidly, with the development of dementia, incoordination, cortical blindness, rigidity and mutism. Apart from mild anaemia, investigations were normal. EEG was abnormal, but not characteristic of CJD. She died two months after the onset of symptoms. At post mortem examination of the brain the characteristic changes of sCJD were identified.

Case 8

A 73 year-old woman presented with blurred vision, photophobia and occasional double vision. She was referred by her GP to an eye clinic and underwent left eye cataract extraction. After the operation she developed confusion and memory loss and was admitted to hospital. She subsequently deteriorated rapidly with the development of myoclonic jerks and on examination cortical blindness was noted. CT scan was normal. EEG showed the 'characteristic' appearances of CJD and 14-3-3 protein in the cerebrospinal fluid was positive. She died in a state of akinetic mutism and post-mortem examination confirmed sCJD. Genetic analysis showed methionine homozygosity at codon 129 of the PrP gene.

Case 9

A 73 year old woman presented with bilateral progressive visual deterioration and occipital headache. She had had two cataract operations a few years earlier. On examination she had bilateral thickened posterior capsules, and a laser capsulotomy was performed. Her eyesight worsened and she developed visual hallucinations. She became confused and agitated. Examination showed cognitive impairment, extrapyramidal features and a supranuclear gaze palsy. MRI was normal, EEG was characteristic of CJD and the 14-3-3 protein was positive. She died 7 months after the onset, and the post mortem examination confirmed sCJD. Genetic analysis showed methionine homozygosity at codon 129 of the PrP gene.

The frequency of past eye surgery was compared with the control groups in sCJD and vCJD (Table 3). In the hospital control group for sCJD, 31 (14%) out of 226 had a history of ophthalmic surgery, with an average of 1.25 procedures per patient and in the community control group for sCJD 14 (13%) out of 106, with an average of 1.43 procedures per patient. In the hospital control group for vCJD (15%) 10 out of 67 had a history of ophthalmic surgery and in the community control group for vCJD 5 (3%) out of 155 had had eye surgery. There were no significant differences between the frequencies of past eye surgery between the cases and any of the control groups.

Details of the year and hospital of each surgical procedure were listed and in the great majority there was no temporal or geographic link between operations. A group of 6 cases of sCJD had been operated on in one hospital and in 2 pairs of cases the procedures had been carried out in the same year. Enquiry about the specific dates of these procedures, however, indicated that the operations had been carried out months apart.

Table 3: Past eye surgery in sporadic CJD compared with control groups

| | Cases (n=510) | Hospital Controls (n=226) | Community Controls (n=106) |
|------------------------------|--------------------------|--------------------------------------|---|
| Patients with eye operations | 58 (11%) | 31 (14%) | 14 (13%) |
| Total number of operations | 78 | 39 | 20 |
| Average per patient | 1.34 | 1.26 | 1.43 |

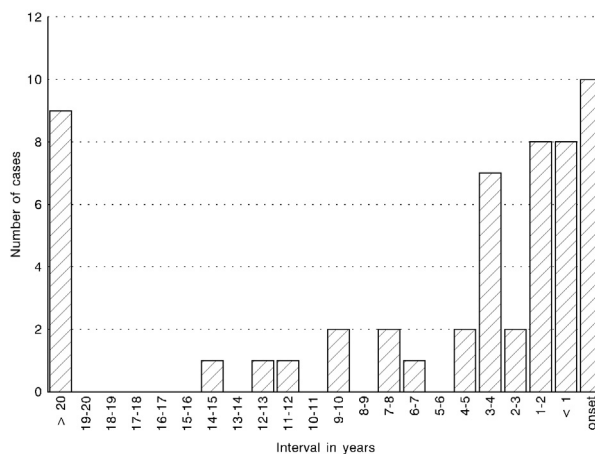


Figure 2: Interval in years between last eye surgery and onset of symptoms in sporadic CJD.

*In four cases information was not available.

Discussion

The aim of this paper is to document the frequency of past eye surgery in CJD and to determine whether there is evidence of transmission of CJD through contaminated ophthalmic instruments. About 10% of cases of sCJD have a history of eye surgery, of which about 70% involved open surgery on the anterior chamber of the eye. In the great majority of cases the surgical instruments were re-used on subsequent patients, usually because CJD developed years after the original procedure. Despite this, the evidence in this paper does not suggest that there is onward iatrogenic transmission of sCJD through eye surgery, a finding consistent with some previous studies.^{2,9}

An important question is whether cases of CJD caused by this type of transmission would be identified by this study. The clinicopathological features and incubation period of iatrogenic CJD vary according to the route of transmission, with, for example, a cerebellar syndrome and only limited cognitive impairment in human growth hormone related CJD. In CJD caused by corneal transplantation the clinical and pathological phenotype is similar to sCJD and the incubation periods in the 3 cases were 15 months, 18 months and 30 years.^{10,11} The surveillance system for CJD in the UK is efficient at identifying cases as judged by annual incidence rates of sCJD around 1 case per million and it is likely that cases with a typical phenotype would be identified. The period of observation of CJD from 1990-2002, with additional information from 1980-1989, suggests that there is the potential to identify case-to-case transmission through past eye

surgery, on the assumption that the incubation period of such cases would be months or years rather than decades.

The data from our case-control study shows no significant risk related to a past history of eye surgery in sCJD in comparison to two control groups. The methodology of the case-control study in our analysis is not ideal. There is a deficit in the numbers of controls in comparison to the numbers of cases and an unmatched analysis was undertaken. The results of our study are consistent with the results of a previous case-control study in Europe,^{2,9} but contrast with the results of an Australian study in which prior cataract/eye surgery was associated with an over 6-fold increase in the risk of sporadic CJD.³ The studies used different types of control group, the European study hospital controls and the Australian study community controls. However, in a reanalysis of the European data using a community control group eye surgery was again found not to increase the risk of sporadic CJD. The disparity in outcome in the two studies relates primarily to the frequency of prior eye surgery in the control groups (European studies 34/406, 37/325: Australian 24/784) rather than the cases (European studies 33/401, 37/328: Australian 24/241). Although this type of study cannot exclude the possibility of rare instances of iatrogenic transmission of CJD through eye surgery, the balance of evidence does not support the hypothesis that exposure to potentially contaminated ophthalmic instruments is a significant risk for developing CJD.

Evidence of transmission of sCJD through contaminated neurosurgical instruments rests on the close temporal relationship between operations carried out on CJD cases and unaffected individuals who subsequently developed CJD.^{12,13} An analysis of the dates and sites of eye operations in cases of sCJD in this study showed no such relationship. The elegant experiments by Weismann et al⁷ raise the possibility that surgical instruments contaminated during neurosurgery might pose a significant risk of iatrogenic transmission. In this context it is of interest that a number of cases of sporadic (and iatrogenic) CJD have undergone brain surgery (12/400 cases in the European study), but there is no evidence from case-control studies that prior neurosurgery increases the risk of developing sporadic CJD. This is despite the fact that potentially contaminated neurosurgical instruments have inadvertently been reused on other patients on a number of occasions.

The data on vCJD is limited and the period of observation is shorter than in sCJD. Although there is currently no evidence of transmission of vCJD through contaminated ophthalmic instruments, this possibility cannot be excluded, not least because the incubation period in vCJD is unknown.

The 10 cases of sCJD undergoing eye surgery in the early clinical stages of the illness may represent the greatest risk of onward transmission of infection, because there are probably higher levels of infection in the eye late in the incubation period due to centrifugal spread of infection along the optic nerve.⁴ The diagnosis of sCJD can be very difficult in the early stages of the clinical illness and this is particularly true of cases with isolated cortical visual symptoms (the Heidenhain variant of sCJD), which affected 4 of these 10 cases. The presence of confusion, memory impairment, or other neurological

signs may raise the suspicion of a neurodegenerative disorder.

Although the evidence in this paper does not suggest that contaminated ophthalmic instruments represent a risk of onward transmission of sporadic CJD, this conclusion should be treated with caution. The eye contains significant levels of infection in sCJD and vCJD and even limited exposures can result in the iatrogenic transmission of CJD.¹⁴ It is essential to follow current guidelines in relation to surgical, including ophthalmic and neurosurgical, instruments used in suspect cases of CJD of all types.¹⁵ Such instruments should be destroyed or quarantined until a definitive diagnosis is available and all cases of suspect CJD should be reported to the local consultant in communicable diseases in order that appropriate measures to protect public health are instituted, including a review of previous surgery.

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3.3

A polymorphism in the regulatory region of PRNP is associated with increased risk of sporadic Creutzfeldt-Jakob disease

Abstract

Creutzfeldt-Jakob disease (CJD) is a rare transmissible neurodegenerative disorder. An important determinant for CJD risk and phenotype is the M129V polymorphism of the human prion protein gene (PRNP), but there are also other coding and non-coding polymorphisms inside this gene. We tested whether a non-coding polymorphism located inside the PRNP regulatory region (PRNP G310C) was associated with risk of CJD and with age at onset in a UK population-based sample of 131 sporadic CJD (sCJD) patients and 194 controls. PRNP 310C allele was in strong linkage disequilibrium with PRNP 129V allele. As expected, methionine and valine homozygosity at PRNP M129V increased significantly the risk of sCJD, alone and adjusted by PRNP G310C (OR MM/MV=7.3; 95%CI 3.9 to 13.5 and OR VV/MV=4.0; 95%CI 1.7 to 9.3). Although the crude analysis did not show a significant association between PRNP G310C and sCJD (OR: 1.5; 95%CI=0.7 to 2.9), after adjusting by PRNP M129V genotype, being a C allele carrier at PRNP G310C was significantly ($p=0.03$) associated with a 2.4 fold increased risk of developing sCJD (95%CI=1.1 to 5.4). Cases of sCJD carrying a PRNP 310C allele presented at a younger age (on average 8.9 years younger than those without this allele), which was of statistical significance ($p=0.05$). Our findings support the hypothesis that genetic variations in the PRNP promoter may have a role in the pathogenesis of sCJD possibly due to an increased level of PRNP expression.

Introduction

The polymorphism coding for methionine (M) or valine (V) at codon 129 of the prion protein gene (PRNP M129V) plays a pivotal role in the susceptibility to Creutzfeldt-Jakob disease (CJD), influencing familial, transmitted and sporadic forms of the disease.¹ Moreover, the PRNP M129V genotype, in combination with the type of disease-associated prion protein (PrP^{sc}) deposited in the brain, is a major determinant of the clinical phenotype of sporadic² and genetic forms³ and also susceptibility to variant CJD (vCJD). All vCJD cases studied to date have been methionine homozygous at this locus.⁴

Transgenic animal models^{5,6} suggest that the level of expression of PRNP has a significant influence on the incubation period of disease. This finding has led to the hypothesis that variations in the regulatory region of PRNP, which may lead to an increased expression of the gene, may influence susceptibility and age at onset in sporadic CJD, independent of the influence of PRNP M129V.

In a previous study we defined the PRNP regulatory region, and identified three infrequent single nucleotide polymorphisms, PRNP-C101G located upstream of the transcription start site, and two intronic SNPs: PRNP G310C and PRNP T385C. An initial association study carried out in 25 sporadic CJD (sCJD) and 77 controls showed that a subgroup that carried any of the rare alleles in the regulatory region had an increased risk for sCJD.⁷

Based on our initial results, we genotyped PRNP G310C in a larger population of 131 sporadic CJD patients and 194 healthy controls from the UK. The aims of the study were to assess the association between PRNP G310C polymorphism and the risk of CJD in an independent set of sCJD patients and to examine for differences in age at disease onset between carriers versus non-carriers of the PRNP 310C allele.

Methods

Cases were derived from a population-based survey of CJD in the UK carried out by the UK National CJD Surveillance Unit.⁸ Those with DNA available from 1991 to 2003 were selected for the study. Only patients with sCJD who fulfilled the WHO diagnostic criteria for definite or probable CJD were included. Definite sCJD diagnosis was based on neuropathological examination. Probable cases required an appropriate clinical profile, supported by characteristic findings on EEG or CSF 14-3-3 protein detection.⁹ Whenever possible, all EEGs were reviewed by a member of the surveillance system and scored for the presence or absence of typical or characteristic diagnostic features.¹⁰ The CSF 14-3-3 immunoassays were performed using Western-blotting.¹¹ Randomly selected anonymous blood donors formed the control group; they were collected from two geographical areas in the UK; one in Northern Ireland (Belfast) and one in Scotland (Edinburgh). None of the cases or controls were included in the previous study.

Demographical and clinical data were collected for patients. Information on PrP^{sc} type was included in the database when available.

The PRNP M129V polymorphism and the PRNP regulatory region polymorphism G310C were genotyped in cases and controls as described in a previous article.⁷

Hardy Weinberg proportions of both genotyped polymorphisms, PRNP M129V and PRNP G310C, were tested. We assessed linkage disequilibrium (LD) between the PRNP regulatory polymorphism and PRNP M129V. Haplotype frequencies were estimated using EH program, and Lewontin's D' and r^2 coefficients were subsequently calculated.¹² Due to the low frequency of the rare allele, there were no PRNP 310C homozygous cases or controls. We compared the heterozygotes group (carriers of PRNP 310C) with the wild type homozygotes group (non carriers of PRNP 310C). Firstly, we tested the association between being a carrier of the PRNP 310C allele and the risk of developing the disease. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using logistic regression and adjusted for PRNP M129V genotype. Secondly, we compared differences in age at onset between CJD cases with and without the PRNP 310C allele. Fitting a general linear model, we assessed the differences in age at disease onset between cases, carriers versus non-carriers, adjusted by the molecular subtype (the combination of PRNP M129V genotype and PrP^{sc} type), which is a major determinant of age at onset.^{2,13} All statistical analyses were performed using SPSS 11.0 for Windows 2000 (SPSS, Inc, Chicago, Illinois).

Results

A general description of the 131 cases included in this study is given in Table 1. There was a non-statistically significant predominance of women. For most of the patients (n=110 (84%)) the diagnosis was neuropathologically confirmed. The molecular classification of the case cohort showed a predominance of the MM1 subtype (53%), corresponding to the classical disease phenotype.^{2,13} The most sensitive diagnostic test was the 14-3-3 immunoassay (90%).

None of the polymorphisms genotyped (PRNP M129V and PRNP G310C) deviated significantly from Hardy Weinberg proportions in the control group ($p=0.4$ and $p=0.5$ respectively). In the sCJD group PRNP M129V genotypic distribution deviated significantly ($p<0.001$) from Hardy Weinberg proportions. We found that PRNP 310C was in LD with PRNP 129V, in the control group ($D'=0.88$, $p<0.001$; $r^2=0.072$, $p<0.001$), in the group of s CJD patients ($D'=0.37$, $p=0.01$; $r^2=0.042$, $p<0.001$), and overall ($D'=0.57$, $p<0.001$; $r^2=0.045$, $p<0.001$).

Table 1: Overall descriptives of sporadic CJD patients (n=131)

| Characteristic | |
|--|---------------|
| Gender: female (%) | 77 (58.8) |
| Diagnosis classification: definite (%) | 110 (84.0) |
| Mean age at onset (years)±SD | 65.6±9.5 |
| Mean duration of disease (months)±SD | 6.6±7.0 |
| Molecular subtype | |
| MM1 (%) | 33 (53.2) |
| MM2 (%) | 4 (6.5) |
| MV1 (%) | 1 (1.6) |
| MV2 (%) | 8 (12.9) |
| VV1 (%) | 2 (3.2) |
| VV2 (%) | 14 (22.6) |
| Clinical characteristics: | |
| Typical EEG/total EEGs (%) | 50/125 (40.0) |
| Positive MRI/total MRIs (%) | 12/28 (42.9) |
| Positive CSF 14.3.3 test/total tests (%) | 46/51 (90.2) |

Although the crude analysis did not show a statistically significant difference between the proportions of PRNP 310C carriers in both groups (OR=1.5; 95%CI=0.7 to 2.9; p=0.3), after adjusting by PRNP M129V genotype we found a significant association between this regulatory region polymorphism and the risk of sporadic CJD. Being a C allele carrier at PRNP G310C was significantly (p=0.03) associated with a 2.4 fold increased risk of developing sCJD (95%CI=1.1 to 5.4) (Table 2). The distribution of PRNP G310C, stratified by PRNP M129V genotypes, showed in all three strata a trend to a higher proportion of individuals with PRNP 310C allele in the sCJD cases than in the controls, this difference was highest and of borderline statistical significance (p=0.07) in the MM group (OR=7.2; 95%CI=0.9 to 58.8) (Table 3).

Table 2: Overall distribution of PRNP 310C allele and risk of sporadic CJD

| PRNP 310C allele | Cases (%) | Controls (%) | OR (95% CI) | P- value | OR (95% CI) * | P- value* |
|-------------------------|------------------|---------------------|--------------------|-----------------|----------------------|------------------|
| Carriers | 17(13.0) | 18 (9.3) | 1.5 (0.7-2.9) | 0.3 | 2.4 (1.1-5.4) | 0.03 |
| Non carriers | 114 (87.0) | 176 (90.7) | | | | |

* Adjusted by PRNP M129V polymorphism.

Table 3: Distribution of PRNP 310C allele, stratified by PRNP M129V genotypes, and risk of sporadic CJD

| PRNP M129V genotype | PRNP 310C allele | Cases (%) | Controls (%) | OR (95% CI) | P-value |
|-----------------------|------------------|-----------|--------------|----------------|---------|
| Methionine/methionine | Carriers | 8 (8.2) | 1 (1.2) | 7.2 (0.9-58.8) | 0.07 |
| | Non carriers | 90 (91.8) | 81 (98.6) | | |
| Methionine/valine | Carriers | 4 (23.5) | 12 (13.0) | 2.1 (0.6-7.3) | 0.3 |
| | Non carriers | 13 (76.5) | 80 (87.0) | | |
| Valine/valine | Carriers | 5 (31.3) | 5 (25.0) | 1.4 (0.3-5.9) | 0.7 |
| | Non carriers | 11 (68.8) | 15 (75.0) | | |

Methionine and valine homozygosity at PRNP M129V increased significantly the risk of sCJD, in the crude analysis (OR MM/MV=4.3; 95%CI=2.5 to 7.2 and OR VV/MV=2.9; 95%CI=1.3 to 6.3) and adjusted by PRNP G310C (OR MM/MV=7.3; 95%CI=3.9 to 13.5 and OR VV/MV=4.0; 95%CI=1.7 to 9.3).

When we assessed differences in the age at onset between sCJD patients who were carriers and not carriers of the PRNP 310C allele, we found a statistically significant ($p=0.05$) association. Patients carrying the PRNP 310C allele presented with disease at an earlier age, being on average 8.9 years younger than non-carriers (Table 4). Table 4 shows that the decrease in age at onset was independent of the molecular subtype. The differences were more pronounced in the MM1 and VV1 strata although in the latter only one patient carried the PRNP 310C allele. The numbers of patients were too low and the differences across each molecular subtype strata were not statistically significant, but when we tested the overall means adjusted by molecular subtype this difference was statistically significant ($p=0.05$) (Table 4).

Table 4: Mean age at onset \pm standard errors of sporadic CJD patients across molecular subtypes and PRNP 310C allele distribution

| Molecular subtype | PRNP 310C carriers | N | PRNP 310C non carriers | N | Mean difference (Years) | P-value |
|-------------------|--------------------|---|------------------------|----|-------------------------|---------|
| MM1 | 57.7 \pm 8.1 | 2 | 67.9 \pm 9.1 | 31 | -10.2 | 0.1 |
| MM2 | - | - | 53.8 \pm 6.9 | 4 | - | - |
| MV1 | - | - | 78.1 | 1 | - | - |
| MV2 | 64.8 | 1 | 66.2 \pm 5.4 | 7 | -1.4 | 0.8 |
| VV1 | 41.0 | 1 | 64.0 | 1 | -23 | - |
| VV2 | 64.3 \pm 10.5 | 4 | 64.8 \pm 11.2 | 10 | -0.5 | 0.9 |
| Overall means* | 56.9 \pm 3.8 | 8 | 65.8 \pm 2.4 | 54 | -8.9 | 0.05 |

* Adjusted by molecular subtype (PRNP M129V and PrP^{Sc} type).

Discussion

There are two important findings in this study. Firstly, we found a significant association between the regulatory polymorphism PRNP G310C and the risk of sCJD. Secondly, those patients who carried the PRNP 310C allele presented with disease at an earlier age.

Being a carrier of the PRNP 310C allele increased the risk of developing the disease 2.4 fold independently of the PRNP M129V polymorphism. However, this association was only apparent when we adjusted for the PRNP M129V genotype.

The genetic of CJD is exceptional in that homozygotes of both PRNP M129V alleles are at increased risk of the disease. The protective effect of the PRNP codon 129 MV genotype (47.2% of the controls versus 18.3% of cases) and the predominance of the PRNP 129 MM genotype in sCJD patients (70.4% of cases) implies that the proportion of valine alleles is lower in cases (20.4%) than in controls (33.8%).

In our LD assessment we observed that PRNP 310C is linked to PRNP 129V. However, the association between the two alleles was stronger in controls than in cases. There is an excess of PRNP M129V homozygosity for both alleles in our sCJD cohort; since homozygosity increases LD, the lower LD in cases as compared to controls is unexpected.

The stratified analysis of PRNP G310C across the PRNP M129V genotypes showed a trend in all three strata to a higher proportion of individuals with PRNP 310C allele in the sCJD cases than in the controls. However, comparing the association between PRNP 310C and risk of sCJD across to the three PRNP M129V genotypes, the MM individual carriers of PRNP 310C presented a higher risk than the MV and VV individuals who also carried PRNP 310C (MM group OR=7.2 versus MV group OR=2.1 and VV group OR=1.4) (Table 3).

An interaction between the effects associated to the two SNPs may explain why the PRNP 310C allele is more often found (10 fold) coupled with the PRNP 129M allele in cases than in controls. If this haplotype (PRNP 310C-129M) is associated with an increased risk of sCJD, we will find it over represented in cases. The excess of the PRNP 310C-129M haplotype in sCJD cases is leading to a reduction of LD in comparison to the controls. Moreover, the interaction between the two alleles may also explain why the relationship between PRNP G310C and sCJD is masked when no adjustment is made by PRNP M129V. Although the p-value for the interaction term was not statistically significant in the logistic regression model, the analysis presented in Table 4 highly suggests that there is a non-additive relationship between PRNP 310C and PRNP 129M.

We found that the PRNP G310C polymorphism may influence the age at onset of disease. Patients with sCJD carrying the PRNP 310C allele presented at an earlier age, being on average 8.9 years younger than non-carriers. This difference was of borderline statistical significance ($p=0.05$). In the analysis stratified by molecular subtypes, the highest difference in age at disease onset between PRNP 310C carriers and non-carriers was found in the MM1 group which would also support the hypothesis of an interacting

effect between PRNP 310C and PRNP 129M alleles.

According to the stochastic protein conformational change theory, the risk of developing CJD may increase proportionally with the level of the PRNP product, the cellular prion protein (PrP^C) available.¹⁴ Our finding of an association between the PRNP 310C allele, which is located in the regulatory region of the gene, and an increased risk and earlier age at onset of sCJD suggests that the PRNP 310C allele may increase the expression of PRNP. This hypothesis is in line with experimental studies in animals. Transgenic mice models indicate that the level of expression of PRNP determines the length of the incubation period following inoculation with infectious material. Mice over-expressing murine Prnp have shorter incubation periods.⁵ A similar relationship is observed in mice with decreased levels of PrP^C. Animals with only one functional copy of murine Prnp consistently have longer incubation periods than wild-type mice when inoculated with the same agent.⁶ In addition, a study in inbred mice has identified three loci on chromosomes 2, 11 and 12, that significantly influence the incubation period. The Prnp region in mice, located on chromosome 2, mapped for a peak lod score of 8.2. The mice studied were all identical for the PrP^C amino acid sequence, suggesting that the regulatory region of this gene may play an important role in the length of the incubation period.¹⁵

The molecular subtype of sCJD patients, determined by the combination of the PrP^{Sc} type (type 1 or 2A) with the PRNP M129V genotype, is a major determinant of age at disease onset.^{2,13} For example in our cohort, in MM or MV type 1 cases the disease developed at age 67 years on average whereas the mean age at onset in VV type 1 cases was 52 years ($p=0.004$). Despite these trends, in all molecular subtypes except the MV1 group (in which there were only three cases), there are cases with symptom onset at an age of less than 50 years (data not shown). This suggests that although molecular subtype is an important determinant of age at onset, there are other influencing factors. Although numbers of cases are limited in our study, it is interesting that within each molecular subtype, on average, carriers of the PRNP 310C allele are younger at onset than non-carriers.

In vitro experiments have suggested that the PRNP 129M allele results in a higher propensity of the PrP to form beta-sheet-rich oligomers.¹⁶ This indicates that PRNP 129M may facilitate self-perpetuating conformational changes of the human prion protein. Therefore, an increased expression of PrP may work synergistically with PRNP 129M increasing the risk of sCJD and diminishing the age at symptoms onset.

A possible source of bias in our study is the use as controls of randomly selected anonymised blood donors who are not matched for age or sex with the patients. For example, if the distribution of the genotypes studied differed with age this would introduce bias. However, a recent report indicated that the distribution of the PRNP M129V genotypes in a Finnish population did not differ with gender or age.¹⁷

Although PRNP 310C is a rare allele, and is not present in the majority of patients with sCJD, our findings may offer a new insight into the elusive aetiology of CJD. The results of our study would suggest that a PRNP dosage effect could play a role in the

causal pathway of sCJD. Nevertheless, we have to emphasize that these conclusions are based on the assumption that PRNP 310 is associated to PrP gene expression due to the fact that it is located inside the gene regulatory region. Therefore, functional studies to assess the effect of the PRNP 310C allele should be carried out as a test for this hypothesis.

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3.4

No evidence for association between Tau haplotypic variants and susceptibility to Creutzfeldt-Jakob disease

Abstract

In the present study we test the association between the Tau protein gene (MAPT) haplotypic variants and risk of Creutzfeldt-Jakob disease. We examined 6 haplotype tagging SNPs (htSNPs), which capture 95% of MAPT genetic variability in Caucasians, in a Dutch population-based sample of sCJD patients and a cognitively normal control group of similar age distribution. We genotyped the same polymorphisms in two other sCJD samples from Italy and the UK. Single locus and haplotype analyses did not detect any significant difference between sCJD cases and controls. We did not find any differences in allelic or genotypic distributions between the two groups. When we compared MAPT haplotypes, we found that two of them were represented differently in sCJD and vCJD patients (H1f: 8% in sCJD versus 2% in vCJD; H1j: 1% in sCJD versus 7% in vCJD). However, these two haplotypes were rare in both groups of patients, and taking the small sample sizes into account, we cannot exclude that the differences are due to chance. None of the p-values ($p=0.04$ and $p=0.01$) remain statistically significant after applying a multiple testing correction like Bonferroni. In brief, our study shows no evidence for an association between MAPT gene variations and CJD, and some evidence for an association to vCJD.

Introduction

A polymorphism at codon 129 of the prion protein gene (PRNP M129V) is the only well-known genetic risk factor for sporadic Creutzfeldt-Jakob disease (sCJD).¹ However, there is increasing evidence that other loci outside PRNP open reading frame might play a role in sCJD etiology as well.²⁻⁶ Even though variant CJD (vCJD) is acquired from infected cattle with bovine spongiform encephalopathy (BSE)⁷ its susceptibility is strongly genetically determined. All vCJD patients tested are methionine (M) homozygous at PRNP M129V.⁸ As for sCJD, there are likely to be other genetic factors determining the risk of a particular individual developing vCJD.

