Determinants of Creutzfeldt-Jakob disease

A genetic epidemiologic study

ESTHER CROES

Acknowledgement
The work presented in this thesis was conducted at the Department of Epidemiology & Biostatistics of the Erasmus MC in Rotterdam and was financially supported by the Dutch Ministry of Health, Welfare and Sports (VWS), the Ministry of Education, the Netherlands Organisation for Scientific Research (NWO), a grant of the European Union (CT98-7022), and the Municipality of Rotterdam.

The author gratefully acknowledges the collaboration with the 'European and allied countries Collaborative Study Group of CJD', the 'Extended European Collaborative Study Group of CJD', the Department of Molecular Genetics, Flanders Interuniversity Institute of Biotechnology (VIB8) at the University of Antwerp, the Department of Neurology at the Academic Medical Centre Amsterdam, the Department of Pathology at the University Medical Centre Utrecht and all Dutch neurologists and geriatricians.

The printing of this thesis was supported by the Department of Epidemiology & Biostatistics, Erasmus MC, the Erasmus University Rotterdam, the Dutch Ministry of Health, Welfare and Sports (VWS), Bayer B.V. and Alzheimer Nederland.

Cover: Liesbeth Sluiter en Michiel Rijsenbrij
Printing: Print Partners Ipskamp, Enschede

ISBN 90-9016169-4

Determinants of Creutzfeldt-Jakob disease

A genetic epidemiologic study

Determinanten van de ziekte van Creutzfeldt-Jakob

Een genetisch epidemiologisch onderzoek

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus
Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 25 september 2002 om 13.45 uur

door

Esther Anne Croes

geboren te Amsterdam

Promotiecommissie

Promotoren	Prof. dr. C.M. van Duijn Prof. dr. A. Hofman
Overige leden	Prof. dr. C. Van Broeckhoven Prof. dr. A.D.M.E. Osterhaus Prof. dr. R.G. Will

Contents

Publications and manuscripts based on the studies described in this thesis

Chapter 2
Croes EA, van Duijn CM. Prion diseases. In: Investigating neurological disease. (ed. A Hofman, R Mayeux) 2001. Cambridge University Press.

Chapter 3.2
Croes EA, Roks CMAA, Jansen GH, Nijssen PCG, van Duijn CM. Creutzfeldt-Jakob disease 38 years after diagnostic use of human growth hormone. *J Neurol Neurosurg Psychiatry* 2002;72:792-3.

Chapter 3.3
Croes EA, Jansen GH, Lemstra AW, Frijns CJ, van Gool WA, van Duijn CM. The first two patients with dura mater associated Creutzfeldt-Jakob disease in the Netherlands. *J Neurol* 2001;248:877-80.

Chapter 4.2
The EUROCJD Group. PRNP codon 129 genotype and clinical features in patients with Creutzfeldt-Jakob disease. Findings in the European Collaborative Studies of Creutzfeldt-Jakob disease.

Chapter 4.3
The EUROCJD Group. The effect of the PRNP codon 129 polymorphism on diagnostic investigations in Creutzfeldt-Jakob disease. Findings in the European Collaborative Studies of Creutzfeldt-Jakob disease.

Chapter 4.4
Croes EA, Alizadeh BZ, Bertoli Avella AM, Rademaker TAM, Vergeer-Drop JM, Dermaut B, Wientjens DPWM, Hofman A, Van Broeckhoven C, van Duijn CM. Polymorphisms in the prion protein gene and in the doppel gene increase susceptibility for Creutzfeldt-Jakob disease. (submitted)

Chapter 5.2
Croes EA, Theuns J, Roks CMAA, Dermaut B, Sleegers K, Houwing-Duistermaat JJ, Van den Broeck M, van Harten B, van Swieten JC, Cruts M, Van Broeckhoven C, van Duijn CM. Octapeptide repeat insertions in the prion protein gene and early onset dementia. (submitted)

Chapter 5.3
Croes EA, Roks CMAA, Houben MPWA, Sleegers K, Kornman-van den Bosch HJ, Wientjens DPWM, van Swieten JC, Tijssen CC, van Duijn CM. Genealogical findings in a population-based sample of patients with Creutzfeldt-Jakob disease and early onset dementia. Promising findings for further genetic research. (submitted)

Chapter 5.4
Dermaut B, Croes EA, Rademakers R, Van den Broeck M, Cruts M, Breteler MMB, Hofman A, van Duijn CM, Van Broeckhoven C. Valine homozygosity at prion codon 129 increases the risk for early-onset Alzheimer's disease in a Dutch population-based sample. (submitted)

Chapter 5.5
Croes EA, Dermaut B, Houwing-Duistermaat JJ, Breteler MMB, Hofman A, Van Broeckhoven C, van Duijn CM. Early cognitive decline in the general population is associated with the codon 129 polymorphism of the prion protein gene. (submitted)

Chapter 6
Croes EA, Dermaut B, van der Cammen TJ, Van Broeckhoven C, van Duijn CM. Genetic testing should not be advocated as a diagnostic tool in familial forms of dementia. *Am J Hum Genet* 2000;67:1033-5.

1

Introduction to the thesis

Chapter 1

Creutzfeldt-Jakob disease (CJD) is the most common form of the human transmissible spongiform encephalopathies.[1] The disease is characterised by rapid neurodegeneration leading to death within weeks to months. CJD is a rare disorder with an intriguing etiology, involving genetic and iatrogenic transmission. Ten to fifteen percent of patients with CJD are determined by mutations in the prion protein gene.[2] The clinical expression of these familial forms is highly variable and may range from a 'typical' CJD phenotype with rapid deterioration to a slowly progressive dementia mimicking Alzheimer's disease. Apart from the causative mutations, a common polymorphism within the prion protein gene determines susceptibility to CJD.[3] Subjects who are homozygous for either allele of this polymorphism are at increased risk of CJD. There is growing evidence that this polymorphism further may influence clinical expression of CJD. Finally, the effect of the prion protein may extend to other forms of neurodegenerative disorders. Besides a role in rare diseases such as Gerstmann-Sträussler-Scheinker disease and fatal familial insomnia, the gene may also have an effect on cognitive decline and dementia in the general population. Up to date, findings in this field have been inconsistent. The challenge for genetic epidemiology is to unravel the role of this gene in common neurodegenerative disorders.

In about 5% of patients with CJD, the disease has been caused by iatrogenic transmission. Unintended transmission of CJD occurred in various ways and has led in some instances to epidemics of the disease.[4] The most notorious epidemic started in the mid eighties and was due to human growth hormone administration. A second epidemic was related to dura mater transplantation. The clinical phenotype of patients with the iatrogenic form of CJD may depend on the mode of transmission as well as on the prion protein genotype. As a consequence, for each new epidemic parameters like the minimum effective dosage and the range of the incubation time are to be assessed. Case studies of individual patients may help in establishing the phenotype of the disease, which may facilitate an early identification of cases and ultimately may prevent further transmission of disease from human to human.

In the majority of patients, the etiology of CJD is unknown. Studies aiming to identify environmental risk factors so far remained unsuccessful. The most important problem encountered in CJD research is the fact that the disease is rare (with a prevalence of about one patient per million individuals per year), making it impossible to conduct follow-up studies, while the rapidly progressive dementia complicates retrospective studies in which the patient's history is assessed after the onset of disease. The opportunities of research into the genetics of the disease are more promising. Although the prion protein gene plays a pivotal role in the etiology of CJD, there is evidence that other, so far unidentified, genes are involved. Further, the role of the prion protein gene in neurodegenerative disorders in the general population remains to be disentangled. Last but not least, we need to have a better understanding of the human to human transmission of CJD, to come to safe medical practice in the future.

This study focuses upon medical and genetic risk factors for CJD. The study is based upon a national surveillance network in the Netherlands, which aims to ascertain all patients with human transmissible spongiform encephalopathies (TSEs). The research program of the surveillance network is embedded within a collaborative European study on incidence and risk factors for all human TSEs. The thesis starts with an introduction on the clinical and epidemiological aspects of the human TSEs. Chapter 2 summarises the current knowledge on etiology, incidence, diagnosis and prognosis of these diseases. In chapter 3, three Dutch patients with the iatrogenic form of the disease are described who developed CJD after human growth hormone administration or dura mater transplantation. This work is part of the surveillance network in the Netherlands. Chapter 4 investigates the role of genetic factors in CJD, using the data of the European consortium. The effect of a common prion protein gene polymorphism on the clinical phenotype and diagnostic investigations in CJD is described in chapters 4.2 and 4.3. The central issue in these chapters is the use of the prion protein gene in the diagnostic process of CJD. Chapter 4.4 addresses the association of several polymorphisms in the PRNP region with CJD. This chapter involves an empirical study in the Dutch surveillance register and a meta-analysis of all reports published on one of the candidate genes studied, the doppel gene. In chapter 5 the effect of the prion protein gene on neurodegenerative diseases other than CJD is explored. First, in chapter 5.2, the phenotype is described in a patient with an octapeptide repeat insertion in the prion protein gene. The relation between number of octapeptide repeat insertions and age at onset is further explored in a meta-analysis including all patients with octapeptide repeat insertions in the prion protein reported in the literature to date. In chapter 5.3 we study the link between patients with CJD and other forms of dementia in a historical study on the genealogical background of patients. Finally, in chapter 5.4 and 5.5, the association of the prion protein polymorphism with Alzheimer's disease and decline in cognitive functioning is assessed, using patients with dementia from the Rotterdam Study. In the last chapter the main findings of this study are discussed and the implications of the findings are presented.

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2

Epidemiological aspects of Creutzfeldt-Jakob disease

Introduction

Prion disorders form a group of transmissible spongiform encephalopathies that may afflict humans and other mammals.[1-3] They are *transmissible* within and between species by inoculation of infected tissue in the brain or administration via the blood, and, to a lesser extent, by ingestion of infected material. The most conspicious feature at microscopic brain examination consists of *spongiform* changes due to cell loss. The clinical outcome of the *encephalopathy* consists mainly of dementia, behavioural changes and cerebellar disorders. Human prion disorders include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru. Prion disorders occur in inherited, acquired and sporadic forms.

Despite their rare occurrence, the unique infectious and inheritable etiology of these dementias make them not only highly interesting scientifically, but also important with regard to public health. The causative agent is thought to be a 'prion', which is an acronym for proteinaceous infectious particle.[1] Unlike the 'classical' infectious agents such as bacteria and viruses, the prion contains little or no nucleic acid[1] and shows an unusual resistance to traditional disinfectants, preservatives and ionising radiation, which signifies its potential hazards. This resistance was dramatically illustrated by the outbreak of a bovine spongiform encephalopathy (BSE) epidemic in the UK during the eighties, for which a change in decontamination procedure of ovine and bovine material for animal feeding was held responsible. In this chapter the etiology, diagnosis, occurrence and prognosis of human prion disorders will be reviewed. The emphasis will be on CJD, by far the most common and complex prion disorder in humans.

Etiology

The pathogenesis of prion disorders remains an issue of ongoing debate. The normal, or cellular, prion protein (PrP^{C}) is found in all body cells, but mainly in the brain and spinal cord. Its function is unknown. There is growing evidence pointing to an abnormal isoform of the prion protein (indicated as PrP^{Sc}) as the transmissible factor.[4] PrP^{Sc} is thought to be derived from normal host protein PrP^{C} through a conformational change.[1,4,5] A plausible model proposed is that PrP^{Sc} may act as a template for the conversion of normal host PrP^{C} into PrP^{Sc}.[1,4,5] Since PrP^{Sc} is hardly degradable, this abnormal isoform accumulates and forms aggregates in varying parts of the brain, where it blocks the normal function.

In relation to etiologic subtypes, the three classical forms distinguished in prion diseases are the inherited, acquired, and sporadic forms. Inherited forms of human prion disease constitute up to 5 to 10% of CJD and virtually all GSS and FFI cases.[1-3,6,7] Familial CJD, GSS and FFI are all caused by mutations in the prion protein gene (PRNP).[1-3,6,7] Not for all patients with a dominant PRNP mutation a family history of prion disease is found,[7] suggesting there is incomplete penetrance of the disease. It is important to realise that the clinical presentation of the disease may differ considerably between mutation carriers from a single

family; carriers may present with various neurodegenerative and neuropsychiatric illnesses.[8]

In addition to the endogenous causes of PrP^{Sc} formation, prion diseases may originate from exogenous PrP^{Sc}. The classical example of human to human tranmission is kuru, in which the disease has been transmitted through cannibalistic rituals. Iatrogenic transmission of CJD has occurred through dura mater transplantation,[13] neurosurgery and electro-encephalographic electrode implantation,[14-16] corneal transplantation[17] and administration of human growth[18-20] and gonadotrophin[21] hormone.

Another form of exogenous transmission of PrP^{Sc} is found in a phenotypically distinct form of CJD, referred to as variant CJD (vCJD).[34] This form was discovered in the UK following the BSE epidemic. Experimental studies strongly suggest that vCJD is linked to the occurrence of an epidemic of BSE.[35-37] The most likely route from cattle to humans is through nutrition. At present the spread of vCJD is of major concern. Although the number of BSE cases is now reduced, in the past years many affected animals have entered the human food chain in the UK and elsewhere.

In the large majority of the patients the source of PrP^{Sc} is unknown, which is referred to as the sporadic form of the disease. Studies on risk factors for sporadic CJD have yielded controversial results.[22-28] An increased risk of CJD has been shown for subjects with a history of infection,[22,25] surgery of the head[23,24] and trauma to the head or body[23,24] in some studies but not in others.[27] A retrospective case-control study found a significant association between the development of sporadic CJD after surgical procedures.[29] The risk increased with the number of surgical treatments, to a maximum of three procedures with an odds ratio of 2.3 (95% confidence interval 1.34-3.41). None of these findings were supported in a joint analysis of 5 European studies.[28,30] Although experimental research indicates that the possibility of transmission through blood products cannot be excluded,[31,32] up until now there has been no epidemiological or clinical evidence for transmission of sporadic CJD from human to human through blood transfusion.[27,28,33] Findings of studies on dietary transmission, through the consumption of (organ) meat[22,25,28] or milk products[28] and on exposure to animals[23,26,28] have not yielded consistent results in sporadic CJD.[27]

In the acquired, sporadic and variant forms of CJD a mild genetic variation (or polymorphism) in PRNP has been implicated in the susceptibility for the disease.[1-3,9] This concerns the codon 129 polymorphism. In the Caucasian population 39% of healthy individuals are homozygous for methionine, 51% are heterozygous and 10% are homozygous for valine.[9] Subjects homozygous for methionine at this codon are at significantly increased risk for acquired and sporadic CJD compared to heterozygotes, whereas homozygotes for valine are at an increased risk for early onset acquired and sporadic CJD.[9] Other genetic or non-genetic risk factors may further underlie the disease. The apolipoprotein E gene, the predominant genetic risk factor for the most common cause of dementia, Alzheimer's disease,[10] has been studied in relation to CJD. Although some studies found evidence for an

Table 1
Diagnostic criteria for CJD

	Criteria
	Clinical features
I	rapidly progressive dementia
II	a. myoclonus
	b. visual or cerebellar problems
	c. pyramidal or extrapyramidal features
	d. akinetic mutism
	Investigations
III	typical EEG[‡]
IV	positive 14-3-3 protein test
	possible: I + 2 of II + duration < 2 years
	probable: I + 2 of II + III or *possible* + IV
	definite: neuropathological or immunohistochemical confirmation

[‡] generalised triphasic periodic complexes at approximately one per second

association, a relation to CJD was not confirmed in other investigations.[11,12]

Diagnosis

Prion disorders are characterised pathologically by the triad of spongy degeneration, neuronal loss, and astrocyte proliferation. In all prion disorders accumulation of the abnormal protease-resistant isoform of the prion protein (PrP^{Sc}) is found in the brain.[1] Classical CJD usually presents between 60 and 70 years of age with a rapidly progressive dementia and cerebellar ataxia. Further, extrapyramidal features, cortical blindness, pyramidal signs and myoclonus in the end stage are frequently present. Characteristic EEG changes consist of generalised periodic sharp wave activity occurring at 1-2 Hz. The diagnostic criteria for possible, probable and definite classical CJD are listed in table 1. These criteria are based upon Master's diagnostic criteria.[38] The most recent alteration in these criteria concerns the 14-3-3 protein test in cerebrospinal fluid (CSF). The presence of this protein in CSF reflects rapid neurodegeneration. When this test is applied to patients with a diagnosis of possible CJD, sensitivity and specificity may reach 95%.[39] This is much higher than the reported sensitivity and specificity for the EEG (67 and 86% respectively).[40] New developments may be anticipated with regard to the use of MRI in the diagnosis of CJD. There is evidence for an increased signal in putamen and nucleus caudatus, especially on T2-weighted images and proton density scans, in sporadic CJD patients.[41] Further, it has been suggested that diffusion-weighted MRI shows changes with high intensity in the

Table 2
Diagnostic criteria for variant CJD

	Criteria
I	Progressive neuropsychiatric disorder
	Duration of illness > 6 months
	Routine investigations do not suggest an alternative diagnosis
	No history of potential iatrogenic exposure
	No evidence of familial form of transmissible spongiform encephalopathy
	Clinical features
II	A. Early psychiatric symptoms‡
	B. Persistent painful sensory symptoms‡‡
	C. Ataxia
	D. Myoclonus or chorea or dystonia
	E. Dementia
	Investigations
III	A. EEG does not show the typical appearance of classical CJD or no EEG performed
	B. Bilateral pulvinar high signal on MRI scan#
IV	Positive tonsil biopsy##
	possible: I and 4 of II + IIIA
	probable: I + 4 of II + IIIA + IIIB or I + IV
	definite: IA + neuropathological confirmation of vCJD

‡ depression, anxiety, apathy, withdrawal, delusions

‡‡ frank pain and / or dysaesthesia

tonsil biopsy is not recommanded routinely, but may be useful in suspect cases in which the clinical features are compatible with vCJD and MRI does not show bilateral pulvinar high signal

spongiform change and extensive prion protein deposition with florid plaques, throughout the cerebrum and cerebellum

cerebral cortex.[42] However, sensitivity and specificity of the current assessment of the MRI are lower than those of the 14-3-3 protein test, but are comparable to that of the EEG.[43]

While the clinical presentation of classical CJD is highly variable and may depend on the etiology, a distinct clinical picture is seen for vCJD, which is related to BSE.[34] These patients present at an unusual early age (mean 29 years) with behavioral and psychiatric disturbances and ataxia, rather than dementia.[34] Early psychiatric symptoms in vCJD include depression, anxiety, apathy, withdrawal and

delusions. Persistent painful sensory symptoms are also often present, including both frank pain and unpleasant dysaesthesia. In a later stage of vCJD, dementia and other neurological disorders appear like in the classical form of CJD. The typical EEG abnormalities for classical CJD are absent.[34] The 14-3-3 protein test has a low sensitivity of only 50% in vCJD patients. Findings on brain MRI-scan, tonsil biopsy and pathology also differ from those in classical CJD. In vCJD, on MRI-scanning a bilateral high signal is seen in the thalamus, especially the pulvinar.[44] In tonsil biopsy samples accumulation of the pathogenic protein can be shown.[45] Neuropathology shows extensive plaque formation, surrounded by vacuoles (florid plaques), and an unusual pattern of prion staining throughout the cerebrum and cerebellum.[34] The presentation of the disease has led to a distinct list of diagnostic criteria, which are listed in table 2.
The value of the diagnostic tests in sporadic and vCJD is summarised in table 3. One may conclude that for sporadic CJD the introduction of the 14-3-3 protein

Table 3
The value of diagnostic investigations in CJD

	sporadic CJD	value	variant CJD	value
EEG	typical changes[†]	+	atypical	-
CSF	14-3-3 protein	+	14-3-3 protein and tau	+
MRI	hyperintensity in putamen and caudate nucleus	+	hyperintensity in pulvinar	+
Tonsil biopsy	normal	-	pathological prions	+*
Brain biopsy	[‡]	±	[‡‡]	±
Urine test	pathological prions	-		-
Post mortem brain examination	[‡]	++	[‡‡]	++

- not informative; ± moderately informative; + highly informative; ++ gold standard

[†] generalised periodic bi- or triphasic complexes, 100-600 ms

[‡] spongiform changes, gliosis and neuron loss; immunohistochemistry

[‡‡] spongiform change and extensive prion deposition with florid plaques, throughout the cerebrum and cerebellum

* not recommended routinely, but may be useful in suspect cases in which the clinical features are compatible with vCJD and MRI does not show pulvinar sign

test in CSF and the improving results in cerebral MRI have limited the diagnostic value of the EEG. For vCJD, only the MRI is supporting the clinical diagnosis. For all prion disorders, DNA diagnostics may be used to support the diagnosis (not listed in table 3). Several dominant mutations in the PRNP gene are known to lead to CJD. In patients with possible CJD from families in which prion disease or dementia is segregating as an autosomal dominant disorder, screening of the PRNP gene may elucidate the diagnosis. However, the presence of a causative mutation does not necessarily explain the observed clinical symptoms and other causes of disease should be excluded. GSS and FFI are also typically autosomal dominant disorders, in which DNA diagnostics may be helpful in establishing the diagnosis.[6,46,47] The clinical presentation of patients with each of the PRNP mutations known to date may be extremely diverse, ranging from cerebellar disorders or dementia to progressive insomnia and autonomic, endocrine and motor dysfunction.[46,47]

Incidence

Prion diseases are rare among humans. The most common prion disease, CJD, is reported worldwide with a mortality of around one death per million persons.[7,38,48,49] Increased frequencies have been found in isolated populations in Slovakia and in Libyan Israelis, in both cases due to an increased frequency of mutations in the PRNP gene.[50-52] Most incidence studies have been based on nationwide retrospective searches; therefore the interpretation of the findings is hampered by differences in case-ascertainment and diagnosis. This problem was largely overcome in the European Union, when in 1993 a collaborative study was started in order to monitor the incidence of CJD in Belgium, France, Germany, Italy, the Netherlands, Slovakia and the UK according to a common protocol.[7] The registers aimed to ascertain all patients diagnosed in those countries with definite

Table 4
Mortality rates of spongiform encephalopathies in Europe, 1997-2001

	1997	1998	1999	2000	2001
Austria	0.90	1.03	1.03	1.03	1.24
France*	1.57	1.75	1.78	1.67	1.79
Germany	1.30	1.48	1.23	1.26	1.32
Italy	1.09	1.22	1.51	1.48	1.51
Netherlands.	1.21	1.21	1.27	0.70	1.00
Spain	0.74	1.47	1.22	1.04	1.29
Switzerland.	1.42	1.27	1.13	1.56	2.63
UK*	1.20	1.22	1.18	0.89	0.98

rates per million inhabitants; * not including vCJD

or probable CJD. In 2000, the reported mortality varied from 0.64 to 1.72 deaths per million.[53] From 1993, there has been a modest increase in mortality (see also table 4). This rise may for a large part be attributed to better case ascertainment due to the inclusion of the 14-3-3 protein test in the diagnostic criteria.[54,55] Further, it may be explained by an increased awareness among neurologists since the recognition of vCJD.

Of all CJD patients, 5-10% are classified as familial and up to 5% as iatrogenic, with the remainder being sporadic.[7] These percentages may vary between countries. Iatrogenic CJD is mainly attributed to growth hormone treatment, particularly in the UK and France. Transmission by dura mater transplantation is seen mainly in Japan, but is clearly on the rise in other countries (see also chapter III-3). Up until August 2002, vCJD has been diagnosed in 135 patients, of whom 126 are inhabitants of the UK, 6 of France, 1 of Ireland, 1 of Italy and 1 of Canada. From 1995 to 2000 a statistically significant rise in mortality in vCJD of 33% per year was seen.[56] At present, mortality is still rising and it is not possible to predict the exact size of the epidemic of vCJD in the UK, let alone that elsewhere. Predictions are hampered by many unknown factors, including the incubation period and number of infected animals consumed. The most recent estimates range from a few hundred to tens of thousands of patients.[57-59] These predictions are based upon three major assumptions. First, it is assumed that only exposure to BSE infected cattle will lead to the disease, excluding (for the time being) a possible human to human transmission. Second, only individuals homozygous for methionine at the PRNP codon 129 polymorphism (about 40% of the population) are susceptible. Third, in these susceptible individuals, the incubation period follows a unimodal pattern. In case one of these assumptions will be falsified, numbers can change dramatically.

The other classical prion disorders, GSS, FFI and kuru, are far more rare than CJD. The exact incidence of GSS and FFI is unknown, but estimated to be between 1 and 10 per hundred million.[3] Although an epidemic of several hundred patients of kuru has occurred in the beginning of the twentieth century in the Fore speaking tribes in Papua New Guinea, the disease has virtually disappeared after cessation of ritual cannibalism.[60]

Prognosis and intervention

Within months after diagnosis of CJD, there is a progressive deterioration of the clinical condition to akinetic mutism. The median survival is estimated to be five to six months.[1,2,7] The duration of disease is found to be longer in familial and genetically determined CJD patients.[2,46] In vCJD the median duration between onset of symptoms and death is 14 months.[34] With regard to the other prion disorders, the course of disease in patients with kuru is also devastating, in most cases leading to death within a year.[46] The median duration of illness in GSS patients is estimated to be between 4 and 5 years.[46]

Experimental research has identified several substances which were able to inhibit the formation of pathological prion configurations. These substances, however, proved to be ineffective when administered at the moment of neurological symptoms. Amphotericin-B was found to prevent hamster scrapie,[61] but failed to be successful in humans.[62] Pentosan polysulphate (a heparinoid), used as prophylaxis for thrombotic disease and in the treatment of ecchymoses, was shown to prolong the incubation period in animal scrapie experiments.[63] However, proof for efficacy in CJD was never found. Recently it was suggested that chlorpromazine (currently in use for the treatment of psychoses) and quinacrine (used in the past for treatment of malaria) could prevent the formation of pathological isoforms of the prion protein in neuroblastoma cells cultured in vitro, and could possibly enhance the breakdown of already formed pathological forms.[64] Both substances can pass the blood brain barrier and have the advantage of having been used for human treatment for many decades. Although the therapeutical range and side effects are known from previous experience in treatment of malaria and psychoses, the effective dosage for the treatment of prion disease remains to be determined. Both quinacrine and chlorpromazine are relatively toxic substances. Side effects of chlorpromazine include extrapyramidal symptoms, sedation, anticholinergic effects, ocular changes, gastrointestinal motility disorders, dermatologic hypersensitivity, hematologic disturbances and respiratory depression. Quinacrine causes severe nausea, headache and anxiety. It has a bitter taste and causes yellow discolouration of the skin. In low doses, quinacrine shows a low penetration of the central nervous system. Currently, several dozens of patients have been treated with quinacrine, but the mean survival period of patients does not seem to differ from that in the past (oral communication). One of the main problems in the treatment of these devastating disorders is the advanced stage of disease by the time a diagnosis is made and treatment is started. The extend of neurological complications in patients at the time of diagnosis hampers the chances of success. An important issue is whether any of the above substances can be useful in prevention of disease. The efficacy of preventive treatment in individuals with a known risk (exposure to human growth hormone, mutation carriers), however, is not only difficult to measure in time, but such trials can only be conducted using substances with few side effects.

In the absence of effective therapy, intervention is limited to prevention of transmission. The iatrogenic infection with the CJD agent through human growth hormone has led to a ban on this product after the development of an analogous synthetic compound. The epidemic of CJD patients due to transmission via dura mater transplants has called for preventive measures in the processing of dura mater. A major issue is the prevention of vCJD. In the past years, all European countries have taken measures to prevent contamination of the human food chain. Also in the Netherlands new rules were implemented to guarantee a safe production of food on the one side and to eliminate BSE on the other. For many years, a "passive surveillance" system has been in use which aimed to identify

and test all suspected cattle. In 1990, the import of cattle from the UK was no longer allowed, with the exception of cows which were slaughtered under the age of six months. Import of these cows was stopped in 1996. In light of the role of meat and bone meal as a source of infection, it has been forbidden to feed ruminants (cattle, goats, sheep) with remainders of ruminants since 1989. This regulation was made successively more stringent. Since 1994 feedings for ruminants should no longer contain meat and bonemeal derived from mammals. Further, since January 1, 2001 it is no longer allowed to feed any farm domestic animals with meat and bone meal. The possibility of cross contamination of cattle feedings by feedings for chicken and pigs forms the background for this regulation. Inactivation of the infectious agent in the destruction process is crucial in the prevention of the spread of BSE. In the Netherlands, all material for destruction has been heated to 133° Celsius and a pressure of 3 bar since many years. This has been shown an effective way of killing the BSE agent. In some other European countries, material was only heated to 100° Celsius at atmospheric level, which might have been a significant co-factor in the BSE epidemic. In 1997, regulation on specified risk material was introduced in the Netherlands. Specified risk material comprises all corpses of cattle, goats and sheep which died due to disease or from unknown causes, and all for consumption rejected animal products. It also includes risk organs of the above ruminants (brain, spinal cord, eyes, tonsils, intestines and spleen), which are removed upon slaughter in all these animals aged one year and older. After destruction, this specified risk material is burned. Despite these regulations, it is of major concern whether subclinical forms of BSE can be fully eradicated.[65]

In addition to the transmission through the nutritional chain, the possible animal to human or human to human transmission of prion disorders through medicinal products is a further matter of importance. In BSE and vCJD, infectivity of non-neuronal material is higher compared to classical CJD. Especially lymphoreticular tissue contains infectivity.[45] Because of the long incubation period and absence of a screening method for blood donors, human to human transmission may occur. Preventive measures have been taken by the British blood banks such as the withdrawal of blood and blood products from vCJD patients, collection of plasma from outside the UK, and removal from leucocytes from erythrocyte and thrombocyte concentrates. Also in the Netherlands the possibility of spread of the pathological agent via blood is diminished through leucodepletion and exclusion of blood donors who received a transfusion of cellular blood products. However, the risk of accidental transmission of vCJD may also be increased during other medical procedures. Particularly surgery involving lymphoreticular tissue, eg. appendectomy or tonsillectomy, might convey a risk of transmission. In the Netherlands, no equivalent of the British 'once only' regulation, which orders destruction of all operation equipment used in operations at risk of prion infectivity, exists at the moment.

For surveillance purposes, an obligatory notification system is in preparation in the Netherlands, which may become effective in 2002. Within 24 hours after

clinical diagnosis the disease must be notified to the local health authorities. Prevention of iatrogenic spread and diagnosing the variant form of CJD are the most important reasons for the introduction of this system.

