Brain-specific proteins in the diagnosis of dementia

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Abstract

With the mean age of the population increasing, the number of patients with dementia is likely to increase. Although Alzheimer's disease is the most common form of dementia, it is recognised that other forms of dementia are more prevalent than initially thought. As drug therapies become available, it will become important to obtain an accurate clinical diagnosis. Creutzfeldt-Jakob disease (CJD) is a rare type of dementia but with the emergence of the new variant form of this disease which affects young patients, and is thought to be related to the ingestion of meat or meat products from cattle affected with bovine spongiform encephalopathy (BSE), it is possible that it may become more common. These concerns have led to the search for diagnostic tests which may help in the early diagnosis of these forms of dementia. This study investigated whether measurement of cerebrospinal fluid (CSF) brain-specific proteins could be used in the assessment of patients with dementia. Four proteins were investigated: S-100b, an astrocytic protein; and three neuronal proteins, neurone-specific enolase, tau protein and 14-3-3. A two site sandwich ELISA was developed and evaluated for the measurement of S-100b. Levels of all four brain-specific proteins were raised in the majority of patients with sporadic CJD. The presence of CSF 14-3-3 had the best combination of sensitivity and specificity for detecting sporadic CJD. CSF 14-3-3 has recently been included in the diagnostic criteria for probable sporadic CJD. The value of measurement of these proteins in the new variant, iatrogenic and familial forms of CJD was also investigated. None of the CSF brain-specific proteins investigated was of value in the diagnosis of Alzheimer's disease.

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List of Abbreviations used

AD	Alzheimer's disease
ADC	AIDS dementia complex
AIDS	Acquired immunodeficiency syndrome
Apo E	Apolipoprotein E
BSE	Bovine spongiform encephalopathy
CBD	Corticobasal degeneration
CJD	Creutzfeldt-Jakob disease
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
DEAE	Diethylaminoethyl
FTD	Frontotemporal dementia
GFAp	Glial fibrillary acidic protein
GSS	Gerstmann-Straussler-Scheinker syndrome
HIV	Human immunodeficiency virus
iNOS	inducible nitric oxide synthase
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
MAP	Mitogen-activated protein
MMSE	Mini mental state examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NNE	Non-neuronal enolase
NPV	Negative predictive value
NSE	Neurone-specific enolase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Parkinson's disease
PiD	Pick's disease

PPV	Positive predictive value
PSP	Progressive supranuclear palsy
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SF	Straight filaments
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor α
VP	Ventricular-peritoneal
VZV	Varicella zoster virus

Data Handling

Assessment of the statistical difference between groups of patients

When data from two groups of patients were compared, the Mann-Whitney, U test was used, when data from more than two groups of patients were compared, the one way ANOVA with Bonferoni modification was used. All analyses were performed using SPSS.

Assessment of diagnostic power of the CSF proteins

The assessment of the diagnostic power was calculated using the sensitivity, specificity, positive predictive power, negative predictive power and efficiency. The equations used were as follows:

Sensitivity: $\frac{\text{number of patients with disease with positive test}}{\text{number of patients with disease}}$

Specificity: $\frac{\text{number of patients without disease with negative test}}{\text{number of patients without disease}}$

Positive predictive value: $\frac{\text{number of true positives}}{\text{number of true positives + number of false positives}}$

Negative Predictive value: $\frac{\text{number of true negatives}}{\text{number of true negatives + number of false negatives}}$

Efficiency: $\frac{\text{number of true positives + number of true negatives}}{\text{total number patients tested}}$