Tau protein plays a key role in the pathogenesis of several neurodegenerative disorders. Neurofibrillary tangles (NFTs) consisting of accumulation of truncated and hyperphosphorylated Tau are one of the hallmarks of Alzheimer's disease (AD). NFTs are also present in other neurodegenerative diseases, including progressive supranuclear palsy (PSP), cortico basal degeneration (CBD), Pick's disease, and argyrophilic grain disease and Parkinson's disease (PD).⁹⁻¹² In patients with sporadic CJD, the Tau protein is profusely released to the CSF. This process is most likely related to the rapid neuronal damage. Tau quantification with Enzyme Linked Immunosorbent Assay (ELISA) is a very valuable test for sCJD diagnosis.¹³ In contrast to sCJD, in vCJD there is an increase of phosphorylated-Tau forms in CSF.¹⁴ The pathogenic implications of this finding are unknown, but it suggests that Tau phosphorylation may differ between sCJD and vCJD.

Tau protein is encoded by MAPT gene, located in chromosome 17. Mutations in MAPT have been identified in frontotemporal dementia and pallidopontonigral degeneration.¹⁵⁻²⁰ There are two common MAPT extended haplotypes in Caucasians, H1 and H2. The H1 haplotype has been linked to several sporadic neurodegenerative disorders like PSP²¹⁻²³, CBD²⁴, FTD²⁵, Parkinson's²⁶ and some studies suggest to Alzheimer's disease.²⁷

A recent study using data from the HapMap project (<http://www.hapmap.org>) identified 6 haplotype tagging SNPs (htSNPs) capturing 95% of MAPT genetic variability in Caucasians.²⁸ The same group showed that H1c, one of the H1 sub-haplotypes, was linked to late onset AD.²⁷ In the present study we examined the association between MAPT haplotypic variants and risk of sCJD and vCJD.

Methods

Cases were derived from population-based surveys of CJD carried out by national CJD registries from 1991 to 2005 in Italy (n=194 sCJD), the United Kingdom (UK) (n=48 sCJD and 52 vCJD) and the Netherlands (n=79 sCJD). All three countries are part of the European CJD surveillance network EuroCJD (<http://www.eurocjd.ed.ac.uk>). Only patients of Caucasian origin who fulfilled the WHO diagnostic criteria for definite or probable CJD were included.²⁹ Definite CJD diagnosis was based on neuropathological

examination. Probable cases required an appropriate clinical profile, supported by characteristic findings on MRI, EEG or CSF 14-3-3-protein detection. Whenever possible, all EEGs and MRIs were reviewed by a member of the surveillance system and scored for the presence or absence of typical or characteristic diagnostic features.^{30,31} The CSF 14-3-3 immunoassays were performed using Western-blotting.³² Healthy controls (n=309) were participants of the Rotterdam Study, which is a population-based study of 7385 subjects age 55 years or older.³³ Controls are all cognitively normal from Caucasian origin. Table 1 displays the main characteristics of cases and controls. Signed informed consent to participate in genetic research was obtained from all controls and patients' relatives. The protocols of the studies were approved by the local medical ethical committees.

Table 1: *Descriptive statistics*

| Origin | Series | n | % Females | % Definite diagnosis | Median age (range) | PRNP M129V genotype | | |
|--------|----------|-----|-----------|----------------------|--------------------|---------------------|----------|----------|
| | | | | | | MM n (%) | MV n (%) | VV n (%) |
| NL | sCJD | 79 | 59 | 52 | 67 (34-87) | 48 (67) | 16 (22) | 8 (11) |
| | controls | 309 | 56 | N/A | 71 (55-93) | 135 (44) | 145 (48) | 23 (8) |
| UK | sCJD | 48 | 58 | 50 | 67 (49-81) | 22 (46) | 13 (27) | 13 (27) |
| | vCJD | 52 | 31 | 74 | 25 (12-62) | 52 (100) | 0 (0) | 0 (0) |
| Italy | sCJD | 194 | 57 | 73 | 67 (35-87) | 141 (73) | 31 (16) | 22 (11) |

For all patients and controls DNA has been extracted from peripheral leucocytes according to a standard protocol. We genotyped five polymorphisms, which had been previously shown to tag the haplotype diversity of MAPT in Caucasians.²⁸ Additionally, the H1/H2 clade was defined by typing the SNP g.8117G>A (numbering according to GenBank accession number AC091628.2), with the allele G tagging H1, and the allele A tagging H2.³⁴ DNA samples were genotyped using a TaqMan allelic discrimination assay. Table 2 shows the primers sequence for each of the SNPs.

Table 2: *SNP sequences*

| SNP | Primer sequence |
|-----------|--|
| rs242559 | AGAAAGTTCTCCCAGGAAACAAGAG ATTCCCTAGCTGTTACCAGTCACTG |
| rs242557 | CGTTTCTTCTTCCTTACAAAGCAGTT TGTCACGGGACCAGGG |
| rs3785883 | GCTCAGCGATATTGTCACATGAC AGTGTCCGGCTGGATGGAC |
| rs2471738 | AGTGGCCTGGTTAGAGACCTT TCTGTCCTGTACCGCAGC |
| H1/H2 | GCCGTCCGCCTCTGT CATCGGTCCGGGGCCA |
| rs7521 | CCTGCGTGTCCCATCTACAG TCTTCAGCTTTGAAAAGGGTTACCC |

Hardy-Weinberg equilibrium (HWE) was calculated for the 6 htSNPs genotypes in the control population using χ^2 statistics. We set a significance level of 0.01 in order to take into account the number of tests performed. We assessed pairwise linkage disequilibrium (LD) between the 6 htSNPs using D' and r^2 calculated from the expectation-maximization estimated haplotypes. We used the program SNPStats (<http://bioinfo.iconcologia.net/SNPstats>) for the LD analysis. Single locus analyses were performed using SPSS software version 13. Allelic and genotypic frequencies were compared using χ^2 statistics. Adjusted analyses were performed using multiple logistic regression analysis. Age, gender and PRNP c129 were included in the model as covariates. We first compared Dutch sCJD cases and controls using the Italian and UK sCJD populations as replication samples. In a separate analysis we compared UK sCJD with vCJD. Haplotype analyses were performed using the program hplus (<http://qge.fhrc.org/hplus/>). We performed haplotype analysis comparing controls versus all sCJD patients and UK sCJD patients with vCJD patients.

Results

Table 1 shows the basic characteristics of our patients and controls. SCJD patients from the UK presented a significantly different PRNP M129V genotypic distribution in comparison to Italian and Dutch cases ($p=0.005$), the latter populations showing a higher proportion of the M allele.

All htSNPs were in HWE ($p > 0.01$). All 5 htSNPs used to define H1 sub-haplotypes were in complete LD with the htSNP tagging H1/H2. In contrast, among each other, LD was more variable. These observations are in line with the hypothesis that the markers are defining H1 sub-haplotypes.

In the first analysis we tested whether MAPT genetic variations were associated with risk of sCJD. None of the htSNPs genotypes showed a statistically significant association with the disease when comparing the Dutch cases to controls nor when comparing the other patients. We did not find association in any of the different sCJD populations studied independently neither when they all were collapsed. When we adjusted by age, gender and PRNP M129V genotype, we did not find any significant relationship with sCJD risk either (Table 3). When we compared in a separate analysis MAPT genetic variants between UK sCJD and vCJD, we also failed to find any statistically significant association (Table 4).

The htSNPs genotyped allowed us to define the two major clades, H1 and H2, which have been described in Caucasians in several previous studies.³⁵ In order to facilitate comparisons we have adopted the same terminology for H1 sub-haplotypes as that used by Pittman et al.²⁸ We did not find any evidence of association between any of the MAPT haplotypes and sCJD (Table 5). We specifically did not find association with the subhaplotype H1c, which has been previously reported to be related to several neurodegenerative diseases. We also compared MAPT haplotypes between sCJD and vCJD from the UK. We found that the frequency of two rare haplotypes, H1f and H1j, were significantly different in sCJD and vCJD patients ($p=0.04$) and ($p=0.01$) (Table 6). However, these two variants were present in a small proportion of patients, and after adjusting by the number of test performed (14 pairwise haplotypic comparisons were made) the results were not statistically significant ($p=0.56$) and ($p=0.14$).

Table 3: *MAPT* Single locus analysis association with sCJD

| SNP | Major Controls allele | NL sCJD | | Italy sCJD | | UK sCJD | | Overall sCJD | | | |
|-----------|-----------------------|----------|---------|------------|---------|---------|---------|---------------|-----------------|-----------------|-------------------|
| | | n (%) | P-value | n (%) | P-value | n (%) | P-value | Allelic n (%) | Genotypic n (%) | Allelic P-value | Genotypic P-value |
| | | | | | | | | | | | |
| rs242559 | A | 467 (76) | 0.60 | 297 (76) | 0.76 | 69 (72) | 0.37 | 482 (76) | 0.84 | 0.92 | [0.73] |
| rs242557 | G | 382 (63) | 0.71 | 248 (64) | 0.54 | 65 (68) | 0.36 | 408 (64) | 0.60 | 0.85 | [0.66] |
| rs3785883 | G | 501 (82) | 0.41 | 319 (82) | 0.55 | 82 (85) | 0.47 | 535 (84) | 0.29 | 0.57 | [0.36] |
| rs2471738 | C | 495 (81) | 0.65 | 301 (78) | 0.37 | 71 (74) | 0.13 | 496 (78) | 0.20 | 0.46 | [0.58] |
| H1/H2 | G (H1) | 469 (77) | 0.75 | 300 (77) | 0.34 | 69 (72) | 0.30 | 484 (78) | 0.89 | 0.90 | [0.87] |
| rs7521 | G | 330 (54) | 0.59 | 205 (53) | 0.95 | 59 (61) | 0.19 | 352 (56) | 0.61 | 0.84 | [0.77] |

P-values are not corrected for multiple testing.

In brackets p-values adjusted by PRNP M129V genotype, age at onset and gender.

Table 4: *MAPT* single locus analysis UK sCJD and vCJD

| SNP | Major allele | UK sCJD | UK vCJD | P-value | |
|-----------|--------------|---------|---------|---------|-------------|
| | | n (%) | n (%) | Allelic | Genotypic |
| Mrs242559 | A | 69 (72) | 67 (66) | 0.36 | 0.28 [0.26] |
| rs242557 | G | 65 (68) | 64 (63) | 0.55 | 0.39 [0.79] |
| rs3785883 | G | 82 (85) | 90 (87) | 0.84 | 0.99 [0.96] |
| rs2471738 | C | 71 (74) | 80 (78) | 0.51 | 0.80 [0.60] |
| H1/H2 | G (H1) | 69 (72) | 67 (67) | 0.54 | 0.55 [0.27] |
| rs7521 | G | 59 (61) | 61 (60) | 0.88 | 0.65 [0.99] |

P-values are not corrected for multiple testing.

In brackets p-values adjusted by PRNP M129V genotype, age at onset and gender.

Table 5: *MAPT* haplotype association with sCJD

| Haplotype | Alleles | | | | | | Frequency | | P-value |
|-----------|----------|----------|-----------|-----------|-------|--------|------------|--------|---------|
| | rs242559 | rs242557 | rs3785883 | rs2471738 | H1/H2 | rs7521 | % Controls | % sCJD | |
| H1e | A | G | G | C | G | A | 25 | 26 | ref |
| H2a | C | G | G | C | A | G | 23 | 22 | 0.64 |
| H1c | A | A | G | T | G | G | 12 | 15 | 0.46 |
| H1d | A | A | G | C | G | A | 13 | 10 | 0.08 |
| H1f | A | G | A | C | G | G | 5 | 5 | 0.88 |
| H1g | A | A | G | C | G | G | 5 | 5 | 0.91 |
| H1h | A | G | A | C | G | A | 4 | 4 | 0.64 |
| H1i | A | A | A | C | G | A | 3 | 3 | 0.68 |
| H1j | A | A | A | T | G | G | 3 | 2 | 0.45 |
| H1b | A | G | G | C | G | G | 2 | 2 | 0.55 |

P-values are not corrected for multiple testing.

Table 6: *MAPT* haplotype analysis UK sCJD and vCJD

| Haplotype | Alleles | | | | | | Frequency | | P-value |
|-----------|----------|----------|-----------|-----------|-------|--------|-----------|-----------|---------|
| | rs242559 | rs242557 | rs3785883 | rs2471738 | H1/H2 | rs7521 | % UK sCJD | % UK vCJD | |
| H2a | C | G | G | C | A | G | 28 | 31 | ref |
| H1e | A | G | G | C | G | A | 24 | 20 | 0.51 |
| H1c | A | A | G | T | G | G | 16 | 10 | 0.40 |
| H1d | A | A | G | C | G | A | 7 | 16 | 0.18 |
| H1f | A | G | A | C | G | G | 8 | 2 | 0.04 |
| H1j | A | A | A | T | G | G | 1 | 7 | 0.01 |

P-values are not corrected for multiple testing.
Included all haplotypes present in both sCJD and vCJD.

Discussion

Our study assesses the relationship between *MAPT* haplotypic variations and CJD for the first time. We examined 6 htSNPs, which are part of an extended *MAPT* haplotype, in a Dutch population-based sample of sCJD patients and a cognitively normal control group of similar age distribution. We genotyped the same polymorphisms in two other sCJD samples from Italy and the UK. All three populations of cases showed similar demographical characteristics. In the UK sCJD cases we found a higher proportion of VV cases (27% versus 11%). This difference might be due to geographical variations in the genotypic distribution of PRNP gene codon 129. According to published data, the frequency of the M allele increases gradually from north-western to south-eastern European countries. An alternative explanation may be a possible higher percentage of young sCJD patients tested for codon 129 in the UK. Earlier disease onset could be linked to higher proportions of MV and VV patients, who usually tend to start the disease at a younger age than the MM. This might be driven by the higher concern in the UK about vCJD cases, who are younger than sCJD and up until now all homozygotes for M. However, in our study, the median age at onset of the UK sCJD population did not differ from the other countries. Finally, we cannot rule out that the differences may be explained by the small numbers.

Statistical analysis revealed no significant differences between cases and controls, with all p-values higher than 0.05 before correcting for multiple testing. Haplotype analysis still failed to detect any significant difference. We specifically did not find significant association with the sub-haplotype H1c which has been previously reported to be related to several neurodegenerative diseases. We found that H1c was slightly overrepresented in sCJD (15%) versus controls (12%), but this difference was not

statistically significant ($p=0.46$). We did not find significant differences either between Dutch controls and vCJD.

When we compared other MAPT haplotypes between UK sCJD and vCJD patients, we found that two of them were represented differently (H1f:8% in sCJD versus 2% in vCJD; H1j:1% in SCJD versus 7% in vCJD). When we compared the frequency of these haplotypes in vCJD versus the Dutch control population only the difference in H1j (3% in controls versus 7% in vCJD) was of borderline significance ($p=0.06$).

However, these two haplotypes were rare in both groups of patients, and taking the small sample sizes into account, we cannot exclude that the differences are due to chance. In any case, none of the p-values ($p=0.04$ and $p=0.01$) remain statistically significant after applying correction like Bonferroni for the 14 pairwise haplotypic comparisons that were made ($p=0.56$ and $p=0.14$).

One possible explanation for our results may be a lack of statistical power. We think that this is unlikely. As a proof of principle, the association of sCJD with PRNP M129V genotypes in the overall sample yielded a very low p-value ($<10^{-11}$). The sCJD risk of PRNP 129 M homozygotes versus PRNP 129 heterozygotes was 3.8 folds higher (95%CI from 2.6 to 5.5). Without multiple testing corrections we estimated that the statistical power of our genotypic analysis would be of 85% to detect an odds ratio as low as 1.6.

In brief, our study shows no evidence for an association between MAPT gene variations and CJD some evidence for an association to vCJD.

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4

**Diagnostic aspects of
Creutzfeldt-Jakob disease**

4.1

CSF tests in the differential diagnosis of Creutzfeldt-Jakob disease

Abstract

We analysed the diagnostic sensitivity and specificity of various brain-derived proteins (14-3-3, Tau, NSE and S100b) in the CSF of patients with Creutzfeldt-Jakob disease (CJD) and the biological factors that modify these parameters. CSF was tested for 14-3-3, Tau, NSE and S100b in 1859 patients with sporadic, genetic, iatrogenic and variant CJD, and in 1117 controls. The highest sensitivity was achieved for 14-3-3 and Tau in sporadic CJD (85% and 86%), and a combined determination of 14-3-3 and either Tau, S100b or NSE increased the sensitivity to over 93%. A multivariate analysis showed that the sensitivity of all tests was highest in patients with the shortest disease duration, age at onset >40 years, and homozygosity at codon 129 of the prion protein gene. In a group of patients with repeated lumbar punctures, a second test also increased the diagnostic sensitivity. The detection of elevated levels of brain-derived proteins in the CSF in patients with suspected CJD is a valuable diagnostic test. Second lumbar puncture should be preformed in patients with atypical clinical course in whom the first test was negative.

Introduction

The detection of 14-3-3 protein in the cerebrospinal fluid (CSF) plays an important role in the clinical diagnosis of CJD.¹⁻⁵ 14-3-3 proteins are reported to be elevated in 95% of sporadic CJD (sCJD), but the sensitivity is lower in patients with other forms of CJD (such as iatrogenic, variant and genetic CJD).⁵⁻⁷ The sensitivity of 14-3-3 also varies in different sCJD phenotypes.⁸⁻¹⁴ Various other proteins have also been tested: neuron specific enolase (NSE), Tau and phosphorylated Tau and astrocytic (S100b) proteins, prostaglandins, interleukins and A β ₁₋₄₂.^{11,15-22}

A combination of several markers might be used to improve the sensitivity and specificity of clinical diagnosis.^{1,5,14,21,23-26} A rational approach to the early and specific diagnosis of CJD is crucial and needs further consideration given that more biochemical markers have been reported in recent years. The rationale of performing a panel of biochemical tests has not yet been evaluated.

We have focused on the analysis of brain-derived proteins in the CSF in different forms of TSE with respect to sensitivity and specificity and investigated the effect of selected patient characteristics on these parameters.

Methods

Patients and database

The study was conducted within the framework of an EC-supported multinational study. Patients were referred to the national surveillance units for the detection of CSF 14-3-3 protein, during the course of routine clinical diagnosis and surveillance.

Samples were sent to the individual laboratories for the detection of CSF 14-3-3 in participating countries between the years 1998 and 2003. All countries collected clinical and neuropathological data from those patients with clinical suspicion of CJD or related disorders. The corresponding diagnoses were obtained by follow-up. Data and samples contributed by each laboratory, were as follows: Spain 906 (30% of the total), Germany 866 (29%), Italy 523 (18%), UK 443 (15%), Switzerland 164 (5.5%), Poland 39 (1.3%), Greece 20 (0.7%), Slovakia 15 (0.5%).

Core clinical data such as age at onset, gender, disease duration, time point of the lumbar puncture (LP), PRNP codon 129 genotype, PrP^{Sc} isotype and final clinical and neuropathological diagnoses were collected by each centre. A database was set up, which included detailed data on the CSF markers and patients' characteristics for 2976 samples from patients with CJD and control patients. In all participating centres, the diagnoses of CJD and the various CJD subtypes were made according to established, internationally agreed criteria.²⁶⁻²⁸ All participant countries belong to the EURO-CJD Surveillance network with comparable surveillance methods. Control patients were who proved not to have CJD or other prion disease, however definite alternative diagnoses were not always possible. The diagnoses in the control patients (non-CJD patients) were

based on neuropathology (whenever possible) and clinical follow-up information. For further analyses, the control patients were assigned to one of the following groups: neurodegenerative, inflammatory, tumor-associated, ischaemic, psychiatric, metabolic and other. In the analysis, only control patients with a definite clinical or neurological diagnosis were included, and as a result data was excluded from 251 control patients.

CSF was taken by LP during the investigation of the patient at the notifying hospital and tests were conducted in each laboratory according to common agreed standards, described in more detail below. The results were entered into the database.

14-3-3

At the initial stages of the data acquisition, we conducted a ring trial between the participating laboratories in order to ensure the comparability of the results between centers. We followed two approaches:

Ten selected CSF samples were sent to all participating laboratories. These ten CSF samples were from patients with CJD, inflammatory disorders of the central nervous system, Alzheimer's disease and a bloodstained CSF sample. Original 14-3-3 Western blot films were scanned and the participants were asked to perform a visual analysis of the Western blots (judged as negative, weak positive, positive) and the results compared. The different centers were found to agree over the classification of the results and, in addition, no individual centers differed from the whole group in terms of sensitivity and specificity. In most cases, the anti-14-3-3 polyclonal rabbit antibody (SC-629, Santa Cruz Biotechnology) was used as a primary detection antibody. Minor variations in the 14-3-3 detection protocols in individual laboratories had no influence on test results. The median time of CSF storage between LP and 14-3-3 tests was 8 days and the median time between disease onset and the LP was 4 months.

In total, 14-3-3 tests were performed on 2934 samples (including 106 from a repeat LP). This group comprised 1826 samples from patients with various forms of transmissible spongiform encephalopathies (TSE) (1531 samples being from sCJD patients) and 1108 from controls.

Tau

Tau protein levels were determined in 1295 samples from 1211 TSE patients (819 being sCJD cases) and 220 control patients. Tau concentrations were measured by an enzyme-linked immunosorbent assay (Innotest hTau-Ag, Innogenetics, Ghent, Belgium). A previously established cut-off value of 1300 pg/ml was used for further calculations.²³

S100b

S100b protein levels were determined in 966 samples from TSE patients (589 being sCJD cases) and in 162 controls. S100b concentrations were measured by enzyme-linked immunosorbent assays. Tests were performed in three centers (UK, Spain and Germany) using various detection methodologies. In 533 samples, levels were determined using a commercially available test kits (Byk Sangtec), while in the other

433 samples, levels were determined using a previously described in-house ELISA.²⁹ Since test sensitivity and specificity vary between methodologies, we used different cut-off points for each test (Byk Sangtec, 4.2 ng/ml, in-house 0.5 ng/ml).

Neuron specific enolase, NSE

NSE protein levels were determined in 748 samples from TSE patients (517 being from sCJD cases) and in 113 controls. NSE concentrations were measured by enzyme-linked immunosorbent assays. Various commercially available test kits were used. For 359 samples, a Hoffman LaRoche (Cobas-Core NSE EIA kit) test was used, in 312 samples NSE was determined using a Delfia NSE, (Wallac ADL GmbH, Freiburg, Germany) and in 77 samples cases a LIAISON NSE, (DiaSorin S.p.A. Saluggia, Italy). Since test sensitivity and specificity vary among methodologies applied, we used different cut-off points for each test used (Hoffman LaRoche, 35 ng/mL, Wallach 25 ng/mL and Byk Sangtec, 25 ng/mL).^{1,30}

Statistical analyses

Descriptive statistics were reported for sCJD and control patients; χ^2 test and the non-parametric Mann-Whitney test were used to assess differences between categorical and continuous variables. For each CSF marker, sensitivity and specificity were calculated in our overall population of TSE and control patients; χ^2 was used to test differences in these predictive characteristics between CSF markers. We also calculated positive result rates for every subtype of TSE.

A multiple logistic regression model was used to assess the effect of a set of clinically relevant patient characteristics on the sensitivity of the different tests in sCJD patients. We included the following characteristics in our model: disease duration (in months), age at onset (in years), disease stage when the LP was performed (during the first third of the total duration of the disease, during the second or during the third), and PRNP codon 129 genotype; country of sample origin and patient gender were entered also as covariates.

Age at onset was categorized in four clinically meaningful groups of age, being the reference group patients younger than 40 years. Disease duration was categorized using the median as a cut-off point we divided our patients in two groups (short or long duration). PRNP codon 129 genotype was entered in the model using two dummy variables with the MV group as reference. The effects of PRNP codon 129 genotype and PrP^{Sc} isotype on the sensitivity of the tests, adjusted again by sex and country of sample origin, were jointly assessed in another multiple logistic regression model, although, due to small numbers for the rest of the markers, this analysis was performed only for 14-3-3. Again, using multiple logistic regression with gender and country of origin include as covariates, we also analyzed whether particular (or individual) clinical factors influenced the false positive rate in controls. Due to small numbers for the other markers, this analysis was performed only for 14-3-3. Adjusted odd ratios and 95% confidence intervals were generated.

Results

Patient characteristics

The database comprised detailed data on CSF levels of several proteins in 2976 samples. Most of the samples belonged to the various TSE forms (n=1859). A definite neuropathological diagnosis was available in 49% of sCJD, 47% genetic CJD (gCJD), 47% iatrogenic CJD, 61% vCJD, 76% FFI and one GSS patients. One thousand one hundred and seventeen samples were from patients with another diagnosis. Most of the diagnoses were assigned to the group with neurodegenerative disorders (n=604, 20% of the total sample). The second largest group comprised patients with inflammatory disorders of the central nervous system (n=213, 7%). Most of the TSE samples were from sCJD patients (n=1552, 52% of the total group and 85% of the TSE group). This group of TSE patients also had the highest post mortem-rate (49.9%, from 14.3 to 75.9%). Post mortem-rate was lower in control patients (12.4% of the total group) and varied considerably among the subgroups (11.4% in neurodegenerative diseases, 26.9% in ischaemia, and 39.7% in paraneoplastic or tumor associated group).

The group with sCJD patients did not differ significantly from the control patients with respect to gender (53% and 49% females in each group) and age at onset (67 years, range 19-97 in cases and 66 years, range 5-92 in controls).

Information on PRNP codon 129 genotype was available in 1319 cases (1069 sCJD and 250 control patients). Most of the sCJD patients were homozygous for methionine (n=656, 61.4%) or valine (n=175, 16.4%) and only 22.2% (n=238) were heterozygous. In controls, 47% (n=118) were homozygous for methionine, 12% (n=29) for valine, and 41% (n=103) were heterozygous.

The PrP^{Sc} isotype was available in 251 sCJD cases, of which 175 had the PrP^{Sc} isotype 1 and 76 type 2A.

CSF markers in the diagnosis

Initially, we calculated the sensitivity and specificity for each marker for all TSE forms (Table 1). We worked with quantitative levels for all markers tested except for 14-3-3, for which the test results were rated as positive or negative. One methodological problem with this latter test is the detection of faint immunoreactive bands, which were rated as weakly positive. Therefore, we decided to calculate the sensitivity and specificity of the 14-3-3 tests according to three different strategies to determine which one would be most beneficial in formulating clinical diagnoses in cases of rapidly progressive dementia:

1. To consider only unequivocal results (by exclusion of trace bands)

Following this rule, we calculated an overall test sensitivity of 84.5% for TSE disease (88.8% for sCJD) and specificity of 83.6% but we lost 79 cases (4.2%) (including 61 sporadic cases) in our calculation. Since this approach is impractical in a clinical setting, we analyzed the following strategies:

2. To consider trace bands as positive results or
3. To consider trace bands as negative results

Following the second approach, the overall 14-3-3-test sensitivity was 85.2% (89.3 for sCJD) and the specificity was 79.2%. The third strategy revealed a lower overall sensitivity of 80.7% (85.1% in sCJD) but a gain in the specificity (a 5.3% increase from 79.2% to 84.5%. ($p < 0.01$)). Since the main purpose of our study was set up to develop a strategy for the best clinical CJD diagnosis in context of differential diagnosis of rapid progressive dementia, we selected the third strategy for our subsequent analyses (Table 1).