Implications

Prion diseases are rare disorders with a unique pathogenesis. Major progress has been achieved in unravelling the genetic etiology. The work on the molecular genetics of these disorders has not only elucidated the pathophysiology, but has also led to the recognition that an unexpectedly wide disease spectrum was associated with various PRNP mutations. PRNP testing may be used as a diagnostic test. However, results may have major implications for relatives, and because of psychological and socio-economic implications testing may be restricted to patients with a clearly positive family history of neuropsychiatric disorders.[66] The diagnosis of sporadic CJD has further improved by the introduction of the 14-3-3 protein test and MRI-scanning of the brain.

With regard to the etiology of prion disorders, little is known of risk factors for sporadic CJD. The major challenges in CJD research in Europe will be related to the BSE epidemic. Possible transmission of vCJD through other animals and medicinal products remains an issue of great concern. An important issue to tackle in future research of risk factors is the exposure assessment. Putative models for the transmission from cow to man includes nutrition, medication and animal exposure. None of these exposures are easily quantified in epidemiologic research. To assess exposure to BSE infected tissue specifically is particularly difficult. Up until now it has been impossible to trace the products of BSE cows in food products or medication. An important point for clinical research concerns the variability of disease expression. As signified by vCJD, disease expression may be atypical. An important question to be answered by neurologists in the near future is whether patients without the methionine-methionine genotype at codon 129 of the PRNP gene will develop vCJD, and what their clinical phenotype will be. The twentieth century has shown that the expression of prion diseases is still unpredictable.

Prion disorders are rare disorders. The tragic disasters of transmission of the disease by growth hormone, dura mater transplants and the transmission of BSE to humans have shown that they are highly relevant. The possibility of transmission of any form of CJD, in particular through blood products and surgical procedures, remains an issue of concern from a clinical point of view. In the light of iatrogenic transmission, early diagnosis, preferably preclinically, is important for surgery, blood transfusion and organ donation policies. This applies not only to vCJD, but also concerns classical forms, as evidenced by the recent epidemic of iatrogenic CJD transmitted by dura mater. Assessment of exposure risks to possibly contaminated agents like nutrition, medication and animals will also be an important issue in the coming years.

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3

Iatrogenic risk factors

3.1

Introduction

In the late fifties of the twentieth century the human to human transmission of the spongiform encephalopathies was first suspected in kuru, a disease which was only prevalent in Papua New Guinea. Kuru is a rapidly neurodegenerative disease which was found to be transmitted through ritual cannibalism. The first iatrogenic human to human transmission in western societies was only recognised twenty years later, when CJD was reported in a patient who received a corneal graft derived from a deceased person with CJD. Medical intervention has since been considered a serious risk factor in the etiology of CJD.[1] Stereotactic electroencephalography, contaminated neurosurgical instruments, administration of human growth hormone, cadaver-derived gonadotrophin therapy and dura mater grafting have been shown to transmit the disease. Extensive research has been performed to test whether different forms of surgery might also be involved, but findings have been contradictory.[2-4]

Two major epidemics of iatrogenic transmission of CJD have occurred, one involving transmission through human growth hormone, the second involving transmission through dura mater. This chapter describes the iatrogenic transmission in CJD identified in the Dutch CJD surveillance program. In chapter 3.2 we describe a patient with an extreme long incubation period after administration of a low dose of human derived growth hormone. In chapter 3.3 two Dutch patients are described who developed CJD after cadaveric dura mater transplantation.

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3.2

Creutzfeldt-Jakob disease 38 years after diagnostic use of human growth hormone

Abstract

A 47 year old man is described who developed pathology proven Creutzfeldt-Jakob disease (CJD) 38 years after receiving a low dose of human derived growth hormone (hGH) as part of a diagnostic process. The patient presented with a cerebellar syndrome, which is compatible with iatrogenic CJD. This is the longest incubation period described so far for iatrogenic CJD. Furthermore, this is the first report of CJD after diagnostic use of hGH. Since our patient was one of the first in the world to receive hGH, other iatrogenic CJD patients can be expected in the coming years.

Introduction

The spongiform encephalopathies are potentially transmissible. In the western world, human to human transmission was first reported in 1974, when a 55 year old woman was described who developed symptoms of Creutzfeldt-Jakob disease (CJD) 18 months after a corneal transplant.[1] Since then, transmission has been reported after stereotactic electroencephalographic depth recording, human growth hormone (hGH) and gonadotrophin treatment, and dura mater transplantation.[2-5] More than 267 iatrogenic CJD patients are known today and their number is still growing.[6] The most important iatrogenic cause of CJD is still contaminated cadaveric hGH. Exposure to contaminated hGH has taken place before 1985, when recombinant growth hormone became available. In a recent study, incubation periods in 139 hGH associated CJD patients were found to range from 5 to 30 years, with a median of 12 years.[6] One of the factors influencing incubation time is genotype at the polymorphic codon 129 of the prion protein gene.[7] Individuals homozygous for either methionine or valine at this polymorphism have a significantly shorter incubation time.[7]

We describe the second hGH related CJD patient in the Netherlands. The patient developed the disease 38 years after hGH injections. To our knowledge, this is the longest incubation period described for any form of iatrogenic CJD. Furthermore, our patient was not treated with hGH, but only received a low dose as part of a diagnostic process.

Case report

This patient presented at age 47 years with paresthesia in both arms since 6 months, difficulty with walking since 4 weeks, and involuntary movements of mainly the upper extremities since 2 weeks. He did not notice any change in cognitive function, although minor memory disturbances were noticed by his twin sister. There was no family history of neurological disease. During childhood, patient experienced a growth delay compared to his twin sister as well as to the average in the Netherlands. At the age of 9 years, a nitrogen retention test using 6 IU hGH during 5 days was performed to exclude growth hormone deficiency. Since the result was not decisive, a quantitative amino acid test was performed, which measures 30 amino acids fasting, and 1, 2, and 3 hours after growth hormone injection. No abnormal amino acid concentrations were found making the diagnosis primordial dwarfism most likely. Therefore no treatment with hGH was given.

On neurological examination at age 47 years we found a slight dysarthria without aphasia. Cranial nerve function was normal. Walking was unstable and wide-based. During movements of the upper extremities myoclonic jerks were present. Sensation, muscle tone and strength were normal. Co-ordination was impaired in all four limbs with a disturbed balance. Tendon reflexes were brisk at the arms and elevated at the legs with a clonus in the ankle reflex. Plantar responses were both normal. On mini mental state examination (MMSE) patient scored 30/30.

Routine laboratory investigation as well as thyroid function, vitamin levels (B1, B6, B12, and E) and copper metabolism were normal. On admission, electroencephalographic (EEG) examination showed generalised arrhythmic slow activity with diffuse spikes and spike waves. EEG examination 2 months later showed a further slowing of the rhythm with bilateral diphasic sharp waves, but was not typical for CJD. Cerebral magnetic resonance imaging (MRI) was normal. Cerebrospinal fluid (CSF) examination showed 1 cell per 3 μl, normal glucose and protein levels, and a strongly positive 14-3-3 protein test. The patient was homozygous for methionine on the PRNP codon 129 polymorphism. On clinical grounds the diagnosis CJD was made.

Within one month the patient's condition rapidly deteriorated and due to severe disturbances in co-ordination and progressive myoclonus he became bedridden. An eye movement disorder developed with slow saccadic and dysmetric eye movements. Temperature became unstable with peaks of 39°C without an infectious focus, for which a disorder of autoregulation was presumed. Until a far advanced stage cognitive function was intact. The patient died 5 months after admission.

The diagnosis CJD was confirmed at autopsy. The brain weighed 990 gram and showed clear cortical and cerebellar atrophy. Spongiosis, neuronal loss and gliosis were found predominantly in the putamen, caudate nucleus, basotemporal and cerebellar cortex; the cerebellum being the most severely affected of these. Vacuoles ranged from 2 to 12 μm. No amyloid or kuru plaques were found. Immunohistochemistry (3F4 antibody 1:1000, Senetek, USA) showed a clear positive staining for prion protein accumulation in a 'synaptic' distribution. Most deposition was found in the stratum moleculare of the cerebellum.

Discussion

We described a 47 year old patient who developed pathology proven CJD 38 years after hGH injections. The patient was never treated with hGH, but received a small dose as part of a diagnostic process. The onset of CJD was signalled by prodromal symptoms of paresthesia followed by a rapidly progressive ataxia. The disease presentation and course with predominantly cerebellar and eye-movement disorders is compatible with iatrogenic CJD caused by hGH treatment.[6,8]

Growth hormone treatment was first described in 1958, but was only produced on a larger scale from human pituitary glands since the beginning of the sixties. In the Netherlands growth hormone extraction started in 1963 and was soon centrally coordinated. Until 1979 the extraction of growth hormone from pituitaries was carried out by a pharmaceutical company on a non-commercial basis. In 1971 commercial products became available also. Our patient was one of the first receiving hGH in the Netherlands and no record exists on the origin of this product. A causal relationship can therefore not be established with full certainty, but coincidental occurrence of growth hormone reception and the development of this very rare disease is unlikely. Since the clinical course in this

relatively young patient is in accordance with an iatrogenic cause, we think the probability is high that the hGH injections explain the development of CJD in this patient.

The first Dutch hGH related CJD patient died in 1990.[9] During several periods from 1963 to 1969 she received intramuscular injections with hGH. During an unknown period the hGH was derived from South America. At age 39, 27 years after start of the therapy, she developed an ataxic gait, slurred speech, sensory disorders and myoclonus, but her cognitive function remained normal. Post mortem examination of the brain confirmed the diagnosis CJD.[9] Following the identification of this patient, a retrospective study was started to trace all 564 registered hGH recipients who were treated before May 1985. Until January 1995, none of these were suspected of CJD.[10] Since 1993 prospective surveillance for all forms of human prion disease is carried out in the Netherlands and, apart from the patient described above, a further two iatrogenic CJD patients were identified, who developed the disease after dura mater transplantation (see chapter 3.3).[11]

An incubation period as long as 38 years has never been reported for iatrogenic CJD before. Huillard d'Aignaux et al. studied the incubation period for 55 hGH related CJD patients in a cohort of 1361 French hGH recipients.[7] The median incubation period was between 9 and 10 years. Under the most pessimistic model the upperbound of the confidence interval for the 95th percentile varied between 17 and 20 years. Although the infecting dose cannot be quantified, it can be speculated that the long incubation period of our patient is partly explained by the administration of a limited amount of hGH. This hypothesis is supported by experimental models, in which higher infecting doses usually produce shorter incubation periods.[6] Since our patient was one of the first in the world receiving hGH, this case indicates that still more iatrogenic CJD patients may be expected in the coming years. Another implication of our study is that CJD can develop even after a low dose of hGH. This case once more testified that world-wide close monitoring of any form of iatrogenic CJD is mandatory.

Acknowledgement

We are grateful to M.Jansen, MD, PhD for his search for the origin of the growth hormone and P.P.Taminiau, MD. CJD surveillance in the Netherlands is carried out as part of the EU Concerted Action on the Epidemiology of CJD and the EU Concerted Action on Neuropathology of CJD, both funded through the BIOMED II programme, and is supported by the Dutch Ministry of Health. This surveillance would not have been possible without the co-operation of all Dutch neurologists and geriatricians.

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3.3

The first two patients with dura mater associated Creutzfeldt-Jakob disease in the Netherlands

Abstract

Creutzfeldt-Jakob disease (CJD) can be transmitted through human growth hormone or gonadotrophin administration, dura mater or cornea transplantation, depth EEG monitoring and the use of contaminated neurosurgical instruments. We describe the first two dura mater associated CJD cases in the Netherlands. Ten and fourteen years before onset of symptoms both patients received a Lyodura implantation. Findings are discussed in light of the growing epidemic of CJD among dura mater recipients.

Introduction

The transmissibility of the human spongiform encephalopathies in primates was proven for kuru and Creutzfeldt-Jakob disease (CJD) in 1966 and 1968 respectively.[1,2] The first iatrogenic human to human transmission was seen in 1974 when a 55 year old woman was reported who developed the disease 18 months after a corneal transplantation.[3] The donor of this corneal graft was diagnosed with CJD. Since then, iatrogenic cases have been described in relation to stereotactic electroencephalography, suspected contaminated neurosurgical instruments, administration of human growth hormone, cadaver-derived gonadotrophin therapy and dura mater grafting.[4-8] Up to now, at least 267 patients with the iatrogenic form of CJD are diagnosed, accounting for up to 2% of all CJD cases.[9]

In 1987, the first iatrogenic case was reported in which dura mater was the vehicle of transmission.[7] Dura mater grafts are used in neurosurgery for sealing dura defects after tumor resection, skull fractures and occasionally for spinal repairs. Since the 1950's cadaveric dural homografts have been used. Allogeneic dura has been non-commercially manufactured by registered tissue banks, but also on a commercial basis. Here we present the first two dura mater associated CJD patients in the Netherlands, identified in 1998 and 1999. The findings are discussed in light of the diagnostics and epidemiology of iatrogenic CJD.

Case reports

In 1997 patient 1, a 51 year old man, presented with increasing complaints of a pre-existent diplopia. In 1983 hemorrhage of a small arteriovenous malformation (AVM) in the fourth ventricle caused a right abducens nerve paresis, followed by a right peripheral facial paresis and a cerebellar syndrome on the right. The patient underwent a suboccipital trepanation to the fourth ventricle with subsequent repair using allogeneic dura mater. On the right side some hearing loss and a partial facial and abducens nerve paralysis remained, as well as a slight disturbance of equilibrium.

By the end of 1997 he developed new complaints. He became unsteady to such an extent that he was in constant need of support while walking. He also was forgetful and bradyphrenic. His voice became hoarse and his speech was indolent. In the beginning of 1998 his memory impairments increased. He could no longer fulfil his job and had difficulty with daily activities. Hospital admission took place. On neurologic examination an emotionally withdrawn man was seen, oriented in place and slightly disoriented in time. His speech was very slow. He had a partial abducens paresis, a slight peripheral facial paresis and some hearing loss, all on the right side. Myoclonic twitches in chin and right upperarm were visible. The deep tendon reflexes were slightly elevated at the right. Dysmetria and intention tremor were severe in all limbs and he walked with a broad based ataxic gait. There were no sensory deficits.

Blood chemistry showed no abnormalities. The last EEG was performed three weeks before his death and showed a generalized slowing and steep waves especially in frontal and midcentral areas, not specific for CJD. Earlier EEG recordings were abnormal but even less specific. In the cerebrospinal fluid a slightly elevated total protein (0.99 g/l) and a strongly positive reaction for the 14-3-3 protein were found. Brain MRI showed the AVM without signs of recent hemorrhage or oedema. The patient was homozygous for methionine at codon 129 of the prion protein gene.
During his hospital stay the patient developed myoclonic jerks in both arms. The cognitive decline was progressive. He became bedridden and died March 1998, six months after the onset of symptoms.
Post mortem examination was performed within 12 hours and showed a brain weight of 1430 grams. No atrophy was present. The base of the brain showed an orange discoloration at the right cerebellopontine angle, due to previous hemorrhage. Within the pons an AVM was present. The neocortex, basal ganglia and cerebellar cortex each showed spongiosis, neuronal loss and gliosis combined with a fine granular staining on immunohistochemistry using the prion protein specific 3F4 antibody (1:1500, Senetek USA).[10,11] Amyloid plaques were not detected; the PrP isotype was not investigated. Most vacuolisation was found in the visual cortex. The cerebellar vermis also showed vacuoles in the stratum moleculare with Bergmanns gliosis and Purkinje cell loss. Findings were consistent with the diagnosis of CJD.

In 1988 patient 2 received a dura mater transplant after resection of a hemangioblastoma in the left cerebellum at the age of 44. His recovery was complete. Ten years later, in December 1998, he complained of diplopia. One month later he noticed his walking was uncontrolled. He also had slight concentration problems and a slurred speech. He continued his work until half February 1999. Two weeks later he was admitted to the hospital. By that time his impaired gait was the predominant symptom.
On physical examination a man with intact intellectual functions was seen who had a pronounced dysarthria. Diplopia on left lateral gaze and a first degree nystagmus were noted. He had a severely impaired gait and was hardly able to stand without support. No sensory disturbances were noticed. Deep tendon reflexes were slightly elevated on the left. Plantar reflexes were normal. During the next month his clinical state deteriorated showing a progressive cerebellar syndrome and a rapidly progressive dementia leading to akinetic mutism.
Routine hematological and biochemical tests were normal. CSF examination showed a strongly positive 14-3-3 protein test. A diffuse abnormal pattern was seen on EEG recording with slow activity and triphasic spikes, typical for CJD. Two MRI scans during his hospital stay showed evidence of recurrence of a 4 mm sized hemangioblastoma between the medulla oblongata and the right tonsil with no further abnormalities. The patient was homozygous for methionine at codon 129 of the prion protein gene. A clinical diagnosis of iatrogenic CJD was made.

When he died four months after the onset of symptoms, the diagnosis of CJD was confirmed on post mortem examination. The brain weighed 1320 grams and showed no atrophy. The left cerebellar hemisphere was adherent to the dura mater and showed the effects of previous surgery. On histological examination prominent findings were neuronal loss, accompanied by gliosis and spongiosis most noteworthy in neocortex, thalamus and cerebellum. No amyloid plaques were detected and PrP isotyping was not performed. Vacuoles ranging from 4 to 30 μ were found most in the parietal cortex. The cerebellum revealed, besides gliosis in the surgical area, spongiosis in the stratum moleculare with loss of Purkinje cells. Fine granular 'synaptic' and some perivacuolar staining was found on immunohistochemistry for the prion protein, confirming the diagnosis of CJD.[10,11]

Discussion

The two patients described here developed CJD fourteen and ten years after a dura mater transplantation. In both patients the first symptom was diplopia, followed by unsteadiness of gait and ataxia. Myoclonus and cognitive decline appeared only at a later stage. Both patients were able to continue their work up to two months before death. EEG recordings were abnormal, but in one patient the EEG lacked the specific periodic sharp wave complexes. In the cerebrospinal fluid the 14-3-3 protein test was strongly positive and both patients were homozygous for methionine at codon 129 of the prion protein gene.

The course of disease in these Dutch patients is in accordance with earlier case reports. Symptomatology of dura associated CJD patients was reviewed in 1998 by Lang et al.[12] They described cerebellar signs, gait disorder, mental deterioration, dysarthria and visual/oculomotor signs during the early stage and myoclonus as the most salient feature later on, while in sporadic cases memory loss, disturbed higher cerebral functions and extrapyramidal signs are the predominant features.

Both our patients were homozygous for methionine at codon 129 of the prion protein gene. It has been shown that homozygosity at codon 129 predisposes to sporadic and iatrogenic CJD.[13] In dura mater-related CJD homozygosity for methionine is the most important genotype, whereas in growth hormone-related CJD valine homozygotes contribute considerably.[9] The median incubation period for homozygotes described by Brown et al. was 108 months (16-195).[9] In our patients, the intervals between exposure and onset of neurologic symptoms of 14 and 10 years respectively are compatible with these observations. Although the EEG is often abnormal in CJD patients, specific changes may not always be found. In sporadic patients classical EEG changes are present in only 67%.[14] Only one of our iatrogenic patients showed the characteristic periodic sharp waves complexes. Lang et al. found periodic EEG activity in the course of the disease in 71% of dura-associated cases (n=21) and triphasic patterns in only 29%.[12] Detection of the 14-3-3 protein in CSF is not yet described in detail in iatrogenic CJD patients.

Zerr et al. analysed CSF samples of 289 suspect CJD cases.[15] Only one dura mater recipient was described in this series, who was positive for the 14-3-3 protein.
Including our patients, at least 114 persons developed CJD after receiving a dura mater transplant by 2001.[9] When considering the period 1993-2000, in which CJD monitoring was established in Europe, dura mater related CJD is the most frequent cause in iatrogenic CJD, except in France and the UK. In the latter two countries, most iatrogenic cases are caused by transmission through human growth hormone.
In both our patients Lyodura® was used. Lyodura®, processed by B.Braun Melsungen AG in Germany, was distributed worldwide since 1968. In the commercial processing this material was exposed to 10% hydrogen peroxide, acetone and ionizing radiation (25 kGy), which is not sufficient to remove CJD infectivity. In May 1987 two CJD patients were identified who were highly suspected of being infected through Lyodura®. Since then, dura mater was also exposed to 1N NaOH during one hour. Seventy two of 75 dura associated CJD patients are traced back as having received this product.[12] One patient in Italy has received dura mater from another manufacturer.[16] Two dura grafts in Japanese patients were of unknown origin, but Lyodura® was very likely in one case and possible in the other.[17] Our second patient received the dura allograft in January 1988, after the announced change of decontamination procedures. Probably his dural graft was harvested before May 1987 and was not withdrawn. This indicates that also in patients with CJD who received a dura mater graft after 1987 iatrogenic disease should be considered. Because of the long latency period still more patients can be expected.
In this report we describe two patients with CJD who were most probably infected through the implantation of dura mater homografts. Autopsy confirmed the diagnosis of CJD. Clinical signs and auxiliary investigations, including the CSF test on the 14-3-3 protein, EEG recordings and genetic testing, were compatible with iatrogenic CJD. In number, dura mater related CJD moved into a silent epidemic, in most countries outnumbering the other forms of iatrogenic and variant CJD. Awareness of iatrogenic CJD including alternative transmission routes is mandatory.

Acknowledgement

CJD surveillance in the Netherlands is carried out as part of the EU Concerted Action on the Epidemiology of CJD and the EU Concerted Action on Neuropathology of CJD, both funded through the BIOMED II programme, and is supported by the Dutch Ministry of Health (VWS). This surveillance would not have been possible without the co-operation of all Dutch neurologists and geriatricians.

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4

The role of the prion protein gene complex in Creutzfeldt-Jakob disease

4.1

Introduction

The prion protein gene (PRNP) plays a major role in spongiform encephalopathies. A common polymorphism at codon 129 in this gene has been found to influence susceptibility to the classical familial, iatrogenic and sporadic forms of the disease as well as to variant CJD, the form related to bovine spongiform encephalopathy.[1,2] There is growing evidence that the codon 129 polymorphism may also affect the clinical features and outcome of diagnostic investigations. Due to the rarity of the disease, most studies assessing the relation of the codon 129 genotype with phenotype, have been hampered by small numbers to be studied. The Collaborative European Study Group on Spongiform Encephalopathies aims to overcome this problem by collecting information according to a standardised protocol in European countries, thereby creating a large collection of data. Despite the classical role of the prion protein gene mutations and the codon 129 polymorphism in CJD, it is likely that other mutations are involved. In and outside the prion protein complex, several candidate genes have been studied, but these studies have yielded inconsistent results.[3,4]

The aim of this chapter is two-fold. First, the effect of PRNP in the classical diagnosis is assessed using the data of the Collaborative Study Group. Second, the role of candidate genes other than the PRNP codon 129 polymorphism is assessed using the data from the Dutch surveillance system and a meta-analysis of reports published to date. In chapter 4.2 the relation between the PRNP codon 129 polymorphism and clinical features in the sporadic form of CJD is examined. The next chapter aims to assess the effect of this polymorphism on the outcome of electroencephalogram, magnetic resonance imaging of the brain and the 14-3-3 protein test in cerebrospinal fluid samples. Chapter 4.4 describes an association study in which the relation of a polymorphism upstream of PRNP and three polymorphisms in the prion protein-like doppel gene to the sporadic form of CJD is explored.

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4.2

PRNP codon 129 genotype and clinical features in patients with Creutzfeldt-Jakob disease. Findings in the European Collaborative Studies of Creutzfeldt-Jakob disease I

Abstract

A polymorphism at codon 129 of the prion protein gene (PRNP) is a major determinant of risk and disease phenotype of Creutzfeldt-Jakob disease (CJD). We studied the relation between PRNP codon 129 genotype and clinical features in 292 sporadic CJD patients derived from a prospective collaborative European study. According to a common protocol, clinical features at onset were studied, including dementia, cerebellar, visual and oculomotor, and (extra)pyramidal features. We found patients homozygous for methionine to have more often visual or oculomotor signs at onset. In contrast to other reports, our study suggests that dementia or forgetfulness is not associated with this genotype. Gait disturbances were significantly more often present at onset in cases homozygous for valine, resulting in a sensitivity of 78%. Although valine homozygotes were on average significantly younger, age could not explain this finding. Patients heterozygous at the polymorphic PRNP codon 129 were found to have a significantly longer disease duration. Although visual disturbances were found in excess in carriers of genotype MM, the sensitivity of this symptom is low (42%). The results of our study demonstrate that phenotypic heterogeneity can at least partly be attributed to the PRNP codon 129 genotype. However, the key characteristic of CJD, a rapidly progressive dementia, occurs independent from the PRNP codon 129 genotype.

Introduction

The prion protein (PrP) plays a major role in the pathophysiology of the spongiform encephalopathies. PrP is encoded by the prion protein gene (PRNP) on chromosome 20. In this gene a common polymorphism is located at codon 129, coding for either methionine (M) or valine (V). This polymorphism plays a key role in human spongiform encephalopathies. In sporadic, iatrogenic and the new variant form of Creutzfeldt-Jakob disease (CJD) it influences susceptibility.[1-3] The codon 129 polymorphism also determines the clinical phenotype, which shows a wide variety in signs and symptoms in familial and sporadic forms of the disease. The different clinical outcome of the PRNP Asp178Asn mutation with either a V allele on the same chromosome (familial CJD), or with an M allele (fatal familial insomnia) illustrates the effect of the PRNP codon 129 polymorphism.[4] The polymorphism on codon 129 of PRNP may also influence the phenotypic outcome in sporadic forms of CJD.[5-7] Homozygosity for the M allele, which is the most common genotype in patients with sporadic CJD, has been reported to lead to the typical CJD phenotype, consisting of short disease duration, dementia and typical periodic sharp wave complexes on the electroencephalogram (EEG).[8] Patients homozygous for the V allele may have an atypical presentation, with ataxia and without the typical EEG changes.[7] Further, these patients are characterised by a younger age at onset.[9] This complicates the interpretation of studies of VV carriers, since an early onset by itself is associated with an atypical clinical expression. This issue has not been addressed in previous studies. Also, no earlier study has addressed the effect of the PRNP codon 129 polymorphism on the sensitivity of various clinical features in the diagnosis of CJD.

Using a common protocol, we studied a series of 292 patients with the sporadic form of CJD. In this group, we studied the association between PRNP codon 129 genotype and clinical features, including a detailed analysis of the effect of age at onset on the relation between genotype and phenotype. The aim of the study was to examine the effect of the PRNP codon 129 polymorphism from a clinical perspective, i.e. the effect on the sensitivity of the clinical features of CJD in the diagnostic process.

Patients and methods

The study was embedded within the European Union (EU) collaborative studies monitoring the incidence of CJD in Belgium (Flanders only), France, Germany, Italy, the Netherlands, and the United Kingdom.[10,11] The national registers aimed to ascertain all patients diagnosed with definite or probable CJD. Clinical suspect cases were directly notified to the reference centre, mainly by neurologists. Classification was standardised according to a diagnostic protocol based on Masters et al.[12,13] The diagnosis of definite cases was neuropathologically confirmed, based on spongiform degeneration of the cerebral grey matter, neuronal loss and astrocytosis and / or by immunohistochemical staining for the prion protein.[14] Probable cases showed a rapidly progressive dementia, a typical

EEG with periodic sharp wave complexes and at least two out of four clinical features (myoclonus, visual or cerebellar symptoms, extrapyramidal or pyramidal symptoms, akinetic mutism).

All patients who died between January 1, 1993 and December 31, 1995 were eligible for this study. When possible, the patients were visited and examined by a medical doctor from the national reference center. Further, the medical record was checked to verify the clinical presentation and symptoms at onset of the disease (defined as all symptoms occuring in the first month of the disease, excluding prodromal signs). Blood was drawn for DNA collection after written informed consent of a close relative. Genetic analysis included determination of the PRNP codon 129 genotype and a search for known mutations of PRNP. Patients with either a positive family history for CJD or a mutation in the PRNP gene were classified as familial and were excluded from the analyses. Patients with a known exposure to human growth hormone, dura mater or cornea grafts or any other known iatrogenic source were also excluded. Five hundred eleven patients with sporadic definite or probable CJD died during the study period. Information on PRNP codon 129 genotype and clinical features was available for 292 patients and ranged from 92% (rapidly progressive dementia) to 87% (pyramidal signs). Most common reasons for non-availability of DNA were failure to obtain permission for inclusion in the study or, in cases without post mortem examination, death before DNA collection took place.

The raw data of the participating countries were centralised and analysed. Chisquare statistics were used to compare the distribution of genotypes over country, disease classification in definite or probable and over gender. Age at onset was compared in the genotypes using analysis of variance. Disease duration was logarithmically transformed for the analyses and age adjusted values were computed using a general linear model. Logistic regression models were fitted to calculate the odds ratios (ORs) and the 95% confidence interval (95% CI) as a proxy for the relative risk for the PRNP codon 129 genotype subgroups to develop any of the clinical features under study. The MM genotype was used as the reference category, since it is the most common genotype in patients. To adjust for possible confounding, ORs were corrected for country, sex and age at onset. For those symptoms and signs in which the OR for MV and VV had the same direction, the risk for MM carriers was calculated with MV and VV patients combined as reference group. In the positive findings, we tested whether age determined the clinical feature within the genotype by classifying patients as young (age at onset before 55 years) or old (age at onset 55 years or over). Presence or absence of clinical features was compared between young and old patients using a chisquare statistic.

Results

Ninetysix patients from France, 67 from Germany, 47 from Italy, 23 from the Netherlands and Belgium and 59 patients from the United Kingdom were included

Table 1
General characteristics of the study population

	MM N = 218	**MV** N = 40	**VV** N = 34
Percentage	74.7	13.7	11.6
Diagnosis CJD			
Definite (%)	123 (69.5)	26 (14.7)	28 (15.8)
Probable (%)	95 (82.6)	14 (12.2)	6 (5.2)
Sex: N female (%)	129 (59.2)	27 (67.5)	20 (58.8)
Median age at onset in years (range)	66.6 (18.2 - 88.1)	64.6 (28.6 - 84.1)	58.7 ‡ (32.6 - 80.0)
Median disease duration in months (range)	4.0 (0.2 – 43.0)	6.5 † (1.0 – 31.0)	5.0 (1.0 – 27.0)

‡ p = 0.01; † p = 0.001 (reference group is MM)

in the analyses. Table 1 lists the PRNP codon 129 genotype distribution in the overall group and in patients with a definite and probable diagnosis. The VV genotype was overrepresented in patients with a definite diagnosis compared to patients with probable CJD (p=0.01). There was no difference in codon 129 distribution between the countries (p=0.34) or between men and women (p=0.60). The median age at onset was significantly lower in VV carriers (p=0.01; see table 1). Median disease duration was longer in patients carrying the MV genotype than in M homozygotes (p=0.001; see table 1).