Chapter 1 - Introduction

1.1 Background

Dementia syndromes affect 3-5% of the population (Jorm et al., 1987), and with the mean age of the population rising this frequency is likely to increase. Although Alzheimer's disease is the most common form of dementia, it is becoming increasingly recognised that other forms of dementia are more common than previously thought (Neary et al., 1988; Knopman et al., 1990). Many of these dementia syndromes have characteristic clinical features, but in the early stages of the disease accurate clinical diagnosis can be difficult. With the development of drug therapies such as the cholinesterase inhibitors, donepezil hydrochloride and rivastigmine for Alzheimer's disease, it is important that an accurate clinical diagnosis be made early in the disease course. The definitive diagnosis of these dementia syndromes is obtained by histological examination of the brain. The underlying pathology is one of selective neuronal loss with the deposition of characteristic cell inclusion bodies in many forms of dementia. The concentration of many brain-specific proteins have been reported to be elevated in the cerebrospinal fluid of patients with diseases associated with neuronal loss such as strokes (Scarna et al., 1982; Sindic et al., 1982; Mokuno et al., 1983; Hay et al., 1984; Persson et al., 1987). The aim of this study was to develop sensitive and specific methods for measuring brain-specific proteins and to evaluate whether these proteins are useful in the assessment of patients with dementia. The work focused on four brain-specific proteins: S-100b, tau protein, 14-3-3 and neurone-specific enolase; and four

dementia syndromes namely, Alzheimer's disease, frontotemporal dementia, Creutzfeldt-Jakob disease and AIDS dementia complex.

1.2 Brain-specific proteins

The concept of brain-specific proteins was first proposed by Moore and McGregor in 1965 (Moore and McGregor, 1965). These workers separated proteins from the liver and brain of rabbits, cattle, rats and monkeys using diethylaminoethyl (DEAE) cellulose chromatography followed by starch gel electrophoresis. Three low molecular weight, acidic proteins were consistently found in high concentrations in the brain fractions but were either absent or found in low concentrations in the liver fractions. These proteins were isolated and two were named 14-3-2 and 14-3-3, after their migration position in DEAE cellulose and starch gel electrophoresis, and the third, was named S-100 since it was soluble in 100% ammonium sulphate (Moore and Perez, 1968). Other structural proteins specific for the central nervous system have since been isolated. Tau protein is a microtubule-binding protein whose function is to stabilise neuronal morphology (Goedert et al., 1991a). A hyperphosphorylated form of this protein has been isolated from the paired helical filaments (PHF) of neurofibrillary tangles (NFTs) which are one of the pathological hallmarks of Alzheimer's disease (AD). Hyperphosphorylated tau has also been shown to be the major constituent of other intraneuronal inclusion bodies such as Pick bodies in Pick's disease (PiD), corticobasal bodies in corticobasal degeneration (CBD) and straight filaments (SF) in progressive supranuclear palsy (PSP). Many so called brain-specific proteins are not specific for the central nervous system, but

present in the brain at much higher concentrations than other tissues, and as such these proteins can be used as markers of brain damage.

1.3 S-100 protein

For many years after its initial discovery S-100 was thought to be the first and most abundant brain-specific protein. It is now known that S-100 belongs to a family of low molecular weight acidic calcium binding proteins, which are highly conserved throughout the vertebrate species (Donato, 1991). Initially these proteins were thought to be brain-specific but later studies have shown that S-100 proteins are found in other tissues or cells, albeit in much lower concentrations than those found in the central nervous system (Hidaka et al., 1983; Kindblom et al., 1984; Takahashi et al., 1985). These proteins contain no carbohydrate, lipid, nucleic acid or phosphate (Fano et al., 1995).

S-100 exists as homodimers or heterodimers of two subunits α and β which have molecular weights of 10.5 and 10.4 kDa respectively. These subunits contain hydrophobic regions in both the C and N termini and a Ca^{2+} binding site of the EF-type at the C-terminus (Hiezmann et al., 1991). The binding of Ca^{2+} to S-100 causes conformational changes in the protein with exposure of some aromatic amino acids, hydrophobic residues and two sulphydryl groups (Baudier and Cole, 1988a). They share 45% sequence homology, but have been shown by complement fixation and cross-immunofixation to be antigenically different (Mahadik et al., 1979). These subunits are also the product of two separate genes, the locus for the α subunit being

1q21 and that for the β subunit being 21q22.2-21q22.3. The classical S-100 proteins are S-100b (ββ), S-100ao (αα) and S-100a (αβ).