Table 1: Sensitivity (positives/total) by subtypes of disease

| Disease subtype | 14-3-3* | 95%CI | Tau† | 95%CI | S100b† | 95%CI | NSE† | 95%CI |
|-----------------|----------------|----------|--------------|----------|--------------|----------|--------------|----------|
| sCJD | 85 (1240/1457) | (83, 87) | 86 (704/819) | (83, 88) | 82 (483/589) | (79, 85) | 73 (379/517) | (69, 77) |
| iCJD | 75 (15/20) | (51, 90) | 53 (8/15) | (27, 78) | 81 (13/16) | (54, 95) | 44 (4/9) | (15, 77) |
| vCJD | 40 (37/93) | (30, 50) | 24 (21/86) | (16, 35) | 62 (55/88) | (51, 72) | 24 (6/25) | (10, 45) |
| gCJD | 78 (109/139) | (70, 85) | 82 (47/57) | (70, 91) | 82 (28/34) | (65, 93) | 60 (21/35) | (42, 76) |
| GSS | 0 (0/7) | (0, 44) | 50 (1/2) | (3, 97) | 100 (1/1) | (5, 100) | 0 (0/2) | (0, 80) |
| FFI | 9 (2/23) | (1, 29) | 0 (0/12) | (0, 30) | 22 (2/9) | (4, 60) | 0 (0/10) | (0, 34) |
| Total | 81 (1403/1739) | (79, 82) | 79 (781/991) | (76, 81) | 79 (582/737) | (76, 82) | 69 (410/598) | (65, 72) |

*Trace result considered as negative.

†Cutoff point for Tau protein was 1300 pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec kit and 0.5ng/ml and for the in house kit used in the UK; for NSE we used 35ng/ml for the Hoffman La Roche kit and 25ng/ml for Byk-Diasorin, Byk-Sangtec and Wallac kits.

The sensitivity of 14-3-3 varied among TSE subtypes (Table 1). For sCJD, the sensitivity was 85%. Iatrogenic and gCJD patients had elevated 14-3-3 levels in 75% and 78% of the cases. In variant CJD (vCJD), only 40% had elevated 14-3-3 levels. All GSS patients were negative and only two FFI patients were 14-3-3 positive.

For Tau levels, we observed a similar distribution (Table 1). The sensitivity was the highest in the group of sCJD patients, followed by genetic and iatrogenic cases. Using the cut-off value of 1300 pg/ml, as suggested for sCJD, the sensitivity of Tau in vCJD was 24%. However, by using a different cut-off point of 500 pg/ml, as suggested previously,⁵ the sensitivity for vCJD increased to 86%.

Elevated S100b levels were observed in 82% of samples from sCJD patients. In other TSE forms higher rates of positive results were observed with S100b than with other markers (Table 1). However, the gain of sensitivity of S100b has to be balanced against the lower specificity observed for this marker (76%).

The CSF marker with the lowest sensitivity for sCJD was NSE (73%), which also gave the highest specificity (95%). Again, in iatrogenic and gCJD, sensitivity was lower (44% and 60%). The lowest NSE sensitivity was seen with vCJD (24%). None of the GSS and FFI patients were positive in this test.

Once the sensitivity had been determined for each marker, we analyzed the rate of 'false-positive' results by subgroups (Table 2). Inflammatory, ischaemic and neoplastic disorders of the brain revealed a high false-positive rate. Usually, those conditions can be ruled out by routine CSF tests and brain imaging. The truly problematic differential diagnoses for CJD are other neurodegenerative disorders, however, when comparing CJD and this subgroup, the specificity was high (90% for 14-3-3 and 89% for Tau).

Table 2: Proportion of positive marker results (positive/total) in control patients

| Diagnostic category | 14-3-3 [†] | Tau [‡] | S100b [‡] | NSE [‡] |
|--------------------------|----------------------|--------------------|--------------------|------------------|
| Neurodegenerative | 10 (62/591) | 11 (11/102) | 34 (22/65) | 3 (1/38) |
| Inflammatory | 27 (56/208) | 5 (3/58) | 10 (5/50) | 2 (1/43) |
| Paraneoplastic/CNS tumor | 27 (21/77) | 22 (5/23) | 44 (8/18) | 20 (2/10) |
| Stroke | 27 (14/51) | 18 (3/17) | 7 (1/14) | 0 (0/11) |
| Epileptic fit | 31 (11/36) | 36 (4/11) | 12 (1/8) | 22 (2/9) |
| Psychiatric | 0 (0/32) | 0 (0/9) | 29 (2/7) | 0 (0/2) |
| Metabolic | 6 (2/33) | - | - | - |
| Other | 5 (3/61) | - | - | - |
| Total | 16 (169/1089) | 12 (26/220) | 24 (39/162) | 5 (6/113) |
| Specificity % (95% CI) | 84 (82, 86) | 88 (8, 92) | 76 (68, 82) | 95 (88, 98) |

[†]Trace result considered as negative.

[‡]Cutoff point for Tau protein was 1300 pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec kit and 0.5ng/ml and for the in house kit used in the UK; For NSE we used 35ng/ml for the Hoffman La Roche kit and 25ng/ml for Byk-Diasorin, Byk-Sangtec and Wallac kits.

None of the individual markers could distinguish between the different forms of TSE (data not shown).

In the next step, we attempted to identify the value of each marker for each sCJD subtype. The results are given in Table 3. We found that all markers were less sensitive in patients with PrP^{Sc} isotype 2A protein than with isotype 1. The lowest sensitivity was found in the MV2 subtype. Another attempt was made to classify CJD patients into 'classic' and 'atypical' subtypes according to previously suggested definitions given.⁹ For all markers, the sensitivity was higher for 'classic' subtypes (Table 3).

Table 3: Test sensitivities in sporadic CJD subtypes

| Subtype | 14-3-3 | | Tau | | S100b | | NSE | |
|----------------------------|---------|------|---------|------|---------|------|---------|------|
| | +/n | Sens | +/n | Sens | +/n | Sens | +/n | Sens |
| MM 1 | 129/140 | 92 | 92/94 | 98 | 74/81 | 91 | 48/64 | 75 |
| MV 1 | 10/11 | 91 | 4/6 | 67 | 3/5 | 60 | 5/5 | 100 |
| VV 1 | 6/6 | 100 | 4/5 | 80 | 2/6 | 33 | 2/4 | 50 |
| MM 2 | 7/9 | 78 | 6/7 | 86 | 5/5 | 100 | 2/3 | 67 |
| MV 2 | 20/31 | 65 | 10/19 | 53 | 13/20 | 65 | 6/12 | 50 |
| VV 2 | 27/30 | 90 | 14/16 | 88 | 13/13 | 100 | 10/11 | 91 |
| classical* | 411/443 | 93 | 271/290 | 93 | 187/210 | 89 | 163/206 | 79 |
| non-classical ^o | 350/410 | 85 | 245/294 | 83 | 180/231 | 78 | 150/220 | 68 |

Sens: Sensitivity

* sporadic CJD, codon 129 genotype MM or MV and duration less than 8 month.

^o sporadic CJD, codon 129 genotype VV (all), codon 129 genotype MM or MV and duration longer than 8 month.

Patient characteristics effects on test sensitivity and specificity

Univariate analysis revealed that all but one of the patient characteristics studied significantly influenced the sensitivity of all tests in sCJD patients i.e, disease duration, PRNP codon 129 genotype, age at onset and time of the LP (the single exception being the disease stage at the time of LP) (Table 4).

Table 4: Factors influencing sensitivity of CSF markers in sCJD patients

| Factors | 14-3-3† | Tau‡ | S100b‡ | NSE‡ |
|-------------------------|------------------|------------------|-------------------|------------------|
| | OR° (95% CI) | OR° (95% CI) | OR° (95% CI) | OR° (95% CI) |
| PRNP c129 MV genotype | ref | ref | ref | ref |
| PRNP c129 MM genotype | 2.1 (1.4, 3.2)* | 2.7 (1.6, 4.4)* | 2.5 (1.4, 4.6)* | 3.1 (1.9, 5.1)* |
| PRNP c129 VV genotype | 3.9 (1.9, 7.9)* | 8.7 (3.3, 22.7)* | 7.9 (2.9, 21.3)* | 6.5 (2.9, 14.5)* |
| Age at onset <40 | ref | ref | ref | ref |
| Age at onset 40-59 | 6.1 (3.2, 11.6)* | 3.8 (1.8, 7.6)* | 3.4 (1.5, 7.6)* | 6.3 (2.3, 17.0)* |
| Age at onset 60-79 | 7.8 (4.3, 14.3)* | 8.8 (4.4, 17.6)* | 13.0 (5.7, 29.5)* | 9.5 (3.5, 25.6)* |
| Age at onset >80 | 4.4 (1.7, 11.5)* | 2.5 (0.8, 7.9) | 3.7 (0.9, 14.4) | 5.2 (1.4, 19.6)* |
| Duration below 6 months | ref | ref | ref | ref |
| Duration above 6 months | 0.5 (0.3, 0.7)* | 0.3 (0.2, 0.5)* | 0.4 (0.3, 0.8)* | 0.5 (0.3, 0.8) |

Adjusted by country of origin and gender.

Ref : reference

° OR odds ratio

* p-value<0.01

†Trace result considered as negative.

‡Cutoff point for Tau protein was 1300pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec kit and 0.5ng/ml and for the in house kit used in the UK; for NSE we used 35ng/ml for the Hoffman La Roche kit and 25ng/ml for Byk-Diasorin, Byk-Sangtec and Wallac kits.

In the multivariate analysis, when we included all these characteristics plus the covariates, all remained significant. When we analyzed disease stage at time of LP, adjusted for the rest of the variables, we observed a trend to have higher sensitivity in 14-3-3 in later stages: 87.6% in the first, 88.0% in the second and 91.3% in the third ($p=0.07$) and this pattern was the same for all markers. We found that in patients younger than 40 years all tests had a significantly lower sensitivity than in older patients. In patients with disease duration longer than six months, all tests had significantly lower sensitivity than in patients with shorter disease duration (and therefore a more aggressive course). Finally, in patients heterozygous for PRNP codon 129 genotype all tests significantly showed lower sensitivity than in homozygous patients (Table 4). From a different perspective, the predicted probability of having a positive 14-3-3 test in sCJD patients was higher when the test was performed in an individual homozygous for the PRNP codon 129 genotype, older than 40 years and with short disease duration. Similar results were obtained for Tau, S100b and NSE (Figure 1).

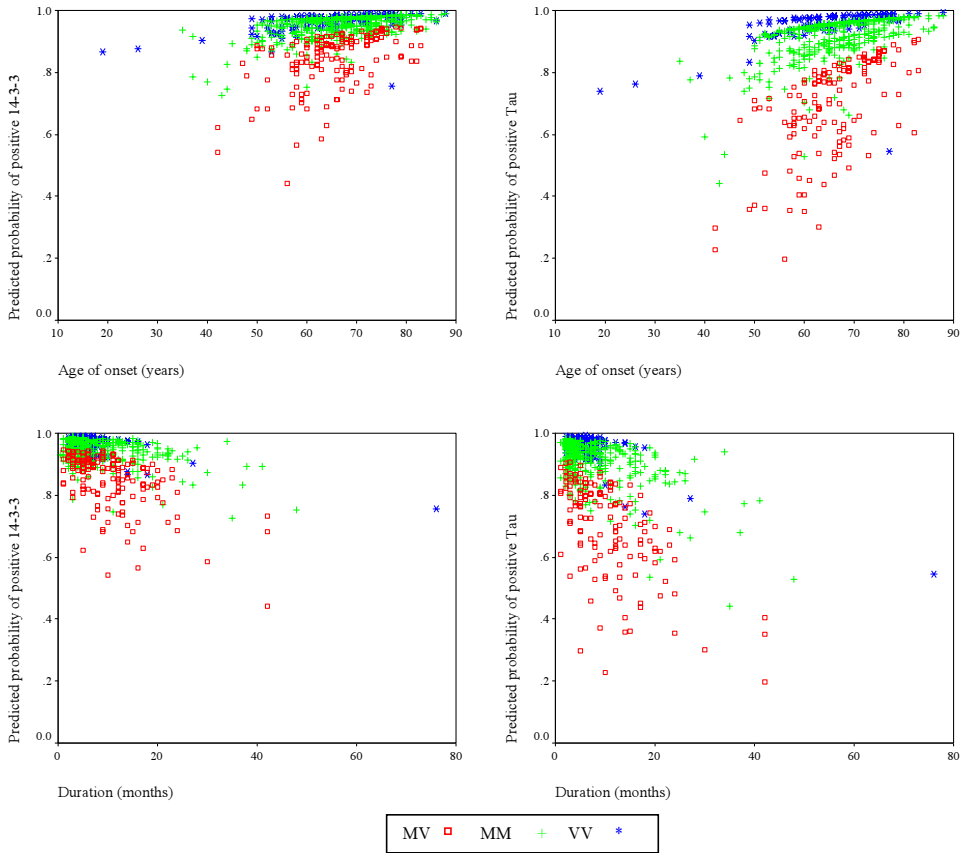


Figure 1: Comparison of predicted probabilities of a positive 14-3-3 or Tau CSF test according to age at onset and duration of illness stratified by PRNP codon 129 genotype.

We also studied the influence of both PRNP codon 129 genotype and PrP^{Sc} isotype on the sensitivity of 14-3-3. Including both variables in the same model showed that they individually had a significant effect on 14-3-3 sensitivity. 14-3-3 had a significantly lower sensitivity in patients with PrP^{Sc} isotype 2A than in patients with type 1 (OR=0.16; 95% confidence interval: from 0.04 to 0.63; $p < 0.01$). There was also a significantly lower sensitivity in patients heterozygous at PRNP codon 129 than in patients homozygous for valine (OR=0.15; 95% confidence interval: from 0.003 to 0.66; $p = 0.012$); but no significant differences were found between heterozygotes and methionine homozygotes.

Univariate analysis revealed that multiple clinical factors were related to an increase in the false positive rate for the 14-3-3 test, namely: diagnostic category

($p < 0.001$), older age at onset ($p = 0.054$), shorter duration of the disease ($p < 0.01$), shorter time from disease onset to LP ($p < 0.05$), and high number of cells in the CSF ($p < 0.001$). In the multivariate analysis, the following remained as significantly associated to a false positive rate: having an inflammatory ($p < 0.01$), or a paraneoplastic/CNS-tumor disease ($p < 0.001$) in comparison to a neurodegenerative disorder; and an increased number of cells in the CSF ($p < 0.05$).

All analyses have been repeated using data on definite sCJD patients only with similar results.

Marker combination

In order to analyze whether a combination of two markers might improve the sensitivity and specificity of the clinical diagnosis in patients with rapidly progressive dementia, we analyzed all possible test combinations (Table 5). The highest sensitivity was achieved by a combination of 14-3-3 with elevated levels of either Tau, S100b or NSE, without significant differences or by the combination of Tau and S100b. Concerning different sCJD subtypes, the combination of two CSF markers revealed the highest sensitivity in patients with PrP^{Sc} isotype 1 or in 'classic' phenotypes. Comparing with a single marker, a combination of two tests increased the sensitivity in both, 'classic' and 'atypical' phenotypes. The test sensitivity for each single marker in 'classic' phenotypes is already very high, but for 'atypical' phenotypes, the combination of two CSF markers led to increased test sensitivity.

Concerning all non-TSE patients, the highest specificity was achieved with normal Tau and NSE levels (88%). In a further analysis, we looked at the marker combination in the subgroup of neurodegenerative diseases only (Table 5). Here we found also the highest specificity by combination of negative Tau or NSE levels (91%), followed by negative 14-3-3 or NSE (82%). For neurodegenerative disorders, a combination of two markers revealed higher specificity as for whole non-TSE group except for test combinations which included S100b.

Table 5: Comparison of all possible two test combinations for sCJD patients and control patients

| Test Combination | Sensitivity % (positive/all) (95% CI) | | | | | | Specificity % (positive/all) (95% CI) | | | | |
|-------------------|---------------------------------------|-----------|-----------|-----------|------------|-------------|---------------------------------------|------------------------------------|-----------------------|----------------------|----------------------------|
| | MM1 | MV1 | VV1 | MM2 | MV2 | VV2 | classical % (positive/all) (95% CI) | atypical % (positive/all) (95% CI) | all TSE patients | all non TSE patients | Neurodegenerative diseases |
| 14-3-3* or Tau† | 99 (93/94) | 83 (5/6) | 100 (5/5) | 86 (6/7) | 68 (13/19) | 100 (16/16) | 98 (283/290) (95, 99) | 91 (262/289) (86, 94) | 94 (757/806) (92, 95) | 68 (71/219) (61, 74) | 72 (73/102) (62, 80) |
| 14-3-3* or S100b† | 99 (80/81) | 80 (4/5) | 100 (6/6) | 100 (5/5) | 85 (17/20) | 100 (13/13) | 98 (206/210) (95, 99) | 93 (211/228) (88, 95) | 96 (557/583) (93, 97) | 57 (70/161) (48, 64) | 58 (38/65) (46, 70) |
| 14-3-3* or NSE† | 97 (62/64) | 100 (5/5) | 100 (4/4) | 67 (2/3) | 83 (10/12) | 100 (11/11) | 97 (200/206) (93, 99) | 89 (195/218) (84, 93) | 94 (480/513) (91, 95) | 68 (36/112) (58, 76) | 82 (31/38) (65, 92) |
| Tau† or S100b† | 99 (79/80) | 80 (4/5) | 80 (4/5) | 100 (5/5) | 76 (13/17) | 100 (12/12) | 97 (197/203) (93, 99) | 90 (201/223) (85, 94) | 93 (521/562) (90, 95) | 69 (47/154) (61, 76) | 65 (40/62) (51, 76) |
| Tau† or NSE† | 97 (59/61) | 100 (5/5) | 75 (3/4) | 67 (2/3) | 73 (8/11) | 100 (10/10) | 97 (187/193) (93, 99) | 87 (170/196) (81, 91) | 91 (430/473) (88, 93) | 88 (13/108) (80, 93) | 91 (32/35) (76, 98) |
| S100b† or NSE† | 95 (53/56) | 100 (4/4) | 50 (2/4) | 100 (1/1) | 50 (5/10) | 100 (8/8) | 92 (147/159) (87, 96) | 80 (140/174) (74, 86) | 86 (348/407) (82, 89) | 82 (17/93) (72, 89) | 77 (24/31) (66, 84) |

Trace result considered as negative.

†Cutoff point for Tau protein was 1300pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec kit and 0.5ng/ml and for the in house kit used in the UK; for NSE we used 35ng/ml for the Hoffman La Roche kit and 25ng/ml for Byk-Diasorin, Byk-Sangtec and Wallac kits.

Sens, sensitivity; Spec, specificity

* sporadic CJD, codon 129 genotype MM or MV and duration less than 8 month

° sporadic CJD, codon 129 genotype VV (all), codon 129 genotype MM or MV and duration longer than 8 month

Repeated lumbar punctures

In 84 patients, at least two LP were performed. The median time between both 14-3-3 tests was 6 weeks. In these patients, 14-3-3 was positive in the first LP in 65% of the CJD cases, but the sensitivity was higher in the second sample taken at a later stage of the disease (79%). Specificity increased too when results of the second LP were taken into account (63% and 69%). Table 6 shows a detailed analysis of repeated LP for various brain-derived proteins. According to this, we observed a general trend for further increase of levels of brain-derived proteins with disease progression in CJD patients.

Table 6: *Repeated 14-3-3 tests: comparison of first LP versus subsequent LP*

| Test | TSE patients | | Control patients | | Sens | | Spec | |
|-----------------------|--------------|----|------------------|----|------|----------|------|-----------|
| | + | - | + | - | % | 95%CI | % | 95%CI |
| First 14-3-3* test | 44 | 24 | 6 | 10 | 65 | (52, 76) | 63 | (36, 84) |
| Repeated 14-3-3* test | 54 | 14 | 5 | 11 | 79 | (67, 88) | 69 | (41, 88) |
| First Tau† test | 37 | 16 | 3 | 2 | 70 | (55, 81) | 40 | (7, 83) |
| Repeated Tau† test | 38 | 15 | 2 | 3 | 72 | (57, 83) | 60 | (17, 93) |
| First S100b† test | 20 | 18 | 0 | 4 | 53 | (36, 69) | 100 | (40, 100) |
| Repeated S100b† test | 25 | 13 | 1 | 3 | 66 | (49, 80) | 75 | (22, 99) |
| First NSE† test | 20 | 26 | 1 | 4 | 43 | (29, 59) | 80 | (30, 99) |
| Repeated NSE† test | 32 | 14 | 0 | 5 | 70 | (54, 82) | 100 | (46, 100) |

*Trace result considered as negative

†Cutoff point for Tau protein was 1300pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec kit and 0.5ng/ml and for the in house kit used in the UK; For NSE we used 35ng/ml for the Hoffman La Roche kit and 25ng/ml for Byk-Diasorin, Byk-Sangtec and Wallac kits.

Sens: sensitivity; Spec: specificity

Discussion

In our study, we tested a panel of brain-derived proteins in the CSF in patients who were suspected cases of CJD to determine: (1) their diagnostic utility either combined or alone, (2) the results in single CSF or in repeated CSF samples, and (3) to determine the effects of selected patient characteristics on the test sensitivity and specificity.^{1,3,5-7,14,24,31}

Amongst the brain-derived proteins tested, 14-3-3 had the highest sensitivity for all types of TSE. In sCJD, the sensitivity was 85%. This is lower than reported previously.^{2,3,7,21,31} The lower sensitivity might be related to a higher number of patients tested in this study, but also to the very rigid strategy of defining 14-3-3 'trace' results as negative. We cannot exclude that the high rate of 'false-negative' 14-3-3 tests might also be related to some atypical CJD subtypes which are increasingly recognized in systematic surveillance studies (such as the MV2 subtype). The sensitivity of all tests was highest for the sporadic group, lower for iatrogenic and genetic cases and the lowest in vCJD, FFI and GSS patients.

Most patients were analyzed for 14-3-3 after it had been adopted as part of internationally agreed clinical diagnostic criteria.^{26,27} Although it is a common view that this marker should be tested in only truly suspect cases of CJD, it has been widely used for screening in dementia.^{3,32} Currently, it is an accepted tool for patient recruitment in surveillance studies and its use has been reported to lead to a substantial improvement in epidemiological figures.³³⁻³⁵ This approach has to be weighed against the concomitant drop in specificity. In the former studies, specificity was high, as only truly suspect CJD patients were tested.^{2,3,36} Subsequently, as the control group was extended with a variety of acute neurological disorders, the specificity figures for those surrogate markers dropped. Referring only to neurodegenerative disorders as potential differential diagnoses of CJD, the previously reported specificity figures continue to apply (Table 2).^{2,3,36}

A combination of 14-3-3 with another marker increases sensitivity.^{1,5,21,23,24} The highest sensitivity was achieved by a combination of 14-3-3 with elevated levels of either Tau, S100b or NSE, but the highest specificity was seen, when normal Tau and NSE levels were combined.

We observed a substantial increase in sensitivity and specificity for several markers, when an additional LP was performed at later disease stages. This observation is in agreement with published single case reports.^{10,13,21,37,38} In our analysis, both sensitivity and specificity increased when the results of the second LP were taken into account. The results of a second CSF test might help to exclude CJD, since with time, levels of brain-derived proteins decline in some other conditions, but usually increase further in CJD. Thus, in cases of doubt, an additional LP several weeks after the initial one is recommended to help support or exclude the diagnosis of CJD.

PRNP codon 129 genotype, PrP^{Sc} isotype, disease duration and age at onset influenced the test sensitivity in sCJD patients.

PRNP codon 129 heterozygous patients have lower sensitivity in all tests performed in the CSF. In addition, the PrP^{Sc} isotype 2A is an independent influencing factor and the effect is even more pronounced in patients with at least one methionine allele. The influence of the PRNP codon 129 genotype and PrP^{Sc} isotype was assessed in previous studies, but due to a lower number of cases, the results did not reach significance levels at that time.^{8,9,21}

We found a trend towards a higher sensitivity in 14-3-3 when the LP is performed in the last third in the course of the disease, but the results were not statistically

significant. Moreover, as shown in our analysis, the sensitivity of markers increased, when a second LP was performed later in the disease. This is in line with previous reports, where the disease stage was reported to correlate with levels of 14-3-3⁹ or 14-3-3, Tau and A β ₁₋₄₂.²¹

The question of disease duration and sensitivity of tests has been addressed in several studies.^{5,8} In general, many of the currently available tests are more sensitive in patients with a typical short disease duration. This has been shown for the detection of periodic sharp and slow wave complexes in the EEG, 14-3-3 in CSF and hyper-intense basal ganglia in MRI or phosphorylated Tau concentrations in the CSF.^{9,39-41} In our study, disease duration of less than 6 months was significantly associated with higher sensitivity for 14-3-3, Tau and S100b proteins.

Age at onset correlated significantly with test sensitivity, being lower in those patients with disease onset before the age of 40. The reasons for this observation are not clear at the moment, but some disease modifying factors might account for this observation. One can assume that atypical CJD subtypes present more frequently at younger age, such as MV2 subtypes. Survival is longer in young patients, which is also related to a less progressive disease course and therefore potentially for a less acute neuronal damage in those patients.⁴² On the other hand, in the logistic regression analysis, age presented as an independent variable. Other factors, which remain to be proven, include an age-dependent neuronal vulnerability that leads to a more pronounced release of the brain-derived proteins into the CSF.

To conclude, in front of a single negative 14-3-3 test in suspected CJD patients, a second test (such as Tau) should be requested. The determination of the codon 129 genotype is extremely helpful for the interpretation of the negative CSF results. In heterozygous patients, the negative test must be interpreted with more caution and other diagnostic tests such as magnetic resonance imaging including diffusion weighted imaging for detection of hyperintense basal ganglia must be more intensely pursued.^{8,40} In addition, a second lumbar puncture at least six weeks after the initial one should be considered. A negative result in patients younger than 40 years at onset should be interpreted with extreme caution.

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4.2

Influence of timing on CSF tests value for Creutzfeldt-Jakob disease diagnosis

Abstract

The analysis of markers in the cerebrospinal fluid (CSF) is useful in the diagnosis of sporadic Creutzfeldt-Jakob disease (sCJD). However, the time at which the study of these markers is most sensitive remains controversial. The objective of our study is to assess the influence of time of sampling on the value of CSF tests in the diagnosis of sCJD. In the framework of a multinational European study, we studied the results of 14-3-3, S100b, neurone specific enolase (NSE) and Tau protein in 833 CSF samples from sCJD patients at different stages of disease and in 68 sequentially repeated lumbar punctures (LP). 14-3-3 and Tau protein increased in sensitivity from onset (88%, 81%) to the advanced stage (91%, 90%). This was significant in the MV group of patients. The absolute levels of S100b ($p < 0.05$), NSE and Tau protein increased in the last stage of disease. High levels of Tau protein, NSE and S100b were associated with shorter survival times ($p < 0.01$). Sixty-eight sCJD patients underwent repeated LP. These sCJD patients were younger, had longer disease durations and were more frequently MV at codon 129 ($p < 0.001$) than the whole group. 14-3-3 sensitivity increased from 65% to 79% in the second LP ($p = 0.06$) and 88% sCJD patients had at least one positive result. Sensitivity and absolute levels of CJD markers increased with disease progression and were modulated by the codon 129 genotype. Early negative results should be interpreted with caution, especially in young patients or those who are MV at codon 129.