The distribution of cardinal symptoms and signs over the PRNP codon 129 genotype subgroups is summarised in table 2. There were no significant differences between the three genotypes in presentation with rapidly progressive dementia or forgetfulness at onset. Overall, cerebellar signs and gait disturbances were found significantly more often in VV carriers. When adjusting for age the frequency of gait disturbances at onset remained significantly increased in carriers of a VV genotype (OR 3.10, 95% CI 1.22-7.87). Within this genotype, onset with gait disturbances was found to be sensitive both in young patients (70%, n=10) and in old patients (82%, n=22) (p=0.51). Visual and oculomotor signs were observed to be 2.40 times (95% CI 1.22-4.73) more frequent in MM homozygous individuals. Also visual disturbances were found 2 times (95% CI 1.05-3.73) more often in MM carriers. The onset with visual or oculomotor signs was not influenced by age at onset (40% in young cases, n=20; 38% in old cases, n=172; p=0.89). (Extra-)pyramidal signs, involuntary movements, myoclonus, sensory signs, seizures, headache, vertigo, pseudobulbar signs and speech disturbances were equally distributed among the groups (data not shown).

Table 2
Sensitivity of symptoms and signs at onset by PRNP codon 129 genotype

	MM positive/tested (sensitivity)	**MV** positive/tested (sensitivity)	**VV** positive/tested (sensitivity)
Rapidly progressive dementia	104/202 (51.5)	15/36 (41.7)	19/32 (59.4)
Forgetfulness	111/196 (56.6)	19/36 (52.8)	20/32 (62.5)
Cerebellar signs	88/193 (45.6)	14/33 (42.4)	21/32 (65.5) *
Gait disturbances	106/196 (54.1)	20/32 (62.5)	25/32 (78.1) *
Visual/oculo-motor signs	74/192 (38.5) *	7/34 (20.6)	7/31 (22.6)
Visual disturbances	84/199 (42.2) *	12/36 (33.3)	7/32 (21.9)
Pyramidal signs	21/190 (11.1)	1/33 (3.0)	2/31 (6.5)
Extrapyramidal signs	22/189 (11.6)	7/36 (19.4)	4/32 (12.5)
Myoclonus	38/197 (14.3)	5/34 (14.7)	5/31 (16.1)

* $p<0.05$

When comparing the presence of clinical features in the codon 129 genotype groups for patients with a definite diagnosis and for those with a probable diagnosis separately, the excess of visual signs and symptoms remained significant in definite cases with genotype MM ($p=0.04$), but not in probable sporadic CJD patients ($p=0.09$). In patients with a definite diagnosis and genotype VV, the high prevalence of gait disturbances (78%) remained significant ($p=0.01$), suggesting this is a sensitive symptom for CJD in VV carriers specifically. In none of the other patients the sensitivity of any feature was higher than 64%. In MM carriers, the most common genotype in sporadic CJD, only forgetfulness reached a sensitivity of 61% in patients with a definite diagnosis. However, the sensitivity for most features was below 50%.

Discussion

We found the PRNP codon 129 genotype significantly influencing age at onset, disease duration and several clinical features in sporadic CJD. In our study, the clinical phenotype of M homozygous individuals showed a short disease duration and visual or oculomotor signs at onset when compared to patients with the MV or VV genotype. However, visual or oculomotor signs were found in 39% or fewer of the patients. Patients with genotype VV were significantly younger and had significantly more often a disease onset with cerebellar symptoms and gait disturbances. We showed this occurred independent of age: gait disturbances were equally present in young and old V homozygotes. Heterozygous patients were found to have a prolonged disease duration. We did not find dementia to be associated with any of the PRNP codon 129 genotypes.

In this study, PRNP codon 129 V homozygous individuals were underrepresented in patients with a probable diagnosis. The fact that CJD patients with the VV genotype often lack the typical periodic sharp wave complexes on electroencephalograms, which was mandatory for a probable diagnosis at the time of study, may explain the underreporting of V homozygous patients with a probable diagnosis. As this may have introduced bias in our study, we analysed the data separately for definite and probable patients. We found an excess of dementia and forgetfullness at disease onset in patients with a probable diagnosis, suggesting these patients are overrepresented in the group without pathological confirmation. However, the excess of cerebellar symptoms and gait disturbances in patients carrying the VV genotype was found in definite patients, as was the excess of visual problems in carriers of genotype MM. Thus, there is no evidence that these findings are biased through the ascertainment of patients.

Two other studies assessed the relation between phenotype and PRNP codon 129 genotype.[8,9] These studies included only patients with post mortem examination. Table 3 lists the distribution of dementia, cerebellar or ataxic signs, and visual disturbances in the codon 129 genotypes in the three studies. Our study found a rapidly progressive dementia equally present for all genotypes in 40-50% of the definite patients (uncorrected $p=0.34$). In contrast, Zerr ($p=0.01$) and Parchi ($p<0.01$) found dementia associated with the M allele. The sensitivity of dementia in MM carriers in the present study is much lower than that reported by Zerr et al. and Parchi et al. Findings of the studies of Zerr and Parchi have been controversial in particular with regard to sensitivity for cerebellar signs or ataxia in MV carriers (80% versus 20%) and visual disturbances. Including our study, all studies observed an association between cerebellar disorders or ataxia with homozygosity for the V allele, which reached significance in the study of Parchi et al. ($p<0.01$) and in the present study. However, estimates of sensitivity in our study in definite patients were closer to those of Zerr. In our study gait disturbances and in the one of Parchi et al. cerebellar signs have a high sensitivity in VV carriers (more than 60%). The sensitivity was much lower in the study of Zerr et al. (41%). In neuropathological studies, severe cerebellar involvement has been described as the characteristic lesion profile in V homozygotes, which

Table 3
Comparison of symptoms and signs at onset in three studies

		n	% in MM	% in MV	% in VV
Dementia	this study	270	51.5	41.7	59.4
	definite	170	54.6	41.7	55.6
	probable	100	47.0	41.7	80.0
	Zerr[9]	108	76.7	61.1	41.2
	Parchi[8]	294	70.6	68.6	31.3
Cerebellar	this study	258	45.6	42.4	65.6
signs / ataxia	*definite*	165	40.5	39.1	61.5
	probable	93	53.2	50.0	83.3
	Zerr[9]	108	19.2	22.2	41.2
	Parchi[8]	294	33.2	80.0	93.8
Visual	this study	267	42.2	33.3	21.9
disturbances	*definite*	170	38.7	29.2	22.2
	probable	97	47.5	41.7	20.0
	Zerr[9]	108	39.7	5.6	17.6
	Parchi[8]	294	24.6	2.9	0

supports our findings and those of Parchi et al.[5,6] Finally, visual disturbances were observed most frequently in patients with genotype MM. Again, estimates of the sensitivity differed considerably between Zerr et al. (40%) and Parchi et al. (25%). Our study suggests that the sensitivity (39% in patients with a definite diagnosis) is close to that of Zerr et al., with exception of the MV carriers, who showed a higher prevalence in our study. The neuropathological substrate for the relation between M homozygosity and visual symptoms has been described by Hauw et al. who observed an increased number of lesions in the occipital cortex of M homozygotes.[6] Based on the PRNP codon 129 genotype and two PrP isotypes Parchi et al. and Zerr et al. further classified six different disease profiles. However, from a clinical perspective this subclassification is not relevant, as it requires brain tissue. Although this can be obtained during life by brain biopsy, the complications for the patients and the limited information for the diagnostic work-up argue against this procedure.

The results of our study confirm that phenotypic heterogeneity is at least partly associated with the PRNP codon 129 genotype. For clinicians it will be important to realise that the presentation of symptoms may vary according to the PRNP codon 129 genotype. For example, CJD patients typically have a rapid disease progression, but may be substantially prolonged in heterozygous patients. Further, gait disturbances appear to be highly sensitive for CJD in VV carriers. Thus, genotyping of the PRNP codon 129 polymorphism may help in interpreting the symptoms during the diagnostic work-up of patients suspected of CJD. It

remains to be resolved whether other host genetic factors or molecular properties are involved in the clinical presentation of the spongiform encephalopathies.

Acknowledgement

This research was performed under the auspices of the EU Concerted Action on the Epidemiology of CJD, funded through the Biomed I program. The epidemiological surveillance of CJD in Europe is only possible through the collaboration of neurologists and neuropathologists. The study in France was supported by INSERM and the Direction Générale de la Santé. The German study was supported by a grant from the Bundesministerium für Gesundheit. The registry of CJD in Italy was supported by the Istituto Superiore di Sanità. The study in the Netherlands was supported by the Netherlands Institute for Health Sciences (NIHES), the Netherlands Organisation of Scientific Research and the Ministry of Health, Sciences and Sports. The study in the United Kingdom was funded by the Department of Health and Scottish Office Department of Health.

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4.3

The effect of the PRNP codon 129 polymorphism on diagnostic investigations in Creutzfeldt-Jakob disease. Findings in the European Collaborative Studies of Creutzfeldt-Jakob disease II

Abstract

In recent years, the diagnostic tests for the diagnosis of Creutzfeldt-Jakob disease (CJD) have improved considerably. There is increasing evidence suggesting test results of electroencephalogram (EEG), magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) tests are in part determined by a polymorphism at codon 129 of the prion protein gene (PRNP), which can either encode methionine (M) or valine (V). In this collaborative study, we assessed the influence of the PRNP codon 129 polymorphism on the sensitivity of the three most often used diagnostic investigations in CJD (EEG, cerebral MRI and the CSF 14-3-3 test) in a population-based sample of 1495 sporadic, 197 familial and 93 iatrogenic CJD patients. In sporadic CJD, we found the highest sensitivity of the EEG in M homozygous individuals (80%, $p<0.001$), but the lowest sensitivity of cerebral MRI in this subgroup (48%, $p=0.03$). Carriers of the V allele showed a low sensitivity at EEG (MV 51.5% and VV 31.4%). The CSF 14-3-3 protein test was found to perform worst in heterozygotes for this polymorphism ($p<0.01$). However, the sensitivity for the CSF test was still 85% in MV carriers. In familial cases, sensitivities showed the same trend for EEG ($p=0.01$) and for the CSF 14-3-3 test ($p=0.05$) as seen in patients with sporadic forms of CJD. In the human growth hormone induced CJD patients, typical EEG findings were absent, irrespective of the genotype. Our study shows that overall sensitivities for diagnostic tests for CJD may differ considerably over the codon 129 genotypes.

Introduction

In recent years, the diagnostic tests for the diagnosis of Creutzfeldt-Jakob disease (CJD) have improved considerably.[1-4] In the sporadic form of CJD cerebrospinal fluid (CSF) tests, among which the 14-3-3 protein test, and cerebral magnetic resonance imaging (MRI) have been incorporated in the diagnostic battery in addition to the classical test for periodic sharp wave complexes (PSWC) at the electroencephalogram (EEG).[2-4] There is increasing evidence that test results in CJD are in part determined by a polymorphism at codon 129 of the prion protein gene (PRNP), which can either encode methionine (M) or valine (V).[5-8] In sporadic CJD, the diagnostic sensitivity of the PSWC on EEG was found to be highest in M homozygotes, reduced in heterozygotes and lowest in VV carriers.[5,8,9] The sensitivity of the 14-3-3 protein test in CSF was found to be reduced in CJD patients with the sporadic form of the disease who were heterozygous for the PRNP codon 129 polymorphism.[6,8] In contrast, hyperintensive changes of the basal ganglia at cerebral MRI were reported to be more prevalent among heterozygous patients with sporadic CJD.[4,8] In familial forms of the disease, the effect of the PRNP codon 129 polymorphism was only studied in small numbers of patients.[6,10-12] Also the data for iatrogenic CJD are scarce.[13]

In this study, we assessed the effect of the PRNP codon 129 polymorphism on the sensitivity of EEG, MRI and the 14-3-3 test in a population-based sample of 1495 patients with sporadic, 197 with familial and 93 patients with the iatrogenic form of CJD.

Patients and methods

The study was embedded in the prospective CJD surveillance programme of the European Union. In 1993, registers were started in France, Germany, Italy, the Netherlands and Flanders, Slovakia and the United Kingdom, which aimed to ascertain all patients diagnosed with probable or definite CJD in their country.[14] The study was extended in 1998 with the inclusion of Australia, Austria, Canada, Spain and Switzerland. A standardised diagnostic protocol was used based on the criteria by Masters et al.[14,15]

Table 1 summarises the joint diagnostic criteria. All patients with a probable or definite diagnosis of sporadic, familial or iatrogenic CJD who died between 1993 and 2000 were eligible for this study. Patients with variant CJD, Gerstmann-Sträussler-Scheinker disease and fatal familial insomnia were not included in the present paper. The pooled dataset comprised 2749 CJD patients. For a total of 1785 subjects (1207 with a definite and 578 with a probable diagnosis) data on the PRNP codon 129 genotype were available. This patient series consisted of 10 cases from Australia, 20 from Austria, 44 from Canada, 507 from France, 531 from Germany, 266 from Italy, 25 from the Netherlands, 273 from the UK, 39 from Slovakia, 60 from Spain and 10 from Switzerland. Based on the etiology of CJD the sample was divided in 1495 patients with the sporadic form, 197 with the familial form and 93 with iatrogenic forms. In the familial cases, 114 subjects

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 extrapyramidal 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 recognised exposure risk
	probable	I progressive cerebellar syndrome in a pituitary hormone recipient or
		II probable CJD case with a recognised exposure risk

carried a PRNP codon 200 mutation, 47 subjects a codon 210 mutation and the remaining subjects had either another known mutation in the PRNP gene (n=17), or no mutation specified (n=19). Of the iatrogenic cases, 14 had developed the disease following dura mater transplantation, 78 patients after human growth hormone (hGH) treatment and 1 had a cornea transplant in the past.

Whenever possible all diagnostic investigations were reviewed by a member of the surveillance system and scored for the presence or absence of typical features. EEG records were scored positive when the periodic activity had a variability of less than 500 ms, the periodic complexes had a bi- or triphasic morphology, lasted 100-600 ms and were found generalised or lateralised on the EEG.[2,4] On cerebral MRI, high signal changes in putamen and caudate head were considered as positive findings in classical forms of CJD.[2,4] In all sites, the CSF 14-3-3 protein test was performed in an expert centre using Western blotting.[16] For 1592 CJD patients information on EEG assessment was present; in 1154 patients who died after the introduction of the CSF 14-3-3 protein test (1998) CSF test results were available and for 426 patients data on MRI were available. MRI data in iatrogenic patients were only present for 4 subjects, and were therefore excluded from the analyses.

All data were centralised and analysed collaboratively. Basic characteristics of patients with sporadic, familial and iatrogenic forms of the disease were compared with chisquare statistics and the non-parametric Kruskal-Wallis test. The sensitivity (number of positive test results in total number of patients) of EEG, MRI and the CSF 14-3-3 test was calculated for each PRNP codon 129 genotype. The association of the PRNP codon 129 genotype and the presence or absence of typical changes on EEG, MRI and the CSF 14-3-3 test was further assessed with a multiple logistic regression model, adjusting for country, sex, classification as definite or probable, year of death, age at death and disease duration. The MM genotype was used as reference, since it is the most common genotype in CJD patients. Stratified analyses were performed for type of disease (sporadic, iatrogenic or familial). Further, patients with the familial form of the disease were stratified according to the PRNP mutation (mutation 200, 210 or other mutations). In iatrogenic CJD separate analyses were conducted for patients exposed to hGH or dura mater.

Results

Table 2 shows the characteristics of the CJD study population and the presence of EEG, MRI and CSF 14-3-3 protein test data. Sporadic CJD patients had the highest post mortem examination rate (70%) and were on average older (median age at death 66.6 years) than patients with a familial or iatrogenic form of the disease. Only half of the iatrogenic CJD patients had autopsy ($p<0.001$) and their median age at death was 27.9 years ($p<0.001$). Sixty percent of sporadic and familial patients were female, compared to 30% in the iatrogenic patients ($p<0.001$). Disease duration was short in sporadic and familial CJD (median 5.0 months)

Table 2
General characteristics of the study population

	Sporadic CJD	Familial CJD	Iatrogenic CJD	p-value
Number	1495	197	93	
Definite diagnosis (%)	1042 (69.7)	117 (59.4)	48 (51.6)	<0.001
Female (%)	879 (58.8)	119 (60.4)	28 (30.1)	<0.001
Median age at death in years (range)	66.6 (20.1 – 88.8)	60.9 (30.8 – 86.8)	27.9 (12.8 – 73.8)	<0.001
Median disease duration in months (range)	5.0 (1-114)	5.0 (1-206)	13.0 (4-46)	<0.001
PRNP codon 129 genotype				<0.001
MM (%)	1017 (68.0)	142 (72.1)	49 (52.7)	
MV (%)	238 (15.9)	42 (21.3)	23 (24.7)	
VV (%)	240 (16.1)	13 (6.6)	21 (22.6)	

compared to that observed in iatrogenic patients (13.0 months; p<0.001). The distribution of codon 129 genotypes differed between sporadic, familial and iatrogenic CJD (p<0.001). This difference was explained by an excess of VV carriers in patients with sporadic and iatrogenic forms.

In sporadic CJD, the codon 129 genotype had a significant effect on the sensitivity of the EEG (table 3), which ranged from 80% in M homozygotes to 31% in V homozygotes (p<0.001). After adjusting for the possible confounders (country, sex, classification, year of death, age at death and disease duration), the prevalence of PSWC on EEG remained significantly decreased in heterozygotes (p<0.001) and V homozygotes (p<0.001). In contrast, the sensitivity of the MRI was higher in patients carrying the MV (66.1%) and VV genotype (59.4%) than in those carrying MM (48.4%) (p=0.03). After adjusting for possible confounders, the difference with homozygotes for the M allele remained significant for heterozygotes (p<0.01) and homozygotes for the V allele (p=0.01). Although the sensitivity of the CSF 14-3-3 protein test was high for all three genotypes, it was lower in heterozygotes (85%) in the unadjusted (p<0.01), and adjusted analyses (p=0.03).

Overall, in patients with an iatrogenic form of the disease the EEG test did not perform well (table 4). In none of the 72 patients in whom the disease was induced by hGH, typical EEG changes were found. Overall, and in those patients in whom the disease was induced by hGH, positive 14-3-3 protein test results

Table 3
Sensitivity of EEG, MRI and CSF 14-3-3 protein test by PRNP codon 129 genotype in sporadic CJD*

	MM	MV	VV	Crude p-value§	Adjusted p-value¤
Sensitivity EEG	79.7% (749 / 940)	51.5% (103 / 200)	31.4% (60 / 191)	<0.001	<0.001
Sensitivity MRI	48.5% (110 / 227)	66.1% (39 / 59)	59.4% (38 / 64)	0.03	0.004
Sensitivity CSF 14-3-3 test	93.4% (622 / 666)	85.0% (136 / 160)	93.3% (154 / 165)	0.002	0.03

Table 4
Sensitivity of EEG, MRI and CSF 14-3-3 protein test by PRNP codon 129 genotype in iatrogenic CJD*

	MM	MV	VV	Crude p-value§	Adjusted p-value¤
Sensitivity EEG					
All	14.6% (7 / 48)	5.9% (1 / 17)	5.3% (1 / 19)	0.42	0.42
Dura mater	66.7% (6 / 9)	100% (1 / 1)	100% (1 / 1)	0.63	0.63
Human growth hormone	0% (0 / 38)	0% (0 / 16)	0% (0 / 18)	1	1
Sensitivity CSF 14-3-3 test					
All	65.0% (13 / 20)	61.5% (8 / 13)	87.5% (7 / 8)	0.42	0.65
Dura mater	71.4% (5 /7)	100% (1 / 1)	100% (1 / 1)	0.69	0.64
Human growth hormone	58.3% (7 / 12)	58.3% (7 / 12)	85.7% (6 / 7)	0.41	0.67

* In brackets positive test results divided by total number of patients for which test result is available within genotype

§ P-values comparing the sensitivities across subgroups

¤ P-values comparing the sensitivities across subgroups adjusted for country, sex, pathological confirmation, year at death, age at death and disease duration

were found mainly in V homozygotes. However, due to small numbers, the differences between sensitivities in the codon 129 genotypes were not significant. The number of patients in whom the disease was attributed to dura mater transplantation was small. Our findings in MM carriers suggest that the EEG yields a sensitivity of 67%, while the CSF 14-3-3 protein test had a sensitivity over 71%. In table 5 data on diagnostic investigations are summarised for patients with the familial form of the disease. In the overall group the sensitivity of the EEG was highest in M homozygous individuals (p=0.01). In the three groups based on type of mutation (codon 200, 210 and other mutations) the same trend was present. There was no significant difference in sensitivity of the MRI between the codon 129 genotypes (p=0.23). However, sensitivity was low for PRNP codon 129 MV carriers, carriers of a codon 210 mutation and carriers of the 'other' mutations. The sensitivity of the CSF 14-3-3 protein test was high. In codon 210 carriers the test was positive in all patients. For the patients carrying the codon 200 mutation a similar pattern was observed as in those with the sporadic form: the sensitivity was reduced in the heterozygotes (p=0.05).

Discussion

In this study we showed an effect of the PRNP codon 129 polymorphism on the sensitivity of EEG, MRI and the 14-3-3 protein in CSF. Overall, the sensitivity of the CSF 14-3-3 protein test was high (>71%) in all etiologic subgroups, with exception of the familial patients with mutations other than on codon 200 or 210 and iatrogenic patients due to hGH who were heterozygous for PRNP codon 129. Although the sensitivity of the CSF 14-3-3 protein test was high in patients with sporadic CJD carrying the MV genotype (85%), it was significantly lower compared to that in homozygotes. This is in line with findings reported earlier.[8] Since the 14-3-3 test is a reflection of the destruction of brain cells, heterozygotes, who have a longer disease duration, may have lower levels of this protein which are more difficult to detect in an early phase.[8] Also in iatrogenic forms of CJD, which have a long disease duration, the sensitivity of the 14-3-3 tests was reduced. The EEG does not perform well in carriers of the V allele. In patients with sporadic CJD, the sensitivity of the EEG was particularly poor in MV (52%) and VV genotype (31%), which is in line with other studies, reporting a sensitivity of 33% and 21% in MV carriers and 0% and 7% in VV carriers.[8,9] This trend was also seen in the familial form. In all groups studied, sensitivities for MRI were low. Of the MM carriers with sporadic CJD, only half had positive findings on MRI.

Our study may be hampered by the fact that recording of EEG, cerebral MRI scanning or CSF collection may have been conducted at different stages in the disease course in different patients. PSWC appear on the EEG only late in the disease course.[2] It is likely that findings on the MRI scan and in the CSF are also time dependent.[4,12,13] Despite these differences between patients, our study reflects the situation in clinical practice, where patients are entering a primary or

Table 5
Sensitivity of EEG, MRI and CSF 14-3-3 protein test by PRNP codon 129 genotype in familial CJD*

	MM	MV	VV	Crude p-value§	Adjusted p-value¤
Sensitivity EEG					
All	75.8% (100 / 132)	63.9% (23 / 36)	33.3% (3 / 9)	0.01	0.18
Codon 200	70.7% (58 / 82)	65.0% (13 / 20)	0% (0 / 1)	0.29	0.66
Codon 210	88.6% (31 / 35)	77.8% (7 / 9)	-	0.40	0.89
Other mutations	73.3% (11 / 15)	42.9% (3 / 7)	37.5% (3 / 8)	0.17	0.17
Sensitivity MRI					
All	61.9% (26 / 42)	53.3% (8 / 15)	100% (4 / 4)	0.23	0.55
Codon 200	71.4% (15 / 21)	57.1% (4 / 7)	100% (2 / 2)	0.49	1.0
Codon 210	56.3% (9 / 16)	57.1% (4 / 7)	-	0.49	1.0
Other mutations	40.0% (2 / 5)	0% (0 / 1)	100% (2 / 2)	0.37	0.37
Sensitivity CSF 14-3-3 test					
All	93.9% (77 / 82)	76.9% 20 / 26)	87.5% (7 / 8)	0.05	0.12
Codon 200	91.1% (41 / 45)	76.9% (10 / 13)	100% (2 / 2)	0.33	0.59
Codon 210	100% (26 / 26)	100% (8 / 8)	-	1	1
Other mutations	90.9% (10 / 11)	40.0% (2 / 5)	83.3% (5 / 6)	0.63	0.63

* In brackets positive test results divided by total number of patients for which test result is available within genotype

§ P-values comparing the sensitivities across subgroups

¤ P-values comparing the sensitivities across subgroups adjusted for country, sex, pathological confirmation, year at death, age at death and disease duration

secondary referral hospital at different stages of disease. Second, we did not include prion protein isotyping in the present analyses. Although glycosylation patterns may be of interest when interpreting test results, isotyping is too specialised to perform during life as a standard test in clinical practice. Further it will require brain biopsy, which is an invasive procedure with major drawbacks for the patient. Third, we limited our study to sensitivity in patients with probable and definite CJD. We do not have data on specificity, because as a referral centre we do not have reliable population-based data on tests being negative in patients a postiori diagnosed with another disease. The same problem holds for patients with a possible diagnosis of CJD, who were also not ascertained reliably. Our findings are therefore only indicative for the sensitivity of the various tests, but we may overestimate this test characteristic.

Despite the limitations of our study, this report is the largest prospective study assessing the relation between the PRNP codon 129 genotype and diagnostic investigations in CJD patients, which also takes into account the etiological background. Our findings have important implications for clinical practice. Although the EEG has been the classical standard in pre-mortem CJD diagnosis, we show that EEG findings are negative in 50% of carriers of one V allele at PRNP codon 129 and in 31% of the carriers of two alleles. This means that a neurologist cannot rely on the EEG in these patients but may rather focus on MRI or CSF testing. Of the heterozygous patients without PSWC on EEG in our study, 77% had positive findings on MRI and 79% had a positive 14-3-3 protein test. For the VV carriers without PSWC on EEG these percentages were 66 and 91 respectively. The sensitivity of CSF testing was high, but the presence of a positive 14-3-3 test was reduced to 85% in heterozygotes. In individuals with a negative CSF test, the diagnosis was confirmed with PSWC on EEG recording in only 24% but in 86% MRI findings pointed to the diagnosis CJD. With regard to familial CJD, CSF testing is in particular useful in carriers of the PRNP 210 mutation. However, in these patients, the diagnosis may depend largely on DNA testing. In general, the MRI performed poorest in terms of sensitivity, but MRI diagnostics are still being improved. Up until now, we found MRI scanning to be less sensitive in M homozygotes. However, of the individuals with negative findings on MRI, 81% had PSWC on EEG and in 94% the 14-3-3 protein was found in CSF.

In conclusion, our study shows that it will be important for clinicians to realise that overall sensitivities for diagnostic tests for CJD may differ considerably over the codon 129 genotypes. Diagnostic test results have to be interpreted based on the PRNP codon 129 genotype.

Acknowledgement

The European and allied countries Collaborative Study Group of CJD is funded by a Biomed II grant. The group includes the following members:

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4.4

Polymorphisms in the prion protein gene complex increase susceptibility for Creutzfeldt-Jakob disease

Abstract

The prion protein gene (PRNP) plays a central role in the origin of Creutzfeldt-Jakob disease (CJD), but there is growing interest in other polymorphisms that may be involved in CJD. Candidates of interest are polymorphisms which may modulate the prion protein expression and polymorphisms in the prion-like doppel gene (PRND). In a population-based sample of 52 patients with sporadic CJD and 250 controls we investigated the PRNP M129V polymorphism, which is consistently associated with CJD, and a single nucleotide polymorphism (SNP 1368) located upstream of PRNP; in PRND we studied the T26M, P56L and T174M polymorphisms. Given the inconsistencies of the existing studies on PRND codon 174, we analysed our data separately and in a meta-analysis. For statistical analyses we used a generalised linear mixed model with random effects, which uses an expectation maximisation (E-M) algorithm to estimate haplotype frequencies for a multiple locus system in samples without parental information. For SNP 1368 and PRNP codon 129 we found a significant evidence for linkage disequilibrium. No evidence was found for a relation of SNP 1368 to CJD independent of PRNP codon 129. We further found a significant increased risk for V homozygotes at PRNP codon 129 and for M homozygotes at PRND codon 174, when adjusting the analyses for the other genotypes. In the haplotype analyses, a significant increased risk was observed in persons homozygous for PRNP codon 129 M and PRND codon 174 M (OR 4.35, 95% CI 1.05-18.09; p=0.04). The meta-analysis on the studies assessing the relation between the PRND codon 174 polymorphism and CJD did not show a consistent effect across populations, raising the question whether PRND 174 M is causally related to CJD, or whether the PRNP allele is in linkage disequilibrium with another polymorphism related to CJD. Our study suggests that homozygosity for the M allele at PRND codon 174 is a predictor of CJD independent of the PRNP codon 129 polymorphism in the Dutch population.

Introduction

The prion protein gene (PRNP) plays a central role in the origin of Creutzfeldt-Jakob disease (CJD). Susceptibility for the disease is influenced by a polymorphism at codon 129 of PRNP.[1] At this site, homozygosity for methionine increases the risk for sporadic, iatrogenic and variant CJD.[2-4] In recent years there has been growing interest in other genetic polymorphisms involved in CJD. Since animal studies have shown that higher levels of PRNP expression lead to shorter incubation times,[5,6] polymorphisms in the regulatory region of PRNP may be of interest. Recently several polymorphisms were identified in intronic and upstream regions of human PRNP.[7,8] Strong evidence was found for association of a single nucleotide polymorphism (SNP) upstream of PRNP exon 1, designated SNP 1368.