S-100b is found in the Schwann cells of the peripheral nervous system and within the of astrocytes of the central nervous system where it reaches a concentration of 10 μM (Moore, 1988). Although the majority of astrocytic S-100b is located within the cytoplasm, 5-7% is membrane bound (Rusca et al., 1972). At least 80-90% of the total S-100b pool is found within the brain, with the remainder being located in other non-neuronal tissues or cells such as testes, melanocytes and T-lymphocytes (Hidaka et al., 1983; Kindblom et al., 1984; Takahashi et al., 1985). S-100ao is found in neuronal cells, particularly hippocampal neurones, of the central nervous system in much lower concentrations than S-100b (Fano et al., 1995). Outside the central nervous system S-100ao is found in high concentrations in cardiac and skeletal muscle, and within the kidneys (Kato and Kimura, 1985; Kato et al., 1986; Takashi et al., 1988). S-100a is found within astrocytes but not Schwann cells (Fano et al., 1995).

1.3.1 Physiological functions of S-100b

The S-100 family of proteins have both intracellular and extracellular functions. It is thought that the intracellular actions of S-100b are mediated through their ability to modulate the activity of other proteins, termed target proteins, by binding to them in a calcium-dependent manner (Fano et al., 1995; Zimmer et al., 1995). S-100b has been shown to have effects on cell-signalling systems, cell metabolism and cell structure. In the presence of GTP, S-100b can stimulate the activity of cerebral

cortex membrane adenylate cyclase system and inhibit membrane bound phospholipase-C (Fulle et al., 1989; Fulle et al., 1992) thus influencing membrane induced cell transduction. S-100b has been shown to bind to fructose-1,6-bisphosphate aldolase to increase its V_{max} *in vitro* and thus may play a role in regulating cell glycolytic energy production (Zimmer and Van Eldik, 1986). S-100b can influence cell morphology by binding to tau protein to prevent its phosphorylation by calcium dependent protein kinase II (Baudier and Cole, 1988b), and by its ability to bind to glial fibrillary acidic protein (GFAp) to prevent its polymerisation (Bianchi et al., 1993).

S-100b expression by astrocytes has been reported to be stimulated by interleukin-1 (IL-1) and by cAMP (Zimmer and Van Eldik, 1989; Kahn et al., 1991; Wu et al., 1993). The extracellular effects of S-100b are to stimulate astrocyte proliferation and hypertrophy (Whitaker-Azmitia et al., 1990; Selinfreund et al., 1991), promote neuritic growth (Kligman et al., 1985), increase neuronal survival (Azmita et al., 1990), and elevate neurone and glial intracellular calcium concentrations (Barger et al., 1992). S-100b has also been reported to cause apoptosis (Fano et al., 1993). The effect of S-100b on cell proliferation appears to be dose dependent with cells increasing in number at lower doses of S-100b but when the concentration is increased the effect is reversed, resulting in cell death (Selinfreund et al., 1991). S-100b has been shown to stimulate inducible nitric oxide synthase (iNOS) in rat cortical astrocytes (Hu et al., 1996), and to cause the death of neurones co-cultured with astrocytes by the production of nitric oxide (Hu et al., 1997).

1.3.2 Methods of S-100b measurement

The methods used to measure S-100b in blood and CSF have been based on immunological assays and include microcomplement fixations tests (Moore, 1966), particle counting immunoassay (PACIA) (Sindic et al., 1982), enzyme-linked immunoassay (Kato et al., 1982), radioimmunoassay (Persson et al., 1987; Griffin et al., 1993) and enzyme-linked immunosorbent assay (ELISA) (Aurell et al., 1989; Griffin et al., 1993; Missler et al., 1995). Many of the earlier assays used antisera which could not distinguish between the different isoforms of S-100 and used a mixture of S-100b and S-100ao as calibrants, therefore they measured total S-100 rather than S-100b. This has resulted in marked differences in the references ranges quoted in the literature. A radioimmunoassay and immunoluminometric assay specific for S-100b are commercially available from Sangtec, Sweden.