Introduction

Creutzfeldt-Jakob disease (CJD) is a rapidly progressive neurodegenerative disease. Presently, the definite diagnosis of CJD requires neuropathological confirmation. Cerebrospinal fluid (CSF) analysis plays an important role in the diagnosis of CJD. After the WHO consultation in 1998, a positive 14-3-3 result is considered as a criterion of probable sporadic CJD (sCJD).¹ Different studies have shown that in the appropriate clinical circumstances a positive 14-3-3 is highly sensitive and specific for the diagnosis of sCJD.² However, the question remains open as to whether 14-3-3 is more sensitive in the initial or late stages of disease. Increased levels of other brain-derived proteins, such as microtubule-associated Tau protein³, S100b⁴ or neurone specific enolase (NSE)⁵ have also been reported to be elevated in CSF in CJD. The published data about how the levels of these proteins are modified during the course of disease are contradictory.^{6-8,9-11,12-13} In addition, the time point during the disease, when biochemical markers are expected to have the highest sensitivity, is not well-defined. These topics have not been studied systematically, especially because data from sequential CSF analyses are rarely available.

In the framework of an European Union supported multinational study on CJD markers, we performed an analysis of the results obtained in different stages of the disease and in repeated lumbar punctures (LP) to assess the influence of time of sampling on the value of CSF tests in the diagnosis of sCJD.

Methods

Study overview

This study was conducted in the framework of a European Union supported multinational study (Early clinical diagnosis of human spongiform encephalopathies by analysis of biological fluids (CJDmarkers), QLG3-CT-2002-81606). All participant countries belong to the EURO-CJD Surveillance network with comparable surveillance methods. CSF samples were sent to the individual laboratories for 14-3-3 analysis in the participating countries between 1998-2003 and clinical and pathological data were collected. CSF was obtained by LP and the analyses were conducted in each laboratory following standard protocols.¹⁴ An analysis of test validity and comparability between laboratories revealed high agreement between laboratories.¹⁴ Previously published cut-off values were used for the quantitative markers.^{3,5}

In all countries, clinical and neuropathological data from patients with clinical suspicion of CJD or related disorders were collected. The diagnoses of CJD and various CJD subtypes were made according to the established criteria in all participating centers. Core clinical data such as age at onset, gender, disease duration, time point of the LP, codon 129 genotype, PrP^{Sc} type and final clinical and neuropathological diagnoses were centralized by each center. A database was set up, which included detailed data on the

CSF markers and patients' characteristics. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Statistical analyses

Since the clinical duration within sCJD patients varies widely depending upon several variables (e.g., age at onset, gender, codon 129 polymorphism of the PRNP gene, and PrP^{Sc} type), the time interval between LP and disease onset is not an appropriate estimate of the stage of disease when the LP was performed. To overcome this problem, we normalized the time of LP to disease onset in each patient by the duration of disease. Thus, we classified patients in three categories according to whether they underwent LP in the first, second or third stage of disease. We analyzed the sensitivity of each marker at different stages of the disease. The differences in sensitivities across strata were analyzed using a logistic regression model adjusting for possible confounders and covariates. We also performed a stratified analysis by codon 129 PRNP genotype. In a similar way we assessed the absolute levels of quantitative markers across the different stages of the disease.

In order to evaluate the correlation between Tau protein, S100b or NSE levels and disease duration we used the non-parametric correlation coefficient Spearman's Rho. Differences in CSF Tau protein levels between patients with disease duration above or below the median (5 months) were compared using the Mann-Witney test. For the second part of the study, we selected patients who underwent more than one LP during the course of disease and then compared the sensitivity and evolution of each of the CJD markers. Differences in sensitivities were assessed using χ^2 test.

Results

One thousand five hundred and fifty two samples from sCJD patients were studied. The time of LP and disease onset were available in 833 patients and the median time from onset to LP was 3 months (range 0.1-84.5). The LP was performed during the first stage of the disease in 16% (n=137), during the second stage in 38% (n=316), and in the final stage in 46% (n=380). The data on test sensitivity for each marker stratified by the disease stage are given in Table 1. The sensitivity of 14-3-3 tended to increase from the first (88%) to the third stage of the disease (91%). When stratifying by codon 129 of PRNP, this trend became significant in the group of MV patients (p=0.01).

Similar results were obtained for Tau protein (sensitivity of 81% in the first stage and 90.5% in the third stage, p<0.05, n=342) (Table 1). When examining the sCJD patients as a whole there were no significant differences found between the median level of Tau protein across the three disease stages. However there was a trend of a gradual increase of Tau levels in the MV patients, with the highest levels in the last stage (median 1909 pg/mL in the second and 2601 in the third) and in VV patients (median 5949 pg/mL in the second and 10422 in the third stage of the disease).

S100b levels were analysed in 297 samples, 37 in the first stage (12%), 112 in the second (38%), 148 in the third (50%). The disease stage clearly influenced the S100b sensitivity with a significant increase ($p=0.01$) in the latest stage (Table 1). The absolute levels of S100b (Byk-Sangtec® kit) also increased across the disease ($p<0.05$).

NSE levels were assessed in 217 samples, and those obtained in the last stage gave a significantly higher sensitivity (83%) when compared to the second stage (67%, $p=0.01$).

Table 1: Sensitivity of markers in sporadic Creutzfeldt-Jakob disease at different disease stages, in the whole group and stratified by codon 129 PRNP genotype

| | First stage† | N | Second stage† | N | Third stage† | N | P for trend* |
|-----------------|--------------|---------|---------------|---------|--------------|---------|--------------|
| Positive 14-3-3 | 87.6 | 120/137 | 88.0 | 278/316 | 91.3 | 347/380 | ns |
| MM | 94.0 | 79/84 | 90.6 | 155/171 | 93.3 | 166/178 | ns |
| MV | 60.0 | 15/25 | 69.8 | 37/53 | 89.2 | 58/65 | 0.01 |
| VV | 100.0 | 6/6 | 95.7 | 44/46 | 92.2 | 47/51 | ns |
| Positive Tau | 80.9 | 38/47 | 84.9 | 107/126 | 90.5 | 153/169 | ns |
| MM | 83.9 | 26/31 | 88.2 | 60/68 | 92.9 | 79/85 | ns |
| MV | 42.9 | 3/7 | 65.2 | 15/23 | 80.6 | 25/31 | 0.048 |
| VV | 100.0 | 1/1 | 100.0 | 17/17 | 96.0 | 24/25 | ns |
| Positive S100b | 73.0 | 27/37 | 81.3 | 91/112 | 93.9 | 139/148 | 0.01 |
| MM | 78.6 | 22/28 | 81.4 | 48/59 | 97.1 | 68/70 | 0.02 |
| MV | 25.0 | 1/4 | 66.7 | 10/15 | 78.6 | 22/28 | ns |
| VV | 0.0 | 0/1 | 89.5 | 17/19 | 100.0 | 23/23 | ns |
| Positive NSE | 82.5 | 33/40 | 67.1 | 53/79 | 82.7 | 81/98 | 0.01 |
| MM | 86.7 | 26/30 | 68.9 | 31/45 | 84.9 | 45/53 | 0.02 |
| MV | 50.0 | 2/4 | 41.7 | 5/12 | 72.7 | 16/22 | ns |
| VV | 0.0 | 0/1 | 76.9 | 10/13 | 87.5 | 14/16 | ns |

*Adjusted by country of origin, gender, disease duration, age of onset.

† percentages

MM= methionine homozygous; MV= methionine/valine; VV= valine homozygous

NSE= neurone specific enolase

The cut-off value for Tau was 1300pg/ml; for S100b 4.2ng/ml, for the Byk-Sangtec® kit and 0.5ng/ml for the non-commercial kit used in the United Kingdom; and 35ng/ml for the Hoffman La Roche® kit and 25ng/ml for Byk-Diasorin®, Byk-Sangtec® and Wallac® kits for NSE.

Levels of Tau protein, S100b and NSE in CSF were negatively correlated with disease duration (non-parametric correlation coefficient Spearman's Rho= Tau innogenetics -20% ($p<0.001$), S100b Byk-Sangtec® -17% ($p=0.002$), S100b in house -18% ($p=0.01$), NSE Hoffman-la Roche® -21% ($p=0.001$)). Patients with a median disease duration of 5 months or less had a median Tau protein level of 6407 pg/mL (75-3623), whilst patients with a disease duration of longer than 5 months had a median Tau protein level of 4411 pg/mL (75-47150), (Mann-Witney test $p<0.001$).

Sixty-eight out of 1552 (4%) sCJD patients underwent more than one LP (at least two). The median time lapse between the LPs was 6 weeks. Forty-four (65%) of these cases were positive for 14-3-3 in the first LP (Figure 1). This value was different from the sensitivity in the whole group (85%, $p<0.01$). Fifty-four (79%) of sCJD cases had a positive 14-3-3 in the second sample ($p=0.06$). Thirty-eight (56%) sCJD patients had a positive 14-3-3 in both samples, and 60 (88%) patients had at least one positive 14-3-3. Twenty-two sCJD patients (32%) changed their result for 14-3-3 in the second LP. Sixty-six percent of false negative sCJD became positive but 14% of those positive for 14-3-3 became negative in the second LP.

The sensitivity of the other markers was also significantly lower in the first LP when compared to the whole group of sCJD ($p<0.05$). However, the sensitivity of these markers increased in the second sample when compared to the first, although not significantly. Eighty-one percent of sCJD cases had positive Tau protein levels in one of the two LPs, but only 60% of cases had positive levels in both of them (Figure 1). With respect to S100b and NSE, 66% and 70% of sCJD patients had positive results in one of the two LPs and 53% and 33% in both of them, respectively.

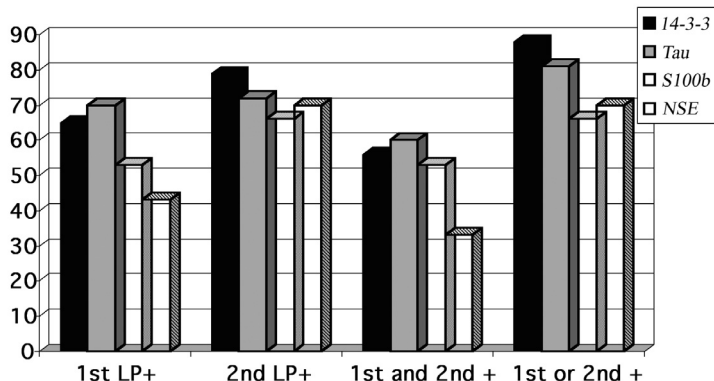


Figure 1: Sensitivity of markers for sporadic Creutzfeldt-Jakob disease diagnosis in repeated lumbar punctures (LP) and combined results in both LP.

LP= lumbar puncture, + = positive, NSE= neurone specific enolase.

We also found that the absolute levels of all three quantitative markers tended to increase in the second LP. This tendency was significant for S100b (median levels: 4.5 ng/ml (1-112) in the first LP, median levels: 7 ng/ml (1-38) in the second; $p=0.027$) (Figure 2). In 39 out of 53 (74%) samples the levels of Tau protein were higher in the second LP than in the first LP. NSE levels were higher in the second LP when compared to the first in 32 out of 46 (70%) samples, and S100b levels were higher in the second LP when compared to the first in 27 out of 38 (71%) samples.

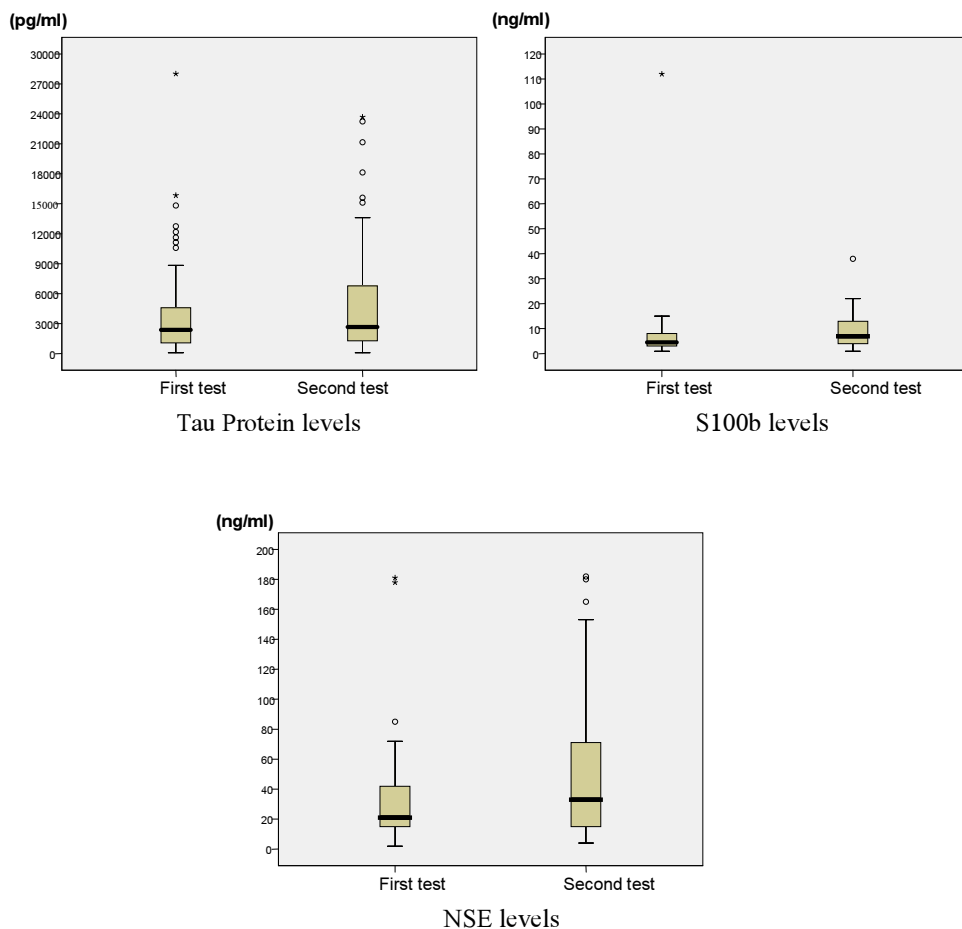


Figure 2: Levels of quantitative markers in repeated lumbar punctures.

When we compared the characteristics of the sCJD patients who underwent repeated LP to the overall group of sCJD we found that these patients were younger at

onset (median/mean age: 61 years SD 9.8 vs. 66 years SD 9.6) and more frequently under 40 years (6% vs. 1%, $p < 0.001$). They had a longer disease duration (median/mean: 12.8 months SD 8.7 vs. 7.9 months SD 7.8) and were more frequently heterozygous at codon 129 ($p < 0.001$).

Discussion

Previous data about the influence of timing on the levels of CSF markers for CJD diagnosis are contradictory. The studies vary with respect to their methodology and often, only single patients were included in the analysis. Jimi et al⁹ showed in a sequential study that the levels of CJD markers increased during the course of the disease and returned to normal or mildly elevated levels in the terminal stage. Mollenhauer et al¹¹ also discussed the difficulties of CJD diagnosis in advanced cases presenting one sCJD case that became 14-3-3 negative in the final stage of the disease. On the other hand, Giraud et al⁸ reported an increase in the levels of 14-3-3 proteins during the course of the disease in a limited number of sCJD cases. Brandel et al⁷ performed a sequential study in iatrogenic CJD, and observed that 14-3-3 was rarely detectable within the first 3 months of the disease but always positive after 7 months associated with aggravation of the disease and the occurrence of dementia. The genotype at PRNP codon 129 influenced the timing of the rise of 14-3-3 in the CSF in these cases. Kropp et al¹⁰ studied NSE levels in 16 sCJD patients with repeated LP. In 15 of them, NSE levels were higher in the second LP with respect to the first, but no other markers were studied and the disease duration or the PRNP genotype were not analysed. Finally, in a recent study, van Everbroeck et al¹³ reported that in 42 sCJD patients the concentrations of 14-3-3 or Tau protein were higher in the middle point of the disease (arbitrarily determined between the 25% and 85% of the total disease duration) than in the early or late stage.

In the framework of a multinational project, we studied the influence of timing on the CSF marker results in 833 sCJD patients. As the disease duration differs among CJD patients, we decided to analyse the influence of the stage of the disease, and not the absolute time to the disease onset or death, on the results of the CSF markers in order to avoid a bias towards the results in patients with shorter disease duration. On the other hand, in order to study the influence of timing on the different PRNP genotypes we also performed a stratified analysis by the PRNP genotype.

Our study showed a tendency for the sensitivity of all CSF markers to increase and for the absolute levels of the quantitative markers to rise during the course of the disease, using both the multivariate analysis and the assessment of the repeated LPs. Thus, CSF markers had the highest sensitivity at advanced stages of disease. The codon 129 PRNP genotype modified this effect. The trend for a higher sensitivity of 14-3-3 and Tau protein for sCJD diagnosis in the later stages of disease became significant in the group of codon 129 MV patients. In addition, we found that higher levels of Tau protein were

associated with a shorter survival, similar to the results of another study, where CSF phosphorylated Tau protein levels were analysed.¹² High levels of other brain-derived proteins (S100b, NSE) were also significantly associated with shorter survival. These brain-derived proteins are released into the CSF as a result of cell damage or death, and as such the levels of these markers reflect the rate of cell death are probably inversely related to disease duration. In those sCJD patients who had more than one LP performed, we found that the sensitivity of all CSF markers increased in the second LP when compared to the first LP. In more than 70% of all sCJD cases tested the absolute levels of the three quantitative markers also increased in the second LP. Sporadic CJD patients who underwent a second LP differed from the whole group, in that they were younger, had a longer disease duration and were more frequently heterozygous for PRNP codon 129 when compared to the whole group. These characteristics made them a more difficult group to diagnose and may explain why a second LP was performed. In this group of atypical sCJD patients a second LP increased the 14-3-3 sensitivity by 14%.

In conclusion, in our study we found that the sensitivity of the CSF markers for sCJD diagnosis increased during disease progression and that this was modulated by the codon 129 genotype. High levels of CSF brain-derived proteins were associated with a shorter survival. A negative CSF 14-3-3 result in the early stages should be interpreted with caution, especially in MV patients. Although performing a second LP is not usual clinical practice for the diagnosis of CJD, it may be useful to perform a second LP, 4-6 weeks after the initial one, in young patients, and those patients with long clinical course or MV genotype.

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4.3

CSF analysis in patients with sporadic CJD and other transmissible spongiform encephalopathies

Abstract

Patients with suspected Creutzfeldt-Jakob disease (CJD) often have routine cerebrospinal fluid (CSF) analysis performed to exclude treatable inflammatory conditions, however little information is available about the range of results obtained for CSF tests in patients with sporadic CJD and other transmissible spongiform encephalopathies (TSE). The objective of our study was to examine the CSF white cell counts, total protein concentrations and the presence of oligoclonal IgG in patients with sporadic CJD and TSEs. Data from 450 patients with sporadic CJD and 47 patients with other TSEs were collected as part of an EC-supported multinational study. Raised cell counts of >5 cells/ μ l were found in three patients out of 298 patients with sporadic CJD, but none had a cell count of >20 cells/ μ l. Total protein concentrations of >0.9 g/l were found in 5 of 438 patients with sporadic CJD, although none had a concentration of >1 g/l. CSF oligoclonal IgG was detected in eight out of 182 sporadic CJD patients. In the patients with other TSEs none had CSF cell counts above 20 cells/ μ l or total protein concentrations of >0.9 g/l and only 1 patient with fatal familial insomnia had detectable oligoclonal IgG. None of the patients with sporadic CJD or other TSEs had abnormalities in all three tests. When investigating patients with suspected TSEs the presence of a CSF cell count of more than 20 cells/ μ l or a total protein concentration of 1g/l or more suggests an alternative diagnosis.

Introduction

The analysis of cerebrospinal fluid (CSF) in patients with suspected sporadic Creutzfeldt-Jakob disease (CJD) is an important investigation in the diagnostic workup. The detection of CSF 14-3-3 is a useful diagnostic test for this condition provided it is used in the appropriate clinical circumstances.^{1,2} The major differential diagnosis of sporadic CJD is other neurodegenerative diseases such as Alzheimer's disease, however a small proportion of patients with suspected sporadic CJD may have inflammatory conditions that are potentially treatable.³ Standard CSF examination has an important utility in identifying these conditions in patients with suspected transmissible spongiform encephalopathies (TSEs). There have been a number of studies showing that there are no substantial inflammatory changes within the CSF of patients with CJD, but most of these reports have only included small numbers of cases or are single case reports.⁴⁻⁸ In this study we describe the results of the white cell count, total protein and oligoclonal IgG found in neuropathologically confirmed cases of sporadic CJD, genetic CJD, variant CJD and fatal familial insomnia.

Methods

The study was conducted in the framework of an EC-supported multinational study. The countries involved were Greece, Germany, Italy, Poland, Spain, Slovakia, Switzerland and the United Kingdom. CSF lumbar punctures were performed as part of the routine diagnostic investigation of the patient by each of the requesting hospitals. As a part of this examination CSF white cell count, total protein and the detection of oligoclonal IgG were performed. CSF samples were also sent to national laboratories for 14-3-3 analysis and the results of the white cell count, total protein and oligoclonal IgG were obtained by the centres from the notifying hospitals. The diagnoses of sporadic CJD and the other forms of TSE were made according to recognized neuropathological criteria and genetic analysis.^{9,10} Core clinical data such as age at onset, gender, disease duration, codon 129 genotype and neuropathological diagnoses were centralized by each centre. A database was set up which included detailed data on the CSF tests and patients characteristics.

CSF results were available from 450 patients with sporadic CJD, 32 patients with genetic CJD, 7 patients with variant CJD, and 8 patients with fatal familial insomnia (Table 1). All CSF samples included in the study were clear and colourless; any blood-stained CSF samples were excluded. The patient demographics are shown in Table 1. A white cell count greater than 5 cells/ μ l was considered to be raised and a total protein of greater than 0.6g/l was considered to be abnormal.

All statistical analyses were performed using SPSS 11.0 for Windows 2000 (SPSS Inc, Chicago, Illinois). Fisher's exact and Mann-Whitney tests were used to assess differences between qualitative and quantitative variables respectively.

Table 1: Patient demographics and number of patients investigated for each of the cerebrospinal fluid parameters, for each TSE group

| | N | Females (%) | Median age at onset in years (range) | Median disease duration in months (range) | Number tested for white cell count | Number tested for total protein | Number tested for presence of oligoclonal IgG |
|--------------|-----|-------------|--------------------------------------|---|------------------------------------|---------------------------------|---|
| Sporadic CJD | 450 | 245 (54.4) | 66 (19-88) | 5 (1-48) | 298 | 438 | 182 |
| Genetic CJD | 32 | 19 (59.4) | 60 (41-77) | 6 (2-34) | 23 | 32 | 10 |
| FFI | 8 | 0 (0) | 54 (24-63) | 15 (8-23) | 3 | 8 | 5 |
| Variant CJD | 7 | 2 (28.6) | 33 (25-43) | 14 (10-27) | 6 | 7 | 0 |

FFI: fatal familial insomnia

Results

Raised white cell counts were detected in three out of 298 of patients with sporadic CJD, three out of 23 patients with genetic CJD, one out of six patients with variant CJD and two out of three patients with fatal familial insomnia (Table 2). Of the three patients with sporadic CJD with raised cell counts, only one patient had a cell count of greater than 10 cells/ μ l, being 20 cells/ μ l. In neither the FFI nor the genetic CJD cases did the cell count exceed 20 cells/ μ l. In none of these cases were there any unusual features to account for presence of a raised white cell count.

Total protein concentrations of greater than 0.6g/l were found in 44 out of 438 patients with sporadic CJD, although only five of these patients had levels of greater than 0.9g/l and none had a value of greater than 1g/l. These five patients had a median age at disease onset of 75 (range: 66-82) and a median disease duration of nine months (range: 2-15). Age at disease onset was significantly higher than that found in the sporadic CJD group with total protein concentrations of <0.9g/l, (median 66 years, range 19-88), $p < 0.05$. Two of these patients were homozygous for MM at codon 129 and the remaining three were heterozygous for methionine and valine. Only one patient with genetic CJD and one patient with FFI had total protein concentrations of greater than 0.6g/l but in neither of these cases did the protein concentration exceed 0.9g/l. Oligoclonal IgG was detected in 4.4% of patients with sporadic CJD and in one patient with FFI, but not in any patient with genetic CJD or variant CJD, (Table 2).

Table 2: Frequency of abnormal CSF white cell counts, raised total proteins and the presence of oligoclonal IgG

| Diagnosis | CSF white cells count >5/ μ l (%) | CSF total protein >0.6g/l (%) | CSF total protein >0.9 g/l (%) | Presence of oligo- clonal IgG (%) |
|--------------|--|----------------------------------|-----------------------------------|--------------------------------------|
| Sporadic CJD | 3 (1.0) | 44 (10.0) | 5 (1.1) | 8 (4.4) |
| Genetic CJD | 3 (13.0)* | 1 (3.1) | 0 (0) | 0 (0) |
| FFI | 2 (66.7)* | 1 (12.5) | 0 (0) | 1 (20.0) |
| Variant CJD | 1 (16.7) | 0 (0) | 0 (0) | 0 (0) |

* Significantly different from sporadic CJD, $p=0.01$. Fisher exact test 2-sided.

When the patients were considered together as a TSE group and the results of the tests combined, only 0.6% of patients had a raised total protein concentration and an elevated white cell count. Only 1% of patients had a raised total protein concentration plus detectable oligoclonal IgG. None of the patients had abnormalities in all three CSF parameters (Table 3).

Table 3: Number of patients with abnormalities in more than one CSF parameter

| Test combination | Number tested | Number of patients with abnormalities (%) |
|--|------------------|--|
| Raised white cell count + total protein >0.6 g/l | 319 | 2 (0.6) |
| Raised white cell count + total protein >0.9 g/l | 319 | 1 (0.3) |
| Oligoclonal IgG + total protein >0.6 g/l | 194 | 2 (1.0) |
| Oligoclonal IgG + total protein >0.9 g/l | 194 | 0 (0.0) |
| Raised white cell count + oligoclonal IgG | 136 | 0 (0.0) |
| Raised white cell count + total protein >0.6 g/l + oligoclonal IgG | 134 | 0 (0.0) |

Discussion

A raised CSF total protein concentration was most common abnormality found in this study, with 10% of sporadic CJD patients having concentrations of greater than 0.6g/l, however only 1.1% of patients had a total protein concentration of greater than 0.9g/l. Interestingly the median age at onset of disease of these patients was significantly higher than that of the rest of the sporadic CJD group. There is evidence that the concentrations

of both albumin and IgG within the CSF increase with age,^{11,12} and as both of these are the major proteins in CSF it suggests that the concentration of total protein will also increase with age. It has also been proposed that reduced CSF flow rate may increase total protein concentrations.^{13,14} As body movement aids the CSF flow rate being bed-bound may reduce the CSF flow rate and, as a result increase CSF total protein concentrations. In this study we found that the median disease duration in sporadic CJD patients with a CSF total protein concentration of greater than 0.9g/l, was longer than those with total protein concentrations of less than 0.9g/l. Therefore it is possible that the raised total protein concentrations found in these patients reflect the age and immobility of the patient rather than any underlying pathology.

The prevalence of CSF oligoclonal IgG in sporadic CJD was found to be 4.4%, which is less than that found in a previous study that reported a prevalence of oligoclonal IgG of 8%.⁸ Interestingly, a study investigating patients with primary neurodegenerative dementia also found oligoclonal IgG in 7% of their patients, although only a few of these patients had neuropathological examination.¹⁵ All of the patients in our study had neuropathological examination and none had evidence of an inflammatory disorder.