Another gene of interest is the *do*wnstream *p*rion *p*rotein *l*ike gene, or doppel gene (PRND). This gene shows a 24% coding sequence identity with PRNP and is located 27 kb downstream of PRNP, suggesting it arose by gene duplication.[9] Both the prion protein (PrP) and the doppel protein (Dpl) are expressed on the cell surface via a glycophosphatydilinositol (GPI) anchor. The function of both proteins is still unresolved. Possibly, PrP and Dpl have antagonistic biological properties, e.g. competitive binding to a common receptor. Disregulation of this balance may lead to pathology.[9] In humans, several polymorphism have been described in PRND. However, findings on the association of these polymorphisms with CJD are inconsistent.[10-12]

In order to study the effect of these potential susceptibility genes for CJD, we have analysed the association of several polymorphisms in PRNP and PRND with CJD. In PRNP, we investigated the M129V polymorphism and the upstream located SNP 1368; in PRND we studied the T26M, P56L and T174M polymorphisms (figure 1).[7,10,11] We performed this association study in a population-based sample of 52 patients with sporadic CJD and 250 controls. Given the inconsistencies of studies on PRND codon 174, we analysed our data separately and in a meta-analysis, including all studies in sporadic CJD published to date.

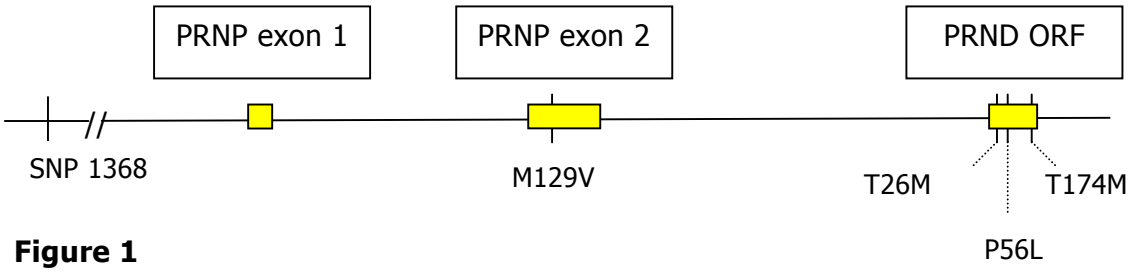

Figure 1
Position of the polymorphisms in this study in PRNP and PRND

Material and methods

Study population. CJD patients were derived from a population-based study in the Netherlands, which aimed to ascertain all patients with this disease. The patients in this study (n=52) were diagnosed with the sporadic form of CJD and were classified as definite or probable according to established criteria.[13,14] Mean age of the patients was 63 years (range 30-82) and 63% were female. The patients were visited by a medical doctor from the reference centre and blood was drawn for DNA collection after informed consent of a relative. CJD patients were compared with a control group, free of dementia and randomly drawn from a population-based study, the Rotterdam Study, for which all inhabitants of a suburb of Rotterdam were invited to participate.[15] Mean age of the control group was 67 years (range 55-87) and 57% was female.

Laboratory analyses. Genomic DNA was extracted from leucocytes following standard procedures.[16]

PRNP codon 129 genotype was determined as described in detail elsewhere.[17] Briefly, DNA was amplified by polymerase chain reaction (PCR) producing a 722-bp fragment. A second, 'nested' PCR reaction produced a 95-bp fragment. The restriction enzyme BbrPI digested the PCR fragment producing a 75-bp fragment in case of codon 129 V and 95-bp fragment in case of a codon 129 M allele. The products were fractionated on a 3.5% agarose gel stained with ethidium bromide.

PRND codon 174. Amplification of a 141-bp product in the PRND open reading frame (ORF) was performed with the forward primer 5'-CTC TGC CTT CTG GCT TTG AT-3' and the reverse primer 5'-CTG GGC ACC TTC AGA ACA C-3'. The PCR product was digested by the restriction enzyme NlaIII into two fragments of 111-bp and 30-bp, when the M allele was present. The restriction product was fractionated on a 3.0 % agarose gel stained with ethidium bromide.

PRND codon 26, codon 56 and PRNP SNP 1368. The 528-bp coding sequence of PRND was amplified by PCR using the forward primer 5'-GAC CCA CCG CCG TTT CTCT-3' and the reverse primer 5'-CGT GGG TTT GGG GGA GAA CA-3' in standard conditions.[10] For PCR amplification of SNP 1368 we designed the forward primer 5'-AAC CAG AAA CAT GGG GTG TT-3' and the reverse primer 5'-AAA CCC TGG CTT TTA CAA AGA A-3', producing a 167-bp fragment. The PCR products were purified with Sap 1U/μl and Exo1 10U/μl. A multiplex SNaPshot reaction (Applied Biosystems) was performed with the single base extension (SBE) primer for codon 26 5'-CCA CCT CTC TGC GGT CCA GA-3' and the primer for codon 56 5'-polyT CCA GGT GGC TGA GAA CCG CC-3'. For SNP 1368 we used the primer 5'-polyT TGT TAA ATC AAT TAC AGG AG-3'. The SNaPshot products were electrophoresed on an ABI 3100 automated DNA sequencer (16, filter E, LIZ 120 as size standard, from Applied Biosystems). All SNPs analyses were performed with Genotyper Version 3.7.

Statistical analyses. Genotype frequencies were tested for Hardy Weinberg equilibrium (HWE) proportions with a HWE program.[18] To test for linkage

disequilibrium between the polymorphisms in PRNP and PRND and, second, to investigate association between haplotypes and CJD, we used a generalised linear mixed model, which is suitable for the analysis of population-based genetic data.[19] Since parental information was missing, an expectation maximisation (E-M) algorithm was used to estimate the haplotype frequencies in a multiple locus system in the samples.
First, we tested for association of any of the polymorphisms with CJD. We used a Wald test for calculating odds ratios (OR) and their corresponding 95% confidence intervals (CI), while adjusting for the other polymorphisms. Based on findings of previous studies assessing the risk of PRNP codon 129, we selected the heterozygotes as the reference group, as this genotype is consistently associated with the lowest risk of CJD.[2] For the other genotypes, we considered the most frequent allele in controls as the wild type. Next, we assessed whether linkage disequilibrium existed between the polymorphisms within and between PRNP and PRND. For this, we used the imputed haplotype frequencies estimated with an E-M algorithm by the program. Finally, we constructed haplotypes for 2 loci (PRNP codon 129 and PRND codon 174). We studied the association of CJD with the combination of two homologous haplotypes. We calculated the ORs to show the strength and the direction of the associations. The 95% CIs were constructed from the profile likelihood using a constrained log-linear model.[19]

Meta-analysis. For the meta-analysis on the association between sporadic CJD and the PRND T174M polymorphism four studies (including this study) were available, leading to a total of 239 patients and 584 controls.[10-12] Genotype frequencies were not in HWE proportions in the controls in one study (p=0.002).[12] The meta-analysis was performed including and excluding this study. The literature does not uniformly assign a risk allele. We therefore conducted the analyses without prior hypothesis in three ways: TM versus TT, MM versus TM and MM versus TT. Following the hypothesis that the prion protein and doppel protein may have similar functions, we also analysed homozygotes versus heterozygotes. Heterogeneity in allele frequencies between the studies was tested with a chisquare test. In the comparison of MM versus TM we found significant heterogeneity between the studies both when the study by Schröder et al.[12] was included (p=0.0009) and excluded (p=0.033). In the comparison of homozygotes versus heterozygotes from the four studies, heterogeneity was also present (p=0.003). In these instances we used a random effect model, which is valid when between study variation exists.[20] In all other comparisons we used a fixed effect model.

Results

With exception of the PRNP codon 129 polymorphism in CJD patients, all genotype frequencies were in HWE proportions, both in the case and the control sample. In CJD patients, those homozygous for the PRNP codon 129

Table 1
Distribution of PRND codon 26 and 56 genotypes in patients with the sporadic form of CJD and controls

PRND T26M	n	TT (%)	TM (%)	MM (%)	P for HWE
Sporadic CJD	48	47 (97.9)	1 (2.1)	0 (0)	0.94
Controls	244	234 (95.9)	10 (4.1)	0 (0)	0.75
PRND P56L	n	PP (%)	PL (%)	LL (%)	P for HWE
Sporadic CJD	38	37 (97.4)	1 (2.6)	0 (0)	0.93
Controls	217	215 (99.1)	2 (0.9)	0 (0)	0.95

polymorphism were overrepresented (unadjusted OR 2.20, 95% CI 1.07-4.92; p=0.03). PRND T26M and P56L were found to be rare polymorphisms (table 1), which were not associated with the disease. In table 2 the allele and genotype distributions of SNP 1368, PRNP codon 129 and PRND codon 174 are shown. After adjusting for the other genotypes, the risk for PRNP codon 129 M and V homozygotes combined was almost two times increased, although this finding was not significant (OR 1.90, 95% CI 0.89-4.03; p=0.09). We found a more than three times increased risk for carriers of PRNP codon 129 VV genotype compared with heterozygotes (OR 3.22, 95% CI 1.00-10.45; p=0.05). A similar increase was found for carriers of PRND codon 174 genotype MM compared to the reference group TT (OR 2.86, 95% CI 1.10-7.48; p=0.03). We found no association with disease for SNP 1368.

In table 3 linkage disequilibrium among the polymorphisms and the association with the disease is explored. For SNP 1368 and PRNP codon 129 we found both in cases (p=0.0003) and controls (p=0.0001) a significant evidence for linkage disequilibrium, with an overrepresentation of the combination SNP 1368 t with codon 129 V and SNP 1368 c with codon 129 M. However, the distribution did not differ between cases and controls (p=0.24). In the second model, haplotypes of all three polymorphisms were constructed. In cases (p=0.003) as well as in controls (p=0.005) linkage disequilibrium between the three loci was significant. The linkage disequilibrium between the three loci was for a large part explained by the linkage disequilibrium between SNP 1368 and PRNP codon 129, since we found no evidence for linkage disequilibrium between PRNP codon 129 and PRND codon 174 (model 3; p=0.31 in cases and p=0.17 in controls). We observed a significant association between haplotype PRNP codon 129 M - PRND codon 174

Table 2
Allelic and genotypic distribution and the risk of CJD for PRNP SNP 1368, PRNP M129V and PRND T174M

Polymorphism			CJD (%)	Control (%)	OR (95% CI)	P-value
SNP 1368	Alleles	t	52 (56.5)	293 (49.8)	Reference	-
		c	40 (43.5)	203 (40.9)	1.11 (0.69-1.78)	0.69
	Genotypes	tt	16 (34.8)	93 (37.5)	Reference	-
		tc	20 (43.5)	107 (43.2)	1.05 (0.47-2.31)	0.90
		cc	10 (21.7)	48 (19.4)	0.74 (0.24-2.32)	0.60
M129V	Alleles	M	60 (71.4)	341 (68.2)	Reference	-
		V	24 (28.6)	159 (31.8)	0.88 (0.64-1.29)	0.55
	Genotypes	MV	12 (28.6)	117 (46.8)	Reference	-
		MM	24 (57.1)	112 (44.8)	1.69 (0.77-3.71)	0.19
		VV	6 (14.3)	21 (8.4)	3.22 (1.00-10.45)	0.05
T174M	Alleles	T	46 (48.9)	219 (57.0)	Reference	-
		M	48 (51.1)	165 (43.9)	1.38 (0.86 - 2.23)	0.16
	Genotypes	TT	14 (29.8)	64 (33.3)	Reference	-
		TM	18 (38.3)	91 (47.4)	1.25 (0.51-3.07)	0.61
		MM	15 (31.9)	37 (19.3)	2.86 (1.10-7.48)	0.03

ORs are adjusted for all alleles or genotypes

Table 3
Linkage disequilibrium between PRNP SNP 1368, PRNP codon 129 and PRND codon 174 and the association with CJD

Model	Haplotype			Haplotype frequencies*		
	SNP 1368	**M129V**	**T174M**	**CJD patients**	**Controls**	**OR (95% CI)**
1				N=80	N=496	
	t	M	-	27	173	Reference
	t	V	-	23	120	1.12 (0.90-2.10)
	c	M	-	29	166	1.10 (0.82-1.92)
	c	V	-	1	37	0.21 (0.11-1.29)
	P-value for linkage disequilibrium			**0.0003**	**0.0001**	
2				N=78	N=384	
	t	M	T	11	62	Reference
	t	M	M	17	81	1.23 (0.62-3.39)
	t	V	T	10	61	1.04 (0.52-2.87)
	t	V	M	12	27	2.53 (0.95-7.90)
	c	M	T	11	81	0.80 (0.40-2.01)
	c	M	M	15	43	2.13 (0.80-5.58)
	c	V	T	2	15	0.59 (0.29-3.23)
	c	V	M	0	15	-
	P-value for linkage disequilibrium			**0.005**	**0.003**	
3				N=80	N=384	
	-	M	T	21	143	Reference
	-	M	M	35	123	1.88 (1.41-3.28)
	-	V	T	13	76	1.10 (0.55-2.48)
	-	V	M	11	42	1.81 (0.91-4.08)
	P-value for linkage disequilibrium			**0.31**	**0.17**	

* Haplotype frequencies are imputed by the program, numbers are chromosomes

M and sporadic CJD (OR 1.88, 95% CI 1.41-3.28; model 3). Also the frequency of the PRNP codon 129 V and PRND codon 174 M haplotype was increased, but not significantly.

In table 4 the haplotype interaction is assessed. In the haplotypes, the first locus is the PRNP polymorphism and the second locus the PRND polymorphism. We used the MT-VT genotype as the reference group, because we found the M allele to be the risk allele in PRND T174M and T the wild type allele. The lowest risk was observed for the heterozygous MT-VM and MM-VT carriers (OR 0.94, 95% CI 0.20-4.54). We found a significantly increased risk in carriers of MM-MM (OR 4.35, 95% CI 1.05-18.09; p=0.04), which is compatible with the significantly increased frequency of this haplotype in table 3. The V-M haplotype was found relatively more often in carriers than in controls, although both groups consisted only of one individual.

The meta-analysis on the studies assessing the relation between the PRND codon 174 polymorphism and CJD did not result in significant findings in any of the three genetic models (figure 2), both in the analyses including and excluding the study of Schröder et al.,[12] in which control genotypes were not in HWE. P-values for the overall effect of the T174M genotype on CJD ranged from 0.4 to 1.0. Also when we tested homozygosity versus heterozygosity, we did not observe an effect of the T174M polymorphism on CJD, neither in the analysis including, nor excluding the study of Schröder et al. (data not shown).

Table 4
The risk of CJD for haplotype interaction, constructed from PRNP codon 129 and PRND codon 174

Sister haplotypes*			Risk		P-value
Haplotype 1	**Haplotype 2**	**N case / control**	**OR**	**95% CI**	
M-T	V-T	3 / 29	Reference	-	-
M-T	M-M	9 / 42	2.07	0.52 – 8.31	0.30
M-T	V-T	4 / 28	1.38	0.28 – 6.73	0.94
M-T or M-M	V-M or V-T	4 / 41	0.94	0.20 – 4.54	0.94
M-M	M-M	9 / 20	4.35	1.05 – 18.09	0.04
M-M	V-M	5 / 16	3.02	0.64 – 14.32	0.16
V-T	V-T	2 / 7	2.76	0.39 – 19.81	0.31
V-T	V-M	3 / 8	3.63	0.61 – 21.53	0.16
V-M	V-M	1 / 1	9.67	0.47 – 197.28	0.14

* first locus in the haplotype is the PRNP M129V polymorphism, second locus is the PRND T174M polymorphism

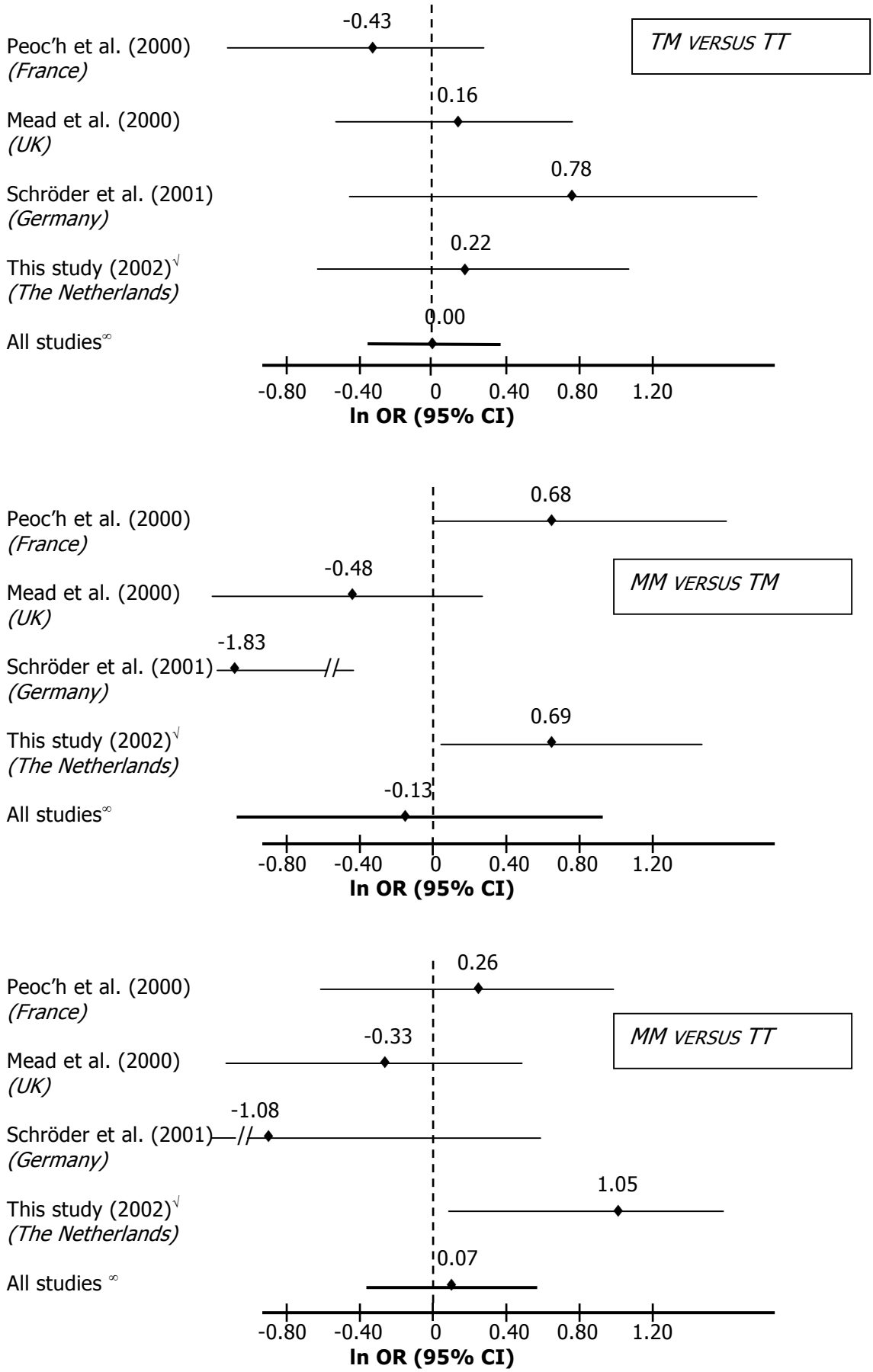

Figure 2
Meta-analysis assessing the risk on CJD associated with PRND codon 174
√ OR adjusted for PRNP codon 129; ∞ based on unadjusted values

Discussion

We found a significant relation of PRNP codon 129 and PRND codon 174 with the sporadic form of CJD. Consistent with the existing literature, we found homozygosity for PRNP codon 129 associated with an increased risk for the sporadic form of CJD.[1] We found the increase most pronounced in V homozygotes, in that the OR for VV was higher than that of MM carriers, and in contrast to the MM genotype, significantly increased in cases. The major finding of this paper was that the PRND codon 174 polymorphism was significantly associated with CJD, independent of the effect of the PRNP codon 129 polymorphism.

Taking into account the effect of the PRNP codon 129 polymorphism, we found an independent significant effect of PRND codon 174, resulting in an almost three-fold increased risk for M homozygotes. This finding is in contrast to earlier reports, which either showed no significant relation[10,11] or a significant increased risk for the heterozygotes.[12] A problem with the latter study is that the controls were not in HWE (p=0.002). Deviations from HWE may be the result of selection bias or genotyping errors, which is a major drawback for interpretation of the data of this study.

Two other studies assessing the relation of the upstream region of human PRNP with CJD have been conducted.[7,8] In one of these studies, the effect of SNP 1368 was assessed, which was found to be related to CJD.[7] This study observed a strong linkage disequilibrium between SNP 1368 and PRNP codon 129, which was confirmed in our study. In both studies, the SNP 1368 c allele was found most frequently in combination with codon 129 M.[7] However, we did not confirm the previously reported association of SNP 1368 with sporadic CJD.[7]

A problem in the interpretation of our data is the small number of patients. Despite the lack of power, our study suggests a significant effect of PRND T174M. The strength of our study is its population-based design in both the case and control series. Although the distribution of the genotypes in our study is in accordance with earlier reports,[7,10,11] the meta-analysis suggests significant differences of the effect of the genotypes across studies, also when the study in which the controls were not in HWE was excluded.[12] There may be various explanations for these differences. The small size of each of the studies may have resulted in random fluctuations. Another explanation is that the PRND codon 174 is not the causal locus, but may be in linkage disequilibrium with a nearby locus involved in CJD. Linkage disequilibrium may differ across populations and this may explain the differences observed between the studies. Further cross cultural studies are needed to resolve this issue.

Our findings suggest that Dpl is involved in the sporadic form of CJD. The function of Dpl remains unknown, but may overlap the function of PrP. In animal studies it has been shown that mice lacking PrP remain healthy throughout life, indicating that another protein may take over the function of the missing PrP.[21] On the other hand, overexpression of Dpl in PrP knock-out mice with more extensive deletions in PRNP have been shown to result in progressive ataxia.[22]

These findings suggest that PrP and Dpl may have a complementary function and that interference of this balance is leading to pathology. Possibly, in humans the balance between the PrP and the Dpl protein is more easily disturbed in carriers of genotype MM at PRND codon 174.
In conclusion, our study suggests that the T174M polymorphism in PRND may increase the risk for CJD. This finding contributes to the growing evidence pointing to overlapping functions of PrP and Dpl. However, given the small size of our study and those of others, further research on the role of PRND in the etiology of CJD is needed.

Acknowledgement

This study would not have been possible without the cooperation of all Dutch neurologists and the kind help of the family members of the patients. This work was supported by the Dutch Ministry of Health, Welfare and Sports (VWS), an EU grant (CT98-7022) and the Netherlands Organisation for Scientific Research (NWO); the Fund for Scientific Research Flanders (FWO-F), DWTC Interuniversity Attractionpoles (IUAP), the International Alzheimer's Research Foundation (IARF), the Alzheimer Association (AA) and the University of Antwerp (UIA), Belgium. Bart Dermaut is a PhD fellow of the FWO-F.

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5

The role of the prion protein gene in dementia

5.1

Introduction

Although the function of the prion protein is largely unknown, it may play a role in maintaining neuronal integrity by protecting the cell from oxidative stress.[1] Loss of its function may therefore lead to neurodegeneration and thus it is conceivable that many neurodegenerative processes may be associated with the prion protein. Various mutations in the prion protein gene (PRNP) have been described leading to a wide variety of clinical symptoms. Not only missense mutations and a single pathogenic deletion have been found, but also octapeptide repeat insertions were shown to be involved in the disease.[2,3] The function of the prion protein and its potential role in various types of dementia make PRNP an interesting candidate gene to study in relation to common neurodegenerative disorders such as Alzheimer's disease.

The aim of the research described in this chapter is to understand the role of CJD genes in rare atypical forms of dementia in the general population, as well as the role of these genes in common forms, such as Alzheimer's disease. Chapter 5.2 describes a study in a series of patients with early onset forms of dementia. In particular the role of the PRNP octapeptide repeat insertions in the clinical phenotype is addressed. To answer the question whether CJD and Alzheimer's disease may share underlying genes (PRNP or others), we studied the genealogy of 59 patients with CJD and 49 with early onset dementia (chapter 5.3). Finally, in chapters 5.4 and 5.5 we examined the role of the PRNP codon 129 polymorphism, the most important susceptibility gene for CJD, in Alzheimer's disease and cognitive decline in the general population.

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5.2

Octapeptide repeat insertions in the prion protein gene and early onset dementia

Abstract

Insertions in the prion protein gene (PRNP) have been described consisting of one, two and four to nine extra octapeptide repeats. The octapeptide repeat insertions have been found in patients with a wide spectrum of neurodegenerative disease. So far, findings on the effect of the length of the octapeptide repeat and age at disease onset have been inconsistent. We sequenced the coding region of PRNP in 17 patients with early onset dementia and found a PRNP two octapeptide repeat insertion in a 69 year old male with atypical dementia. We further conducted a meta-analysis of all patients with PRNP octapeptide repeat insertions (n=59) to assess the relation of number of repeats with age at disease onset and duration of illness. We used a linear mixed effects model to study the relation with age and found an increasing number of repeats to be significantly associated with younger age at onset (p=0.0001). When adjusting for PRNP codon 129 genotype, a polymorphism determining susceptibility and clinical phenotype in Creutzfeldt-Jakob disease, the part of the total variance which remained unexplained by between family factors was reduced to 10%. We used a Cox proportional hazards analysis to study the relation with duration of disease and found a significant decrease in disease duration with the length of the octapeptide repeat (p=0.04), when adjusting for age at onset and PRNP codon 129 genotype. Our findings show a significant association of the length of the octapeptide repeat with age at disease onset and disease duration in the spongiform encephalopathies.

Introduction

The familial forms of the human spongiform encephalopathies are explained by mutations in the prion protein gene (PRNP), located on the short arm of chromosome 20. Mutations in PRNP may lead to different clinical phenotypes, including familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease and fatal familial insomnia.[1-4] These mutations can be missense mutations, deletions or insertions.[5,6] Insertions are only found in the octapeptide repeat region located between codons 51 and 91 of PRNP, which normally contains one nonapeptide repeat and four octapeptide repeats.[7] The insertions identified so far comprised of one, two and four to nine extra octapeptide repeats.[8]

Expansion of an octapeptide repeat or 24-nucleotide repeat region is rare, but expansion of trinucleotide repeats are found in other neurodegenerative disorders, including Huntington's disease and the spinal cerebellar ataxias.[9-11] The expansion of the polyglutamine tract in these disorders is associated with accumulation and higher toxicity of the protein.[12] For several disorders with an expanded polyglutamine repeat an inverse correlation was found between repeat length and age at onset.[13,14] The relation between the number of octapeptide repeats and onset of disease in human spongiform encephalopathies has not been studied.

We sequenced the coding region of PRNP in a population-based sample of 17 patients with early onset dementia and identified in one patient an insertion of two octapeptide repeats. Here we describe the clinical phenotype of the patient. We further present a meta-analysis in which we studied the relation of the number of inserted repeats with age at onset and duration of disease in all patients with PRNP octapeptide repeat insertions reported in the literature to date.

Methods

Since 1998 we are ascertaining patients with early onset dementia in a defined geographical region in the northern part of the Netherlands. A medical doctor visited the patients and the diagnosis of dementia was independently confirmed by a member of the research team and a neurologist using a standardised protocol based on the DSM-III R criteria.[15] After written informed consent of a relative, blood was drawn for DNA collection. In this study, we ascertained 17 patients with early onset dementia who were extensively studied for mutations in genes known to be involved in dementia. In these patients, we analysed exons 16 and 17 of the amyloid precursor protein (APP) gene, and exons 3 to 12 of the presenilin 1 and 2 genes by direct sequencing.[16] In one patient, we observed a single base change predicting an amino acid change at codon 301 (T301M) in the presenilin 2 gene. Currently, we are analysing the effect of T301M on APP processing and β-amyloid secretion in an in vitro assay to determine if it may be a pathogenic mutation.

We analysed the single coding exon (exon 2) of PRNP by direct sequencing of 3 overlapping PCR fragments (A, B and C) spanning the coding region.[17] In one patient, visualisation of the PCR fragments on a 1.5 % agarose gel stained with ethidium bromide demonstrated an insertion as an extra band of about 100 bp. To further characterise the insertion we cloned the PCR fragments in the pCR4-TOPO vector (Invitrogen, Carlsbad CA). Clones carrying the insertion were selected by colony PCR and the insert was sequenced using vector primers.

Meta-analysis. We used a Medline search with a combination of the words 'mutation', 'insertion', 'octapeptide', or 'repeats' and 'prion', 'PRNP', 'Creutzfeldt-Jakob disease', 'Gerstmann-Sträussler-Scheinker disease' or 'spongiform encephalopathy' to identify all patients with octapeptide repeat insertions in the PRNP gene. We included patients suspected of a prion disease with an octapeptide repeat extension disclosed by DNA analysis, patients with post mortem examination supporting the diagnosis of spongiform encephalopathy and a relative with an octapeptide repeat insertion, and patients with both post mortem examination and DNA analysis. Patients from PRNP insertion families suffering from an unidentified neurodegenerative disease, without PRNP analysis or post mortem examination, were not included.
For statistical analyses, a linear mixed effects model fitted by restricted maximum likelihood (REML) was used to assess the relation between number of inserted octapeptide repeats and age at disease onset, while adjusting for dependence of observations within families. The natural logarithm of age was modeled with the number of inserted octapeptide repeats as a covariate and with and without the PRNP codon 129. To study the association between number of inserted octapeptide repeats and disease duration we used a Kaplan Meier analysis. To assess the effect of the octapeptide repeats adjusted for age at onset and PRNP codon 129 genotype, we performed a Cox proportional hazards regression analysis. Robust standard errors were used which take into account dependence of the data. Analyses were performed with the number of inserted octapeptide repeats as a continuous variable.

Results

The patient with a two octarepeat insertion. At age 59 years, the patient developed an atypical dementia, starting with a gradual loss of initiative and bradyphrenia. At onset, the patient showed a minor decline in short-term memory. Neurological examination 6 years later revealed a remarkable apathy, decline in cognitive functions and the presence of primitive reflexes. The decline in memory was still minor and visual spatial abilities were only slightly disturbed. Electroencephalography did not show the periodic sharp wave complexes typical for CJD. Cerebral computerised tomography scanning showed symmetrical atrophy and calcifications of the basal ganglia. At the age of 69 years, the condition of the patient further deteriorated into a mute and incontinent state,

although he was still mobile and able to feed himself. Myoclonus, cerebellar and visual disturbances were absent. The family history showed atypical dementia in the patient's mother, who died at age 82 years. Post mortem examination was not performed in the mother and no DNA was available. The patient's father died at 79 years of age without evidence for dementia. The patient's siblings (aged 82, 77 and 74 years) have no signs of dementia.
The octapeptide repeat insertion in PRNP contained an identical double repeated 24-nucleotide sequence located between the fourth (normally R3) and fifth (R4) repeat and included a silent nucleotide substitution from GGG to GGA in the seventh triplet (coding for glycine in both cases). The nucleotide sequence of this octapeptide repeat insertion has been designated R2a.[18] The nucleotide sequence in the third and fourth octapeptide repeat in our patient also changed, and was identical to that described by van Harten et al. in a patient of Dutch origin (R1 R2 R2a R2 R2a R2a R4).[19] The normal order is R1 (the nonapeptide), R2, R2, R3, R4. The patient was heterozygous for the polymorphic PRNP codon 129, carrying the valine (V) allele on the chromosome with the octapeptide repeat insertion, which is identical to the disease haplotype in the patient described by Van Harten.[19]
Genealogical data from our patient and the patient described by Van Harten et al. was collected using the municipal registers. We were able to trace back the ancestors of both patients for 6 generations, till 1780, but it was not possible to link both patients to a common ancestor within these 6 generations. Genealogical data were complete, except for one branch in the fifth generation of the patient described here, for which no information was available. The dead end in one branch in the fifth generation, non-paternity, or a common ancestor before 1780 may explain our failure to establish a genealogical link.

Meta-analysis. Twenty-four articles reporting PRNP octapeptide repeat insertions in 22 different families were identified.[7,8,18-39] In total 59 patients fulfilled our inclusion criteria. Key clinical features were a progressive dementia, ataxia or other cerebellar disturbances and abnormal behaviour, mood disorders or other psychiatric symptoms. The table shows the age at onset, disease duration and PRNP codon 129 genotype as described in the papers. The figure gives the findings of a meta-analysis on the association between the number of inserted octapeptide repeats and the age at disease onset. We found a significant inverse relation between the number of inserted octapeptide repeats and age at disease onset ($p=0.0001$ in the model unadjusted for PRNP codon 129 genotype and $p<0.0001$ after adjusting for this genotype). Overall, the codon 129 genotype contributed significantly to the model ($p=0.0003$). Compared to carriers of genotype MV as reference group, patients with genotype MM tended to have an earlier onset and carriers of genotype VV a later onset. The model adjusting for the codon 129 genotype reduced the part of the total variance which remains unexplained by between family factors from 32% to 10%.
In the analyses adjusting for age at onset and codon 129 genotype, survival was significantly prolonged in carriers of the shorter repeats ($p=0.04$). We found

Table
Age at disease onset, disease duration and PRNP codon 129 genotype in patients with PRNP octapeptide repeat insertions

article	*Number of repeats*	*Number of patients**	*PRNP codon 129* [∝]	*Age at onset (years)*	*Disease duration (months)* [√]
Laplanche [28]	1	1	MM	73	4
Goldfarb [18]	2	2	MM	58 / 75	3 / 156+
Van Harten [19]	2	1	VV	61	84
This article	2	1	MV	59	120+
Laplanche [28]	4	1	VV	82	4
Rossi [35]	4	1	MM	65	6
Campbell [20]	4	1	MM	56	2
Yanagihara [8]	4	1	MM	56	5
Goldfarb [25]	5	2	n.a.	31 / 45	60 / 180
Cochran [22]	5	3	MM	26 to 44	84 to 168+
Skworc [36]	5	3	MV	51 to 61	4 to 96
Oda [32]	6	3	MM	33 to 34	60 to 84+
Capellari [21]	6	2	MV	38	48 / 120
Nicholl [31]	6	2	n.a.	34 / 46	3 / 60
Poulter / Collinge [23,34]	6	13	MM [8] / MV [3]	22 to 47	24 to 156[§]
Goldfarb [25]	7	3	n.a.	23 to 35	120 to 156
Dermaut [39]	7	2	MM	24 / 31	132
Goldfarb [26]	8	3	MM	35 to 54	3 to 24
Laplanche [29]	8	5	MM	21 to 32	36 to 144+
Van Gool [37]	8	3	MV [1] / VV [2]	21 to 54	12+ to 72
Moore [30,42]	8	4	n.a.	23 to 41	n.a.
Owen [33]	9	1	n.a.	53	30
Krasemann [27]	9	1	MM	32	72+

* patients with mutation analysis, or patients with autopsy and a blood relative with mutation analysis, or patients with mutation analysis as well as autopsy, are reported
[∝] M: methionine; V: valine; the numbers in superscript denote the number of carriers
[√] for patients alive at time of publication, the disease duration is indicated with a '+'
[§] duration is missing for patients alive at time of publication
n.a.: not available

the disease duration lowest in VV carriers and highest in the heterozygotes. However, we could not find a statistically significant difference in survival between the three genotypes.

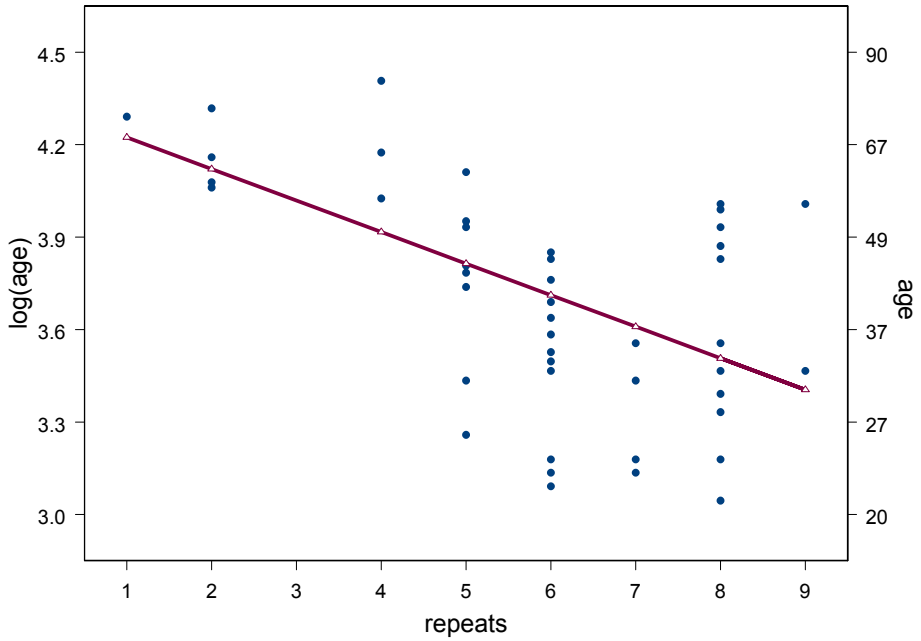

Figure
Relation between the number of insertions in the PRNP octapeptide region and the natural logarithm of age at onset, adjusted for PRNP codon 129 genotype. The best fit line is shown

Discussion

Mutations in PRNP may significantly contribute to early onset atypical forms of dementia.[40] We therefore screened this gene in a series of 17 patients with early onset dementia in the Netherlands. We detected a mutation in PRNP in 1 of 17 patients with early onset dementia, indicating that the contribution of mutations in the PRNP gene to dementia in this sample was limited.

The PRNP two octapeptide repeat insertion we observed in this patient led to an atypical form of dementia with disease duration of more than ten years. Repeat insertions of two extra octapeptide repeats were described previously.[18,19] Van Harten et al. also described a patient with a two octapeptide repeat insertion with atypical dementia in the Netherlands.[19] We could not find a link between the two Dutch patients within 6 generations. However, the rarity of the PRNP insertion mutations, the presence of the V allele on the PRNP insertion haplotype, and particularly the identical nucleotide sequence of the inserted octapeptide repeats in both patients, suggest that the patients are related and developed the disease due to the same mutation.

The second patient was described by Goldfarb et al.[18] The young patient (58 years) showed a severe neurological deterioration leading to mutism and comatose state within weeks. However, the mother of the patient, carrying the same mutation, showed a gradual cognitive decline at the age of 75 years leading to mutism 13 years later.[18] Also in the family described by us there is a major

difference in onset age between the patient (59 years) and the mother (79 years). In the spongiform encephalopathies, the stability of the octapeptide repeat insertions found in PRNP was shown to be high in several generations, suggesting that a difference in length of the inserted octapeptide repeat may not explain the difference in onset age between first degree relatives.[33] This suggests that the disease penetrance is highly variable and that other factors than the mutation itself may determine the onset and progression of the disease, even in a single family. The late onset in some carriers may explain also why the family history appears negative in a considerable number of patients. If the disease may express as late as age 75 years, carriers may already have died of other disorders, particularly in past centuries.

The discordance in age at onset between families of patients carrying PRNP octapeptide repeat insertions prompted us to perform a meta-analysis to examine the relation between number of inserted octapeptide repeats in PRNP and age at disease onset. Despite the large differences found in families, we found a significant inverse relation between number of inserted octapeptide repeats and age at disease onset. The model including octapeptide repeat length and PRNP codon 129 genotype explained about 90% of the variance observed in age at onset between the families, leaving only 10% of the total variance unexplained, which may either be caused by genetic or shared environmental factors. The meta-analysis also shows that the 10 years disease duration in our patient with two extra octapeptide repeats and the heterozygous MV genotype is exceptional. However, the decreased survival which is observed in carriers of shorter octapeptide repeat insertion is for a large part attributable to the older age at onset. In the analyses taking into account the age at onset, we found a significantly prolonged survival associated with the shorter repeats. The duration of illness in our patient is also compatible with the findings of Van Harten et al., who described a disease duration of 7 years against a background of PRNP codon 129 V homozygosity, which may lead to a decreased survival compared with the heterozygotes.

Animal studies have shown that prion protein molecules containing a larger octapeptide repeat region aggregate more readily and have a higher protease-resistance.[41] In line with these findings, our study suggests that longer insertions in the octapeptide repeat region in PRNP may lead to the inhibition of turnover resulting in a faster accumulation of the protein, earlier onset of disease, and reduced survival.

In conclusion, in a Dutch population-based sample of 17 patients with early onset dementia we identified one patient carrying a two octapeptide repeat insertion in PRNP. We further showed in a meta-analysis that the number of inserted octapeptide repeats in PRNP is inversely related to age at onset and the duration of disease. Our meta-analysis shows that the length of the octapeptide repeat insertion in combination with the PRNP codon 129 genotype explains a large part of the variation in age at onset observed. The challenge in the future will be to determine the biological mechanism underlying this process.

Acknowledgement

We thank Mrs Hilda Kornman and Mr W. Pasveer for collection of the genealogy data. This work was supported by the Dutch Ministry of Health, Welfare and Sports (VWS) and the Netherlands Organisation for Scientific Research (NWO); the Fund for Scientific Research Flanders (FWO-V), the InterUniversity Attraction Poles (IUAP) programme P5/19 of the Federal Office for Scientific, Technical and Cultural Affairs (OSTC), and a concerted action of the University of Antwerp (UIA), Belgium. Bart Dermaut is a PhD and Jessie Theuns a postdoctoral fellow of the FWO-F.

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5.3

Genealogical findings in a population-based sample of patients with Creutzfeldt-Jakob disease and Alzheimer's disease. Promising findings for further genetic research

Abstract

In Creutzfeldt-Jakob disease (CJD), there is strong evidence for a genetic component in the etiology. However, most of the patients with the genetic form of CJD do not have a positive family history for the disease, indicating that the penetrance of the mutations may be low. On the other hand, in relatives of patients with the sporadic form of the disease, neurodegenerative disorders have been found. Given the low penetrance, we hypothesised that CJD and dementia patients with a negative family history may be linked to each other, or to other patients with early onset forms of dementia, more distantly. In this study we examined the genealogy of a population-based series of 59 patients with CJD and 49 patients with early-onset dementia for evidence of a common ancestor. Further, we studied the genealogy of 16 patients with glioma as control sample. We found 9 patients with CJD related to each other by common ancestors 5 to 7 generations ago. We also found two couples of CJD and Alzheimer's disease patients with a common ancestor within 6 generations. In the patients with glioma, 4 were linked 11 generations ago, while none of the patients shared an ancestor with CJD or Alzheimer's disease patients. In two couples of CJD patients, we found a remarkable resemblance in clinical presentation, which, taken together with the link to a common ancestor, strongly suggests the presence of a shared mutation. No mutation in the prion protein gene, amyloid precursor proteine gene or the presenilin genes was found, indicating that the disease is most likely explained by another, yet unidentified gene. In conclusion, our study underscores the role of genetics in CJD and indicates that genes other than PRNP may be involved.

Introduction

Creutzfeldt-Jakob disease (CJD) is a transmissible encephalopathy. Iatrogenic forms of the disease exist which have been transmitted from human to human by treatment with human growth hormone and dura mater transplantation.[1] Also transmission from cattle (bovine spongiform encephalopathy) to humans (variant CJD) has occurred.[2] The transmission of the disease among and across species has fuelled research of clusters of disease in time and place.[3-6] Yet, this route can explain only a small proportion of patients (5% with an iatrogenic cause and 130 patients with variant CJD up to date).[7] In addition to the acquired form of the disease, there is strong evidence for a genetic component. In 14.5% of the patients, mutations are found in the prion protein gene (PRNP), leading to a wide clinical spectrum of neurodegenerative disease.[8] Most of these patients do not show any family history for neurodegenerative disorders, suggesting a low penetrance in carriers.[8] Genetic susceptibility also plays a pivotal role in intra- and interspecies transmission.[2] However, for the majority of cases the origin of the disease is unknown. Although these patients are referred to as having the sporadic form of the disease, neurodegenerative disorders have been found in relatives of these patients, suggesting that familial factors may be involved.[8,9]

Given the low penetrance, we hypothesised that patients with a negative family history may be linked to each other, or to other patients with rare forms of dementia, through a common ancestor. In this study we examined the genealogy of a population based series of 59 patients with CJD and 49 patients with early onset-dementia for evidence of a common ancestor. We used a set of 16 patients diagnosed with a non-dementing disorder, in this case glioma, as a control group. Finally, we examined whether known mutations involved in early onset dementia could explain the familial aggregation.

Methods

CJD patients were derived from a population-based study in the Netherlands, which was established in 1993. Patients were ascertained through a voluntary notification system, aiming to ascertain all patients with transmissible spongiform encephalopathies annually. A medical doctor visited the patient to verify the diagnosis. For this study, patients with a definite, probable or possible diagnosis of CJD were included (n=59).

Early onset dementia patients were derived from a Dutch population-based study of early onset Alzheimer's disease in the 4 northern provinces of the Netherlands. The patients were sampled during two study periods. The original sample was collected between 1980 and 1987 and has been described in detail elsewhere.[10] The initial study was extended between 1997 and 2000 with the same sampling criteria. The current sample comprises 49 patients for whom genealogical information was available.

As a control series we included patients with glioma derived from a population-based study in a defined area in the south of the Netherlands. All patients with

glioma (n=16) were identified through the local neuro-oncological institute, where all patients with glioma from this area are registered.

Genealogical information on the parents and grandparents of all patients was obtained from a first degree relative or a spouse of the patient, or, in case of the glioma patients, from the patient himself. Further genealogical data were collected using the municipal records. Genealogical data comprised full names, date and place of birth, date and place of marriage, full name of spouse and date and place of death. We aimed to collect data for a minimum of five generations. For this study, we searched for a distant common ancestor among patients. There were no patients from the same nuclear family for any of the disorders.

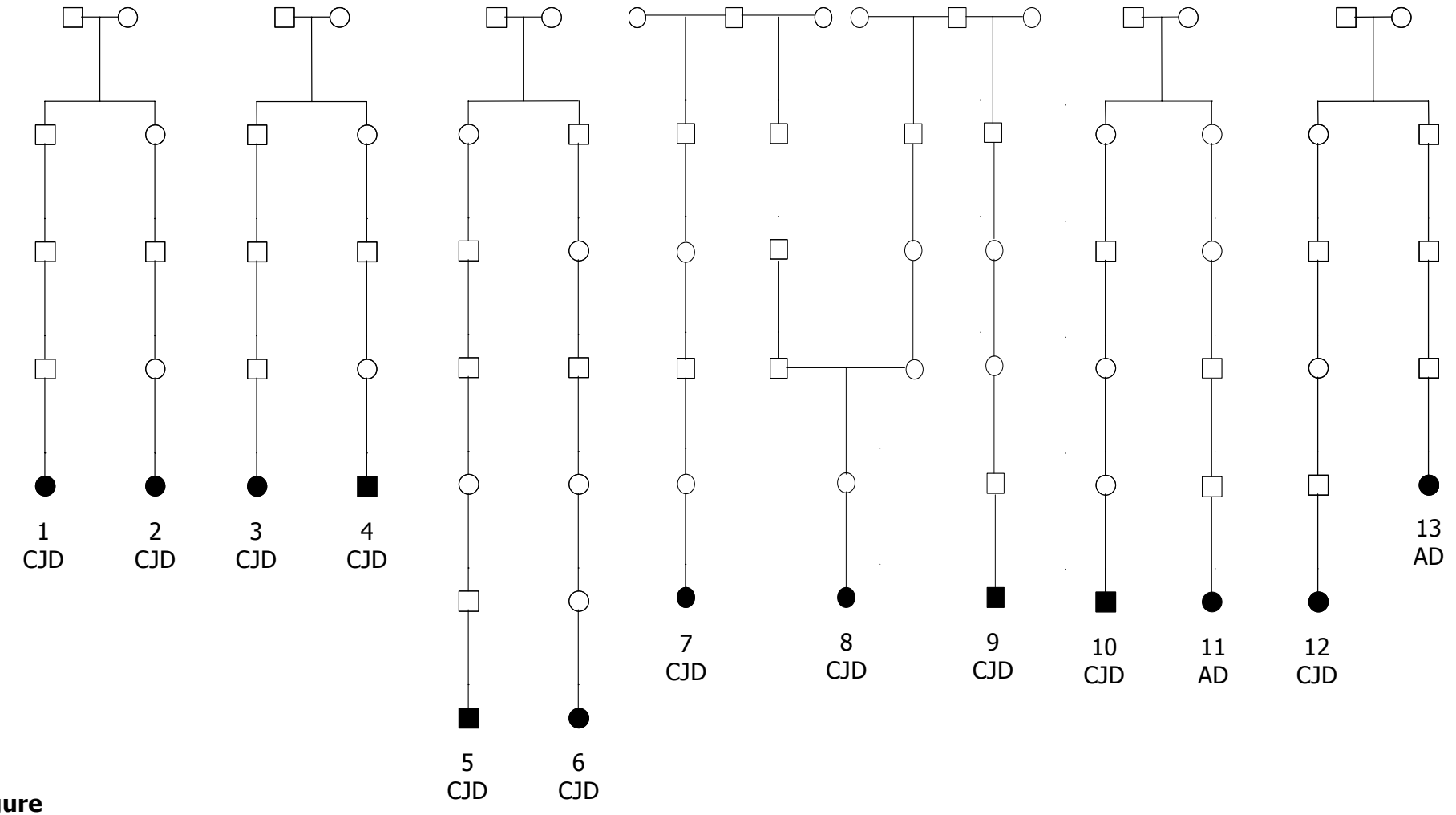
In the total dementia group (including the patients with CJD), information on 823 (95%) great-grandparents was collected, 1728 (84%) ancestors from the fifth generation, 1760 (51%) ancestors from the sixth generation and 262 (4%) ancestors from the seventh generation (table 1). In the glioma patients, these percentages ranged from 90% in the fourth generation to 40% in the seventh generation (table 1). Reasons for incomplete data were missing files from the municipal registers, which for a large part was due to demolition during the second world war, loss to follow-up due to migration to another municipality, offspring from unmarried women and origin from neighbouring countries.

PRNP mutation screening of the single coding exon 2 was performed by direct sequencing of 3 overlapping PCR fragments spanning the coding region. For M129V genotyping a pyrosequencing assay with PCR primers 5'-TGG GGG GCC TTG GCG GCT AC-3' (biotinylated), 5'-GTT TTC ACG ATA GTA ACG GTC C-3' and sequencing primer 5'-CTG CTC ATG GCA CTT CCC A-3' was used. A mutation analysis of all coding and 5'-non-coding exons of the presenilin-1 and -2 genes was performed as described before.[11] Mutation screening of the amyloid precursor protein gene and analysis of the apolipoprotein E genotypes was done as described elsewhere.[10,12]

Table 1

Presence of genealogical information in the fourth to seventh generation of patients diagnosed with CJD, early onset dementia and glioma

Gene-ration	CJD patients (n=59)		Dementia patients (n=49)		Total dementia (n=108)		Glioma patients (n=16)	
	present	**%**	**present**	**%**	**present**	**%**	**present**	**%**
4	441/472	93	382/392	97	823/864	95	115/128	90
5	726/944	77	717/784	91	1443/1728	84	168/256	66
6	776/1888	41	984/1568	63	1760/3456	51	242/512	47
7	244/3776	6	18/3136	1	262/6912	4	409/1024	40



Figure

Pedigrees showing common ancestry in patients with CJD and Alzheimer's disease

Results

The diagnosis CJD was classified 'definite' in 42 patients, 'probable' in 12 and 'possible' in 5 patients. For one CJD patient, an iatrogenic cause of the disease could not be excluded.[13] All other patients were classified with the sporadic form of the disease. In the early onset dementia patients, 46 cases were diagnosed with Alzheimer's disease and the remaining three with frontal temporal lobe dementia.

We found two pairs of CJD patients who were related to each other within five generations and one pair of CJD patients with a common ancestor within seven generations (figure 1). We identified one CJD patient who shared a common ancestor within six generations through grandpaternal side with a second CJD patient and through grandmaternal side with a third (figure 1). As these patients are derived from an isolated area these ancestors may be related to each other. We further identified two couples of CJD and early onset dementia patients who were linked to a common ancestor (figure 1). The diagnosis in all 9 CJD patients was confirmed at autopsy. None of the patients with CJD or early onset dementia was found to have a common ancestor with patients diagnosed with glioma and none of the glioma patients were related to each other within seven generations. However, four of the sixteen glioma patients shared a common ancestor within eleven generations.

Table 2 summarises the clinical features of the patients sharing a common ancestor. We identified two pairs in which patients showed a remarkable similarity in clinical presentation. The first pair (patients 1 and 2 in table 2 and figure 1) was characterised by early onset (57 and 53 years) and long disease duration (2.7 and 1.3 years). The initial symptoms were psychiatric disturbances, followed by a progressive dementia. For one of the patients (number 2) a family history of psychiatric disturbances was found in a cousin, who was reported to suffer from psychoses. In the second pair (patients 8 and 9 in table 2 and figure 1) a rapid disease course was observed in which sensory disturbances and involuntary movements were the prominent features at onset of the disease. The family history in both patients was positive for dementia. When considering the two pairs of patients with diagnosis CJD and early onset Alzheimer's disease linked to a common ancestor, no similarities were found within the pairs. However, the presentation of the disease in both patients with definite CJD was predominated by a stroke-like onset, including aphasia and hemiplegia, which are rare presenting symptoms of the disease. Also in one of the patients with probable Alzheimer's disease, aphasia was a presenting symptom. In three of the four patients, the family history was positive for early or late onset dementia.

When taking both sets of patients (CJD-CJD and CJD-Alzheimer's disease), 12% of the patients were linked to a common ancestor. None of the five patients with common ancestry in whom DNA analysis was performed carried a mutation in PRNP, the amyloid precursor protein gene or in the presenilin genes. The genotypes of PRNP codon 129 and APOE are listed in table 2. For four of the seven pairs with common ancestry, clustering would not have been anticipated on

Table 2
Clinical features, genotype and family history in patients with CJD and Alzheimer's disease with a common ancestor

Patient[√]	Diagnosis	Sex	Age at onset (years)	Disease duration (years)	Clinical features	PRNP codon 129 genotype*	APOE genotype	Family history	Type of disease in family	Relation of relative	Age at onset of relative
1	definite CJD	F	57	2.7	psychiatric onset, progressive dementia	MM	2 / 3	-			
2	definite CJD	F	53	1.3	psychiatric onset, progressive dementia, blindness	n.a.	n.a.	+	psychiatric	cousin	n.a.
3	definite CJD	F	77	0.2	visual disturbances, pyramidal features, myoclonus	n.a.	n.a.	n.a.			
4	definite CJD	M	47	0.5	cerebellar onset, rapidly progressive dementia, myoclonus	VV	n.a.	-			
5	probable CJD	M	63	0.5	extra-pyramidal onset, myoclonus	n.a.	n.a.	-			
6	definite CJD	F	73	0.3	psychiatric onset, rapidly progressive dementia	n.a.	3 / 3	+	dementia	mother	90
7	definite CJD	F	70	0.3	visual disturbances, ataxia	MM	3 / 3	-			
8	definite CJD	F	60	0.2	cerebellar onset, sensory disturbances, involuntary movements	MM	3 / 3	+	dementia	father	86
9	definite CJD	M	61	0.2	sensory disturbances, involuntary movements	n.a.	n.a.	+	dementia	mother grandfather	75 n.a.
10	definite CJD	M	71	0.2	rapidly progressive dementia, hemiplegia, aphasia	n.a.	n.a.	-			
11	probable AD	F	63	>10	slowly progressive dementia, apraxia	MM	4 / 4	+	dementia	mother	<65
12	definite CJD	F	71	0.2	depressive symptoms, progressive dementia, hemiplegia, aphasia	n.a.	n.a.	+	dementia	mother	90
13	probable AD	F	61	>6	slowly progressive dementia, aphasia	MV	3 / 4	+	dementia	sister	78

[√] see figure 1
* M: methionine; V: valine
AD: Alzheimer's disease; n.a.: not available

geographical grounds, since their residence at diagnosis was in different provinces at least 50 kilometers apart.

Discussion

We described a genealogical search in a population-based series of 59 patients with CJD and 49 patients with early onset dementia and found a common ancestor within seven generations in seven couples consisting of at least one CJD patient. In four of the seven couples, the link was not expected from the residence of the patients. Based on the findings in four of the sixteen glioma patients, who shared a common ancestor eleven generations ago and were not found to be related to the CJD and Alzheimer's disease patients, the link appears to be specific for CJD and Alzheimer's disease.

The range in clinical features in CJD is wide. However, there was a remarkable clinical resemblance of two of seven couples with an infrequent presentation. One couple presented with psychiatric symptoms, the other with sensory disturbances and involuntary movements. In both sets of patients a family history for neurological or psychiatric disorders was found. In the first couple, presenting with psychiatric symptoms, this concerned a cousin suffering from psychoses. In the patients presenting with sensory disturbances and involuntary movements this concerned dementia, which was accompanied by a severe tremor in the mother of patient number 9. The clinical resemblance of these patients together with the link to a common ancestor strongly suggest the presence of a shared mutation. However, a mutation in PRNP was not found, indicating that the disease is most likely explained by another, yet unidentified gene. Of interest are also the two sets of patients with a mixed pathology (CJD and Alzheimer's disease). The finding of familial clustering of Alzheimer's disease and CJD is in line with studies suggesting familial aggregation of both disorders.[9,14] In both patients with CJD, the diagnosis was neuropathologically confirmed. Also the disease in these patients could not be explained by a known mutation in CJD and Alzheimer's disease related genes, suggesting there is a common gene involved affecting neurodegeneration in both diseases. In the CJD patients, in whom the diagnosis was confirmed at autopsy, the disease phenotype was characterised by an atypical onset, followed by the key clinical feature of CJD, a rapidly progressive dementia. The presentation of the disease in both patients with Alzheimer's disease with a slowly progressive dementia was unremarkable and suggests that an underlying unknown gene may be involved in other patients as well.

The fact that no clustering was found of patients with CJD or Alzheimer's disease with patients suffering from glioma, suggests this clustering is specific to both neurodegenerative diseases. Also the finding that the glioma patients could not be linked to each other up to eleven generations, while patients with CJD and Alzheimer's disease were linked much earlier, is compatible with the view of a close genetic link between the patients in this set. The finding of a negative family

history in some of the patients indicates that the penetrance of the mutation will be low.

Our study showed that extensive genealogical research may unravel familial connections between patients that were not anticipated based on family history. In low penetrance disorders such as CJD it may be worthwhile to conduct genealogical research in order to establish links between patients. Although the pivotal role of the PRNP gene in CJD has long been recognised, our findings imply that the role of genetic factors may be underestimated in that other genes may be involved. In fact we may even underestimate the role of genetics, as part of our genealogical data was incomplete and non-paternity may conceal links between patients. Further, we did not consider links between CJD patients and patients with a late onset form of Alzheimer's diseases. In empirical data on aggregation of CJD and dementia there is no evidence that the link of CJD specifically concerns early onset dementia. However, the late onset forms of dementia are common and more difficult to study for genealogical history in the absence of computerised genealogy data. Thus, unknown genes and other familial factors may explain more than the 14.5% of patients estimated.[8]

In conclusion, our study underscores the role of genetics in CJD. The study suggests that we may underestimate the role of genetics other than PRNP in the etiology of CJD. Finding these genes may be important as it will reveal new proteins involved in CJD. The problem in identifying new genes is the lack of families segregating CJD. Our data may open new opportunities to identify new genes in families.

Acknowledgement

This study would not have been possible without the cooperation of all Dutch neurologists and the kind help of the family members of the patients. We are very grateful to Mr W Pasveer, Mr MHM Starkenburg, Mr AGM Heijmerikx, Mr HJE Hartog, Mr M Kijf, Mrs E Rentmeester de Haas and Mr W de Bakker, who helped in providing the genealogical data. This work was supported by the Dutch Ministry of Health, Welfare and Sports (VWS) and the Netherlands Organisation for Scientific Research (NWO).