In the other sub-types of TSE investigated patients with FFI and genetic CJD had a higher frequency of raised white cell counts when compared to those with sporadic CJD. FFI patients also had elevated total protein concentrations and a higher frequency of oligoclonal IgG. These results may reflect the small numbers of FFI and genetic CJD patients investigated when compared to sporadic CJD. It is of interest that only a small number of patients in this study had more than one abnormality and none had abnormalities in all three of the parameters investigated.

This study has shown that whilst a small percentage of patients with CJD may have isolated increases in CSF white cell count or total protein the magnitude of these changes are small and that the presence of CSF oligoclonal IgG is also rare. None of the patients investigated had abnormalities in all three of these parameters which suggests that the isolated changes seen are not due to a classical inflammatory response. Raised white cell count and total protein in the CSF is unusual in TSE. The presence of more than 20 cells/ μ l and/ or 1g/l of proteins in the CSF suggests an alternative diagnosis.

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4.4

Determinants of diagnostic investigation sensitivities across the clinical spectrum of sporadic Creutzfeldt-Jakob disease

Abstract

To validate the provisional findings of a number of smaller studies and explore additional determinants of characteristic diagnostic investigation results across the entire clinical spectrum of sporadic Creutzfeldt-Jakob disease (CJD), an international collaborative study was undertaken comprising 2451 pathologically confirmed (definite) patients. We assessed the influence of age at disease onset, illness duration, prion protein gene (PRNP) codon 129 polymorphism (either methionine or valine) and molecular sub-type on the diagnostic sensitivity of EEG, cerebral MRI and the CSF 14-3-3 immunoassay. For EEG and CSF 14-3-3 protein detection, we also assessed the influence of the time point in the patient's illness at which the investigation was performed on the likelihood of a typical or positive result. Analysis included a large subset of patients (n=743) in whom molecular sub-typing had been performed using a combination of the PRNP codon 129 polymorphism and the form of protease resistant prion protein (type 1 or 2 according to Parchi et al¹) present in the brain. Findings for the whole group paralleled the subset with molecular sub-typing data available, showing that age at disease onset and disease duration were independent determinants of typical changes on EEG, while illness duration significantly influenced positive CSF 14-3-3 protein detection; changes on brain MRI were not influenced by either of these clinical parameters but overall, imaging data were less complete and consequently conclusions are more tentative. In addition to age at disease onset and illness duration, molecular sub-type was re-affirmed as an important independent determinant of investigation results. In multivariate analyses that included molecular sub-type, time point of the investigation during a patient's illness was found not to influence the occurrence of a typical or positive EEG or CSF 14-3-3 protein result. A typical EEG was most often seen in MM1 patients and was significantly less likely in the MV1, MV2 and VV2 sub-types, whereas VV2 patients had an increased likelihood of a typical brain MRI. Overall, the CSF 14-3-3 immunoassay was the most frequently positive investigation (88.1%) but performed significantly less well in the very uncommon MV2 and MM2 sub-types. Our findings confirm a number of determinants of principal investigation results in sporadic CJD and underscore the importance of recognizing these pre-test limitations before accepting the diagnosis excluded or confirmed. Combinations of investigations offer the best chance of detection, especially for the less common molecular sub-types such as MV2 and MM2.

Introduction

Pre-mortem diagnosis of the rare, transmissible neurodegenerative disorder, sporadic Creutzfeldt-Jakob disease (CJD), largely relies on an appropriate clinical profile supported by characteristic findings on routine investigations such as the electroencephalogram (EEG) and cerebrospinal fluid (CSF) analysis.² Neuropathological examination of the brain remains necessary to achieve a definite diagnosis. Cerebral magnetic resonance imaging (MRI) may also demonstrate highly suggestive changes and is assuming a greater role in CJD evaluation but is not currently included in diagnostic criteria for surveillance purposes.³⁻⁶ Although the diagnostic utility of these investigations (particularly the EEG and CSF 14-3-3 protein detection) has been supported by a number of studies⁷⁻⁹, they remain imperfect surrogate markers, with apparent non-uniform sensitivity across the clinical spectrum of sporadic CJD.¹

Different clinical and pathological sub-types of sporadic CJD have been linked to polymorphism status at codon 129 of PRNP combined with the type (1 or 2) of protease-resistant prion protein (PrP^{Sc}) found in the brain, and together these probably define different human prion strains.^{1,10,11} The most widely used scheme distinguishes only two PrP^{Sc} types,¹ while another commonly employed nomenclature delineates a greater number.¹⁰ Employing this nosology to sub-type sporadic CJD, the EEG has been shown to vary widely between the specific molecular sub-types in demonstrating characteristic or typical changes.^{1,12} Generally, the likelihood of characteristic changes on the EEG was reported to be highest in MM homozygotes, reduced in heterozygotes and lowest in VV carriers. Immunoassay for 14-3-3 proteins in CSF, increasingly utilized in the diagnostic evaluation of patients with suspect CJD,⁹ has also been shown to vary in rates of positivity according to molecular sub-type, with the likelihood of noticeably reduced detection rates in heterozygous patients with type 2 PrP^{Sc} (MV2).¹²⁻¹⁴ A notable difficulty with all previous reports assessing rates of positive diagnostic investigations in molecular sub-types of sporadic CJD is the small numbers of patients studied, especially the less common phenotypic sub-types. Such small patient numbers militate against the reliability of the observations and have precluded multivariate analyses to assess for covariates and probable confounding factors. As a corollary, unexplored so far, is whether within each of the molecular sub-groups and across the phenotypic spectrum of sporadic CJD, there are additional independent variables influencing diagnostic test results. Consequently, there remains a need to more confidently establish the diagnostic utility of these investigations across the various molecular sub-types of sporadic CJD and define further determinants of their sensitivity and specificity across the entire clinical range. More clearly defining additional factors influencing the sensitivity and specificity of these principal investigations has important practical implications when investigating individual patients.

As part of a collaborative, multi-national, CJD surveillance program (EUROCJD) initiated in 1993, we assessed the effect of patient clinical features such as age at onset, illness duration and PRNP codon 129 polymorphism on the diagnostic sensitivity of

EEG, CSF 14-3-3 immunoassay and cerebral MRI in a large, population-based sample of 2451 pathologically confirmed (definite) sporadic cases of CJD, employing harmonized case definitions and data sets. Further, similar analysis was undertaken in a subset of patients (n=743) in whom molecular sub-typing using brain PrP^{Sc} isotype and codon 129 polymorphism had been determined. Inclusion of this data allowed detailed assessment of the variation in positive test results across the molecular sub-types, as well as whether patient parameters such as of age at onset, illness duration and time point at which the investigation was performed were additional independent determinants of investigation results.

Patients and Methods

The present study was undertaken as part of the ongoing activities of the prospective CJD surveillance program (EUROCJD) conducted by the European Union and allied countries. In 1993, national surveillance registers commenced in France, Germany, Italy, the Netherlands, Slovakia and the United Kingdom, with the aim of ascertaining all patients diagnosed with probable or definite CJD in the respective countries. The study was extended in 1997 and again in 1998 to include Australia, Austria, Canada, Spain and Switzerland. EUROCJD collaborative study methods utilizing standardized case definitions and centralized harmonized demographic data sets were as described previously.^{15,16}

The study comprised all patients (n=2451) with definite sporadic CJD who died between December 31st, 1992 and December 31st, 2002; 136 cases from Australia, 68 from Austria, 146 from Canada, 491 from France, 450 from Germany, 342 from Italy, 100 from the Netherlands, 438 from the UK, 18 from Slovakia, 183 from Spain and 79 from Switzerland. A diagnosis of definite sporadic CJD required neuropathological confirmation most commonly performed post-mortem but occasionally through brain biopsy. Analysis of the PRNP open reading frame was performed in the majority of patients to exclude genetic CJD, as clinical differentiation from sporadic CJD is not always possible.^{17,18} Of the 2451 patients, 746 had their brain PrP^{Sc} isotype determined, which in combination with PRNP codon 129 genotyping allowed molecular sub-typing according to the system of Parchi et al.¹ Whenever possible, PrP^{Sc} typing was based on analysis of a number of brain regions, including cerebral cortex, striatum or thalamus and cerebellum, although the preferred number and precise location of sampling sites varied between participating countries.

Whenever possible, all EEGs and MRI brain scans were reviewed by a member of the surveillance system and scored for the presence or absence of typical or characteristic diagnostic features. EEG records were scored positive or characteristic when they fulfilled validated criteria:⁸ sustained periodic sharp wave complexes (PSWC) with a variability of less than 500 ms, with the periodic complexes (lasting 100-600ms) demonstrating a bi- or tri-phasic morphology and seen in a generalized or

lateralized distribution. The CSF 14-3-3 immunoassays were performed in each of the national surveillance centres using Western blotting, with conformity of testing methods and results interpretation confirmed by blinded sample exchange programs. On cerebral MRI, high signal in the putamen and caudate nucleus when using long-repetition time pulse sequences was considered a positive finding for sporadic CJD;³ however, there was no systematic use of particular techniques such as fluid attenuated inversion recovery (FLAIR) or diffusion weighted imaging (DWI). For assessment of the influence of the timing of the investigation during a patient's illness on the test result, data sets for EEG and CSF 14-3-3 protein detection were sufficient to allow this type of analysis. We chose to divide the symptomatic phase into thirds (initial, middle and last) rather than into a greater number of epochs. This was to ensure avoiding the creation of illness periods of such brevity, especially at the onset of symptoms, that they would be unlikely to correspond to a clinically meaningful phase of a patient's illness during which investigations would usually be undertaken, and if undertaken because of the development of symptoms suggesting neurological dysfunction, that these features were sufficiently developed that the investigations had a reasonable likelihood of displaying abnormalities. All data were centralized and analyzed collaboratively.

Statistical analysis

Descriptive statistics were calculated for the whole sample and for every molecular sub-type of the disease. Fisher's Exact and Mann-Whitney tests were used to assess differences between qualitative and quantitative variables, respectively. The number of positive findings, (acknowledging patient may have undergone multiple tests) over the total number of tested CJD cases for EEG, MRI and the CSF 14-3-3 immunoassay was determined for each stratum. The frequency of positive results (number of positive test results in total number of patients) was then calculated for EEG, MRI and the CSF 14-3-3 test for each stratum.

In order to analyze the independent effect of each factor on positive test results, taking into account possible confounders, we fitted two multiple logistic regression models with the test result as output. Both models included age at onset, and disease duration as predictors, and included country of origin, year of death and gender as covariates.

In the first model PRNP codon 129 genotype was entered as a predictor, while in the second model we used the molecular sub-type (combination of PRNP codon 129 genotype and PrP^{Sc} type). Age at onset was categorized into clinically meaningful groups, with patients younger than 50 years serving as the reference group. Disease duration was also categorized with the same criteria into three groups: duration <6 months, 6 months to <1 year, and longer than 1 year. PRNP codon 129 genotype was entered in the model using two dummy variables, with MM as the reference group; for molecular sub-types we created eight dummy variables (MM2, MM1/2, MV1, MV2, MV1/2, VV1, VV2, and VV1/2) with MM1 group as reference. Adjusted odd ratios and 95% confidence intervals were generated. All statistical analyses were performed using SPSS 11.0 for Windows 2000 (SPSS, Inc, Chicago, Illinois).

Results

Table 1 summarizes the salient features of all definite sporadic CJD cases. Of the 2451 patients identified, 1329 were females (54.2%). The median age at death was 68 years (range 20-95 years), while the median disease duration was five months (range 1-81 months).

Table 1: Summary of clinical features of all patients, including the subset undergoing molecular sub-typing

| Total number | All patients | Patients with molecular sub-typing |
|--|------------------|------------------------------------|
| Female (%) | 1329 (54.2) | 392 (52.8) |
| Median of age at onset (range) | 67.2 (15.6-94.9) | 66.2 (15.6-90.0) |
| Age at onset <50 (%) | 116 (5.1) | 39 (5.5) |
| Age at onset 50-59 (%) | 412 (18.2) | 141 (19.7) |
| Age at onset 60-69 (%) | 867 (38.3) | 286 (40.0) |
| Age at onset 70-79 (%) | 729 (32.2) | 212 (29.7) |
| Age at onset >80 (%) | 137 (6.1) | 37 (5.2) |
| Median duration of illness in months (range) | 5 (1-81) | 5 (1-62) |
| Patients with durations <6months (%) | 1332 (58.8) | 404 (56.5) |
| Patients with duration 6-12 months (%) | 611 (27.0) | 179 (25.0) |
| Patients with durations >12months (%) | 321 (14.2) | 132 (18.5) |
| Codon 129 genotype | | |
| Met-Met/Total (%) | 1061/1604 (66.1) | 504/743 (67.8) |
| Met-Val/Total (%) | 272/1604 (17.0) | 117/743 (15.7) |
| Val-Val/Total (%) | 271/1604 (16.9) | 122/743 (16.4) |
| Patients with PRNP gene sequenced (%) | 1492 (63.6) | 650 (87.5) |
| PrP isotype | | |
| type 1 | 495/743 (66.6) | 495/743 (66.6) |
| type 1 and 2 | 44/743 (5.9) | 44/743 (5.9) |
| type 2 | 204/743 (27.5) | 204/743 (27.5) |
| EEG | | |
| Typical/Total (%) | 1216/2083 (58.4) | 371/666 (55.7) |
| 14-3-3 protein in CSF | | |
| Positive/Total (%) | 1340/1521 (88.1) | 486/554 (87.7) |
| MRI | | |
| Characteristic findings/Total (%) | 405/1036 (39.1) | 150/387 (38.8) |

An EEG was performed on 2083 patients (85%). Of those undergoing EEG, 1216 cases showed typical PSWCs (58.4%) (Table 1). The codon 129 genotype had a significant effect on the likelihood of a positive result of the EEG in sporadic CJD patients (Table 2), which ranged from 73.2% in MM homozygotes to 21.5% in VV homozygotes. After adjusting for possible confounding variables (country, sex, year of death, age at onset and disease duration), the prevalence of PSWC on the EEG was significantly decreased in heterozygotes ($p<0.001$) and VV homozygotes ($p<0.001$). The median age of death of sporadic CJD patients with a typical EEG was 69.0 years (range 34 to 92) versus 66 years (range 20 to 88) in patients with an atypical EEG ($p<0.001$). The presence of PSWC was further analyzed by age at onset and disease duration (Table 3). The likelihood of a typical EEG steadily increased with age ($p<0.001$, adjusted for the same variables), while there was an inverse correlation with disease duration ($p=0.001$).

Table 2: Summary of principal investigation findings for all patients who underwent PRNP codon 129 genotyping

| | EEG | MRI | 14-3-3 protein in CSF |
|---------------|-------------------|-------------------|-----------------------|
| | Typical/Total (%) | Typical/Total (%) | Positive/Total (%) |
| PRNP genotype | | | |
| M/M | 710/970 (73.2) | 172/474 (36.3) | 709/796 (89.1) |
| M/V | 99/245 (40.4) | 65/134 (48.5) | 147/191 (77.0) |
| V/V | 50/233 (21.5) | 71/139 (51.1) | 184/196 (93.9) |
| P-value | <0.001 | 0.004 | 0.002 |

Adjusted by: age at onset, disease duration, sex, country of origin, year of death.

CSF 14-3-3 protein analysis was performed on 1521 cases (62.1%) of sporadic CJD. Of these, 1340 cases were positive (88.1%) (Table 1). Although the frequency of a positive CSF 14-3-3 immunoassay was high for all three codon 129 genotypes, heterozygotes had a significantly lower rate (77.0%) in adjusted analyses than VV ($p<0.001$) and MM ($p<0.05$) (Table 2). Age at disease onset did not influence CSF 14-3-3 protein detection but as for the EEG a positive result was less likely with longer disease duration (Table 3) ($p<0.001$).

Table 3: *Principal investigation findings for all patients according to age at disease onset and duration of illness*

| | EEG | MRI | 14-3-3 protein in CSF |
|--|--------------------------|--------------------------|------------------------------|
| | Typical/Total (%) | Typical/Total (%) | Positive/Total (%) |
| Age at onset | | | |
| Age at onset <50 (%) | 35/104 (33.7) | 18/58 (31.0) | 62/77 (80.5) |
| Age at onset 50-59 (%) | 197/371 (53.1) | 83/196 (42.3) | 222/260 (85.4) |
| Age at onset 60-69 (%) | 457/796 (57.4) | 166/415 (40.0) | 472/545 (86.6) |
| Age at onset 70-79 (%) | 438/675 (64.9) | 126/328 (38.4) | 452/492 (91.9) |
| Age at onset >80 (%) | 81/124 (65.3) | 12/37 (32.4) | 73/83 (88.0) |
| P for trend | <0.001 | 0.3 | 0.4 |
| Disease duration | | | |
| Patients with durations <6 months (%) | 814/1228 (66.3) | 203/587 (34.6) | 779/840 (92.7) |
| Patients with durations 6-12months (%) | 283/558(50.7) | 125/287 (43.6) | 358/408 (87.7) |
| Patients with durations >12months (%) | 115/288 (39.9) | 77/160 (48.1) | 146/211 (69.2) |
| P for trend | 0.001 | 0.4 | <0.001 |

Adjusted by: PRNP codon 129 genotype, sex, country of origin, year of death.

MR brain imaging was performed on 1063 patients (42.3%). Of these patients, 405 patients showed characteristic changes (39.1%) (Table 1). In contrast with the EEG, the likelihood of a positive MRI scan was higher in cases with the MV ($p < 0.05$) and VV genotypes ($p = 0.002$) in comparison to those with an MM genotype (Table 2). MRI results were not significantly associated with age at onset or disease duration (Table 3).

Analysis of sporadic CJD patients according to molecular sub-type

In 743 patients their codon 129 polymorphism and brain PrP^{Sc} isotype were determined allowing molecular phenotypic sub-stratification (Table 1).¹ In 87 % of these patients the PRNP gene was sequenced, ruling out pathological mutations associated with genetic CJD. In comparison to the entire group, the median age at onset, median disease duration and PRNP codon 129 polymorphism distributions were similar, and there were no significant differences in the overall rates of positive results for each of the investigations. The most common PrP^{Sc} isotype was type 1 (67%); type 2 was present in 27% of the sample, and in 44 cases (6%) there was coexistence of both type 1 and 2.

The relative frequency of the various molecular sub-types is provided in Table 4. There were occasional significant differences in demographic characteristics between sub-groups. MM1 patients were the oldest at disease onset (median 67.8 years) and VV1 patients the youngest (median 47.2 years). The duration of the disease was significantly shorter in MM1 patients (median 4 months) in comparison to the rest, especially the MM2 (median 12.5 months) and MV2 (median 12 months) sub-groups, with these groups also demonstrating a higher percentage of patients with disease lasting more than one year (57% and 52%, respectively). Of note, albeit the small patient numbers preclude statistically significant differences, patients with co-existence of PrP^{Sc} types 1 and 2 (MM1/2, VV1/2, and MV1/2) tended to have intermediate phenotypes but generally aligning more to the most common molecular PrP^{Sc} sub-type within each PRNP codon 129 genotype.

Overall, determinants of investigation findings in patients who underwent molecular sub-typing were the same as for the entire group. Independent of disease duration and molecular sub-type, age at disease onset correlated significantly with a positive EEG result ($p = 0.007$) (Figure 1). Patients who developed symptoms below age 50 years had a lower rate of typical PSWCs (22%) than patients presenting after age 60 years. The median age at onset of the patients with an atypical EEG was 64 years (range 16 to 86), compared with 68 years (range 31 to 90) in patients showing the typical findings. CSF 14-3-3 protein immunoassay and MRI results were not significantly correlated with patient's age at disease onset.

Table 4: Summary of age at disease onset and illness duration for all patients undergoing molecular sub-typing

| | Sporadic CJD molecular sub-types | | | | | | | | | |
|----------------------------------|----------------------------------|-------------------|------------------|------------------|-------------------|------------------|-------------------|------------------|------------------|--|
| | MM1 | MM2 | MMI/2 | MV1 | MV2 | MV1/2 | VV1 | VV2 | VV1/2 | |
| Total number (%) | 444 (59.8) | 31 (4.2) | 29 (3.9) | 37 (5.0) | 73 (9.8) | 7 (0.9) | 14 (1.9) | 100 (13.5) | 8 (1.1) | |
| Median of age at onset (range) | 67.8 (31.1-89.4) | 60.3 (37.7-82.3)* | 65.6 (48.9-89.9) | 65.5 (15.6-84.2) | 63.6 (40.8-81.1)* | 66.3 (56.7-77.9) | 47.2 (23.6-72.7)* | 66.3 (40.2-86.3) | 59.7 (18.7-72.7) | |
| Age at onset <50 (%) | 12 (2.8) | 5 (16.7)† | 1 (3.8) | 1 (2.8) | 2 (2.9) | 0 (0.0) | 7 (53.8)† | 9 (9.3)† | 2 (25.0)† | |
| Age at onset 50-59 (%) | 78 (18.2) | 9 (30.0) | 3 (11.5) | 9 (25.0) | 17 (24.6) | 1 (14.3) | 2 (15.4) | 20 (20.6) | 2 (25.0) | |
| Age at onset 60-69 (%) | 172 (40.1) | 11 (36.7) | 12 (46.2) | 10 (27.8) | 37 (53.6)† | 3 (42.9) | 3 (23.1) | 35 (36.1) | 3 (37.5) | |
| Age at onset 70-79 (%) | 143 (33.3) | 4 (13.3)* | 7 (26.9) | 13 (36.1) | 12 (17.4)* | 3 (42.9) | 1 (7.7) | 28 (28.9) | 1 (12.5) | |
| Age at onset >80 (%) | 24 (5.6) | 1 (3.3) | 3 (11.5) | 3 (8.3) | 1 (1.4) | 0 (0.0) | 0 (0.0) | 5 (5.2) | 0 (0.0) | |
| Median duration (months) (range) | 4.0 (1.0-39.0) | 12.5 (3.0-38.0)† | 6.0 (2.0-33.0) | 5.0 (1.0-54.0)† | 12.0 (3.0-62.0)† | 13.0 (2.0-30.0) | 11.0 (2.0-29.0)† | 6.0 (2.0-25.0)† | 5.5 (3.0-62.0) | |
| Durations <6 months (%) | 307 (71.6) | 9 (30.0)* | 11 (42.3)* | 21 (58.3) | 5 (7.2)* | 3 (42.9) | 4 (30.8)* | 40 (41.2)* | 4 (50.0) | |
| Durations 6-12months (%) | 78 (18.2) | 4 (13.3) | 11 (42.3)† | 7 (19.4) | 28 (40.6)† | 0 (0.0) | 3 (23.0) | 47 (48.5)† | 1 (12.5) | |
| Durations >12months (%) | 44 (10.3) | 17 (56.7)† | 4 (15.4) | 8 (22.2)† | 36 (52.2)† | 4 (57.1)† | 6 (46.2)† | 10 (10.3) | 3 (37.5)† | |
| Female (%) | 239 (53.8) | 15 (48.4) | 14 (48.3) | 18 (48.6) | 35 (47.9) | 5 (71.4) | 5 (35.7) | 56 (56.0) | 5 (62.5) | |

*Significantly lower than MM1 (p<0.05).

†Significantly higher than MM1 (p<0.05).

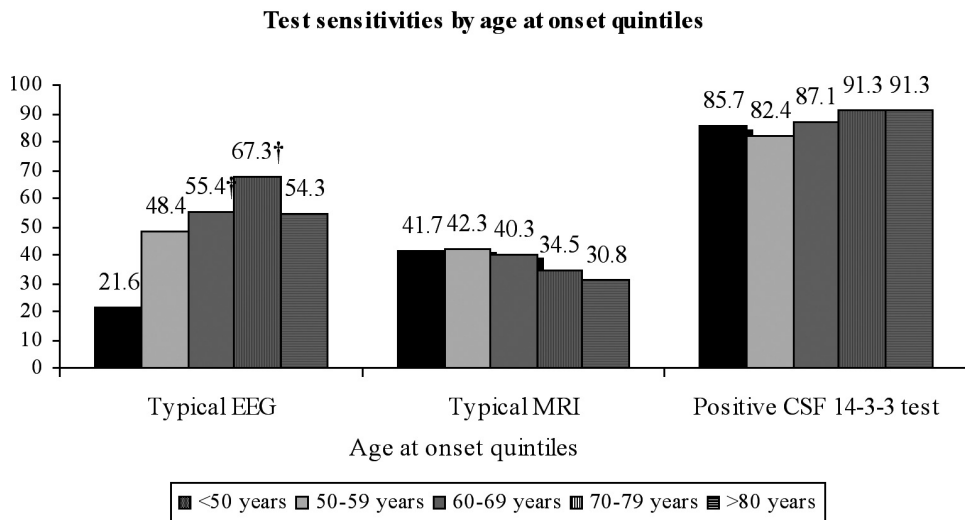


Figure 1: The principal investigation results for EEG, brain MRI, and CSF 14-3-3 protein analysis were correlated with age at disease onset in sporadic CJD for all patients undergoing molecular sub-typing. Patients were grouped by age at onset; <50 years, 50-59 years, 60-69 years, 70-79 years, and > 80 years. Only the EEG showed a significant correlation with age at disease onset.

† Significantly lower than in <50years group ($p < 0.05$). Adjusted by molecular sub-type, disease duration, sex, country of origin and year of death.

The duration of disease was independently associated with the likelihood of PSWCs. Patients with disease duration shorter than 6 months had a significantly higher rate ($p = 0.02$) of a typical EEG than patients with duration longer than 12 months (65% versus 35%, respectively). The median disease duration of patients with an atypical EEG was 6 months (range from 1 to 62) versus 4 months (range from 1 to 39) in patients with typical EEG. Disease duration significantly ($p = 0.004$) influenced the likelihood of a positive CSF 14-3-3 test result. Those patients with disease duration shorter than 6 months had a higher rate of positive test results than those with duration longer than 12 months (91% versus 72%, respectively). On average, patients with 14-3-3 proteins detectable in their CSF had shorter disease durations (median 5 months, range from 1 to 39) than patients with a negative test result (median 11 months, range from 2 to 54). MRI results did not correlate with disease duration (Figure 2).

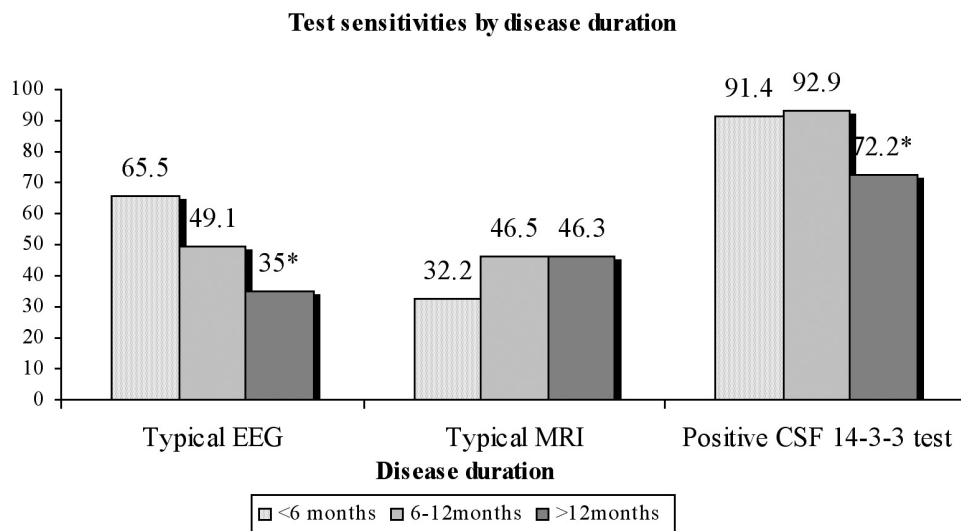


Figure 2: The principal investigation results for EEG, brain MRI, and CSF 14-3-3 protein analysis were correlated with disease duration in sporadic CJD for all patients undergoing molecular sub-typing. Disease duration was arranged as; <6 months, 6-12 months, and >12 months. For both the EEG and CSF 14-3-3 protein detection there was a significant inverse correlation with disease duration of greater than 12 months.