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5.4

Valine homozygosity at prion codon 129 increases the risk for early onset Alzheimer's disease in a Dutch population-based sample

Abstract

Homozygosity at codon 129 (M129V) of the prion protein gene is a genetic risk factor for Creutzfeldt-Jakob disease. Familial clustering of sporadic Creutzfeldt-Jakob disease with other types of dementia suggests that common genetic risk factors might predispose to Creutzfeldt-Jakob disease and other neurodegenerative brain diseases. We investigated the role of M129V in early onset Alzheimer's disease in a Dutch population-based case-control sample (123 cases, 282 controls). Using the MV genotype as reference, we observed a significant association between early onset Alzheimer's disease and homozygosity at prion codon 129 (OR 1.9, 95% CI 1.1-3.3; p=0.02) with the highest risk for V homozygotes (OR 3.2, 95% CI 1.4-7.1; p<0.01). In early onset Alzheimer's disease patients with a positive family history the risk further increased to 2.6 (95% CI 1.3-5.3; p<0.01) for homozygosity of either the M or V allele and to 3.5 (95% CI 1.3-9.3; p=0.01) for V homozygotes. This study suggests that homozygosity at codon 129 of the prion protein gene, particularly of the V allele, is a genetic risk factor for early onset Alzheimer's disease that is strongest in cases with a positive family history of dementia.

Introduction

Alzheimer's disease (AD) is the most common form of dementia and is neuropathologically characterised by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles. Genetic factors are important in both the rare early onset type of AD (EOAD; onset ≤ 65 years) and late onset AD (LOAD; onset > 65 years).[1] Although missense mutations in the amyloid precursor protein gene (APP) and the presenilin 1 and 2 genes (PSEN1, PSEN2) account for 20 to 70% of autosomal dominant EOAD,[2,3] their contribution to AD in the general population is probably less than 0.1%.[4] On the population level, the APOE □4 allele modulates the risk of both EOAD and LOAD[5,6] and explains up to 17% of AD in the general population.[7]

The gene encoding the prion protein (PRNP) is a well established risk gene for Creutzfeldt-Jakob disease (CJD), a rapidly progressive dementia characterised by the deposition of proteinase resistant prion proteins in brain. Individuals homozygous for the M or V allele at codon 129 (M129V) are at increased risk of developing sporadic,[8,9] iatrogenic[10] and variant CJD.[11]

Although AD and CJD are clearly distinct disease entities, several lines of evidence suggest that a detailed molecular genetic study of PRNP in AD might be relevant. First, several epidemiological studies have reported familial clustering of sporadic CJD with other types of neurodegenerative dementia,[12-14] suggesting shared genetic risk factors. Second, familial CJD caused by PRNP mutations can clinically mimic a wide range of neurodegenerative disorders including AD.[15] Finally, a French study showed an association between V homozygosity at PRNP codon 129 and cognitive performance in the elderly.[16] Although not confirmed by others,[17] this may also imply a role of PRNP in dementia and AD. Together with increasing evidence showing an important role of prion proteins in normal neuronal functioning (e.g. protection against oxidative stress),[18] these observations suggested that PRNP might be a pleiotropic genetic susceptibility factor with implications in a broader spectrum of neurodegeneration of the central nervous system.

In order to further address these questions, we have screened the PRNP coding region in a Dutch population-based series of EOAD and performed a case-control association analysis with the PRNP codon 129 polymorphism.

Methods

EOAD patients were derived from a Dutch population-based study of EOAD in the 4 northern provinces of the Netherlands and metropolitan Rotterdam. The patients were sampled during two study periods. The original sample was collected between 1980 and 1987 and has been described in detail elsewhere.[6,19]. The initial study was extended between 1997 and 2000 in a genetically isolated part of the previously described area with the same sampling criteria (Croes et al., unpublished data). The current sample comprises 101 patients from the original group with an addition of 22 cases from the extended study. Median age at onset

Table 1
General descriptives of the EOAD patients and controls

	EOAD	Controls
Number of individuals	123	282
Median age at onset / median age	58.0*	60.6
at examination in years (range)	(33-65)	(55-66)
% Female	77*	52
% Familial AD	64%	n.a.

*p<0.001 (compared to controls); n.a.: not available

in this sample was 58.0 years (range 33-65) and 77% was female (table 1). The diagnosis of probable AD was independently confirmed by a member of the research team and a neurologist using a standardised protocol consistent with the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD.[20] Forty four patients were classified as sporadic and in 79 patients the criteria for familial were met (at least one first degree relative with dementia),[6,19] of which 10 cases fulfilled our criteria of autosomal dominant inheritance.[21] Seven patients were identified carrying a PSEN missense mutation (5 in PSEN1 and 2 in PSEN2)[2] (and Croes et al., unpublished data). However, recent preliminary studies investigating the effect of these mutations on APP processing and Aβ secretion in an in vitro assay, showed that some might not be pathogenic (Theuns et al., unpublished data). EOAD cases were compared to 282 control individuals (median age at examination of 60.6 years; range 55-66; 52% female) from the population-based Rotterdam Study (table 1).[22] All controls were screened for cognitive dysfunction and potential control subjects with dementia were excluded. PRNP mutation screening of the single coding exon 2 was performed by direct sequencing of 3 overlapping PCR fragments spanning the coding region.[23] For M129V genotyping we developed a pyrosequencing assay[24] with PCR primers 5'-TGG GGG GCC TTG GCG GCT AC-3' (biotinylated), 5'-GTT TTC ACG ATA GTA ACG GTC C-3' and sequencing primer 5'-CTG CTC ATG GCA CTT CCC A-3'. APOE genotyping was performed as previously described.[6]

Genotype and allele frequencies were compared between cases and controls using the χ^2 statistic. Hardy Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested with the HWE and EH programs.[25] Odds ratios (ORs) were calculated to assess the strength of association. ORs are presented with their corresponding 95% confidence intervals (CI) and were corrected for age, gender and APOE genotype using logistic regression. In accordance with the prion model assuming faster disease propagation in case of PRNP M129V homozygosity, ORs were calculated using the heterozygous MV genotype of PRNP M129V as reference. Onset ages for the different PRNP genotypes were compared using ANOVA.

Table 2
PRNP M129V allele and genotype frequencies in EOAD patients and controls

	EOAD		Controls		
	N	**%**	**N**	**%**	**p-value**
M	155	63	368	65	0.54
V	91	37	196	35	
	246		*564*		
MM	54	44	114	40	0.03
MV	47	38	140	50	
VV	22	18	28	10	
	123		*282*		

Results

Direct sequencing of the PRNP coding region in EOAD cases revealed 3 known single nucleotide polymorphisms (SNPs): the known amino acid substitution at codon 129 (c.385A>G; M129V), an intronic SNP (IVS1-31G>A) and a silent coding SNP at codon 117 (c.351A>G; V117V).[26] Both the A allele of IVS1-31G>A (3%) and G allele (4%) of c.351A>G were only observed in the heterozygous state, and were in nearly complete LD with each other and the V allele of M129V. No causative mutations were found. PRNP M129V genotyping was performed in EOAD patients and controls (table 2). In the EOAD patients, the genotyping results were in total agreement with the sequencing data. No significant deviation from HWE was observed in the control group (p=0.11). In EOAD patients the M129V genotype distribution was significantly different from controls due to an increased homozygote frequency (62% versus 50% in controls). Homozygosity was associated with a statistically significant 2 times increased risk for developing EOAD (table 3). Separate risk calculations for MM and VV genotypes showed that individual risks were highest for V homozygosity. Stratification of the EOAD cases according to family history showed that risk estimations were consistently higher in EOAD cases with a positive family history. When the 7 PSEN mutation carriers were excluded (n=72) similar results were obtained for homozygosity (OR 2.3; 95% CI 1.1-4.7; p=0.02), VV (OR 3.2; 95% CI 1.2-8.7; p=0.02) and MM genotypes (OR 2.0; 95% CI 1.0-4.3; p=0.07). Equivalent analyses in sporadic EOAD showed a nearly 3 times non-significantly increased risk for VV carriers while MM and overall homozygote frequencies were not increased.

In order to test for interaction between PRNP M129V and APOE, we stratified the EOAD sample for the presence of an APOE ε4 allele. PRNP M129V genotype or allele distributions were not significantly different between APOE ε4(+) and APOE ε4(-) strata in EOAD cases (data not shown), suggesting no interaction between PRNP and APOE. Analysis of variance indicated that there was no statistically significant difference in age at onset between PRNP genotypes in EOAD (p=0.17).

Table 3
Association between PRNP M129V and EOAD

Population	N	Risk genotype	Frequency (cases/controls)	OR	95% CI
EOAD	123	MM + VV	0.62 / 0.50	1.9*	1.1-3.3
		MM	0.44 / 0.40	1.6	0.9-2.9
		VV	0.18 / 0.10	3.2**	1.4-7.1
fEOAD	79	MM + VV	0.66 / 0.50	2.6**	1.3-5.3
		MM	0.46 / 0.40	2.4*	1.1-5.0
		VV	0.20 / 0.10	3.5*	1.3-9.3
sEOAD	44	MM + VV	0.52 / 0.50	1.2	0.6-2.5
		MM	0.39 / 0.40	0.9	0.4-2.0
		VV	0.14 / 0.10	2.7	0.9-8.2

Odds ratios (OR) are presented with their 95% confidence intervals (CI), calculated with MV as reference and corrected for gender, age and APOE genotype
fEOAD: familial EOAD; sEOAD: sporadic EOAD
* $p \leq 0.02$; ** $p < 0.01$

Discussion

We observed a statistically significant association between homozygosity at PRNP codon 129 and EOAD. The risk to develop EOAD was higher for V homozygotes than for M homozygotes. When the analysis was restricted to EOAD patients with a positive family history of dementia, the risks for overall, V and M homozygosity were consistently higher.

Although difficult to exclude, it is unlikely that population admixture explains our association, since no statistically significant differences in age, sex, family history of AD and PRNP or APOE distributions were observed between cases derived from Rotterdam or the northern part of the Netherlands (data not shown). Another concern may be that our population harbours undiagnosed familial or sporadic CJD cases that explain the association. It is of interest that, like in our EOAD cases, V homozygotes are also overrepresented (41%) in sporadic CJD cases aged 49 years or less.[9] However, although such cases often have an atypical clinicopathological presentation,[27] it is unlikely that such sporadic CJD cases have been diagnosed as EOAD and included in our series. In general, the low incidence ($1/10^6$/year) and shorter disease duration of sporadic CJD compared to EOAD make it unlikely that undiagnosed sporadic CJD cases explain the observed association. The absence of disease related variations in the PRNP coding region further excludes the possibility that our EOAD sample contains familial CJD cases that clinically presented as EOAD. Moreover, all 15 patients in our EOAD series that came to autopsy had a proven neuropathological diagnosis of AD.

Based on our current knowledge of AD pathogenesis, it is difficult to understand how PRNP homozygosity might contribute to AD pathology. In CJD, susceptibility due to PRNP homozygosity strongly supports the 'prion model', which assumes that interaction between homologous prion proteins enhances the conversion of normal prion protein (PrP^{c}) to the pathologic protease resistant form (PrP^{Sc}).[28] It is striking that, similar to sporadic CJD, the association in EOAD is also explained by an increased frequency of homozygotes. However, in contrast to what the prion model would predict, PrP^{Sc} deposits are normally not found in AD brains. An alternative explanation might therefore come from studies that have looked into the role of prions in normal neuronal functioning. In line with numerous studies showing an important role for normal PrP^{c} in protecting neurons against oxidative stress,[18] a recent study found an apparent reactive upregulation of normal PrP^{c} in AD brains[29] while another study demonstrated PrP^{c} immunostaining in senile □-amyloid plaques.[30] The finding of poorer cognitive performance of V homozygotes in a French population-based study on cognitive aging (the EVA study) also supports a role of PrP^{c} in normal cognitive functioning.[16] A possible explanation of our association data could be that variation(s) in the PRNP regulatory region in LD with the V allele results in altered PrP^{c} expression and hence increased sensitivity of neurons to oxidative stress.

While the precise underlying biological mechanism remains to be clarified, we here show that homozygosity at PRNP codon 129, particularly of the V allele, might be a genetic risk factor for EOAD that is modulated by family history of dementia. Although the prion protein has a well established role in neurodegeneration and possibly an important role in normal neuronal functioning, independent replication studies in other EOAD samples are needed to further validate our findings.

Acknowledgement

We are grateful to Hubert Backhovens and Dirk Van den Bossche for their skilled technical assistance in the genetic analyses. We thank Jeannette Vergeer, Wilma Luiten and Bianca de Graaf for their help in the laboratory analysis and Gerwin Roks for the sampling of patients. Financial support was received from the Fund for Scientific Research Flanders (FWO-F), the InterUniversity Attraction Poles (IUAP), program P5/19 of the Federal Office for Scientific, Technical and Cultural Affairs (OSTC) Belgium, the International Alzheimer's Research Foundation (IARF) Belgium, a concerted action of the University of Antwerp (UIA), the Alzheimer Association (AA) USA, the Netherlands Organisation for Scientific Research (NWO), the Dutch Ministry of Health and a EU grant (CT98-7022). Marc Cruts is a postdoctoral fellow and Rosa Rademakers and Bart Dermaut are PhD fellows of the FWO-F.

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5.5

Early cognitive decline in the general population is associated with the codon 129 polymorphism of the prion protein gene

Abstract

The codon 129 polymorphism in the prion protein gene (PRNP) is an established determinant of Creutzfeldt-Jakob disease, but findings on its role in other forms of dementia and cognitive decline have been conflicting. We studied the role of this polymorphism in cognitive decline in 965 subjects aged 55 years and over from a prospective population-based study (the Rotterdam Study). Participants were sampled in four ten-years age categories. The association of the PRNP codon 129 polymorphism (M129V) to Mini Mental State Examination (MMSE) at baseline (n=878) and decline during follow-up (n=418) was assessed using linear regression analysis adjusting for age, gender, education and ε4 allele of the apolipoprotein E gene (APOE). Carriers of genotype VV were more often diagnosed with dementia (p=0.009). Median MMSE scores at baseline were not significantly related to PRNP genotype. Besides the expected annual decline in MMSE with age (p<0.001), the age-stratified analyses comparing cognitive decline in the three genotypes revealed a significantly increased decline in cognitive performance in V homozygous individuals aged 55-64 years (p<0.01). In the 55-64 years age categories, cognitive decline in V homozygotes was similar to that seen in subjects 65-74 years old. We found no significant evidence for effect modification of this relation by APOE genotype or family history of dementia. Our findings strongly support the hypothesis that the PRNP codon 129 polymorphism is involved in various neurodegenerative brain processes.

Introduction

The abnormal isoform of the prion protein plays a key role in the spongiform encephalopathies. A common polymorphism in the gene encoding the prion protein (PRNP) is known to determine susceptibility in sporadic, iatrogenic and the new variant form of Creutzfeldt-Jakob disease (CJD).[1-3] This polymorphism at codon 129 either encodes for methionine (M) or valine (V). Since the prion protein may have neuroprotective properties, there is increasing interest in the role of this protein in other neurodegenerative processes.[4] Various studies have reported inconsistent associations between genetic variability at PRNP and cognitive performance. Berr et al. observed a significant increased prevalence of cognitive impairment in PRNP codon 129 V homozygotes.[5] The effect was similar to that of the ε4 allele of the apolipoprotein E gene (APOE*4). Two subsequent studies did not confirm a relation between the PRNP codon 129 genotype and sporadic Alzheimer's disease.[6,7] However, one of these studies did suggest a significant faster deterioration in patients with sporadic Alzheimer's disease carrying the PRNP codon 129 V allele.[7] We recently observed a significant increase in patients homozygous for the V allele in early onset Alzheimer's disease. This finding was most pronounced in patients with a positive family history.[8]

In this paper we address the question whether genetic variability of the PRNP codon 129 polymorphism plays a role in cognitive decline and whether this relation is age dependent.

Material and methods

Study population. Participants of this study were derived from the Rotterdam Study. For this population-based, single-centre cohort study on chronic and disabling diseases in the elderly, all inhabitants of a suburb of Rotterdam aged 55 years and over were invited to participate. The design of the study is described in detail elsewhere.[9] The medical ethical committee of the Erasmus MC approved the study protocol. In total 7983 participants (response rate 78%) were examined between 1990 and 1993. Cognitive performance was assessed by the Mini Mental State Examination (MMSE) (Dutch version, maximum score 30) and the Geriatric Mental State Schedule. Screen positive persons were further evaluated, by interviewing a close relative, neuropsychological tests, neurological examination and neuro-imaging. Two neurologists independently diagnosed dementia based on the DSM-III-R definition.[10] For this study, we used the MMSE score as an indicator of cognitive performance.

We randomly selected from the baseline population 978 individuals from four age categories (55 to 64 years; 65 to 74; 75 to 84 and 85 years and over), to maximise the power for detecting an age specific gene effect. From all participants, blood was drawn and genomic DNA was extracted from leukocytes. The APOE and PRNP genotypes were determined as previously described.[11,12] At the second follow-up period 418 (43%) participants of our random sample were re-examined. For this group, mean follow-up was 6.5 ± 0.6 years. Reasons for

loss to follow-up were death (63%), refusal (33%) and inability to undergo the MMSE (4%).

Statistical analyses. Baseline characteristics for the 3 PRNP codon 129 genotype groups (MM, MV and VV) were compared using analysis of variance (age), the non-parametric Kruskal-Wallis test (baseline MMSE), a chi-square test (gender and prevalence of dementia at baseline) and the Mann-Whitney U test (baseline MMSE in the presence or absence of APOE*4 allele(s)). To test for a survival effect of the PRNP codon 129 genotype, which may result in an age dependent change of allele frequencies, a polytomous logistic model was fitted, with age (logarithmically transformed) as covariate. Genotype MV was used as reference category, since it is the most common genotype in the general population and associated with the lowest risk for CJD.
For the assessment of cognitive decline in each subject with follow-up data, all prevalent demented individuals were excluded from the analyses. In these subjects, progression of dementia cannot be assessed using the MMSE. The median annual change in MMSE score was calculated and a linear regression model was fitted adjusting for age at baseline, sex, highest education attained, baseline MMSE score and APOE*4 to compare the effect between the reference group MV and genotype MM or VV. The effect of the PRNP codon 129 genotype on the annual MMSE change was studied in separate age categories.

Results

The PRNP codon 129 genotypes were determined in 965 individuals. Genotype and allele frequencies were in Hardy Weinberg equilibrium in the total group, as well as in the separate age categories. Table 1 summarises the baseline characteristics according to PRNP codon 129 genotypes. There were no significant differences in sex or age across genotypes. We found no evidence for a shift in genotype frequencies with age (p=0.14). Significantly more carriers of genotype VV were diagnosed with dementia (13.3% in genotype MV, 17.4% in MM and 26.5% in VV; p=0.009) (table 1). This resulted in a 2.34 fold (95% confidence interval 1.33 - 4.12) increase in dementia in carriers of genotype VV compared to those with the MV genotype.
Table 2 shows the characteristics of the dementia patients. The mean age at onset was decreased in carriers of genotype VV, but this was not significant (p=0.42). APOE genotypes were available for 918 participants ($\varepsilon2\varepsilon2$, n=10; $\varepsilon2\varepsilon3$, n=143; $\varepsilon2\varepsilon4$, n=26; $\varepsilon3\varepsilon3$, n=495; $\varepsilon3\varepsilon4$, n=232; $\varepsilon4\varepsilon4$, n=12) and followed the Hardy Weinberg equilibrium. The frequency of APOE*4 was similar in patients with PRNP codon 129 MM (30%) and MV (29%) genotypes (table 2). Although the frequency of APOE*4 was increased in VV carriers (45%), no significant evidence for a difference with other codon 129 genotype carriers was found.
Data on MMSE scores at baseline were available for 878 individuals and at follow up for 418 individuals. The baseline MMSE score did not differ between the

Table 1
Characteristics of the study population by PRNP codon 129 genotype

	MV	MM	VV	P-value
Number	440	435	90	
(% of total)	(45.6)	(45.1)	(9.3)	
Female	313	296	62	0.65
(% in genotype)	(71.1)	(68.3)	(68.9)	
Mean age (SD)	74.7 (11.0)	76.0 (10.8)	74.8 (11.6)	0.23
Median baseline MMSE	28	27	28	0.37
score (range)	(6-30)	(6-30)	(1-30)	
Dementia				
At baseline §	33 / 412	43 / 408	12 / 83	0.15
(% in genotype)	(8.0)	(10.5)	(14.5)	
At follow-up §	22 / 379	28 / 365	10 / 71	0.05
(% in genotype)	(5.8)	(7.7)	(14.1)	
Total §	55 / 412	71 / 408	22 / 83	0.009
(% in genotype)	(13.3)	(17.4)	(26.5)	

§ Number of individuals divided by total number of individuals within genotype for whom the information is available

Table 2
Age at onset, presence of APOE*4 allele and family history of dementia according to PRNP codon 129 genotypes in patients with dementia

	MV	MM	VV	P-value
Mean age at onset (SD)	85.4	86.3	84.4	0.42
	(5.9)	(5.8)	(7.4)	
APOE*4 carriers §	16 / 54	20 / 68	9 / 20	0.39
(% in genotype)	29.6%	29.4%	45.0%	
Family history of dementia §	8 / 43	7 / 60	2 / 16	0.60
(% in genotype)	18.5%	11.7%	12.5%	

§ Number of demented patients divided by total number of patients within genotype group for whom the information was available

genotypes in the overall analyses or in any of the age categories (table 3). As expected, the median MMSE scores decreased with age ($p<0.001$). A borderline significant association between APOE*4 and MMSE was found ($p=0.08$). Table 4 shows the median annual decline for the three genotypes in each age category. Overall, the annual decline in MMSE increased with age ($p<0.001$). Comparing cognitive decline between the three genotypes, we found a significantly higher decline in cognitive performance in V homozygous individuals aged 55-65 years

Table 3
MMSE scores at baseline by PRNP codon 129 genotype in four age categories

Age category	Codon 129	Number (%)	Median	Range
55-64	MV	114 (51.6)	28.0	23 – 30
	MM	86 (38.9)	28.5	22 – 30
	VV	21 (9.5)	29.0	25 – 30
65-74	MV	91 (42.1)	29.0	22 – 30
	MM	105 (48.6)	28.0	18 – 30
	VV	20 (9.3)	28.0	20 – 30
75-84	MV	96 (45.5)	27.0	14 – 30
	MM	100 (47.4)	27.0	7 – 30
	VV	15 (7.1)	28.0	25 – 30
85-95	MV	97 (42.2)	25.0	6 – 30
	MM	107 (46.5)	25.0	6 – 30
	VV	26 (11.3)	22.0	1 – 29

Table 4
Annual decline in MMSE score by PRNP codon 129 genotypes in four age categories

Age category	Codon 129	Number	Median decline	Range	P value*
55-64	MV	80	0.00	-0.65 to 0.60	reference
	MM	66	0.00	-0.64 to 0.90	n.s.
	VV	15	0.16	-0.16 to 0.93	<0.01
65-74	MV	70	0.14	-0.64 to 1.21	reference
	MM	77	0.15	-0.72 to 4.41	n.s.
	VV	14	0.15	-0.43 to 0.71	n.s.
75-84	MV	33	0.14	-0.32 to 2.54	reference
	MM	34	0.16	-0.32 to 6.45	n.s.
	VV	8	0.20	-0.16 to 1.38	n.s.
85-95	MV	8	0.38	0.00 to 1.51	reference
	MM	12	0.58	-0.46 to 5.12	n.s.
	VV	1	2.71	n.a.	n.s.

n.a.: not applicable; n.s.: not significant

* after adjusting for age at baseline, sex, highest education attained, baseline MMSE score and presence or absence of APOE*4 allele

(p<0.01). In the other age categories, no significant effect was observed. The figure shows that cognitive decline in VV carriers aged 55-64 years was similar to that seen in the age category 65-74 years, while in MV and MM carriers, cognitive decline increased with age.

When stratifying the data on the absence or presence of APOE*4 no evidence for effect modification of the relation between PRNP and MMSE was found on baseline cognitive functioning. The median baseline MMSE in VV carriers in the APOE*4 positive group was 27 (range 9-30) and in the APOE*4 negative group 28 (range 1-30) (p=0.53). Although annual cognitive decline was highest in VV carriers who also carried APOE*4 (0.16; range –0.43 to 1.38), we could not show a significant difference (p=0.09) in the overall analysis compared with the decline in VV carriers without APOE*4 (0.07; range –0.28 to 0.77), nor in the separate age groups.

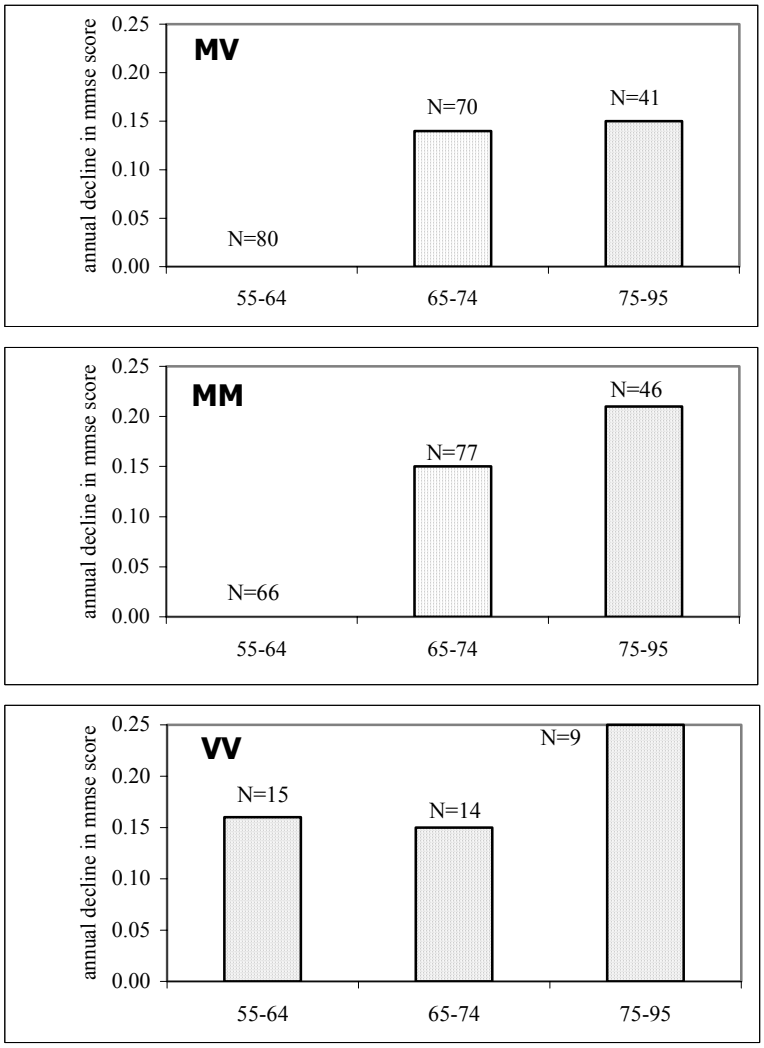

Figure
Annual decline in MMSE score in relation to the PRNP codon 129 polymorphism, in three age categories

Age categories 75-84 and 85-95 years were combined to increase the number

Discussion

In this population-based series of 965 individuals aged 55 to 95 years we found that V homozygosity at PRNP codon 129 was significantly related with increased annual decline in cognitive functioning in an at baseline non-demented population aged 55-64 years. We further showed that significantly more carriers of genotype VV were diagnosed with dementia. The prevalence of dementia in VV carriers was twice that found in patients who were heterozygous at PRNP codon 129.

Before interpreting our findings, some methodological issues need to be addressed. Since we only included individuals aged 55 years or over, we cannot assess the role of the PRNP codon 129 genotypes at early age. However, cognitive decline is usually subtle before age 55 years and the statistical power to study decline in younger age is therefore limited. Further, at early age MMSE cannot be used to assess decline of cognitive functioning. Second, many individuals in the study did not survive the follow-up period. This may have introduced bias if survival is related to PRNP codon 129 genotypes, in particular in VV carriers at old age, when most new patients with dementia occur. Given the fact that allele frequencies were stable over age categories in this study, it is unlikely that our findings are biased selectively in VV carriers.

Follow-up data on cognitive functioning in relation to the PRNP codon 129 genotypes have not been reported before. In previous studies, a cross-sectional approach was used to study either the relation with cognitive performance or with Alzheimer's dementia.[5-8] The study of Berr et al. assessed the influence of the PRNP codon 129 genotypes on cognitive abilities in a community-based series of 1163 elderly. They observed significantly lower MMSE scores in individuals aged 66-71 years who were homozygous for V at PRNP codon 129.[5] In line with these findings, we found an increased decline in MMSE score in the age category 55-64 years. Although the findings were not significant, the decline was higher in V homozygotes who also carried an APOE*4 allele. Our own finding of an overrepresentation of V homozygosity among early onset Alzheimer's disease patients is in line with the present findings on cognitive decline.[8] In the cross-sectional analyses, no significant difference in mean MMSE scores was found in the present study. However, in our population a significant association was also not seen for APOE*4, an established determinant of dementia and cognitive decline.[19] It remains to be explained why the PRNP codon 129 VV genotype only has an effect on annual cognitive decline in those aged 55-64 years. Interestingly, in sporadic CJD patients with genotype VV at PRNP codon 129, the risk for developing the disease differs significantly with age.[1] In patients aged 49 years or younger, 41% are V homozygotes, compared with 5% in patients aged 80 years and over.[1] Our data suggest that in the general population carriers of the PRNP codon 129 genotype VV have already an annual cognitive decline at middle age (55-64 years) similar to that seen in elderly (65-74 years), i.e., there is a shift in cognitive decline towards early age.

Epidemiological studies provide accumulating evidence for a role of the prion protein in general neurodegenerative processes in the brain, not limited to prion

disorders. These findings are supported by neuropathological studies showing an overexpression of prion protein in senile plaques and increased prion immunoreactivity in hippocampus, subiculum and temporal cortex in patients with Alzheimer's disease.[13,14] However, the effect is small, which may explain why several other reports did not show an effect of the PRNP polymorphism on early or late onset Alzheimer's disease.[6,7]

The function of prions is still undetermined, but is suggested to be neuroprotective.[4] The prion protein may play a role in trace-element binding and may thereby have anti-oxidative properties.[15,16] Loss of this anti-oxidative function may lead to neurodegeneration.[15] Also the role of the PRNP codon 129 V allele in transmissible spongiform encephalopathies is not clearly understood. It has been shown that the codon 129 polymorphism alters protein stability resulting in changes in protein conformation.[17] However, the switch to the disease causing protein conformation was found enhanced in carriers of the PRNP codon 129 M allele, which is in line with the increased susceptibility for CJD in M homozygotes, but conflicting with findings in other forms of dementia and cognitive functioning.[18]

In conclusion, our data support the view of a role of the prion protein in neurodegeneration. Independent studies are needed to explore its relation with other neurodegenerative diseases.

Acknowledgement

This work was supported by the Netherlands Organisation for Scientific Research (NWO), the NESTOR Stimulation Program for Geriatric Research in the Netherlands (Ministry of Health, Welfare and Sports and Ministry of Education), the Municipality of Rotterdam, a EU grant (CT98-7022), the Fund for Scientific Research Flanders (FWO-F), the InterUniversity Attraction Poles (IUAP) programme P5/19 of the Federal Office for Scientific, Technical and Cultural Affairs (OSTC), and a concerted action of the University of Antwerp (UIA), Belgium. Bart Dermaut is a PhD fellow of the FWO-F.

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6

General discussion

Introduction

Creutzfeldt-Jakob disease (CJD) is a rare disease with an incidence of 1-2 patients per million per year.[1] Despite the rare occurrence of the human transmissible spongiform encephalopathies, research in this field is important for two reasons. First, from an epidemiological perspective, the disease has been unpredictable, in that iatrogenic transmission has resulted into two major epidemics, one due to human derived growth hormone and the other due to dura mater transplantation.[2] Not only has iatrogenic transmission of the disease led to major health problems and premature death of young persons, also the transmission of bovine spongiform encephalopathy (BSE) to man has had major implications for health care, agriculture, economics and politics in Europe.[3] Second, the pathological mechanism underlying CJD may not only concern transmissible spongiform encephalopathies, but may also be involved in other neurodegenerative processes. The findings in the field of the spongiform encephalopathies may therefore have implications far beyond rare disorders as CJD and may concern major health problems in the general population, such as Alzheimer's disease.

In this chapter the findings of the studies in this thesis are discussed in the context of our current knowledge of CJD and related disorders. First, we discuss the methodological issues with regard to the study design and the findings. The main findings of the thesis and their implications are discussed next. Finally, future research in the field of genetic epidemiology is addressed.

Methodological considerations

Iatrogenic research. From an epidemiological perspective, CJD is a difficult disease to study. The disease is rare which makes follow-up studies impossible, even when common exposures, e.g. meat consumption or exposure to therapy, are studied in relation to the risk of disease. The rapidly progressive dementia further complicates retrospective case-control research, since we cannot ask patients about their past exposures or life style habits. Also control selection has been a major problem in case-control research in CJD. For example, findings of case-control studies on surgery and the risk of CJD have been inconsistent due to different origin of the controls.[4-6] An Australian study reported a dose-response relationship between the number of surgical treatments and the risk of CJD using a series of population-based controls.[6] This finding was at odds with the result of a collaborative European study comparing 405 patients to 405 clinic-based controls.[5,7] When repeating the latter study with population-based controls, findings differed from the initial findings and supported the Australian study.[4] The conclusions of the two studies renew the discussion on the possibility that a proportion of cases with the sporadic form of CJD may result from contamination related to surgical events,[4,6] which may be as high as 35% of cases.[4]

Case studies have played a crucial role in CJD research. The first methodological consideration arising from the presented studies in this thesis is related to the value of case reports in epidemiological research. Case studies have been

criticised for being anecdotal, subjective and not representative. Since they are not based on large numbers, they are by themselves not likely to cover the full spectrum of disease. Last but not least, they do not fulfil the current concept of evidence-based medicine. However, one of the main functions of the case report is to recognise unexpected events.[8] In this respect, case reports have been proven to be extremely useful in CJD at several instances. The first iatrogenic transmission of CJD was reported in a woman who developed the disease 18 months after a corneal transplantation. The donor of this graft was diagnosed with CJD after the transplantation had occurred.[9] The linking of this rare exposure to the likewise rare disease probably has prevented further transmission in excluding CJD patients from cornea transplant donation, but also prompted awareness of the possibility of transmission of this disease among humans.

Similar arguments related to prevention may be made for the iatrogenic transmission of CJD through human derived growth hormone and transplantation of dura mater grafts, although in these forms the epidemic has spread further. Due to the long incubation period in these two latter forms, many individuals were infected before the route of transmission had been recognised. Also in the case of the transmission of BSE to humans, leading to a variant form of CJD (vCJD), case reports played a key role. Although BSE had already been transmitted from cows to multiple species, including cats, the possibility of transmission to humans was discounted.[10] A first report consisting of a series of case reports proved to be crucial.[3] After the publication of this report, which was based on 10 patients with an unusual young age at onset and distinct neuropathological findings, necessary actions were taken to prevent BSE infected material to enter the human food chain. Although the relation of BSE and vCJD was disputable at the time of publication of the case series, research on the causative agent of both entities was enhanced and soon the relation was confirmed experimentally.[11,12]

One may argue that the major problem in CJD, its rarity, makes it particularly suitable for research based on case reports. However, there have clearly also been some drawbacks. First, this method is inappropriate for studying common exposure. Successful case reports have concerned only rare exposure such as dura mater transplants and growth hormone administration. It is not possible to address questions on the role of surgery or blood transfusion in human to human transmission, or the role of meat consumption in the transmission of BSE to humans. This implies a major limitation for CJD research, both from a public health and from a clinical perspective. Up until now it has been impossible to exclude surgery beyond doubt as a source for the transmission of CJD, nor has it been possible to sort out how BSE was passed on from cattle to man. To answer these questions through epidemiologic research, extensive historical studies are required using data of subjects who have been exposed. For medical exposure, this will be laborious, but feasible, in that the origin of medicins have to be traced. However, this method is not suitable for nutritional research. For the latter, epidemiology most likely reached its limits, as it will be impossible to track meat and other food products.

Another drawback of case reports is that they may yield contradicting reports. This may be illustrated by the inconsistencies in reports on onset and duration of disease in patients carrying octapeptide repeat insertions in the prion protein gene (see chapter 5.2). This problem may be solved by combining case reports into meta-analyses to derive meaningful results. This is also illustrated in this thesis. In the prion protein gene, octapeptide repeat insertions are rare and have been reported worldwide in a total of 23 families. Combining these case reports in a meta-analysis resulted in the observation of a statistically significant relation of the length of the repeat with the age at onset and disease duration (chapter 5.2). Other authors have combined case reports in human growth hormone and dura mater induced CJD and reviewed the information.[13,14]

Genetic research. A second methodological issue concerns the question how to disentangle the pathogenesis of clustering of CJD. Historically, there has been interest in clustering of the disease in time and place to evaluate the risk of environmental factors, both in the classical form and vCJD.[15-17] This research has not yielded convincing evidence for environmental risk factors. In this thesis, we have taken a new approach by searching for clustering of patients in terms of their genealogy and geographical history (chapter 5.3). We aimed to find patients who developed the disease due to an unknown common gene. The methodology of this method is still poorly developed and the main question to be resolved concerns the size of clustering to be expected in a population under the null hypothesis of no common genetic mutation. In the absence of computerised genealogical data in the Netherlands, we resolved this problem by including a control group consisting of patients with a non-neurodegenerative disease (glioma). The data presented in chapter 5.3 are a first step to identify any new genes, in that it identified patients who have most likely developed the disease due to the same gene, as they are linked to a common, recent ancestor. This approach has proven to be possible in the Netherlands, but also in other smaller populations, such as Iceland. Again this method is most likely to work in rather rare diseases and is limited by the availability of genealogical records.
This thesis focuses for a large part on the genetic susceptibility to CJD, which is a key factor in all forms of the disease, being familial, iatrogenic, sporadic, or the variant form. We performed a candidate gene study on the role of the doppel gene (PRND), a PRNP related gene, and a polymorphism upstream of PRNP, as well as a study on PRNP as a candidate gene for Alzheimer's disease and cognitive decline. Regarding genetic research, the next methodological issue to be discussed concerns its limitations. Candidate gene studies have been widely criticised for the failure to produce results that can be replicated. There may be several reasons for this. In CJD research, most studies have been small, which makes the study susceptible to both false positive and false negative findings (see also chapter 4.4). The solution to this problem may again be to perform a meta-analysis, as is presented in chapters 4.4 and 5.2. Another reason for inconsistent findings of studies is linkage disequilibrium, which may differ between populations

and lead to associations between a polymorphism and disease in some populations, but not in others. Also this possibility is explored in chapter 4.4. Last but not least, population admixture may lead to inconsistent findings between studies. For long it has been assumed that population admixture may explain the high rate of positive findings. However, there is substantial evidence that this problem has occurred only under extreme conditions.[18] More likely, multiple testing of markers without any knowledge of its function may explain the discrepancies between studies. To overcome this problem, studies in this thesis were limited to polymorphisms associated to disease before, yielding a hypothesis for the associations to be tested. Finally, there may be misdiagnoses and genotyping errors in studies, explaining why they cannot be replicated. Genotyping errors are the main reason for deviations from the Hardy Weinberg equilibrium in population studies. The fact that studies with control series not in Hardy Weinberg equilibrium are still published, strongly suggests that genotyping errors contribute to erroneous associations.

Diagnostic studies. The third methodological point concerns the diagnosis of CJD. Up until now several studies have addressed the issue on sensitivity. A topic ignored in research on CJD is the specificity of a test. Examination of the specificity requires that the tests are applied in non-CJD patients. Again, the rarity of disease plays a crucial role here, as well as the complexity of the diagnostic procedure, which is often conducted in a tertiary referral centre. This makes it difficult to derive unbiased samples to study specificity (see chapter 4.3).

A special case of interest to the genetic epidemiologist is the use of genetic screening in the differential diagnosis of CJD. It has been advocated to use a molecular diagnostic program in patients with early onset dementia and a positive family history for dementia.[19] However, there are significant limits on the usefulness of genetic testing.

The major concern with regard to genetic testing is related to the practical problems encountered when applying molecular screening of dementia genes in clinical practice. A large number of mutations, e.g. those in the presenilin genes, are rare and distributed throughout the gene. More than 50% of these presenilin mutations are genetically "private"; that is, they are found only in a particular patient or family.[24,25] Novel mutations are often found in the presenilin genes and in PRNP.[19,26,27] On the one hand, these mutations will easily be missed when only for the known mutations is screened, leading to a false negative finding. On the other hand, the effects of these novel mutations are difficult to interpret and are often misjudged.

A notorious example of misjudging the pathogenicity of a presumed missense mutation is the Glu318Gly substitution in the presenilin-1 gene. This substitution was earlier reported as a causative mutation in patients with familial early onset Alzheimer's disease.[24,25] In 1999, however, Dermaut et al. demonstrated that an elderly group of 256 control subjects included 9 carriers of this substitution who were not demented, indicating that the frequency in control subjects was similar

to that found in patients.[28] Rather than being a pathogenic mutation, the Glu318Gly is a rare allele, that is not associated with either Alzheimer's disease or dementia in general and does not influence the β-amyloid formation. This example illustrates that studies of a large series of controls should be performed or functionality of the mutation assessed, before conclusions are drawn about the pathogenicity and penetrance of a particular mutation. For any untreatable disease with a devastating course, as is the case in Alzheimer's disease and in the spongiform encephalopathies, the burden of an incorrect molecular diagnosis should be prevented by all possible means, since genetic testing does not have implications for the patient alone, but also discloses predictive information to family members, which could influence issues such as life expectancy, possibilities for insurance, and psychosocial well-being. These problems are less an issue of concern when testing for susceptibility polymorphisms, such as the PRNP codon 129 polymorphism and apolipoprotein E. For these genes, the increased risk of disease in carriers is modest, resulting in less pronounced implications for the relatives. Yet, for interpretation of diagnostic tests, these polymorphisms may prove to be relevant (see chapters 4.2 and 4.3).

A special concern in relation to the use of molecular screening is the importance of ascertaining the presence of treatable dementias. The presence of a mutation does not eliminate the possibility of the coexistence of a treatable form of dementia. Conditions such as depression, drug intoxication, vascular dementia, and metabolic disorders can mimic and coincide with Alzheimer's disease, especially in patients with a long disease course. Even when a major mutation is present in the family, tests for treatable causes of dementia should not be omitted in clinical practice.

Overall, it may be concluded that the contribution of genetic testing to clinical diagnosis is small and does not counterbalance the problems associated either with interpretation of any mutation that is found, or with secondary effects on family members. Nevertheless, for scientific reasons, genetic testing is very worthwhile. Testing may increase our knowledge about the different mutations, which could have clinical applications in the future.

Main findings and their implications

Iatrogenic transmission. The iatrogenic transmission of CJD has been puzzling in various aspects. First, the fact that CJD occurs with an incidence of one patient per million inhabitants per year and has a duration of 4 to 6 months makes it *a priori* unlikely that iatrogenic transmission occurs. Yet, the disease has been transmitted unintendly in multiple instances against all odds, raising questions about the number of patients with subclinical forms of the disease. Despite the fact that CJD is a rare disorder, multiple batches of growth hormone may have been contaminated in the past. Second, it is puzzling that the mode of transmission (genetic, human growth hormone injections or dura mater transplantation) of CJD results in such distinct phenotypes.

In this thesis we describe three patients ascertained as part of the CJD surveillance in the Netherlands, in whom the disease was iatrogenically transmitted. One patient received a low dose of human derived growth hormone as part of a diagnostic process 38 years before (chapter 3.2). Two other patients received a Lyodura implantation after trepanation of a bleeding of an AVM and resection of a hemangioblastoma ten and fourteen years before onset of symptoms (chapter 3.3).

Treatment with growth hormone has been recognised as a risk factor for CJD since 1985. Our finding contains novel information in that the patient was given the human growth hormone for diagnostic reasons. Earlier reports have always concerned patients treated with multiple doses, aiming to regulate growth. In contrast, our patient received growth hormone only during one week and for diagnostic purposes. Although the exact dose is unknown, medical records of the patient recorded that a low dose was used. The long incubation time in this patient is in accordance with findings of others suggesting dose dependent incubation times.[13] The source of the growth hormone that was used in the patient described by us is unknown. It is also uncertain whether other children have been infected during this period, asking for an increased surveillance in the Netherlands.

The clinical presentation in iatrogenic CJD (chapters 3.2 and 3.3) differs considerably from the presentation in the sporadic form and is not only determined by the mode of transmission, but also depends on the PRNP codon 129 genotype (chapter 4.2). Each of the three patients was homozygous for methionine (M) at codon 129. The onset of disease in the patients with the dura mater transplantation was at age 54 years and 51 years and in the patient with the human growth hormone related form of CJD 47 years. This is younger than the median age at onset described in CJD patients with the sporadic form and the same genotype (67 years)(chapter 4.2), but older than the median age we found in iatrogenic CJD (28 years)(chapter 4.3). Compared to the other growth hormone patients, the age in our growth hormone patient was high, which may be explained in the former group by the large number of patients who were treated for growth retardation at young age during a long period. Our patient only received a low dose of the growth hormone for diagnostic purposes, which may explain the long incubation period. EEG recordings were abnormal in all three patients, but lacked the specific periodic sharp wave complexes in two of three cases. This finding is compatible with the results of the study described in chapter 4.3, in which typical EEG findings were absent in all growth hormone related patients and in 33% of M homozygotes in whom CJD was induced by a dura mater transplant. The cerebrospinal fluid 14-3-3 protein test was strongly positive in all three patients, compatible with the findings in chapter 4.3.

These three atypical iatrogenic patients underscore that awareness for CJD with iatrogenic etiology is mandatory. More patients may have been exposed to the batch of growth hormone that was used in the patient presented here. One patient with growth hormone related CJD has been identified before in the

Netherlands.[29] However, this patient was treated partially with a privately imported product from South America. The present patient is more relevant for surveillance as the source of the growth hormone was probably located in the Netherlands. Also the two patients with dura mater transmission ask for surveillance. The finding that one of the patients received the dura mater transplant in a period that was assumed to be "safe" illustrates that even preventive measures not always guarantee exclusion of risk.

Genetic risk factors

The codon 129 polymorphism in the prion protein gene. For long the PRNP codon 129 polymorphism has been recognised as an important risk factor for CJD. There is increasing evidence suggesting symptoms of the disease and test results of EEG, MRI and CSF tests are also in part determined by this polymorphism.[30,31] However, studies on CJD assessing the frequency of clinical symptoms across genotype have been based on small numbers of cases. Further, diagnostic interpretation of signs and symptoms differs across studies. In the European Collaborative Study Group of CJD this problem was largely overcome by using a standardised protocol (chapter 4.2). In five countries, the effect of the codon 129 polymorphism on clinical features was assessed in patients with the sporadic form of the disease who were sampled from 1993 to 1995. The study showed a significant effect of the codon 129 genotype on visual or oculomotor signs at onset (more often in M homozygotes) and gait disturbances (in valine (V) homozygous patients). The cerebellar symptoms in carriers of genotype VV were found to be a direct genotypic effect, unrelated to age. The sensitivity of this characteristic is high for V homozygotes (66%). The study suggested that phenotypic heterogeneity is at least partly associated with the PRNP codon 129 genotype. However, the key characteristic of CJD, a rapidly progressive dementia, was shown to occur independent of the PRNP codon 129 genotype. These findings suggest that genotyping the codon 129 polymorphism in patients with CJD may be relevant in the diagnostic work-up.

In this light, the findings of the European Collaborative Study Group on the role of the PRNP codon 129 polymorphism on diagnostic investigations are even more convincing (chapter 4.3). The study showed that overall sensitivities for diagnostic tests for CJD may differ considerably over the codon 129 genotypes. In sporadic CJD, the highest sensitivity of the EEG was found in M homozygous individuals (80%)($p<0.001$), but the lowest sensitivity of cerebral MRI in this subgroup (48%)($p=0.03$). In contrast, carriers of the V allele showed a low sensitivity of the EEG (MV 51.5% and VV 31.4%). The CSF 14-3-3 protein test was found to perform worst in heterozygotes for this polymorphism ($p<0.01$). However, the sensitivity of the CSF test was still 85% in MV carriers. In familial cases, sensitivities showed the same trend in EEG ($p=0.01$) and in the CSF 14-3-3 test ($p=0.05$) as seen in patients with sporadic forms of CJD. In the human growth hormone induced CJD patients, typical EEG findings were absent, irrespective of the genotype. The findings imply that in the diagnostic process of CJD clinical

symptoms and diagnostic test results should be interpreted based on the genotypic background. When using a genetic test for diagnosis, an important question is what the implications are for relatives. For a susceptibility polymorphism, such as the PRNP codon 129, the absolute risk of disease for carriers is that low, that the implications of genetic testing for relatives is negligible.

The large differences in phenotype which are related to the PRNP codon 129 genotype are a unique feature in CJD. Usually, the effect of polymorphisms on the phenotype of the disease is small, e.g. the effect of the apolipoprotein E polymorphism on Alzheimer's disease or the HLA DR2/3 on multiple sclerosis. The highly variable expression of CJD cannot solely be explained by genetic variation at codon 129 of PRNP. Several studies have shown that an interplay between this polymorphism and the glycosylation pattern of the prion protein has a large influence on the phenotypic expression. This finding suggests that not only the sequence of the gene is relevant, but also the subsequent glycosylation of the protein.[30,31] Based on the PRNP codon 129 polymorphism and the glycosylation pattern, Parchi classified six disease variants.[31] The large majority of patients had at least one M allele and glycosylation pattern 1, which led to the classical CJD phenotype, consisting of a predominance of cognitive decline and a wide range of visual disturbances. A quarter of the cases, carrying at least one V allele and glycosylation pattern 2, displayed ataxia. Patients with M homozygosity and glycosylation type 2 displayed a thalamic form of CJD or prominent dementia. A small group, linked to V homozygosity and glycosylation type 1, was clinically characterised by a progressive dementia. However, due to small numbers in some of the subgroups, overlapping clinical features, intermediate mobility of the type 2 protease resistant fragment in the PRNP codon 129 MV group and the co-existence of both isotypes in almost 5% of the cases, this classification can currently not fully explain the phenotypic heterogeneity of sporadic CJD.

A question that remains to be answered is how far the differences observed by Parchi and Zerr are explained by the sequence of the polymorphism at codon 129 of PRNP or by the glycosylation pattern. As part of the European Collaborative Study we simultaneously analysed the glycosylation data and sequence data of 449 patients with sporadic CJD. We assessed the effect of genotype and isotype on diagnostic investigations. For the EEG, we found that both genotype and isotype contributed significantly ($p<0.001$). The PRNP codon 129 genotype did not significantly influence the outcome of MRI of the brain or the 14-3-3 test in CSF, while the effect of the isotype was significant in the 14-3-3 test ($p=0.003$). The sensitivity of the EEG and 14-3-3 test was reduced in patients with glycosylation pattern type 2. Although the finding of the glycosylation patterns is of interest from a scientific point of view, for clinical care determination of the glycosylation pattern will be of limited use, since it has to be performed on brain material. Currently, brain biopsy is not indicated since it does not alter any therapeutic strategies.

The codon 174 polymorphism in the doppel gene. Although PRNP plays a central role in the origin of Creutzfeldt-Jakob disease (CJD), there is growing interest in other polymorphisms that may be involved in CJD. Several candidate genes have been studied, including APOE, HLA and the presenilins. In chapter 4.4 we have studied genes in the prion protein complex. We showed that the prion-like doppel gene (PRND) harbours a polymorphism at codon 174 which has an effect on CJD, independent of the PRNP codon 129 polymorphism. Findings of the studies of PRND in human populations have been controversial (chapter 4.4), even in a meta-analysis combining all data of studies conducted to date. Our study suggests a role of the PRND gene in CJD. The PRND gene may be involved through several mechanisms. Although the function of doppel remains unknown, it is hypothesised to have overlapping functions with the prion protein. One of the problems to elucidate is the finding that the doppel protein is normally hardly present in the brain.[32] Animal studies have shown a complex association of PRNP and PRND. PRNP knockout mice lacking extensive regions around the open reading frame, were shown to have an upregulated production of the doppel protein, which was, surprisingly, found to be enhanced by the PRNP promoter.[32] These mice exhibited ataxia. However, when normal prion protein was introduced, no phenotypic changes were observed, indicating that the absence of prion proteins is more important than the presence of the doppel gene. On the other hand, the presence of normal prion proteins may also give a negative feedback and block the production of doppel. The effect of the T174M polymorphism on the doppel protein is unknown.

The prion protein and other neurodegenerative processes. The function of the prion protein has been puzzling for a long time. Mice lacking the prion protein developed normally and remained healthy throughout life.[33] Recent findings point to a neuroprotective effect of this protein, which may result from anti-oxidative abilities.[34,35] This function may imply that the prion protein is also involved in other neurodegenerative processes. In this thesis, the relation of the codon 129 polymorphism with Alzheimer's disease and cognitive decline in the general population is studied.

The studies presented in chapters 5.4 and 5.5 suggest that the prion protein has a significant effect both on early and late onset Alzheimer's disease and on cognitive decline. Both the PRNP codon 129 MM and VV genotype were associated with early onset Alzheimer's disease. Although we cannot exclude the possibility of misdiagnosis of CJD patients as Alzheimer's disease patients, the low frequency of CJD in the population makes this unlikely. The observation that carriers of a PRNP codon 129 homozygous genotype have an increased risk for Alzheimer's disease, raises a question about the mechanism involved. An increased risk for homozygotes for both alleles does not fit a normal Mendelian gene effect in which one allele is associated with disease. There may be several explanations. The prion theory of Prusiner suggests that homozygosity for either allele results in the production of identical proteins. Propagation of the conformational change in the

prions, which is associated with disease, may be enhanced in proteins with identical structure. However, the prion theory is a likely explanation for the disease mechanism in CJD, but its application to the mechanism in Alzheimer's disease is highly hypothetical. Alternatively, there may be other explanations in which one of the alleles is associated with the disease, while the other is in linkage disequilibrium with a second mutation leading to disease. In the non-CJD dementias, the highest risk to develop late onset Alzheimer's disease was associated with homozygosity for the V allele. However, when considering the effect of the V allele on cognitive decline, the association was limited to individuals aged 65 year or less. Interestingly, the proportion of V homozygotes in CJD patients is highest in the young age categories.[36] V homozygotes in the general population showed cognitive decline under age 65 years at a similar rate as that seen in the subgroup over 65 years, suggesting that neurodegeneration in carriers of genotype VV is high early in life. Despite the high decline, we did not find support for an increase in mortality in VV carriers in a population-based sample of more than 950 individuals (chapter 5.3).

There is still considerable controversy about the function of known mutations in PRNP as well as on the frequency. Finckh et al. have suggested that PRNP mutations are frequent in patients with early onset dementia, harbouring up to 33% of the mutations in known dementia genes (amyloid precursor protein gene, presenilin genes and PRNP).[19] We have not found PRNP mutations in our patients with early onset Alzheimer's disease.[37] When sequencing the prion protein gene in a population-based sample of 17 patients with early onset dementia, we identified one patient with a two octapeptide repeat insertion in PRNP. The phenotype in this patient was atypical (see chapter 5.2), making it difficult to use these mutations for clinical diagnosis. As to the function of mutations, there have been extensive inconsistencies on the clinical phenotype of PRNP octapeptide repeat insertions. Data of case reports on carriers have yielded contradictory results in the effect on age at onset and duration of disease. In chapter 5.2 we present a meta-analysis of all case reports in the literature and show that an increasing number of repeats is significantly associated with younger age at onset ($p=0.0001$). When adjusting for PRNP codon 129 genotype, the unexplained correlation of age at onset between families was reduced to 10%. Duration of the disease decreased significantly with the length of the repeat ($p=0.04$), when adjusting for age at onset and PRNP codon 129 genotype. These findings show a significant association of the length of the octarepeat with age at disease onset and disease duration in the spongiform encephalopathies.

There is no discussion on the fact that the PRNP gene plays a pivotal role in CJD. Not only does the gene influence the risk of familial CJD, it also has a significant effect on the risk of iatrogenic and variant forms of the disease. The same holds for the prion protein. Animals in whom PRNP has been knocked out do not develop a spongiform encephalopathy, suggesting that organisms need to produce their own prion protein in order to develop the disease.[33,38] Yet, both in animals and in humans it is clear that other genes (and proteins) must be

involved. In humans only 50% of the CJD patients with a positive family history for dementia carry a mutation in PRNP, suggesting a major role of other genes. Our finding in chapter 5.3 underlines this hypothesis. When studying the genealogy of 59 patients with CJD, 5 pairs of patients were linked to a common ancestor within 5 to 7 generations, while in a control group of 16 patients with glioma, links were only found after 11 generations. This finding suggests a common genetic origin in CJD patients. However, the disease could not be explained by mutations in PRNP, suggesting other genes may explain this familial aggregation. Of further interest is the familial aggregation of CJD with Alzheimer's disease. In 49 patients with Alzheimer's disease and 59 patients with CJD, we found 2 pairs with a shared ancestor within 6 generations. These findings were specific to CJD and Alzheimer's disease, since we did not find a familial aggregation of these two groups with the glioma patients, which we studied as control group. This finding is in line with that of the European Collaborative Studies suggesting familial clustering of CJD and other forms of dementia.[7] We only investigated the rare early onset form of Alzheimer's disease and not the more common late onset form, which raises the question whether we underestimate the familial aggregation.
Familial aggregation of CJD and early onset Alzheimer's disease could not be explained by mutations in known genes (amyloid precursor protein gene, presenilin genes and PRNP), fuelling speculations that Alzheimer's disease and CJD may have a common etiology. At least this hypothesis appears to hold for a proportion of patients, in our study 12%, but this may be an underestimation given the dead ends in the genealogy, non-paternity, the possibility of more distant links further away than the 7 generations we collected or connections to patients with late onset dementia that we did not study. On the one hand this may concern a mechanism involving prion propagation. On the other hand more general mechanisms may be involved, such as neurodegeneration. The prion protein may have neuroprotective properties so that loss of its function would increase the vulnerability of neurons.[39] Apart from PRNP, many genes may be involved in these processes, which so far remained undisclosed in the etiology of CJD.

Further research

Basic surveillance has proven to be a successful starting point for the identification of new forms of the spongiform encephalopathies and will most likely remain to be a cornerstone for human CJD research in the next years. This concerns research of classical CJD as well as the BSE related variant form of CJD. The crucial questions to resolve in vCJD research will be to find out whether subjects with genotype MV or VV at PRNP codon 129 are susceptible for vCJD and how the disease will express clinically. BSE related CJD will remain a worry throughout Europe. The doubling of the incidence of patients with definite sporadic CJD in Switzerland, a country with a high incidence of BSE, fuels

speculations on the transmission of BSE to humans in other forms than vCJD. The finding of the pathological isoform in muscle tissue imposes the duty of epidemiologic research to further explore the role of meat in the transmission of BSE to humans. Also the infectious nature of BSE asks for further research. BSE may have been transmitted to the sheep population, although there is no empirical evidence. Statistical models predicted a mortality due to ovine related vCJD ranging from 50 to 50.000, or to 150.000 in the worst case scenario.[40]

The first challenge for future research on the classical forms of CJD concerns the diagnosis of disease. Although the sensitivity and specificity of the current tests (14-3-3 protein test in CSF, MRI of the brain and EEG) is high, these test results are still based on diagnostic investigations performed in highly specialised centres. For clinical practice, it would be preferable if tests could be performed in local hospitals. In particular MRI diagnostics may be helpful, which is also more uniformly available. The sensitivity of MRI diagnostics is still low, but improvement is expected, as this test is new as a diagnostic tool in CJD. An issue that needs further evaluation concerns the inclusion of genotyping in the diagnostic process. First, mutation analysis could be used for this, as advocated by some authors.[19] However, the use of mutation screening is controversial. Although screening is technically possible, the problem to be resolved concerns the interpretation of the causality of the mutation. More research is needed to make mutation screening suitable for clinical practice. The other challenge may be to incorporate data on the PRNP codon 129 polymorphism in the diagnostic process, which requires further investigations, not only into sensitivity of clinical features and tests, but also into the specificity.

The second challenge concerns the etiology of the disease. Most of our current knowledge about the pathogenesis of CJD is based on knowledge of the genetics of CJD, as it is derived from knockout mice and chimeric animals. The challenge for the future will be the identification of new genes which may determine susceptibility for the disease. Knowledge of these genes will give a better understanding of the disease process.

A third challenge will concern the prevention of CJD and the control of spread of the disease from human to human. Given the frequency, one of the major issues to be tackled concerns the transmission through surgery and blood products. The first way of transmission is still not excluded and the second is a point of major concern, especially because the lymphoid system is also involved in vCJD. It is not obvious how to tackle this problem in an epidemiological setting. It may be argued that these answers are more likely to come from animal studies, in particular in primates.