* Significantly lower than in <12 months group ($p < 0.05$). Adjusted by molecular sub-type, age at onset, sex, country of origin and year of death.

Certain molecular sub-types showed significant correlations with investigation results. MM1 patients demonstrated the highest frequency of positive EEG results (73%) followed by MV1 patients (53%), although after adjusting for covariates the significance was reduced ($p = 0.01$). VV2 and MV2 patients had infrequent occurrence of a typical EEG, with a positive result only observed in 12.8% and 17.5% of the cases, respectively (Figure 3). Of the sub-groups with coexistence of PrP^{Sc} types, only VV1/2 patients were significantly lower. CSF 14-3-3 immunoassay was the most frequently positive investigation, with an overall positive rate of 88%. However, there were some significant variations across molecular sub-types, with the test significantly more often positive (all $p < 0.05$) for MM1 and VV2 patients (91% and 95%) than for MM2 (61%) and MV2 (71%) (Figure 3). Of the patient groups with coexistence of PrP^{Sc} types, only MM homozygotes had a significantly lower rate of positive results for this test (Figure 3). For MRI, only the VV2 sub-group demonstrated a significantly increased likelihood of a typical positive result. Of note, within each molecular sub-type except VV1, occasional patients did not display typical or characteristic findings with any of the three investigations.

Test sensitivities by sporadic CJD subtypes

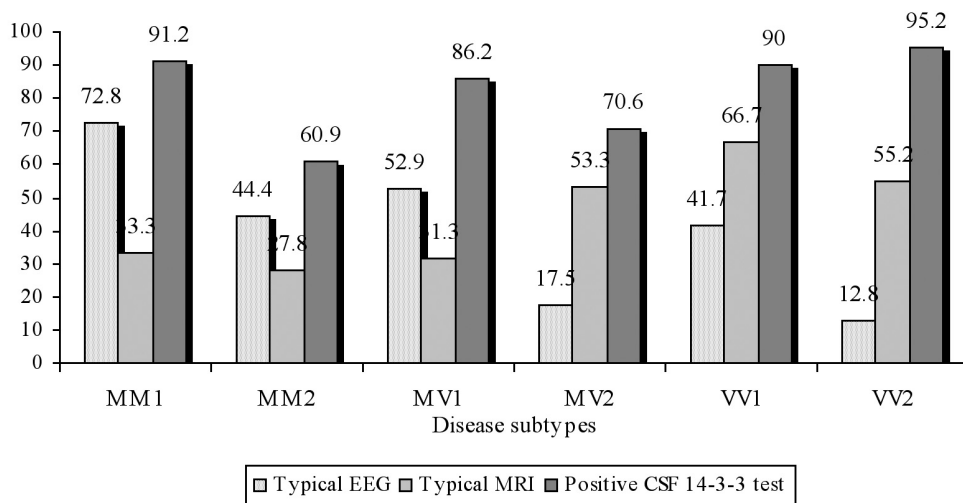


Figure 3: The sensitivities of EEG, brain MRI and CSF 14-3-3 protein detection were assessed according to the sporadic CJD molecular sub-type: MM1, MM2, MM1/2; MV1, MV2, MV1/2; and VV1, VV2, and VV1/2. The MV1, MV2, VV2 and VV1/2 sub-types all showed a significantly reduced EEG sensitivity, while the VV2 sub-type displayed a significantly higher likelihood of a typical MRI result. The MM2, MM1/2, and MV2 sub-types all showed significantly reduced rates of CSF 14-3-3 protein positivity.

*Significantly lower than MM1 ($p < 0.05$).

† Significantly higher than MM1 ($p < 0.05$). Adjusted by age at onset, disease duration, sex, country of origin and year of death.

Assessment of the influence of the time point of the investigation during a patient's illness on the test result

For both EEG and the detection of CSF 14-3-3 proteins, sufficient data were available to also analyse the influence of the time point at which the investigation was performed during the patient's illness on the likelihood of a typical or positive result. With the median duration of illness only 5 months, we chose to divide the symptomatic phase into thirds (initial, middle and last) rather than into a greater number of epochs. This was undertaken to avoid the creation of illness phases of such brevity, especially at the onset of symptoms, that they would be unlikely to correspond to a clinically meaningful period of a patient's illness during which investigations would usually be undertaken and that the test would have a reasonable chance of showing typical abnormalities.

With respect to EEGs, 2425 recordings with a known time point in relation to the

chronology of the patient's illness were available for analysis. When analysed in isolation, the likelihood of a typical EEG was significantly less in the first third of a patient's illness compared to the last third ($p=0.02$). Multivariate analysis (adjusted by country, gender, year of death, age at onset, disease duration and codon 129 polymorphism) also showed a positive EEG was significantly more likely in the last third of a patient's illness compared to the first third ($p=0.001$). When a patient's molecular sub-type was substituted for codon 129 polymorphism in the same regression model however, a typical periodic EEG was no longer significantly more likely in any particular time period of the patient's illness. These results are consistent with a patient's molecular sub-type constituting the dominant determinant of the likelihood of a positive EEG rather than the specific time point at which the test is undertaken in the symptomatic period. Detection of CSF 14-3-3 proteins showed no significant association with the time point of sampling during a patient's illness, regardless of whether assessed in isolation or in analogous multivariate analyses.

Discussion

The current study is by far the largest to date to assess the diagnostic sensitivity of investigations usually employed in the evaluation of suspected sporadic CJD. By virtue of the large number of definite sporadic CJD patients comprising our study we have been able to confidently delineate the sensitivities of the principal investigation results across the entire phenotypic spectrum, especially the less common molecular sub-types, as well as clarify and extend hitherto uncertain independent determinants of investigation results.¹⁶ Analysis of only neuropathologically confirmed CJD patients has allowed us to avoid the inherent difficulties that arise from analysis of investigations that also serve in case definition criteria for probable cases; however, it is noteworthy that separate analysis of all EURO-CJD sporadic CJD patients from this time period, that is, inclusion of clinically probable cases, gave the same results as analysis of just definite cases (data not shown). Multivariate regression modelling demonstrated that age at disease onset and disease duration both act as independent determinants of some investigation results in sporadic CJD, in addition to the influence of molecular sub-type. Consequently, in the clinical evaluation of suspect CJD, our findings reinforce the importance of fully appreciating the limitations of specific investigations relative to these salient patient clinical features, otherwise the heightened likelihood of a negative test result may be underestimated and incorrectly interpreted as undue reassurance against the diagnosis. Specifically, the probability that an EEG will demonstrate PSWCs increases with age and conversely decreases with disease duration, especially if longer than 12 months. Hence, in a patient under 50 years it is most likely that an EEG will not show typical changes, and even in older patients, if their illness is longer than 6 months, the probability of PSWCs is at best only around 50%. In contrast to a prior smaller study¹² disease duration longer than 12 months was also observed to significantly lessen the

likelihood of a positive CSF 14-3-3 result. This underscores the importance of acknowledging illness duration when interpreting a negative result in an individual patient suspected to be manifesting sporadic CJD.

The present study re-affirmed that molecular sub-type, in addition to age at onset and illness duration, is also an important independent determinant of principal investigation results.^{1,4,10,12-14} In contrast to the EEG, where the presence of valine alleles at codon 129 additively reduces the likelihood PSWCs, valine homozygotes (especially VV2 patients) were significantly more likely to demonstrate a typical MRI scan than methionine homozygotes (particularly the MM1 sub-group). These data conform to a pattern whereby sub-types with 'classical' sporadic CJD and predominant cerebral cortical involvement (MM1 and MV1) are more likely to manifest PSWCs on EEG, while those with ataxia and subcortical neuropathological changes (VV2 and MV2) are more likely to demonstrate characteristic basal ganglia changes on MRI. However, in contrast to a prior report suggesting phenotypic homogeneity between the MM1 and MV1 sub-types,¹ we found that the likelihood of a typical EEG was significantly lower and the average illness duration significantly longer in the MV1 patients. The most likely explanation for this difference in findings is the much larger sample of MV1 patients available for analysis in our study.

The CSF 14-3-3 protein immunoassay was the most sensitive investigation across the sporadic CJD phenotypic spectrum, but the influence of molecular sub-type was still evident. Codon 129 heterozygotes, especially the MV2 sub-group, were significantly less likely to be associated with a positive result,^{9,12-14} as were the very uncommon MM2 and MM1/2 sub-groups. In comparison to the simple demographic features of age at disease onset and illness duration, the molecular sub-typing profile is much less likely to be available pre-mortem in individual patients. Nevertheless, if this information is available investigation results would also need to be interpreted in this specific context for the most accurate estimation of excluding or confirming the likelihood of sporadic CJD. Recognizing these overall limitations and variations across the molecular sub-types, from a practical clinical perspective it would appear prudent to utilize all three principal investigations (brain MRI, CSF 14-3-3 protein analysis and EEG), as irrespective of molecular sub-type only very uncommonly (except the rare MM2 sub-group) will all three investigations not show characteristic findings in an individual patient with sporadic CJD.⁵

Our analyses do not support that the time point at which the investigation is undertaken during a patient's illness is an important determinant of the test result. This was particularly evident for CSF 14-3-3 protein detection, for which no association between time point of sampling and a positive result was found regardless of analytical approach. For EEG, the apparent reduced likelihood of a typical or periodic recording in the first third of a patient's illness compared to the last period, when examined in isolation or with one of the multivariate regression models, was no longer evident when the regression model included molecular sub-type. These findings are again consistent with the generally dominant influence of age at onset, illness duration and molecular

sub-type as the key independent determinants of investigation results compared to the time point of test sampling during a patient's illness.

Although molecular sub-typing of sporadic CJD patients has proven a useful phenotypic classification system, there are some difficulties with this approach. Not uncommonly, more than one PrP^{Sc} type may be present in the brain of a patient dying from sporadic CJD¹, and it is likely that the frequency of occurrence increases with the number of regions assessed.^{19,20} At present, there is no consensus on how to classify such patients and whether it is generally better to adopt a dichotomised approach based on predominance of one type when several regions are studied or accept classification based on the analysis of only one or two brain sites. In our study, patients with more than one PrP^{Sc} type were a small minority, but such patients were allowed status as a separate group within each PRNP genotype without attempting to adjust for relative abundance of the two PrP^{Sc} types. Regardless of PRNP genotype, our patients with simultaneous occurrence of both PrP^{Sc} types tended to present a different phenotype, sometimes intermediate between those with a single PrP^{Sc} isotype and the same codon 129 status, but often extending beyond the range observed. This suggests that the simultaneous presence of two PrP^{Sc} types may evoke a third or unique phenotype within each codon 129 genotype, although our small patient numbers militated against achieving significance with these observations. Analysis of investigation results also generally suggests that sub-groups with co-existence of PrP^{Sc} isotypes tended to appear different although not invariably. For CSF 14-3-3 results, the MM1/2 group behaved more like MM2 patients, and for the EEG the VV1/2 group were also more like the VV2 patients. The latter is in keeping with the predominant phenotypic influence of type 2 PrP^{Sc} on these investigations or may simply represent a sampling bias wherein type 2 PrP^{Sc} was under-appreciated as the dominant protease resistant isotype across the brain.

MRI brain imaging is now very commonly employed in the diagnostic evaluation of suspected sporadic CJD but its ultimate sensitivity and specificity is still being defined.^{3-6,21} For a number of reasons, our overall sensitivity for typical changes on MRI scan is lower than that reported in much smaller studies.^{4-6,12,22} Importantly, patient MRI data collected for this study extended over a decade, during which time this imaging technique was increasingly used in the evaluation of suspect sporadic CJD, and there were concurrent improvements in the diagnostic sensitivity of pulse sequences used for this illness.^{3,5,6} Consequently, the overall number of patients studied with MRI was relatively low (42.3%), and the utilisation of optimised MRI techniques such as DWI and FLAIR mainly occurred only in the very latter phase of our study epoch and even then was not uniformly adopted by all neuro-imaging services. Further, during the study period there was progressive appreciation that increased cerebral cortical signal using the more sensitive techniques of DWI and FLAIR is probably a reasonably common feature in sporadic CJD.^{5,6} Consequently, another reason for our low MRI sensitivity relates to our restrictive definition of what changes we considered characteristic of the diagnosis (i.e. signal hyperintensity limited to the basal ganglia). Overall, this combination of factors has compromised our assessment of the true diagnostic

sensitivity and clinical utility of MRI in the evaluation of sporadic CJD. This sub-optimal aspect of the present study is being redressed by a further prospective EURO-CJD collaborative project.

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5

General discussion

Introduction

Due to the unique characteristics of the transmissible agent and its public health implications, the group of transmissible spongiform encephalopathies (TSE) has been object of a considerable amount of attention from the scientific community and even for the general society, especially after the bovine spongiform encephalopathy (BSE) crisis. Taking into account that the estimated incidence of this group of diseases is around 1 to 2 cases per million-year,^{1,2} this is something exceptional. Since the transmissibility of the disease was proven³ and the protein-only hypothesis proposed as the most commonly accepted pathogenic theory,⁴ both achievements awarded with the Nobel Prize, we have learnt a great deal about the mechanisms involved in these diseases. However, important challenges remain, the most important one is perhaps that of developing an effective treatment.

This thesis focuses on two issues: aetiological factors and clinical diagnosis. The data collected have been scrutinized under two main prisms: classical epidemiology in search of environmental factors, and genetic epidemiology in search of genetic determinants and interactions between genetic and environmental factors. The studies are all product of collaborative efforts, fruit of the work of multidisciplinary teams of national and international epidemiological surveillance networks.

The objectives of this chapter are the following to: present the main findings of the studies included in this thesis, put them into perspective of each other and of the current scientific knowledge, discuss the most relevant methodological issues and finally outline the directions for further research in light of the findings of this thesis.

Epidemiology of CJD

The national Creutzfeldt-Jakob disease (CJD) surveillance systems were originally created with the objective of monitoring the disease trends and detecting the potential emergence of atypical cases, especially cases of variant CJD (vCJD).⁵ Moreover, independently of its public health function, the establishment of international surveillance networks has contributed enormously to broaden the scientific knowledge of TSEs, favoring the exchange of knowledge among experts from different countries, encouraging scientific collaborations, and facilitating the generalization and standardization of new diagnostic techniques. Most importantly, it enabled to collect large series of cases and controls using a common protocol and homogeneous procedures.

In chapter 2.1 we discussed methodological issues concerning the set up of a multinational collaborative network like the extended European collaborative study group of CJD (NEUROCID) and the results of their monitoring activities during a 8-years period in Belgium, Denmark, Finland, Greece, Iceland, Ireland, Israel, Norway, Portugal, Sweden and the United Kingdom. The overall annual mortality rate of CJD in

the 11 studied countries from 1997 to 2004 was 1 case per million inhabitants. This figure is in agreement with most of the studies carried out until now.² There were some variations across time and among countries but they were not statistically significant.

The main objective of the CJD surveillance systems is the detection of cases related to the BSE epizootic. These include vCJD cases which are known to be related to BSE, but also new clinical forms of disease. The potential occurrence of these new phenotypes might be determined by factors like host genome and route of infection. We first aimed to unravel how frequent the different clinical presentations of CJD were, starting with the most common subtype: sCJD. The commonest clinical presentations were the rapidly progressive dementia (74%), followed by the ataxic onset (9%). Although much less frequent than the first two, visual presentation (Heidenhein's syndrome) (4%) was also well recognized. Other clinical presentations, psychiatric (3%), sensory (2%), and extrapyramidal (0.4%) were recognized less frequently. Finally, 6% of the cases presented with a slowly progressive dementia syndrome. Furthermore, we defined atypical CJD cases according to three criteria: age at onset < 50 years, illness duration > 1 year and atypical clinical presentation being defined as any other than rapidly progressive dementia, cerebellar or Heidenhein's. During the study period we ascertained 27 sCJD patients (6% of the total) younger than 50 years at onset, 51 sCJD patients (12% of the total) with disease duration superior to one year, and 69 (6%) sCJD patients with atypical clinical onset. Excluding the UK, only one case of vCJD, in the Republic of Ireland, was diagnosed during the period of observation in the countries participating in NEUROCID.

In chapter 2.2 we present a case-report of the first vCJD patient ascertained in the Netherlands. The clinical characteristics of the patient, MRI scan images, neuropathological study, PrP^{Sc} western blot profile and PRNP M129V genotype, were all indistinguishable from that reported in UK vCJD patients. These similarities suggest that our patient shares a common etiological agent with the UK cases. The identification of this patient had important public health implications for the country. The most immediate one was that it contributed to call attention of the medical community. Soon after the description of the first case a second one was identified.⁶

Case-reports are often criticized as anecdotic⁷ and not reliable scientific evidence. However, the history of CJD research is a very good example of their potential importance in the study of rare diseases. Case-reports have played a fundamental role in CJD research. After the first experimental transmission of CJD in 1968 further evidence came through the descriptions of cases transmitted iatrogenically via corneal transplantation and neurosurgical procedures.^{9,10} It was thanks to a series of cases, vCJD was first identified in the UK.¹¹ It also has been an important tool for identifying new vCJD cases linked to blood transfusion.¹² However, case reports have obvious limitations for assessing the effect of common exposures, offering sometimes contradictory evidences. Even for well-established risk factors, it is sometimes very difficult to confirm a solid causal link with the disease. For instance, the second iatrogenic CJD case associated to human growth hormone in the Netherlands received only a very small dose with diagnostic purposes 38 years before the disease onset.¹³

Another question addressed in this thesis (chapter 3.1) is the origin of vCJD cases outside the UK. The fact that the first Dutch vCJD patient never traveled to the UK, and the relatively low BSE incidence in the Netherlands, less than 80 BSE cases¹⁴ had been detected until that time, led us to the conclusion that the most likely cause of our patient's disease had been the bovine material imported from the UK during the BSE epizootic in that country. In chapter 3.1 we assessed the role of UK bovine exports in the spread of vCJD to countries outside the UK. We found a positive correlation between the amounts of material imported from the UK during the 80s and the number of vCJD cases ascertained. Specifically, live bovine seemed to be the main independent factor associated to the occurrence of vCJD outside the UK. The greatest volume of these exports went to France, the Netherlands and Ireland, the three countries with higher number of vCJD cases outside the UK.

The occurrence of vCJD and its causal link with bovine spongiform encephalopathy in cattle¹⁵ have increased interest in the search for possible environmental sources of sCJD. Iatrogenic CJD is rare. Horizontal transmission of the disease has been linked to human corneal grafting, human dura mater grafts, human growth hormone treatment, human pituitary gonadotrophin therapy and neurosurgical procedures or stereotactic EEG electrodes.¹⁶ Epidemiological studies have tried to elucidate the role of hidden environmental factors in the etiology of sCJD.¹⁷⁻³⁰ Despite these efforts, the mode of transmission of sCJD, if any, remains unknown. However, the rarity of this entity, and the fact that in case of transmission the disease develops after a usually very long incubation period, makes its epidemiological study very challenging. In chapter 3.2 we present the results of a case control study carried out in the UK assessing the past history of ophthalmic surgery as a risk factor for sCJD and vCJD. We did not find evidence of any association between past history of ophthalmic surgery and risk of either sCJD or vCJD. The findings of our study are consistent with several previous case-control studies assessing the history of eye surgical procedures as a possible hidden risk factor for sporadic CJD.^{22,23} However, these findings contrast with an Australian study in which cataract/eye surgery increased risk of sCJD more than 6 folds in comparison to community controls.²⁴ One interesting finding in our study is that sCJD patients with early visual symptoms, especially patients with Heidenhein's form of CJD, are often referred at first to ophthalmologists, and not infrequently they are diagnosed with cataracts and often treated. Considering that the eye of CJD patients has proved to be infectious, and the level of infectivity is thought to increase with the clinical course of the disease,³¹ the information included in our study is of potential interest for ophthalmologists and public health.

Epidemiological studies assessing the role of common exposures as CJD risk factors face important methodological challenges. Classically, in CJD research, the case-control design has been the main approach in looking for environmental etiological factors. However, there are important methodological issues. First of all, and due to the special characteristics of the disease, it is very unlikely that at the moment of the diagnosis the patient is able to answer a questionnaire about past exposure. Therefore,

this information comes most of the times from an interview with their relatives, and this should be taken into account when interviewing controls, which ideally should be carried out with identical methodology in order to avoid information bias. Another possible source of information bias is the different means of obtaining the information in cases and controls. For example, in a surgical risk assessment study, community controls were interviewed telephonically by non-medically trained interviewers.²⁴ The selection of controls is always a crucial issue for all case-control studies. A potential problem of the use of hospital controls is that it may lead to bias towards high exposures for medical disorders, surgery or blood transfusion because a person that is already hospitalized is more likely to have a history of previous medical or surgical interventions. On the other hand, in studies with community controls it is likely that some patient's relatives may report more events than controls, simply because they are more willing to thoroughly recall past exposures motivated by the disease in their relative. This would lead us to a recall bias, which is certainly another source of information bias. A possible way to avoid information bias when assessing surgery as a possible risk factor for CJD would be to collect all case and control data directly from the medical records. This implies a considerable effort and would require excellent medical records, which are not likely to be available in all countries. But some countries like the Scandinavian would be suitable, and indeed a study with such characteristics is currently ongoing.

Genetic determinants of CJD

Clustering of CJD has been shown by several studies. Common environmental factors have been proposed as a possible explanation but this clustering might be caused by genetic factors as well.²⁷ Genealogical clustering of Dutch sCJD cases included in our national registry is higher than expected by chance (data not published). Several case-control studies have shown an association with family history of dementia.²² There are animal studies suggesting that genetic loci, other than the prion protein gene (PRNP) open reading frame, might be involved in the disease susceptibility and incubation period.³² In this thesis we have performed two genetic association studies in order to assess other genetic factors independently of the well-known PRNP M129V polymorphism associated with CJD.

Genetic association studies are one of the main tools of genetic epidemiology for identifying genomic variants underlying susceptibility to genetically complex diseases. However, obtaining replication of the initial findings has proved to be difficult.³³ Multiple reasons may explain this problem including inadequacies in study design, small samples, population admixture, multiple testing or genotyping errors. A common concern is multiple testing. Currently, most molecular biology techniques are highly automated. This has made it possible to genotype large numbers of single nucleotide polymorphisms (SNPs) across the whole genome. One of the prices that we have to pay

for this great advance is the increase in false positive rate (type I error) or spurious associations due to the exponential increase of statistical tests with a very low a priori probability. There are several options in order to deal with this problem. It has been proposed to decrease the cutoff point level of what we consider as statistically significant proportionally to the number of tests performed, as the case of Bonferroni correction. Less conservative approaches like the permutation test are being developed. A strategy to possibly minimize type I error due to multiple testing is to always design and interpret the statistical analysis in the light of what we know about biology. In chapter 3.3 we studied the association between CJD risk and genetic variations in the non-coding region upstream PRNP gene. The polymorphisms studied were selected based on their location in a region known to affect the expression of the PRNP gene. By studying functional polymorphisms we increase the a priori probability of our test, hence, diminishing type I error. In our population based study we found that being carrier of the infrequent allele of the regulatory region polymorphism PRNP G310C was associated to increased risk of sCJD and earlier disease onset. Moreover we found statistical evidences of an interaction between the effects associated to the PRNP G310C polymorphism and PRNP M129V. A possible explanation for our findings may stem from in vitro experiments suggesting that the PRNP 129M allele results in a higher propensity of the PrP to form beta-sheet-rich oligomers.³⁴ This indicates that PRNP 129M may facilitate self-perpetuating conformational changes of the human prion protein. Therefore, PRNP 129M may be by itself a potential risk factor for sCJD. Assuming that PRNP 310C allele increases PRNP expression, if the overexpressed prion protein carries M instead of V, this haplotype may work synergistically increasing the risk of sCJD and diminishing the age at symptoms onset.

In chapter 3.4 we performed another genetic association study. We examined the association between the Tau protein gene (MAPT) haplotypic variants and risk of CJD. Although MAPT is a good candidate gene, which has been associated before to several neurodegenerative diseases, its possible relationship with CJD has never been tested before. We examined 6 haplotype tagging SNPs (htSNPs), which capture 95 % of MAPT genetic variability in Caucasians. A Dutch population-based sample of sCJD patients and a cognitively normal control group of similar age distribution were genotyped. We genotyped the same polymorphisms in two other sCJD samples from Italy and the UK. Single locus and haplotype analyses did not detect any significant difference between sCJD cases and controls.

Apart from PRNP 129MM genotype selectivity, susceptibility to vCJD is still largely unexplained. A recent publication showed that CSF levels of phosphorylated-Tau are higher in vCJD patients than in sCJD³⁵ suggesting that there might be some differences in the mechanisms governing Tau protein phosphorylation between sCJD and vCJD. Therefore, we were interested in comparing MAPT variations between sCJD and vCJD. We did not find any differences in allelic or genotypic distributions between the two groups. When we compared MAPT haplotypes, we found that two of them were represented differently in sCJD and vCJD patients (H1f: 8% in sCJD versus 2% in

vCJD; H1j:1% in SCJD versus 7% in vCJD). However, these two haplotypes were rare in both groups of patients, and taking the small sample sizes into account, we think that the differences are likely due to chance. None of the p-values ($p=0.04$ and $p=0.01$) would remain statistically significant after applying a multiple testing correction like Bonferroni. Although our findings are compatible with a role of MAPT in vCJD susceptibility, the evidence is far from convincing at this stage.

Diagnostic investigations of CJD

There have been several studies assessing specificity and sensitivity of the three main diagnostic tests of CJD.³⁶⁻³⁸ An important section of this thesis is devoted to the study of the main tests employed for the clinical diagnosis of human TSEs. This is a crucial issue because, until now, there are no clinical tests allowing a definite diagnosis of CJD. The available diagnostic investigations currently used in clinical practice are imperfect surrogate markers of CJD. Thus, in order to correctly interpret results, it is very important to know which factors are modulating the test sensitivities and specificities. Equally important is to know how these tests do perform across the wide phenotypic spectrum of TSEs, understanding the variation of test sensitivities associated to the disease subtype. Studies addressing these questions are scanty in the scientific literature.³⁹⁻⁴²

Determinants of diagnostic investigations of CJD

Currently, the definite diagnosis of CJD relies on the neuropathological examination of the brain, which is in the majority of cases carried out post-mortem because the performance of brain biopsy needs special precautions and it is rarely indicated during the diagnosis workup. The pre-mortem diagnosis of CJD largely depends on an appropriate clinical context supported by characteristic findings on routine investigations such as the electroencephalogram (EEG) and the cerebrospinal fluid (CSF) analysis. Therefore, when a diagnosis of CJD is suspected, investigations are required first to rule out any alternative diagnosis and to support the diagnosis of CJD. Current diagnostic criteria (Table 1) are based upon the combination of progressive dementia, myoclonus, multifocal neurological dysfunction and a characteristic EEG or CSF detection of 14-3-3 protein.⁴³

Table 1: *Diagnostic criteria for sporadic, familial and iatrogenic CJD*

| Disease type | Classification | Criteria |
|----------------|----------------|--|
| Sporadic CJD | definite | post mortem examination |
| | probable | I progressive dementia and II at least two out of four features (myoclonus, visual or cerebellar disturbances, pyramidal or extrapyramida features, akinetic mutism) and III a. typical periodic sharp wave complexes on EEG or b. (since 1998) positive 14-3-3 CSF test and duration less than two years |
| Familial CJD | definite | I CJD confirmed at autopsy and II a. definite or probable CJD described in a first degree relative and/or b. disease-specific PRNP mutation in the index patient or the first-degree relative |
| | probable | I probable diagnosis of CJD plus definite or probable CJD in a first degree relative or II progressive neuropsychiatric disorder plus disease-specific PRNP mutation |
| Iatrogenic CJD | definite | CJD confirmed at autopsy and patient known with a recognized exposure risk |
| | probable | I progressive cerebellar syndrome in a pituitary hormone recipient or II probable CJD case with a recognized exposure risk |

When assessing the main factors likely to modulate CJD diagnostic tests sensitivities we faced several methodological difficulties. Many of the factors that we have investigated, as possibly influencing tests outputs, are correlated among each other. This complicates the interpretation of our statistical analyses because multiple confounders and covariates have to be taken into account. A multivariate analysis adjusting for all these covariates and confounders is therefore needed in order to assess independently the relation between these factors and the output of diagnostic tests. However, in order to perform this kind of analysis with enough statistical power, we need a large sample of patients. The only possible way to perform these studies with such a rare disease is within the framework of international collaborations which are able to collect sufficient data studied with a homogeneous methodology. In order to make a correct estimate of the sensitivity of a test, a gold standard for comparison is needed. In the case of TSEs the diagnostic gold standard is the neuropathological confirmation. Therefore, in theory, we should include only definite cases for this kind of studies. This restriction considerably reduces the number of available patients. An additional specific problem of sCJD, may be the fact that the probable diagnosis is based on both clinical signs/symptoms and diagnostic tests. Therefore, if we assess the sensitivity of a sCJD diagnostic test with cases that have been defined based on the result of that same test, we are entering a vicious circle. Although conceptually this may look a serious hazard, in practice, our results do not change considerably whith or without excluding probable

cases from our analyses. This is most likely due to the very high reliability of the current CJD clinical criteria. A more difficult question is how to study specificity. It is well known that there are false positive results for all CJD diagnostic tests, but most of the diseases that can yield false positive results can be easily ruled out by the clinical history. Hence, in order to assess how accurately these tests discriminate non CJD patients, it is very important to take the clinical context into account. A clear example of that is the CSF 14-3-3 protein determination. In theory, a positive result of this test in a compatible clinical context is thought to be highly specific.³⁸ However, with the increasing availability of the test, its use is becoming more and more frequent every day, and very often it is employed not only in patients who fulfill clinical criteria for CJD. For example, it is often undertaken as part of the diagnostic workup of any other type of dementia with atypical features or ataxic syndromes. Therefore, it is very difficult to estimate global specificity, because a large sample of prospectively studied cases with a common methodology would be needed. As this would be extremely difficult, in order to study specificity of CSF tests we took a different approach collecting all cases studied in the national surveillance centers that turned out to be no CJD patients, but had a final diagnosis, clinical or pathological. That way we focused on describing the false positive rate of the test for each of the main differential diagnosis of CJD. We believe that this is a more realistic approach and the information obtained is more useful for the clinician who can judge each particular patient in the light of her or his clinical context.

EEG. The presence of characteristic periodic sharp wave complexes (PSWCs) in the EEG is the most classical sign supporting the diagnosis of sCJD, and it has been the part of the original diagnostic criteria.⁴⁴ However, PSWCs are not always present in sCJD patients. Although they are associated with the most common phenotype of the disease, other less common forms usually lack this finding.⁴⁰ EEG plays an important role in the differential diagnosis between sCJD and vCJD, because in the latter only exceptionally PSWCs appear. In fact, there are only two reported cases in the literature in whom, at a very advanced disease stage, PSWCs were detected in the EEG.^{45,46}

We found out that the probability that an EEG demonstrate PSWCs increases with age at disease onset, and conversely decreases with disease duration. Therefore, these two factors should be taken into account when interpreting a negative EEG result. Independently of the previous clinical factors, PRNP V129M modulated EEG sensitivity to demonstrate PSWCs. We found that EEG positive results ranged from 73% in MM patients to 21% in VV. Hence, it seems that the presence of V allele reduces the likelihood of finding PSWCs. This could be explained by the different pathological profiles associated with the three genotypes with predominant cortical involvement in MM patients in comparison with the other genotypes.

We specifically assessed the influence of EEG performance time point during the course of the disease on test sensitivity. We found that the frequency of EEG showing PSWCs was significantly higher when the test was performed in the last third of the total disease duration. However, when we adjusted our analysis by the disease molecular subtype, that difference was not statistically significant. This suggests that the specific time point when the EEG is undertaken during the course of the disease is not influencing its sensitivity independently of molecular subtype.

CSF analysis. The development of a method allowing the detection of PrP^{Sc} in a biological fluid remains as the ultimate aim in the field of CJD diagnosis. Meanwhile, the main diagnostic tool available right now is the detection of brain-derived proteins present in the CSF as a result of neuronal damage. The presence of these proteins in the CSF is not specific of CJD, almost any acute brain insult, like inflammation, stroke or infections can raise the concentration of these markers in the CSF. Therefore they cannot be used for a general screening of CJD. However, in an appropriate clinical context, like the case of a patient who fulfills the clinical criteria for possible CJD, the results of these tests have a very high sensitivity and specificity.³⁸ In 1998 WHO disease criteria included the 14-3-3 determination in the CSF, making the presence of periodic complexes in the EEG for the diagnosis of probable sCJD⁴³ not anymore compulsory. The introduction of the neuronal protein 14-3-3, as part of the CJD diagnostic criteria, has produced a considerable impact, increasing in most countries the number of CJD patients ascertained and allowing the identification of uncommon phenotypes usually lacking PSWCs in the EEG. On the other hand, the use of 14-3-3 test has widely generalized in the neurological practice and the test is frequently performed in patients who fail to fulfill the clinical criteria for possible CJD. As one might have expected, the increase in cases ascertained thanks to the 14-3-3 has paralleled a drop in the global specificity of the test.⁴⁷

Even though some studies have previously assessed sensitivity and specificity of some of these markers,^{38,48-50} several important questions remain unanswered. In particular, the question of which factors influence test sensitivities have been only partially delineated. In order to address some of those important issues an EU-concerted multinational study (EPICJD) was set up led by Professor Inga Zerr from the Georg-August-University of Göttingen, collecting the largest series of patients to date. The studies enclosed in chapters 4.1, 4.2 and 4.3 are all product of this collaboration.

In our study we assessed the independent influence on several clinical parameters like age at disease onset and disease duration on sensitivity of the main CSF markers of CJD: 14-3-3 protein, Tau protein, neuronal specific enolase (NSE) and the glial protein S100b. We could show that being older than 40 years and having short disease duration were independently associated to higher test sensitivity. We also assessed, conjointly with the previous factors, the role of genetic variations like PRNP codon 129 polymorphism. We showed that being heterozygote for PRNP codon 129 polymorphism decreased the test sensitivities independently of other factors. Remarkably, we found similar results for all four markers consistent with a disease dependent effect.

We specifically addressed the question of the influence of the CSF sample point timing during the course of the disease as a factor likely to contribute to test sensitivities of 14-3-3, Tau protein, NSE, and S100b. We observed that 14-3-3 and Tau protein tended to increase from onset to advanced stage, and this trend was significant for the patients heterozygotes for PRNP V129M. A possible explanation for this effect of PRNP V129M polymorphism could be the fact that heterozygote patients may have a lower neuronal death rate. Therefore, the level of the marker in sCJD may need more time to reach the threshold established as the cutoff point for a positive test result. As part of the same study we also analyzed a group of patients who underwent repeated lumbar punctures. The results were also concordant, and on average we found higher sensitivity in the second test than in the first.

Another question that we were able to address was whether a combination of CSF markers might improve sensitivity and specificity. The combined determination of 14-3-3 and Tau increased the sensitivity from 85% and 86% to 93%, at the expense of a specificity drop from 89% to 72% in the group of neurodegenerative diseases.

We also studied the factors modulating specificity when these tests are applied to a population of patients referred to the surveillance centers. Not surprisingly, the main predictors of false positive tests were a final diagnosis of inflammatory or paraneoplastic/CNS tumor disease and the presence of increased cells in the CSF.

In a separate study, but in the same population, we studied routine CSF analysis in TSE patients. The routine analysis of the CSF is very often performed as part of the diagnosis workup of CJD patients. Classically, the CSF of CJD patients do not show inflammatory signs like pleocytosis or significantly high protein elevation. Therefore, the utility of this test is mainly the exclusion of treatable inflammatory conditions. However, in the literature the information about the usual range of these main CSF parameters in TSE patients is scarce as it mostly comes from small studies and it is restricted to sCJD patients. We studied the main parameters of the CSF routine analysis in a series of 450 sCJD cases, and 47 patients with other TSEs, including 8 fatal familial insomnia (FFI) patients, 7 vCJD patients and 32 genetic CJD (gCJD) patients, all of them with a pathologically confirmed diagnosis. Our study showed that an elevated number of inflammatory cells (>20 cells/ μ l) strongly militates against the diagnosis of TSE. Raised levels of total CSF proteins concentration was a common finding in our patients. However, none of them presented concentrations higher than 1g/l. In 4.4% percent of our pathologically defined sCJD patients oligoclonal IgG was detected in CSF, which is lower than that of a previous publication which included cases without a definite diagnosis.⁵¹

MRI scan. The magnetic resonance imaging (MRI) scan is the other fundamental diagnostic test in the diagnostic workup of sCJD. Currently, the main function of MRI scanning is the exclusion of alternative diagnoses. However, characteristic signal alterations in basal ganglia and cerebral cortex are common in sCJD patients⁵²⁻⁵⁵ and their utility for the clinical diagnosis and even their inclusion as part of the sCJD diagnostic

criteria are currently subject of investigation. Furthermore, MRI scan is already the main diagnostic test included in the clinical criteria for probable vCJD. In this TSE subtype the bilateral hyperintensity of the pulvinar nuclei of the thalamus is considered as a very sensitive sign, present in 78 % of vCJD cases.⁵⁶ The specificity of the pulvinar sign is thought to be almost 100%, with just a few false positive cases published in the literature.⁵⁷⁻⁵⁹

In our analysis we found a lower MRI scan sensitivity than that reported in previous smaller studies.^{39,54} However, our data on MRI scans had some important caveats which made our results less reliable. The MRI scan data collection for this study extended for more than one decade. Therefore, the imaging techniques employed were not homogeneous. During the course of the study new sequences with higher sensitivity to detect the typical findings, like FLAIR or DWI, were progressively employed in CJD diagnostics. The other main issue that can explain this discrepancy is that for this study, cerebral cortical increased signal, a sign commonly found in sCJD using the more sensitive techniques as FLAIR and DWI was not considered as part of the typical findings.

Independently of clinical factors, we found out that the probability that a MRI will show hyperintensity in the basal ganglia is strongly modulated by PRNP V129M genotype. In contrast to what we found in EEG, the likelihood of a typical MRI scan was higher in cases with the MV and VV genotypes. A likely explanation for this finding would be again the different pathological profiles associated to PRNP V129M genotypes. In that way, MRI scan seems to be a good complementary test to EEG because is more sensitive in those patients in whom EEG tests do not frequently show PSWCs.

Diagnostic test sensitivities across the phenotypic spectrum of sCJD

TSEs have a very wide phenotypic spectrum. The clinical profile and the sensitivity to the different diagnostic tests are different depending of the disease subtype. Moreover, within each subtype there is also high variability. In general, the disease phenotype depends on the interplay between the host characteristics and the etiological agent. However, the mechanisms underlying this variability are not completely well understood. One good example is the differences between the clinical profile of the classical forms of sCJD and vCJD. In vCJD, PSWCs are absent in the EEG almost in 100% of cases, in contrast to the majority of classical sCJD patients in whom this sign is very a characteristic feature. Another example is the variability of gCJD phenotype depending on the causal PRNP mutation. For example, as we have shown in our study, while patients carrying the PRNP E200K mutation present a clinical profile identical to classical sCJD, with a high percentage of positive 14-3-3 tests, patients carrying any of the Gerstmann-Straussler-Scheinker (GSS) mutations are negative in most cases. In addition to the causal agent, also the route of transmission is important. Iatrogenic CJD (iCJD) presents different phenotypes depending of how the infectious agent reached the brain, via blood, as in the case of human growth hormone linked cases, or directly put in

contact to the CNS as the cases linked to dura mater grafts. Last but not least, the different clinical and pathological subtypes that comprise the wide phenotypic spectrum of sCJD have been linked to the PRNP M129V polymorphism combined with the type (1 or 2) of PrP^{Sc} found in the brain. These combinations probably define the different human prion strains.^{40,60} Some of the phenotypic variants are extremely infrequent and, therefore, having them all represented in a study requires a very large number of patients enrolled. Most studies assessing the variations in sensitivity of the sCJD tests across the phenotypic spectrum of the disease are scarce and of small size. Several of them have found variations in the sensitivity of the three main tests across the different disease subtypes.³⁹⁻⁴² However, these studies did not perform multivariate analysis and did not include a sufficient number of cases for some of the rare subtypes. The studies presented in chapter 4 focus on this fundamental question. We analyzed these variations in sensitivity of the sCJD tests in the light of the molecular classification of the disease. They are all international collaborative efforts and comprise the biggest series of patients ever collected for these purposes.

In order to better understand the interrelations of the different biological factors likely to influence sensitivity, like age at onset, duration of the disease, or time point when the test was undertaken we aimed to perform a multivariate analysis. To this end, we studied, together with the molecular subtypes, all predictors in a multiple logistic regression model.

We found that the molecular subtypes are indeed the strongest determinants of all three test sensitivities. Table 2 displays the different test sensitivities across the molecular classification of sCJD, including as independent groups the combinations with more than one type of PrP^{Sc} in the brain. MM1 and MV1 showed a similar profile, as reported before (ref Parchi). However, we found significantly higher EEG sensitivity in MM1 patients. VV2 and MV2 patients rarely presented PSWCs in the EEG. More frequently than MM1 and MV1 patients they had typical MRI scans. Their main difference was in 14-3-3 test sensitivity, which was higher in VV2 patients. Tests performed on patients belonging to the infrequent VV1 subtype usually presented similar profile than VV2, but the sensitivity of EEG tended to be higher in VV1. However, the numbers were low. Taking into account our results, MM2 patients seem to be the most challenging subgroup for diagnosis. In these patients the 14-3-3 determination in CSF had a sensitivity of only 61%, less than 50% for EEG, whereas MRI was only positive in less than 33% of the patients. Moreover, 5 out of the 16 MM2 patients to whom the three tests were performed were negative for all three investigations, the highest percentage of all molecular subtypes. Finally, although the number of patients included in the combined subgroups was too small to draw solid conclusions, these patients tended to present an intermediate phenotype between those with single PrP^{Sc} type and the same PRNP M129V genotype.

Table 2: Diagnostic tests sensitivities across the molecular subtype of sCJD

| | Molecular Phenotypes | | | | | | | | |
|--|----------------------|------------------|-----------------|-----------------|------------------|---------------|----------------|------------------|----------------|
| | MM1 | MM2 | MM1/2 | MV1 | MV2 | MV1/2 | VV1 | VV2 | VV1/2 |
| EEG | 294/404 | 12/27 | 16/25 | 18/34 | 11/63 | 3/7 | 5/12 | 11/86 | 1/8 |
| Typical/total (%) | (72.8) | (44.4) | (64.0) | (52.9)* | (17.5)* | (42.9) | (41.7) | (12.8)* | (12.5) |
| MRI | | | | | | | | | |
| Characteristic findings/total (%) | 70/ 210 (33.3) | 5/18 (27.8) | 1/16 (6.3) | 5/16 (31.3) | 24/45 (53.3) | 1/3 (33.3) | 6/9 (66.7) | 37/67 (55.2)† | 1/3 (33.3) |
| 14-3-3 protein in CSF positive/total (%) | 300/329 (91.2) | 14/23 (60.9)* | 16/20 (80.0) | 25/29 (86.2) | 36/51 (70.6)* | 4/6 (66.7) | 9/10 (90.0) | 79/83 (95.2) | 3/3 (100.0) |

*Significantly lower than MM1 ($p < 0.05$)

†Significantly higher than MM1 ($p < 0.05$)

Adjusted by age at onset, disease duration, gender and country of origin.

Our results offer some useful knowledge to clinicians that may help them when interpreting these diagnostic investigations. Even though the PrP^{Sc} type is most of the times not available at the moment of the diagnosis, the clinical profile together with the PRNP M129V information may offer the clinicians enough hints to grasp the patient's disease subtype, which may help them to better interpret the test results. This underscores the importance of the PRNP M129V polymorphism testing, especially in atypical patients, adding extremely valuable information to the clinical context. Finally, another important lesson that we have learnt from our analysis is the importance of performing all three tests in the diagnostic workup of patients with suspected TSE, as it increases the diagnostic probabilities.

Further research

Human surveillance has successfully identified new cases of vCJD, however there remain possible new challenges. Recent findings like the discovery of new variants of BSE⁶¹ and the proven transmission of vCJD via blood to genotypes other than PRNP codon 129 MM¹² are a warning call to remain watchful for new disease phenotypes.

The question whether iatrogenic exposure via surgery is involved in a significant proportion of sCJD cases remains without a satisfactory answer. The development of new epidemiological approaches tackling some of the important caveats of the previous case-controls studies would be an interesting step forward in order to clarify this issue. For instance, the use of hospital records instead of interviewing relatives and controls may be an interesting strategy in order to reduce some unavoidable bias.

On the other hand, in the near future it is likely that new genetic determinants of sCJD will be discovered. Transgenic mice studies have shown that other polymorphisms inside or outside the open reading frame of PRNP, and even other genes, might be involved in the disease susceptibility.

The availability of high throughput genome wide SNP genotyping is opening new opportunities for genetic epidemiological research like genome wide association studies. The performance of such studies in large samples of sCJD patients is likely to offer soon important new insights about the genetic determinants of the disease.

In this same field, an obvious next step for us would be to perform functional studies assessing whether the PRNP G310C polymorphism is associated with changes in PRNP expression, and therefore, supporting the findings of our genetic association study.

It is mandatory to clarify the role of MRI in the diagnosis of sCJD, with studies, specifically designed to take into account the phenotypic spectrum of the disease, and to include large numbers of patients. It is important to define what the typical findings for each of the disease subtypes are, which the most reliable image techniques are, and finally, to consider the inclusion of MRI scan as part of the sCJD criteria.

The development of a diagnostic technique enabling an early definite diagnosis of TSEs would be the main goal in terms of diagnostic research. This fundamental step would facilitate the development and testing of new treatments, which remains as the most important challenge in the field.

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Summary

Transmissible spongiform encephalopathies (TSE) or prion diseases constitute a fascinating group of neurological disorders. In spite of their rarity, their unique etiopathological mechanisms and epidemiological aspects have attracted a considerable number of researchers.

This thesis focuses on two facets: etiological mechanisms and diagnostic tests of Creutzfeldt-Jakob disease (CJD). We have searched new insights into these two topics using genetic and classic epidemiological methodology.

For the studies presented in this book, we have used data from the Dutch, Italian and UK CJD National Surveillance Systems, the 'European collaborative Study group of CJD' (EUROCJD), the 'extended European collaborative Study group of CJD' (NEUROCJD), the 'Early clinical diagnosis of human spongiform encephalopathies by analysis of biological fluids-CJD Markers collaboration' (EPICJD), and the 'Rotterdam Study'.

Chapter 1 provides the scope of the thesis. Chapter 2.1 describes the design and characteristics of the extended collaborative study group of CJD (NEUROCJD), and the results of their monitoring activities during an 8-year period. In this chapter we summarize relevant methodological issues and discuss the main problems encountered. The average mortality rate of sporadic CJD during the period of observation was 1 death per million person-year. The main focus of the study is on describing the disease clinical presentation of sporadic CJD and the characteristic of the atypical patients. Variant CJD cases in Ireland and in Portugal were successfully detected by their national surveillance centres. Chapter 2.2 reports the case of the first variant CJD patient identified in the Netherlands. The clinical characteristics of the patient, MRI scan images, neuropathological study, PrP^{Sc} western blot profile and PRNP M129V genotype, were all indistinguishable from that reported in UK vCJD patients.

Chapter 3 explores different hypothesis in relation to the origin of sporadic and variant CJD aetiology. In chapter 3.1 we correlated UK bovine exports with the spread of variant CJD to countries outside the UK. We found a positive correlation between the amounts of material imported from the UK during the 80s and the number of variant CJD cases ascertained. Specifically, live bovine was found to be the main independent factor associated to the occurrence of variant CJD outside the UK ($r_s=0.73$; 95%CI=0.42 to 0.89; $p<0.001$). The greatest volume of these exports went to France, the Netherlands and Ireland, the three countries with higher number of variant CJD cases outside the UK. In chapter 3.2 we assessed the causal role of past history of ophthalmic surgery in sporadic and variant CJD. We found no association with either form of the disease. We observed that a proportion of sporadic CJD patients presenting with visual symptoms were often referred at first to ophthalmologists, and not infrequent they were diagnosed with cataracts and often treated. In chapter 3.3 we studied the association between sporadic CJD risk and genetic variations in the non-coding region upstream the prion

protein gene (PRNP). In our population based study we found that being carrier of the G allele of the regulatory region polymorphism PRNP G310C was statistically associated ($p=0.03$) with a 2.4 fold increased risk of developing sporadic CJD (95%CI=1.1 to 5.4). Cases of sporadic CJD carrying a PRNP 310C allele presented at a younger age (on average 8.9 years younger than those without this allele), which was of statistical significance ($p=0.05$). The polymorphism studied was selected based on their location in a region known to affect the expression of the PRNP gene. Therefore, our findings support the hypothesis that genetic variations in the PRNP promoter may have a role in the pathogenesis of sporadic CJD. In chapter 3.4 we tested the association between the Tau protein gene (MAPT) haplotypic variants and risk of CJD. We examined 6 haplotype tagging SNPs (htSNPs), in a Dutch population-based sample of sporadic CJD patients and a cognitively normal control group of similar age distribution. We genotyped the same polymorphisms in two other sporadic CJD samples from Italy and the UK. Single locus and haplotype analyses did not detect any significant difference between sporadic CJD cases and controls. We did not find any differences in allelic or genotypic distributions between sporadic and variant CJD. However, we found that two of them were represented differently in sporadic CJD and variant CJD patients (H1f: 8% in sporadic CJD versus 2% in variant CJD; H1j: 1% in sporadic CJD versus 7% in variant CJD). Yet, when we corrected for the number of tests that were performed, none of these associations remained statistically significant.

Chapter 4 focuses on the main diagnostic investigations of CJD. In chapter 4.1 we analysed the diagnostic sensitivity and specificity of various brain-derived proteins (14-3-3, Tau, NSE and S100b) in the cerebrospinal fluid (CSF) of patients with CJD and the biological factors that modify these parameters. In the framework of a multinational European study (EPICJD), CSF was tested for 14-3-3, Tau, NSE and S100b in 1859 patients with sporadic, genetic, iatrogenic and variant CJD, and in 1117 controls. The highest sensitivity was achieved for 14-3-3 and Tau in sporadic CJD (85% and 86%), and a combined determination of 14-3-3 and either Tau S100b or NSE increased the sensitivity to over 93%. A multivariate analysis showed that the sensitivity of all tests was highest in patients with the shortest disease duration, age at onset >40 years, and homozygosity at codon 129 of the PRNP. In a group of patients with repeated lumbar punctures (LP), a second test also increased the diagnostic sensitivity. Our results reaffirm that the detection of elevated levels of brain-derived proteins in the CSF in patients with suspected of CJD is a valuable diagnostic test. The second LP should be performed in patients with atypical clinical course in whom the first test was negative. Chapter 4.2 assessed the influence of the time of sampling on the value of CSF tests in the diagnosis of sporadic CJD. We studied the results of 14-3-3, S100b, NSE and Tau protein in 833 CSF samples from sporadic CJD patients at different stages of disease and in 68 sequentially repeated LP. 14-3-3 and Tau protein increased in sensitivity from onset (88%, 81%) to the advanced stage (91%, 90%). This was significant in the group of patients with methionine-valine (MV) PRNP codon 129 genotype. The absolute levels

of S100b ($p < 0.05$), NSE and Tau protein increased in the last stage of disease. High levels of Tau protein, NSE and S100b were associated with shorter survival times ($p < 0.01$). Sixty-eight sporadic CJD patients underwent repeated LP. These sporadic CJD patients were younger, had longer disease durations and were more frequently MV at codon 129 ($p < 0.001$) than the whole group. 14-3-3 sensitivity increased from 65% to 79% in the second LP ($p = 0.06$) and 88% sporadic CJD patients had at least one positive result. Sensitivity and absolute levels of CJD markers increased with disease progression and were modulated by the codon 129 genotype. Early negative results should be interpreted with caution, especially in young patients or those who are MV at codon 129. Chapter 4.3 examined the CSF white cell counts, total protein concentrations and the presence of oligoclonal IgG in patients with human prion diseases. Data from 450 patients with sporadic CJD and 47 patients with other prion diseases were collected. Raised cell counts of >5 cells/ μ l were found in three patients out of 298 patients with sporadic CJD, but none had a cell count of >20 cells/ μ l. Total protein concentrations of >0.9 g/l were found in 5 of 438 patients with sporadic CJD, although none had a concentration of >1 g/l. CSF oligoclonal IgG was detected in eight out of 182 sporadic CJD patients. In those patients with other prion diseases, none had CSF cell counts above 20 cells/ μ l or total protein concentrations of >0.9 g/l and only 1 patient with fatal familial insomnia had detectable oligoclonal IgG. None of the patients with sporadic CJD or other prion diseases had abnormalities in all three tests. When investigating patients with suspected prion disease, the presence of a CSF cell count of more than 20 cells/ μ l or a total protein concentration of 1g/l or more suggests an alternative diagnosis.

In chapter 4.4 we aimed to define the main determinants of the three characteristic diagnostic investigations (EEG, MRI scan and CSF 14-3-3 test) sensitivity across the entire clinical spectrum of sporadic CJD. Our analysis, embedded in the international collaborative study EURO-CJD, comprised 2451 pathologically confirmed patients. Our results showed that age at disease onset and disease duration were independent determinants of typical changes on EEG, while illness duration significantly influenced positive CSF 14-3-3 protein detection. Changes on brain MRI were not influenced by either of these clinical parameters, but overall imaging data were less complete and, consequently, conclusions are more tentative. In addition to age at disease onset and illness duration, molecular subtype (defined as the combination of PRNP codon 129 genotype and PrP type) was re-affirmed as an important independent determinant of investigation results. In multivariate analyses that included molecular subtype, time point of the investigation during a patient's illness was found not to influence the occurrence of a typical or positive EEG or CSF 14-3-3 protein result. A typical EEG was most often seen in MM1 patients and was significantly less likely in the MV1, MV2 and VV2 subtypes, whereas VV2 patients had an increased likelihood of a typical brain MRI. Overall, the CSF 14-3-3 test was the most frequently positive investigation (88.1%) but performed significantly worse in the very uncommon MV2 and MM2 subtypes. Our findings confirm a number of determinants of clinical, laboratory and

imaging investigations results in sporadic CJD and underscore the importance of recognizing these pre-test limitations before accepting the diagnosis excluded or confirmed. Combinations of investigations offer the best chance of detection, especially for the less common molecular subtypes such as MV2 and MM2.