Most of the preventive measures have been targetting to known iatrogenic factors. Within the UK, the site of the largest BSE and vCJD epidemic, preventive measures include the derivation of blood and blood products from other countries and the single use of surgical instruments for high risk operations, i.e. operations involving high amounts of lymphoid tissue, like the appendectomy. Given the development in Switzerland, these measures should also be considered in other

European countries. However, this will have major implications for plasma supply. Already the UK ban has led to shortage of plasma on the world market.
Last but not least, the ultimate challenge in CJD research will be to develop a treatment for the disease. Recent developments in this field include the use of quinacrine and chlorpromazine. Although quinacrine is no longer in use in Europe because of its serious side effects, it has been suggested that this drug may inhibit the formation of the pathological isoform of prions in vitro.[41] Despite the major side effects, several patients with classical and variant forms of the disease have been treated with quinacrine or chlorpromazine. Results of treatment are extremely difficult to interpret. No data from clinical trials on the effectiveness are available, nor are there any data on the effective dosis for this particular disease. The use in patients with CJD has so far not yielded any positive results. A more fruitful approach may be to develop a treatment which is based on knowledge of the etiology of the disease. The current research is targetting to the misfolding and aggregation of the protein, and drugs that may destabilise these aggregates.[42]
CJD is a rare disorder that has sprung from several unexpected sources ranging from human growth hormone treatment and dura mater transplantation to BSE infected material. There is no doubt that mankind will be confronted with this disease also in the near future, despite our growing knowledge and our efforts to prevent transmission of this disease among humans and among farm animals. The failure predicts that eradicating the disease will be difficult. The existence of reservoirs among wild life, e.g. chronic wasting disease, and the transmission of BSE to various animals in the zoo make the prospects of eradication rather pessimistic. If any lesson is to be learned in terms of transmission of the rare spongiform encephalopathies in animals and humans, it is to expect the unexpected.

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7

Summary & Samenvatting

Summary

Creutzfeldt-Jakob disease (CJD) is a rare disease with an incidence of 1-2 patients per million per year. Despite the rare occurrence of the disease, research in this field is important for two reasons. First, CJD is unpredictable and has resulted in major epidemics, both in iatrogenic forms and in the BSE related variant form, and second, the pathological mechanism underlying CJD may not only concern transmissible spongiform encephalopathies, but may also be involved in other neurodegenerative processes.

The thesis focuses upon two aspects: medical risk factors and genetic risk factors. For the results presented in this thesis, data were used from the Dutch surveillance system of CJD, the European Collaborative Study Group of CJD, the Rotterdam Study, and a population-based study on Alzheimer's disease.

Chapter 1 gives a general introduction to the thesis.

Chapter 2 provides a review on the epidemiology of CJD. This chapter describes the various forms of the disease and the search for etiological factors underlying them. Further, the clinical features and the diagnostic procedures are presented. Finally, the incidence of the various forms of the disease, the prognosis and the absence of an effective treatment are mentioned.

Chapter 3 describes the iatrogenic risk factors for CJD. This chapter presents 3 Dutch patients who were identified in the Dutch CJD surveillance program, one patient was infected through human growth hormone, the other two through dura mater transplantation. In chapter 3.2 a 47 year old man is described who developed pathology proven CJD 38 years after receiving a low dose of human derived growth hormone as part of a diagnostic process. This is the longest incubation period described so far for iatrogenic CJD. Furthermore, this is the first report of CJD after diagnostic use of human growth hormone. Since our patient was one of the first in the world to receive growth hormone, other iatrogenic CJD patients can be expected in the coming years. Chapter 3.3 describes the first two dura mater associated CJD cases in the Netherlands. The findings are discussed in light of the growing epidemic of CJD among dura mater recipients.

Chapter 4 focuses upon the genetic factors involved in CJD. The aim of this chapter is two-fold. First, the effect of the prion protein gene (PRNP) in the classical diagnosis is assessed using the data of the European Collaborative Study Group. Second, the role of candidate genes other than the PRNP codon 129 polymorphism is studied using the data from the Dutch surveillance system and a meta-analysis of reports published to date.

In chapter 4.2 the relation between the PRNP codon 129 polymorphism and clinical features is explored. The results of this study confirm that phenotypic heterogeneity is at least partly associated with the PRNP codon 129 genotype. Patients homozygous for methionine (M) were found to have more often visual or

oculomotor signs at onset. Gait disturbances were significantly more often present at onset in cases homozygous for valine (V). Although V homozygotes were on average significantly younger, age could not explain this finding. Patients heterozygous at the polymorphic PRNP codon 129 were found to have a significantly longer disease duration. In contrast to other reports, this study found that the key characteristic of CJD, a rapidly progressive dementia, occurs independent from the PRNP codon 129 genotype.

In chapter 4.3 the influence of the PRNP codon 129 polymorphism on the sensitivity of the three most often used diagnostic investigations in CJD (EEG, cerebral MRI and the CSF 14-3-3 test) is assessed . This study showed that overall sensitivities for diagnostic tests for CJD may differ considerably over the codon 129 genotypes. In sporadic CJD, the sensitivity of the EEG was 80% in M homozygous individuals, compared with 52% in carriers of genotype MV and 31% in V homozygotes ($p<0.001$). In contrast, the sensitivity of cerebral MRI was lowest in M homozygotes (48%, $p=0.03$). The CSF 14-3-3 protein test was found to perform worst in PRNP codon 129 MV carriers ($p<0.01$), but the sensitivity was still 85% in this group. In familial cases, sensitivities showed the same trend in EEG ($p=0.01$) and in the CSF 14-3-3 test ($p=0.05$) as seen in patients with sporadic forms of CJD. In the human growth hormone induced CJD patients, typical EEG findings were absent, irrespective of the genotype.

In chapter 4.4 a polymorphism which may modulate the prion protein production (single nucleotide polymorphism (SNP) 1368) and polymorphisms in the prion-like doppel gene (PRND) (T26M, P56L and T174M) are studied in a population-based sample of 52 patients with sporadic CJD and 250 controls. For SNP 1368 and PRNP codon 129 significant evidence for linkage disequilibrium was found. However, there was no association with disease for SNP 1368. Further, a significant increased risk for V homozygotes at PRNP codon 129 and for M homozygotes at PRND codon 174 was observed, when adjusting the analyses for the other genotypes. In the haplotype analyses, a significantly increased risk was found in homozygotes for PRNP codon 129 M and PRND codon 174 M (OR 4.35, 95% CI 1.05-18.09; $p=0.04$). The meta-analysis on the studies assessing the relation between the PRND codon 174 polymorphism and CJD did not result in significant findings. This study suggests that homozygosity for PRND 174 M is an independent risk factor for CJD.

In **chapter 5** the role of the prion protein is assessed in dementia and cognitive decline. Although the function of the prion protein is largely unknown, it may have a role in maintaining neuronal integrity by protecting the cell from oxidative stress. Loss of its function may therefore lead to neurodegeneration and thus it is conceivable that many neurodegenerative processes may be associated with the prion protein.

In chapter 5.2 a sample of 17 patients with early onset dementia is screened for mutations in PRNP. A PRNP two octapeptide repeat insertion was found in a 69 year old male with atypical dementia. We further conducted a meta-analysis of all patients with PRNP octapeptide repeat insertions (n=59) to assess the relation of

number of repeats with age at disease onset and duration of illness. An increasing number of repeats was found to be significantly associated with a younger age at onset (p=0.0001) and reduced survival (p=0.04), when adjusted for age at onset and PRNP codon 129 genotype.

In chapter 5.3 a promising new approach for the identification of other genes involved in CJD is presented. The genealogy of a population-based series of 59 patients with CJD and 49 patients with early onset dementia is examined for evidence of a common ancestor. Further, the genealogy of 16 patients with glioma is studied as control sample. We found 9 patients with CJD related to each other by common ancestors 5 to 7 generations ago. We also found two couples of CJD and Alzheimer's disease patients with a common ancestor within 6 generations. In the patients with glioma, 4 were linked only 11 generations ago, while none of the patients shared an ancestor with CJD or Alzheimer's disease patients. In two couples of CJD patients, we found a remarkable resemblance in clinical presentation, which, taken together with the link to a common ancestor, strongly suggests the presence of a shared mutation. No mutation in PRNP, the amyloid precursor protein gene or the presenilin genes was found, indicating that the disease is most likely explained by another, yet unidentified gene. The study underscores the role of genetics in CJD and indicates that genes other than PRNP may be involved.

Chapter 5.4 investigates the role of the codon 129 polymorphism of PRNP in early onset Alzheimer's disease in a Dutch population-based case-control sample (123 cases, 282 controls). Using the MV genotype as reference, we observed a significant association between early onset Alzheimer's disease and homozygosity at prion codon 129 (OR = 1.9; 95% CI 1.1-3.3; p=0.02) with the highest risk for V homozygotes (OR = 3.2; 95% CI 1.4-7.1; p<0.01). In early onset Alzheimer's disease patients with a positive family history the risk further increased to 2.6 (95% CI 1.3-5.3; p<0.01) for homozygosity of either the M or V allele and to 3.5 (95% CI 1.3-9.3; p=0.01) for V homozygotes. This study suggests that homozygosity at codon 129 of PRNP, particularly of the V allele, is a genetic risk factor for early onset Alzheimer's disease.

Finally, in chapter 5.5, the role of the codon 129 polymorphism in cognitive decline is assessed in 965 subjects aged 55 years and over from the Rotterdam Study. The association of the PRNP codon 129 polymorphism to Mini Mental State Examination (MMSE) at baseline (n=878) and decline during follow-up (n=418) was assessed. Carriers of genotype VV were more often diagnosed with dementia (p=0.009). Median MMSE scores at baseline were not significantly related to PRNP genotype. Besides the expected annual decline in MMSE with age (p<0.001), the age-stratified analyses comparing cognitive decline in the three genotypes revealed a significantly increased decline in cognitive performance in V homozygous individuals aged 55-64 years (p<0.01). In the 55-64 years age categories, cognitive decline in V homozygotes was similar to that seen in subjects 65-74 years old. We found no significant evidence for effect modification of this relation by APOE genotype or family history of dementia. Our findings

strongly support the hypothesis that the PRNP codon 129 polymorphism is involved in various neurodegenerative brain processes.

In **chapter 6** the methodological considerations of these studies are presented. This chapter highlights several aspects, ranging from the use of case-control studies in epidemiological research to the use of genetic testing in the diagnostic process. The most important findings of this thesis are two-fold. First, it is stressed that the iatrogenic form of the disease can present atypically and that still new patients are diagnosed. Second, this thesis has shown that genetic factors are important in CJD and related disorders. We showed that the PRNP codon 129 genotype plays a major role in the clinical presentation of the disease, but may also underlie more general neurodegenerative processes, like cognitive decline and Alzheimer's dementia. Finally, we have shown that there is increasing evidence pointing to other genes involved in CJD and related disorders.

Samenvatting

De ziekte van Creutzfeldt-Jakob (CJD) is een zeldzame ziekte met een incidentie van één tot twee patiënten per miljoen per jaar. Ondanks de zeldzaamheid van deze aandoening is onderzoek naar deze ziekte belangrijk om twee redenen. Ten eerste is CJD een onvoorspelbare ziekte die al eerder belangrijke epidemieën veroorzaakte, zowel in de iatrogene vorm als in de aan BSE gerelateerde vorm. Ten tweede is het pathologisch mechanisme dat aan CJD ten grondslag ligt mogelijk ook betrokken bij andere neurodegeneratieve processen.
Dit proefschrift richt zich op twee aspecten: medische risicofactoren en genetische risicofactoren. Voor de resultaten die in dit proefschrift worden gepresenteerd zijn gegevens gebruikt uit de Nederlandse surveillance voor CJD, de Europese studie groep voor CJD, het Erasmus Rotterdam Gezondheids Onderzoek (ERGO) en een populatie onderzoek naar de ziekte van Alzheimer.
Na een algemene introductie in **hoofdstuk 1** geeft **hoofdstuk 2** een overzicht van de epidemiologie van CJD. De verschillende vormen van de ziekte worden beschreven en de mechanismen die mogelijk aan de ziekte ten grondslag liggen worden samengevat. Daarnaast worden de klinische verschijnselen en de diagnostische procedures toegelicht. Tenslotte worden de incidentie van de verschillende vormen van de ziekte, de prognose en de behandeling besproken.
Hoofdstuk 3 beschrijft de iatrogene risicofactoren voor CJD. In dit hoofdstuk worden drie iatrogene patiënten gepresenteerd die werden geïdentificeerd in het Nederlandse surveillance programma voor CJD. Eén patiënt was geïnfecteerd via humaan groeihormoon, de andere twee door dura mater implantatie. In hoofdstuk 3.2 wordt een 47-jarige man beschreven die pathologisch bewezen CJD ontwikkelde 38 jaar nadat hij een lage dosis groeihormoon van humane origine had ontvangen, in het kader van een diagnostische procedure. Dit is de langste incubatieperiode die tot dusverre is beschreven voor de iatrogene vorm van CJD. Het is tevens de eerste rapportage van CJD na diagnostisch gebruik van humaan groeihormoon. Aangezien onze patiënt tot de eersten in de wereld behoort die groeihormoon kregen toegediend, kunnen meer patiënten de komende jaren worden verwacht. Hoofdstuk 3.3 beschrijft de eerste twee CJD patiënten in Nederland met een via dura mater implantatie verkregen vorm. De bevindingen worden beschreven in het licht van de groeiende epidemie van aan dura mater gerelateerde CJD patiënten.
Hoofdstuk 4 richt zich op de genetische factoren die een rol spelen in CJD. Dit hoofdstuk heeft een tweeledig doel. Ten eerste wordt het effect van het gen coderend voor het prion eiwit (PRNP), welk eiwit een belangrijke rol speelt in CJD, onderzocht op de klinische presentatie van de ziekte. Hiervoor werd gebruik gemaakt van de gegevens van de Europese studie groep voor CJD. Ten tweede wordt de rol van kandidaat-genen anders dan het polymorfisme op codon 129 van

PRNP onderzocht. Hiertoe werd gebruik gemaakt van gegevens verkregen uit de Nederlandse surveillance en een meta-analyse van alle tot heden gepubliceerde artikelen.
In hoofdstuk 4.2 wordt de relatie tussen het PRNP codon 129 polymorfisme en de klinische verschijnselen onderzocht. De resultaten van deze studie bevestigen dat de heterogeniteit in het fenotype gedeeltelijk geassocieerd is met het genotype van het PRNP codon 129. Bij patiënten homozygoot voor methionine (M) bleek de ziekte vaker te beginnen met visuele of oogbewegingsstoornissen. Loopstoornissen werden significant vaker gezien bij patiënten die homozygoot waren voor valine (V). Hoewel V homozygoten gemiddeld jonger waren, was deze bevinding niet aan een leeftijdseffect toe te schrijven. Patiënten heterozygoot voor het codon 129 hadden een significant langere ziekteduur. In tegenstelling tot eerder onderzoek werd in deze studie aangetoond dat het belangrijkste kenmerk van CJD, een snel progressieve dementie, onafhankelijk van het PRNP codon 129 genotype optreedt.
Hoofdstuk 4.3 bestudeert de invloed van het PRNP codon 129 polymorfisme op de sensitiviteit van de drie meest gebruikte diagnostische testen in CJD (EEG, MRI van de hersenen en de 14-3-3 test in de liquor). De studie toont aan dat de sensitiviteit van de verschillende diagnostische testen sterk kan wisselen tussen de PRNP codon 129 genotypes. In patiënten met de sporadische vorm van de ziekte werd de hoogste sensitiviteit van het EEG in personen homozygoot voor het M allel gevonden (80%, tegenover 52% in dragers van genotype MV en 31% in V homozygoten, $p<0.001$). In de M homozygoten werd daarentegen de laagste sensitiviteit van de MRI gevonden (48%, $p=0.03$). De 14-3-3 liquor test was het minst gevoelig in patiënten met genotype MV ($p<0.01$), hoewel de sensitiviteit in deze groep nog wel 85% was. In de familiaire patiënten werd een vergelijkbare trend in de sensitiviteit van het EEG ($p=0.01$) en de 14-3-3 liquor test ($p=0.05$) waargenomen als in patiënten met de sporadische vorm. In de patiënten bij wie de ziekte door humaan groeihormoon was geïnduceerd waren de voor CJD typische EEG bevindingen afwezig, ongeacht het genotype.
In hoofdstuk 4.4 worden polymorfismen bestudeerd die ofwel de productie van het prion eiwit kunnen beïnvloeden (het stroomopwaarts van PRNP gelegen 'single nucleotide polymorphism' SNP 1368) ofwel gelegen zijn in het prion-achtige doppel-gen (PRND) (T26M, P56L en T174M) in een serie van 52 patiënten met de sporadische vorm van de ziekte en 250 controles. Er werd een significant linkage disequilibrium gevonden tussen SNP 1368 en het PRNP codon 129 polymorfisme. Er was echter geen associatie met de ziekte. Daarnaast werd er een significant verhoogd risico op CJD gevonden voor personen homozygoot voor het V allel op PRNP codon 129 en homozygoten voor M op PRND codon 174. In de analyses van de haplotypes werd een verhoogd risico gevonden voor PRNP codon 129 M homozygotie en PRND codon 174 M homozygotie (odds ratio 4.35, 95% betrouwbaarheidsinterval 1.05-18.09; $p=0.04$). De meta-analyse die de relatie tussen het PRND codon 174 polymorfisme en het risico op CJD onderzocht resulteerde niet in significante bevindingen. De resultaten in dit hoofdstuk wijzen

erop dat homozygotie voor PRND codon 174 M een onafhankelijke risicofactor is voor CJD.

Hoofdstuk 5 onderzoekt de relatie tussen het prion eiwit enerzijds en dementie en cognitieve achteruitgang anderzijds. Hoewel de functie van het prion eiwit grotendeels onbekend is, zou het eiwit een rol kunnen spelen bij het in stand houden van de neuronale integriteit door de cellen te beschermen tegen oxidatieve stress. Verlies van deze functie zou kunnen leiden tot neurodegeneratie en het is daarmee voorstelbaar dat verschillende neurodegeneratieve processen geassocieerd zijn met het prion eiwit.

In hoofdstuk 5.2 is een groep van 17 patiënten met een vroege vorm van dementie onderzocht op mutaties in PRNP. In een 69-jarige man met een atypische dementie werd een insertie van twee octapeptide 'repeats' gevonden. De relatie van het aantal repeats met de leeftijd van eerste verschijnselen en duur van de ziekte onderzochten we vervolgens aan de hand van een meta-analyse. Een toenemend aantal repeats was significant geassocieerd met een jongere leeftijd waarop de eerste verschijnselen zich openbaarden ($p=0.0001$) en een kortere ziekteduur ($p=0.04$). Deze bevindingen tonen een significante relatie tussen de lengte van de octapeptide repeat in PRNP en leeftijd van ontstaan en duur van de ziekteverschijnselen in de spongiforme encefalopathieën.

In hoofdstuk 5.3 wordt een nieuwe en veel belovende manier gelanceerd voor de opsporing van andere genen betrokken bij CJD. Dit hoofdstuk benadrukt de rol van genetische factoren in CJD en wijst erop dat andere genen dan PRNP betrokken zijn. In een serie van 59 patiënten met CJD en 49 patiënten met vroege vormen van dementie werd de genealogie bestudeerd met het doel gemeenschappelijke voorouders te identificeren. Als controle groep werden 16 patiënten met gliomen gebruikt. In negen patiënten met CJD werd een verwantschap gevonden 5 tot 7 generaties eerder. Er waren twee koppels van patiënten met CJD en de ziekte van Alzheimer die 6 generaties eerder een gemeenschappelijke voorouder deelden. In de patiënten met gliomen werden de eerste gemeenschappelijke voorouders pas gevonden na 11 generaties, terwijl geen van deze patiënten een voorouder met CJD of Alzheimer patiënten deelde. In twee koppels van CJD patiënten vonden we een opvallende gelijkenis in klinische presentatie, hetgeen met de verwantschap aan een gezamenlijke voorouder een sterke aanwijzing is voor de aanwezigheid van een gemeenschappelijke mutatie. Er kon echter geen mutatie in PRNP, het amyloid precursor proteïne gen of de beide preseniline genen worden aangetoond, wat erop duidt dat de ziekte waarschijnlijk wordt verklaard door een ander, niet nader geïdentificeerd gen.

Hoofdstuk 5.4 bestudeert de rol van het PRNP codon 129 polymorfisme in vroege vormen van de ziekte van Alzheimer in een serie van 123 patiënten en 282 controles. In de analyses werd het meest frequente genotype in de algemene populatie, MV, als referentie gebruikt. Er werd een significante relatie gevonden tussen vroege vormen van de ziekte van Alzheimer en homozygotie voor het PRNP codon 129 genotype (odds ratio 1.9, 95% betrouwbaarheidsinterval 1.1-3.3;

p=0.02), met het hoogste risico voor V homozygoten (odds ratio 3.2, 95% betrouwbaarheidsinterval 1.4-7.1; p<0.01). In patiënten met de ziekte van Alzheimer en een positieve familieanamnese voor dementie nam het risico verder toe tot 2.6 (95% betrouwbaarheidsinterval 1.3-5.3; p<0.01) in het geval van homozygotie voor M of V en tot 3.5 (95% betrouwbaarheidsinterval 1.3-9.3; p=0.01) for V homozygoten. Dit hoofdstuk suggereert dat homozygotie op codon 129 van PRNP, met name van het V allel, een risicofactor is voor vroege vormen van de ziekte van Alzheimer.

Tenslotte wordt in hoofdstuk 5.5 de rol van het PRNP codon 129 polymorfisme onderzocht in relatie tot cognitieve achteruitgang in 965 personen van 55 jaar of ouder uit ERGO. De associatie van het PRNP codon 129 polymorfisme met de Mini Mental State Examination (MMSE) score aan het begin van de studie (n=878) en de achteruitgang tijdens vervolgonderzoek (n=418) werd bekeken. Dragers van genotype VV werden vaker gediagnostiseerd met dementie (p=0.009). De mediane MMSE score aan het begin van de studie was niet significant gerelateerd aan het PRNP codon 129 polymorfisme. Naast de verwachte jaarlijkse achteruitgang in MMSE score met de leeftijd (p<0.001), toonde de naar leeftijd gestratificeerde analyses een significant grotere achteruitgang in cognitief functioneren in V homozygoten in de leeftijdscategorie 55 tot 64 jaar (p<0.01). In deze leeftijdscategorie was de achteruitgang in cognitief functioneren even groot als in de 65- tot 74-jarigen. Er waren geen aanwijzingen voor effect modificatie door het APOE genotype of de familieanamnese voor dementie. De bevindingen ondersteunen de hypothese dat het PRNP codon 129 polymorfisme betrokken is bij diverse neurodegeneratieve processen in de hersenen.

In **hoofdstuk 6** worden methodologische overwegingen bij de studies gepresenteerd. Dit hoofdstuk belicht verschillende aspecten, variërend van het gebruik van case-control studies in epidemiologisch onderzoek tot het gebruik van genetische testen in het diagnostisch proces. De bevindingen van dit proefschrift zijn tweeledig. Ten eerste wordt benadrukt dat iatrogene vormen van de ziekte zich atypisch kunnen presenteren en dat nog steeds nieuwe patiënten met deze vorm worden gediagnostiseerd. Ten tweede heeft dit proefschrift de boodschap dat genetische factoren een belangrijke rol spelen in CJD en verwante aandoeningen. In dit proefschrift wordt aangetoond dat het PRNP codon 129 genotype een grote rol speelt in de klinische presentatie van de ziekte, maar ook ten grondslag kan liggen aan meer algemene neurodegeneratieve processen zoals cognitief verval en de ziekte van Alzheimer. Tenslotte levert dit proefschrift sterke aanwijzingen voor de betrokkenheid van andere genen in CJD en aanverwante ziekten.

Dankwoord

Nu dit proefschrift op de rede en ik voor pampus lig is het hoog tijd om alle betrokkenen te bedanken.
Over de wetenschappelijke bekwaamheden van mijn eerste promotor, professor Cock van Duijn, valt veel goeds te zeggen, maar zij is bovenal een bijzonder mens. Ik waardeer haar gymnastische geest die telkens als de rand van de aarde zich aandient open vaarwater weet te vinden om de reis volle kracht vooruit voort te zetten. De grote kwaliteit van mijn tweede promotor, professor Bert Hofman, is zijn vermogen om voorbij de horizon te kijken: in de tijd dat CJD nog gewoon de ziekte van Jakob en Creutzfeldt was zette hij zich al in voor Europese surveillance en legde daarmee de kiel voor het huidig onderzoek. Aan dit surveillance programma zijn de 'European and allied countries Collaborative Study Group of CJD' en de 'extended European Collaborative Study Group of CJD' ontsproten. I thank the EURO and NEURO CJD members for their pleasant collaboration. In particular I would like to thank professor A. Alperovitch, professor H. Budka, dr. A. Giulivi and dr. N. Cashman, professor C. Masters and dr. S. Collins, dr. E. Mitrová, dr. J. de Pedro Cuesta, professor M. Pocchiari, professor S. Poser and dr. I. Zerr, professor A. Aguzzi and dr. M. Glatzel and the leaders of the two groups, professor R.G. Will and dr. R. Knight. I am further grateful to the Edinburgh group of professor Bob Will, who's constructive ideas (and interest in art) I much appreciate. I am very happy with the almost daily collaboration with Terri Lindsay and Jan Mackenzie. Een ander belangrijk samenwerkingsverband is dat met de Vlaamse groep van professor Christine van Broeckhoven. Op het gebied van de moleculaire genetica is er door haar kritisch en kundig oog-in-het-zeil synergie ontstaan tussen onze beide afdelingen. Het rak naar Antwerpen en de samenwerking met Bart Dermaut is altijd voor de wind geweest.
Het aanmelden van patiënten door de hele Nederlandse vloot van neurologen en geriaters heeft voor het water onder de kiel van dit onderzoek gezorgd. Ik ben hen veel dank verschuldigd. Een apart woord van dank aan dr. John van Swieten, wegens zijn hulp aan het dementie onderzoek. Het onderzoek bleef drijvende dankzij de welwillende hulp van de familieleden van de patiënten. Een onmisbare bijdrage aan de identificatie van patiënten werd geleverd door professor Pim van Gool, neuroloog in het AMC, en Gerard Jansen, patholoog in het UMCU, die respectievelijk de 14-3-3 liquortest en (voordat hij overboord stapte) de obducties verrichtten. De prettige samenwerking met dr. Jan van Wijngaarden en Trudy van Dijk van het ministerie van VWS dient zeker vermeld te worden.
Deze studie is deels gebaseerd op data uit ERGO. Ik dank daarvoor de ERGO onderzoeksleiders en medewerkers. Naast ERGO zal ook de GRIP studie de komende jaren een belangrijke plaats innemen op de afdeling genetische epidemiologie. Ik dank de onderzoeksleiders van deze studie voor de prettige

samenwerking tijdens het opzetten van de cohort studie. Ook de medewerkenden in Sprundel, onder aanvoering van Leon Testers, kunnen niet onvermeld blijven.
En dan de bemanning van de 22e. Jeanine Houwing monsterde gelijktijdig met mij aan. Zij is mijn klankbord tijdens woelige zeeën gebleken. Marieke Dekker, scheepsmaat, was mijn 'sister in mutiny'. De virtuoze matrozen, Kristel Sleegers, Anna Schut, Ingrid Rietveld, MarieJosee van Rijn en Mark Houben, hebben gelukkig gehoor gegeven aan het 'alle hens aan dek' en hebben een extra zeiltje bijgezet: zij hebben voor dit onderzoek heel Nederland bevaren en Marie ging zelfs overzee. And Omer Njajou Tchikamgoua, cabinmate, when I really needed you, you dropped the anchor in Cameroon in screaming inability to lift it again. Hilda Kornman, supplier van genealogische data, kon zich aanvankelijk een behouden vaart niet voorstellen, maar ging enthousiast overstag toen we op de goede koers bleken te zitten. De data zijn geweldig. Ook Jeannette Vergeer heeft zwaar weer moeten trotseren voordat ze met de juiste primers glorieus de thuishaven bereikte. Gelukkig ben ik met de matrozen Wilma Luijten, Ruud Oskamp en Tessa Rademaker, die in het lab al het zware zwabberwerk hebben verricht, hetgeen echter niet mogelijk zou zijn geweest zonder de hulp van Els, Bernadette, Erik en Florencia. De hulp van Aida Bertoli Avella op de achtergrond was onmisbaar. Gezwabberd en gefourageerd werd er ook door de internationale bemanning bestaande uit Behrooz, Fakhredin, Fernando en Yurii, die op allerhande gebied zijn bijgesprongen. De mede-opvarenden uit het verleden: Arjen Slooter, Gerwin Roks en Norbert Vaessen zijn nog niet vergeten. Tenslotte is er nog Rene Molhoek, benedendeks gehuisvest, die zich inmiddels naast uitvinder van twee (bij tijd en wijle muitende) CJD en een hele grote ERF database ook de vormgever van onze website kan noemen. Hij wordt in het ruim ondermeer bijgestaan door Eric Neeleman en Nano Suwarno.
De betrokkenheid van Mária, Pieter, Ingrid, Babette en van een afstandje Maaike en Natasja, heb ik zeer gewaardeerd; zij hielden vele weekenden lang Annabel en Catharina in depot. De eensgezinde piraterij van mijn drie helden maakt mijn leven tot een geweldig avontuur.

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About the author

Esther Anne Croes was born on December 11, 1966 in beautiful Amsterdam. She attended secondary school in Alkmaar and passed the gymnasium-β in 1985. Esther studied at the University of Amsterdam, obtained a medical degree cum laude in 1994 and graduated as an art historian in modern art in the same year. For four years, she was involved in patient oriented research at the department of surgery at the Academic Medical Centre Amsterdam and worked as a resident in surgery at the "St. Lucas ziekenhuis" in Amsterdam and "ziekenhuis Hilversum". In 1998 she abandoned Amsterdam for "watertown" Rotterdam and started to work at the department of Epidemiology & Biostatistics of the Erasmus MC, where she obtained a Master of Science degree in Genetic Epidemiology and where she will continue to work in the next years. She shares her life with Michiel, Annabel and Catharina Rijsenbrij. Since 1988 they are renovating traditional cargo ships, built in the beginning of the previous century, for living and sailing.