In Chapter 5 the main findings of this thesis are presented and put into perspective of each other, and of the current scientific knowledge. The most relevant methodological issues are discussed, and finally the directions for further research in light of the findings of this thesis are outlined.

Samenvatting

Overdraagbare spongiforme encefalopathie (TSE) oftewel prionziekten vormen een fascinerende groep van neurologische aandoeningen. Ze komen slechts sporadisch voor, maar dankzij hun unieke pathologische mechanismen en epidemiologische aspecten vormen ze een inspiratiebron voor veel onderzoekers.

Dit proefschrift richt zich op 2 aspecten: de etiologische mechanismen en diagnostische tests van de ziekte van Creutzfeldt-Jacob (CJD). Met behulp van zowel de genetische als de klassieke epidemiologische methodologie zochten we naar nieuwe inzichten in deze twee onderwerpen.

Voor het onderzoek dat in dit proefschrift beschreven staan, maakten we gebruik van de Nederlandse, Italiaanse en Britse nationale surveillance systemen voor CJD, de European Collaborative Study Group of CJD (EUROCJD), de Extended European Collaborative Study Group of CJD (NEUROCJD), de Early clinical diagnosis of human spongiform encephalopathies by analysis of biological fluids-CJD markers collaboration (EPICJD) en de Rotterdam Studie.

In hoofdstuk 1 wordt het doel van dit proefschrift uiteengezet. Hoofdstuk 2.1 beschrijft het design en de kenmerken van de extended collaborative study group of CJD (NEUROCJD), en de resultaten van hun monitoring activiteiten gedurende een periode van 8 jaar. In dit hoofdstuk vatten we de relevante methodologische vraagstukken samen en bespreken we de belangrijkste problemen die zich voordeden. Het gemiddelde sterftecijfer van sporadische CJD gedurende de observatieperiode was 1 geval per miljoen inwoners. Het belangrijkste doel van de studie was het beschrijven van de klinische presentatie van de sporadische CJD en de kenmerken van atypische patiënten. Patiënten met variant CJD in Ierland en Portugal werden succesvol geïdentificeerd door nationale surveillance centra. Hoofdstuk 2.2 beschrijft het geval van de eerste variant-CJD patiënt die in Nederland ontdekt werd. Deze vertoont een grote gelijkenis met eerdere patiënten uit het Verenigd Koninkrijk. Dit suggereert een zelfde oorsprong van de ziekte.

In hoofdstuk 3 worden verschillende hypothesen met betrekking tot de etiologie van sporadische en variant CJD getoetst. In hoofdstuk 3.1 correleerden we de export van runderen met de verspreiding van variant CJD naar andere landen dan het Verenigd Koninkrijk. We vonden een positieve correlatie tussen de hoeveelheid materiaal dat geïmporteerd werd uit het Verenigd Koninkrijk tijdens de jaren '80 en het aantal gevallen van variant-CJD. De import van levende runderen leek als belangrijkste onafhankelijke factor gerelateerd te zijn aan het vóórkomen van variant CJD buiten het Verenigd Koninkrijk ($r_s=0.73$; 95% betrouwbaarheidsinterval=0.42-0.89; $p<0.001$). Het merendeel van de export richtte zich op Frankrijk, Nederland en Ierland, de drie landen met het grootste aantal variant CJD gevallen buiten het Verenigd Koninkrijk. In hoofdstuk 3.2 onderzochten we of er een oorzakelijk verband is tussen het ondergaan van oogheelkundige chirurgie en het ontwikkelen van sporadische en variant-CJD. Er werd geen verband gevonden, wel vonden we dat een deel van de sporadische CJD patiënten

die visuele symptomen had vaak in eerste instantie naar oogartsen werd verwezen en daar werd gediagnosticeerd met staar en daarvoor behandeld. In hoofdstuk 3.3 bestudeerden we het verband tussen de kans op sporadisch CJD en genetische variaties in de niet-coderende regio upstream van het prion eiwit gen (PRNP). In ons onderzoek vonden we dat dragers van het zeldzame allel van het regulerende-regio-polymorfisme PRNP G310C een 2.4 maal verhoogde kans hebben op de sporadische vorm van CJD. Dit verhoogde risico was statistisch significant (95% betrouwbaarheidsinterval 1.1-5.4, $p=0.03$). Patiënten met sporadische CJD die drager waren van een PRNP 310C allel werden op jongere leeftijd ziek (gemiddeld 8.9 jaar jonger dan degenen zonder dit allel). Dit leeftijdsverschil was statistisch significant ($p=0.05$). De polymorfismen die we bestudeerden werden geselecteerd gebaseerd op hun lokalisatie in een regio waarvan we weten dat die de expressie van het PRNP gen beïnvloedt. Onze bevindingen ondersteunen de hypothese dat genetische variaties in de promotor van het PRNP gen een rol spelen in de pathogenese van sporadisch CJD. In hoofdstuk 3.4 testten we de associatie tussen haplotype varianten in het gen dat codeert voor het Tau eiwit (MAPT) en de kans op CJD. We onderzochten 6 haplotype tagging SNPs (htSNPs), in een Nederlandse steekproef van sporadische CJD patiënten en een cognitief normale controle groep met een vergelijkbare leeftijdsverdeling. We genotypeerden dezelfde polymorfismen in twee andere series patiënten met de sporadische vorm van CJD uit Italië en het Verenigd Koninkrijk. Met single locus en haplotype analyses werden geen significante verschillen tussen de sporadische CJD cases en controles gevonden. Ook vonden we geen verschillen in de allelische of genotypische verdelingen tussen sporadische en variant CJD. Bij het vergelijken van de MAPT haplotypes ontdekten we dat twee haplotypen verschillend verdeeld waren bij sporadische CJD en variant CJD patiënten (H1f: 8% in sporadische CJD en 2% in variant CJD; H1j: 1% in sporadische CJD en 7% in variant CJD). Echter, na correctie voor het aantal uitgevoerde testen bleek geen van deze associaties statistisch significant te zijn.

Hoofdstuk 4 richt zich op de studie van de belangrijkste diagnostische onderzoeken voor CJD. In hoofdstuk 4.1 analyseerden we de diagnostische sensitiviteit en specificiteit van verschillende eiwitten uit de hersenen (14-3-3, Tau, NSE en S100b) in de cerebrospinale vloeistof (CSF) van patiënten met CJD en biologische factoren die deze parameters modificeren. Binnen een Europese studie (EPICJD) werd het CSF van 1859 patiënten met sporadische, genetische, iatrogene en variant CJD en 1117 controlepersonen getest op 14-3-3, Tau, NSE en S100b. De hoogste sensitiviteit werd gevonden voor 14-3-3 en Tau voor sporadisch CJD (85% en 86%), en een gecombineerde bepaling van 14-3-3, óf Tau, óf S100b óf NSE verhoogde de sensitiviteit tot boven de 93%. Een multivariate analyse toonde aan dat de sensitiviteit van alle tests het hoogst was voor patiënten met een korte ziekte duur, een leeftijd van 40 jaar of ouder bij de manifestatie van de ziekte, en homozygositeit op codon 129 van het PRNP. In een groep patiënten die herhaalde lumbaalpuncties ondergingen, verhoogde een tweede test de diagnostische sensitiviteit. Onze resultaten herbevestigden dat de detectie van

verhoogde niveaus van hersen eiwitten in het CSF voor patiënten met een verdenking op CJD een goede diagnostische test is. Een tweede lumbaalpunctie zou moeten worden uitgevoerd bij patiënten met een atypisch klinisch verloop waarbij de eerste test negatief was. In hoofdstuk 4.2 beschrijven we de invloed van het tijdstip van afname op de waarde van de CSF tests voor de diagnose van sporadische CJD. We onderzochten de resultaten voor 14-3-3, S100b, NSE en Tau eiwit in 833 monsters van sporadische CJD patiënten in verschillende ziektestadia en in 68 sequentieel herhaalde lumbaalpuncties (LP). Tau-eiwit en 14-3-3 verhoogden in sensitiviteit van de beginstadia (onset) (81% en 88%) naar geavanceerde stadia (90% en 91%). Dit was significant voor de groep patiënten met het genotype methionine-valine (MV) PRNP codon 129. De absolute niveaus van S100b ($p < 0.05$), NSE en Tau eiwit namen toe in het hoogste ziektestadium. Hoge niveaus van het Tau eiwit, NSE en S100b waren geassocieerd met kortere overlevingsduur ($p < 0.01$). Achttenzestig sporadische CJD patiënten ondergingen herhaalde LP. Deze sporadische CJD patiënten waren jonger, hadden langere ziekteduren en waren vaker MV voor codon 129 ($p < 0.001$) dan de rest van de groep. De sensitiviteit voor 14-3-3 steeg van 65% naar 79% bij de tweede LP ($p = 0.06$) en 88% van sporadische CJD patiënten had ten minste één positief testresultaat. De sensitiviteit en absolute niveaus van CJD markers namen toe met progressie van de ziekte en werden gemoduleerd door het codon 129 genotype. Vroege negatieve resultaten moeten voorzichtig geïnterpreteerd worden, in het bijzonder als het gaat om jonge patiënten of patiënten die MV zijn voor codon 129. In hoofdstuk 4.3 onderzochten we het CSF op het aantal witte bloedcellen (cell counts), totale eiwit concentraties en de aanwezigheid van oligoclonale IgG onder patiënten met humane prionziekten. Er werden gegevens verzameld van 450 patiënten met sporadische CJD en 47 patiënten met andere prionziekten. Verhoogde cell counts van >5 cellen per microliter werden gevonden bij drie van 298 patiënten met sporadische CJD, maar geen van hen had een cell count van >20 cellen per microliter. Totale eiwitconcentraties van >0.9 gram per liter werden gevonden bij 5 van 438 patiënten met sporadisch CJD. Er werden geen concentraties boven de 1 gram per liter gevonden. CSF oligoclonaal IgG werd gedetecteerd bij acht van 182 sporadische CJD patiënten. Van de patiënten met andere prionziekten had er geen een cell count boven de 20 cellen per microliter of totale eiwitconcentraties boven 0.9 gram per liter, slechts 1 patiënt met fatale familiale slapeloosheid (insomnie) had detecteerbaar oligoclonaal IgG. Bij geen van de patiënten met sporadisch CJD of andere prionziekten werden abnormaliteiten gevonden in de drie tests. Bij het onderzoeken van patiënten met een verdenking op prionziekten suggereert een CSF cell count van meer dan 20 cellen per microliter of een totale eiwitconcentratie van 1 gram per liter of meer een alternatieve diagnose. In hoofdstuk 4.4 onderzochten we de belangrijkste determinanten van de sensitiviteit van drie karakteristieke diagnostische onderzoeken (EEG, MRI scan en CSP 14-3-3 tests) over het hele klinische spectrum van sporadische CJD. Er werd een internationaal samenwerkingsverband (EUROCJD) opgezet waarbij 2451 pathologisch bevestigde patiënten werden geïncludeerd. Onze resultaten toonden

aan dat de leeftijd waarop de ziekte zich manifesteerde en ziekte duur onafhankelijke determinanten van typische veranderingen op een EEG waren, terwijl ziekte duur significant de detectie van CSF 14-3-3 eiwitten verhoogde. Veranderingen op hersen-MRI's werden niet beïnvloed door deze klinische parameters, maar in het algemeen waren de gegevens van de beeldvorming minder compleet en de conclusies hieromtrent deshalve meer speculatief. Naast de leeftijd van ziekte-manifestatie en ziekte duur, werd het moleculaire subtype (gedefinieerd als de combinatie van het PRNP codon 129 genotype en PrP type) herbevestigd als belangrijke onafhankelijke determinant van de testresultaten. Multivariate analyses toonden aan dat moleculair subtype en het tijdstip van bepalingen tijdens het ziekteverloop van de patiënt geen invloed hadden op het vóórkomen van een typisch of positief EEG of CSF 14-3-3 eiwit testresultaat. Een typisch EEG werd het vaakst gezien bij MM1 patiënten en kwam significant minder vaak voor bij de MV1, MV2, en VV2 subtypes, terwijl bij VV2 patiënten vaker een typisch hersen-MRI werd gezien. Een positieve CSF 14-3-3 test kwam het vaakst voor (88.1%) maar deze test deed het minder goed bij de zeer zeldzame MV2 en MM2 subtypes. Onze bevindingen bevestigen een aantal determinanten van belangrijke onderzoeksresultaten voor sporadisch CJD en onderstrepen het belang van het erkennen van de beperkingen van deze tests voordat de diagnose al dan niet bevestigd kan worden. Combinaties van onderzoeken geven de beste detectie-kans, met name voor zeldzame moleculaire subtypes zoals MV2 en MM2.

In hoofdstuk 5 worden de belangrijkste bevindingen van dit proefschrift beschreven en in elkaars perspectief en dat van de huidige wetenschappelijke kennis geplaatst. De meest relevante methodologische aspecten worden bediscussieerd en ten slotte worden toekomstige onderzoeksrichtingen besproken.

Resumen

Las encefalopatías espongiformes transmisibles (EET) o enfermedades por priones constituyen un fascinante grupo de enfermedades neurológicas. A pesar de su baja incidencia, sus singulares mecanismos etiopatogénicos y el complejo perfil epidemiológico han atraído a un considerable número de investigadores.

Esta tesis se centra en dos aspectos: los mecanismos etiológicos y pruebas diagnósticas de la enfermedad de Creutzfeldt-Jakob (ECJ) y otras EET. En esta compilación de trabajos hemos buscado nuevas claves para entender mejor estos dos aspectos de la enfermedad, utilizando herramientas tomadas tanto de la epidemiología genética como de la epidemiología clásica.

En los estudios que se han llevado a cabo se han utilizado datos procedentes tanto de los centros de vigilancia epidemiológica de Países Bajos, Italia y Reino Unido, como de los grupos 'European collaborative study of CJD' (EUROCJD), 'extended European collaborative study of CJD' (NEUROCJD), 'Early clinical diagnosis of human spongiform encephalopathies by analysis of biological fluids-CJD Markers collaboration (EPICJD), y el 'Rotterdam Study'.

En el capítulo 1 se introducen los temas tratados en el libro.

En el capítulo 2 se describe la metodología empleada y los problemas más comúnmente encontrados durante la puesta en marcha de un estudio de colaboración internacional para la vigilancia de la ECJ (NEUROCJD). Los resultados de las actividades de vigilancia epidemiológica quedan resumidos en este capítulo. La tasa de mortalidad durante el periodo de observación fue de 1 caso por millón de habitantes y año. Se prestó especial atención a las distintas formas de presentación clínica de los casos de ECJ esporádica así como a los pacientes con clínica atípica. Casos de la variante de la ECJ fueron detectados con por los servicios de vigilancia de Irlanda y Portugal. En el capítulo 2.2 se describe las características del primer paciente de variante de la ECJ detectado en los Países Bajos. Su perfil clínico, genético y neuropatológico fue en todo punto indistinguible del presentado por los casos británicos, lo que apunta a un origen etiológico común.

En el capítulo 3 se exploran diversas hipótesis etiológicas en relación con la ECJ esporádica y con la variante. En el capítulo 3.1 estudiamos la relación entre el material bovino exportado desde el Reino Unido y la aparición en otros países de casos de variante de la ECJ. En este análisis encontramos una fuerte asociación entre dicho material exportado durante la década de los 80 y el número de casos de variante de ECJ detectados. En concreto el número de animales vivos, de forma independiente, es el factor que tiene una mayor relación con el número de casos de variante aparecidos fuera del Reino Unido ($r=0,73$; 95%CI=de 0,42 a 0,89; $p<0,001$). El mayor número de animales tuvo como destino Francia, Países Bajos e Irlanda, los tres países con mayor incidencia de variante de la ECJ. En el capítulo 3.2 estudiamos el posible papel de la cirugía oftálmica como factor de riesgo para el desarrollo de la ECJ esporádica y de la variante. No encontramos asociación con ninguna de esas dos formas de la enfermedad,

pero una observación interesante fue el hecho de que un grupo de pacientes esporádicos que debutaron con clínica visual fueron remitidos en primer lugar al oftalmólogo, y con frecuencia fueron diagnosticados y tratados de cataratas. En el capítulo 3.3 estudiamos la asociación entre la ECJ esporádica y cierta variante genética en la región no codificante del extremo 5' del gen de la proteína priónica (PRNP). En nuestro estudio poblacional encontramos que el ser portador del alelo G del polimorfismo genético en estudio (PRNP G310C) estaba estadísticamente asociado ($p=0,03$) con un riesgo de enfermedad 2,4 veces mayor (IC95% de 1,1 a 5,4). Los casos de ECJ esporádica portadores del alelo PRNP 310G comenzaron la enfermedad como media 8,9 años más jóvenes que los no portadores ($p=0,05$). El polimorfismo estudiado fue seleccionado en base a su localización en una región asociada a la expresión de PRNP. Nuestros hallazgos apoyan la hipótesis de que las variaciones en la región reguladora de PRNP podrían tener un papel etiopatogénico en la ECJ esporádica. En el capítulo 3.4 estudiamos la asociación entre las diferentes variantes haplotípicas del gen de la proteína Tau (MAPT) y el riesgo de ECJ. En una muestra de población de los Países Bajos examinamos seis polimorfismos de MAPT en enfermos de ECJ y controles cognitivamente sanos, todos de origen caucásico y con similar distribución de edad. Genotipamos los mismos seis polimorfismos en otras dos muestras de ECJ originarios de Italia y del Reino Unido. El análisis de cada polimorfismo en singular y formando haplotipos no detectó ninguna diferencia estadísticamente significativa entre controles y pacientes de ECJ esporádica. Cuando comparamos los haplotipos entre ECJ variante y esporádica observamos diferentes frecuencias en dos de ellos (H1f: 8% esporádicos versus 2% en variante; H1j: 1% esporádicos versus 7% en variante). El significado de estas diferencias es incierto, pues al tener en cuenta el número de tests realizados, las diferencias no son estadísticamente significativas.

El capítulo 4 se centra en el estudio de las principales pruebas diagnósticas de la ECJ. En el capítulo 4.1 estudiamos la sensibilidad y especificidad del análisis de varias proteínas de origen cerebral (14-3-3, Tau, NSE y S100b) en líquido cefalorraquídeo (LCR) para el diagnóstico de la ECJ. También estudiamos los diferentes factores biológicos que modifican esos parámetros. En el marco de un estudio europeo de colaboración multinacional (EPICJD), analizamos 14-3-3, Tau, NSE y S100b en 1859 pacientes con ECJ esporádica, iatrogénica, genética y variante, y 1117 controles. La mayor sensibilidad en ECJ esporádica se alcanzó con 14-3-3 y Tau (85% y 86%); la determinación combinada de 14-3-3 bien con Tau, bien con s100b o NSE incrementó la sensibilidad hasta aproximadamente un 93%. Un análisis multivariante en los pacientes esporádicos mostró que la sensibilidad de todas las pruebas fue máxima en pacientes con duración breve de la enfermedad, edad de inicio superior a los 40 años y homocigotos para el codon 129 de PRNP. En el subgrupo de pacientes a los que se les realizaron repetidas punciones lumbares se observó que la segunda prueba incrementó la sensibilidad diagnóstica. Nuestros resultados reafirman que la detección de niveles altos de estas proteínas de origen cerebral en LCR, en pacientes con sospecha clínica de

ECJ, supone una valiosa herramienta diagnóstica. Se recomienda la repetición de la punción lumbar en aquellos pacientes con clínica atípica y un primer resultado negativo. En el capítulo 4.2 se estudia la influencia del momento en el que se realiza la punción lumbar sobre la sensibilidad de los análisis de LCR. Se estudiaron los resultados de distintas pruebas (14-3-3, Tau, S100b y NSE) en 833 pacientes de ECJ esporádica realizados en distintos estadios de la enfermedad, así como en 68 pacientes en los que se realizaron estudios secuenciales. Se observó que la determinación de las proteínas 14-3-3 y Tau aumenta su sensibilidad progresivamente desde el inicio (88% y 81%) hasta estadios avanzados (91% y 90%). Esa tendencia fue estadísticamente significativa en el grupo de pacientes heterocigotos para el codón 129 de PRNP. Globalmente, los niveles absolutos de Tau, S100b ($p < 0,05$) y NSE se incrementaron en el último estadio de la enfermedad en relación al primero. Niveles altos de Tau, NSE y S100b se asociaron a menor supervivencia ($p < 0,01$). El subgrupo de pacientes en los que se realizó al menos una segunda punción lumbar eran más jóvenes que la media, con duración de enfermedad mayor, y con mayor frecuencia de heterocigotos para el codón 129 de PRNP ($p < 0,001$). La sensibilidad de la detección de 14-3-3 se incrementó de un 65% hasta un 79% en la segunda punción lumbar ($p = 0,06$) y el 88% de los pacientes tuvieron al menos uno de los dos resultados positivos. La sensibilidad y los niveles absolutos de los marcadores de la ECJ en LCR aumentaron con la progresión de la enfermedad modulados por el genotipo del codón 129. Resultados negativos obtenidos en estadios precoces se deben interpretar con cautela, especialmente en pacientes jóvenes o heterocigotos para el codón 129. En el capítulo 4.3 se examina los resultados de los estudios de rutina de LCR (número de células blancas y proteínas totales) y la presencia de bandas oligoclonales IgG en pacientes con EET. Se analizaron datos de 450 pacientes esporádicos y 47 con otros tipos de enfermedades. Un número de células en LCR mayor de cinco cels/ μ l se encontró sólo en tres pacientes esporádicos de los 298 estudiados; ninguno de ellos superaba las 20 cels/ μ l. Concentraciones de proteínas superiores a 0,9 gr/l se encontraron en cinco de los 438 pacientes esporádicos estudiados; ninguno de ellos alcanzó niveles superiores a un gr/l. Se detectaron bandas oligoclonales IgG en ocho de los 182 pacientes esporádicos. Ninguno de los pacientes con otras EET presentó un número de células superior a 20 o concentración de proteínas superior a 0,9 gr/l, y sólo en un paciente con insomnio familiar fatal se detectaron bandas oligoclonales IgG. Ninguno de los pacientes esporádicos o con otra EET presentó anomalías en las tres pruebas. En resumen, la presencia en el LCR de más de 20 cels/ μ l o de concentraciones de proteínas superior a un gr/l debe sugerir un diagnóstico alternativo. El objetivo del capítulo 4.4 es definir los principales determinantes de la sensibilidad de las tres pruebas diagnósticas características de la ECJ en todo su espectro fenotípico: electroencefalograma (EEG), resonancia magnética (RM) y determinación de 14-3-3 en LCR. A este fin realizamos un estudio en el seno del grupo EUROCDJ incluyendo información de 2451 pacientes con diagnóstico definitivo. Nuestros resultados demuestran que tanto la edad de inicio como la duración

de la enfermedad influyen, como factores independientes, en la aparición de los cambios típicos en el EEG; por su parte la duración de la enfermedad lo hace también en la detección de la proteína 14-3-3; la aparición de los cambios característicos en la RM no parece depender de estos dos factores clínicos, aunque los datos a ese respecto son incompletos y por ello las conclusiones más provisionales. Además de los previos, el subtipo molecular de la enfermedad (definido por la combinación del genotipo del codón 129 de PRNP y el tipo de PrP) se reafirmó como el factor más influyente en los resultados de las pruebas. En un análisis multivariante incluyendo todos los factores mencionados con anterioridad se observó que el momento en el que se realiza la prueba diagnóstica no influye de una manera estadísticamente significativa, independientemente del subtipo molecular, en la sensibilidad del EEG o en el resultado de la determinación de 14-3-3. Los pacientes con subtipo MM1 presentaron con mayor frecuencia EEG típico de ECJ esporádico que los pacientes MV1, MV2, y VV2, sin embargo, los VV2 presentaron con mayor frecuencia cambios típicos en la RM. Globalmente, la detección de proteína 14-3-3 en LCR demostró ser la prueba más sensible (88,1%), aunque su rendimiento fue significativamente peor en los subtipos infrecuentes MV2 y MM2. Nuestros hallazgos definen y confirman una serie de factores moduladores del rendimiento de las principales pruebas diagnósticas de la ECJ, y subrayan la importancia de tenerlos en cuenta a la hora del juicio diagnóstico. La combinación de las distintas pruebas incrementa las posibilidades diagnósticas, sobre todo en los subtipos infrecuentes como MM2 o MV2. En el capítulo 5 los principales hallazgos de esta tesis son expuestos y analizados tanto en su conjunto como en el contexto de la literatura científica actual. Se discuten los aspectos metodológicos más relevantes, y por último se mencionan las líneas futuras de investigación a la vista de los hallazgos presentados en este texto.

Acknowledgements: the chronicle of the journey

I'm writing this last bit of my thesis in an airplane, two days before the deadline to hand in the manuscript to the printer. I'm thinking that this is quite an inspiring environment since I see this book as a journey. Indeed, a considerable amount of the work included in it has been done in planes or at airports.

It's been a relatively long period of time, and looking back on my memories I realise that I have met a lot of nice people who, in one way or another, have contributed to my journey. It would be impossible to list all their names; thanks to all of you.

I perfectly remind the precise moment when all this started. You may have forgotten this, Polo. We were having lunch with Charo Carpizo and you two started discussing the CJD cases in Cantabria, and that it would be a good subject for a PhD. I think that the fact that the last diagnosis I made as a resident was a CJD, faded my last doubts away. That was the beginning of my journey, and everything started thanks to you, Polo. Therefore, in chronological order you must be the first person to be acknowledged. Our initial plans of describing the unusually high incidence of CJD in Cantabria are still on standby. In contrast, our friendship has never stopped growing, and you and Carmen have been with me all through this journey, during the good moments and also during the bad ones. Muchas gracias.

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Pascual Sánchez-Juan
Rome, December 2nd 2006

About the author

Pascual Jesús Sánchez-Juan was born on August 4th 1973 in Elche (Spain), a Mediterranean city covered by palm trees. He attended primary school in 'Colegio público León Felipe' where he graduated with honours. He studied secondary school from 1987 to 1991 in 'Colegio Aitana' where he graduated cum laude. He moved to Pamplona, in the North of Spain, to study in 'Universidad de Navarra' where he obtained a medical degree in 1997. He moved again, this time to the green Santander, where he started his Neurology training in 2002 in University Hospital 'Marqués de Valdecilla' under the guidance of Professor José Berciano. After obtaining his title of Consultant Neurologist he was awarded with the first edition of the IFIMAV research grant 'Wenceslao López Albo' for his project on CJD: 'Programa de Vigilancia de la ECJ en Cantabria y Proyecto de Atención Integral a Pacientes con Enfermedades Neurodegenerativas por Depósito de Proteínas', supervised by Professor J.M. Polo. He stayed in UK National CJD Surveillance Unit (Edinburgh) under the supervision of Professor Bob Will from September 2002 to August 2003. Then he moved to Rotterdam where he obtained in the Erasmus MC a Master of Science degree in Genetic Epidemiology. After that, he continued working as part of the staff of the Genetic Epidemiology Unit of the department of Epidemiology & Biostatistics of the Erasmus MC. Since 2004 he has been coordinating the Dutch CJD surveillance, directed by Professor Cornelia van Duijn. As member of the Dutch surveillance system, he is an active part of several international CJD surveillance and research projects like EURO-CJD, NEURO-CJD and EPIC-JD. Currently he is back in the Neurology Service of the University Hospital 'Marqués de Valdecilla'.

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