1.3.3 Clinical use of measurement of cerebrospinal fluid S-100b

Raised concentrations of S-100b have been reported to occur in the cerebrospinal fluid of patients with strokes (Sindic et al., 1982; Mokuno et al., 1983; Persson et al., 1987; Lamers et al., 1995), Creutzfeldt-Jakob disease (Sindic et al., 1982; Noppe et al., 1986; Jimi et al., 1992; Otto et al., 1997a), meningitis (Sindic et al., 1982; Mokuno et al., 1983; Persson et al., 1987; Lamers et al., 1995), Parkinson's disease (Mokuno et al., 1983), brain tumours (Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986), viral infections of the central nervous system (Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986), Guillain-Barré syndrome (Sindic et

al., 1982; Mokuno et al., 1994), epilepsy (Noppe et al., 1986) and dementia (Noppe et al., 1986; Lamers et al., 1995).

There are conflicting reports concerning S-100b in the cerebrospinal fluid of patients with multiple sclerosis (MS), with some reporting an increase (Michetti et al., 1980; Massaro et al., 1985; Noppe et al., 1986; Lamers et al., 1995) and others not (Sindic et al., 1982). This apparent discrepancy appears to be due to the type and clinical status of the patients investigated. Elevated concentrations of CSF S-100b have been found in patients in the acute phase of MS but only marginally increased concentrations in the non-acute phase of the disease (Mokuno et al., 1983; Massaro et al., 1985). A study has shown that CSF S-100b concentrations increase after an exacerbation of MS, reach maximal concentrations at three weeks and then gradually decline (Massaro et al., 1985). It has been suggested that raised concentrations of CSF S-100b favour a diagnosis of relapsing-remitting rather than secondary progressive MS (Jongren et al., 1997).

It has also been suggested that raised CSF concentrations of S-100b reflect underlying cell damage and may be used to either monitor disease activity or to give information about prognosis. Measurement of S-100b in sequential CSF samples in patients with infections of the central nervous system has shown that concentrations fall rapidly in contrast to other CSF components such as cell count and total protein, and therefore reflect more accurately the clinical course in these patients (Mokuno et al., 1983). Raised concentrations of S-100b are found in patients with large cerebral infarcts, as assessed by computed tomography, but not in smaller infarcts or transient ischaemic attacks (Persson et al., 1987). In large infarcts the raised concentrations of

S-100b were related to disease severity and were maximal between eighteen hours and four days post event (Persson et al., 1987). In subarachnoid haemorrhage concentrations of S-100b in cerebrospinal fluid taken twenty-four hours post event were found to be related to clinical outcome as assessed by the Jennett and Bond scale (Jennett and Bond, 1975). A patient who had a large intracerebral haematoma and underwent surgery and later died, had CSF S-100b concentrations which increased prior to clinical deterioration post-operatively. A patient with severe head injury who had an uneventful recovery had CSF S-100b concentrations which rapidly returned to normal after an initial increase, suggesting that CSF S-100b concentrations may be useful in predicting clinical outcome (Persson et al., 1987).

A study investigating CSF S-100b and neurone-specific enolase (NSE) in patients with Guillain-Barré syndrome found that elevated concentrations of these markers were associated with a longer disease duration, whilst normal concentrations of these proteins were associated with an early recovery. There was a significant correlation between CSF S-100b concentrations and recovery time (Mokuno et al., 1994).

CSF S-100b measurement may be useful in the diagnosis of Creutzfeldt-Jakob disease (CJD). In a study measuring S-100b, NSE and CK-BB in sequential CSF samples from patients with CJD, it was found that S-100b was the only marker to remain elevated throughout the whole course of the disease (Jimi et al., 1992). In a larger study it was found that concentrations of CSF S-100b of greater than 8 ng/mL had a sensitivity of 84% and a specificity of 91% for the diagnosis of CJD (Otto et al., 1997a).

1.3.4 Clinical use of measurement of blood S-100b

The earliest report of S-100b concentrations in the blood was by Persson, who reported that increased serum S-100b reflected the increased CSF S-100b in patients with cerebral infarctions, head injury and subarachnoid haemorrhage (Persson et al., 1987). More recent studies have shown that serum S-100b concentrations were increased in patients with acute ischaemic stroke (Abraha et al., 1997; Buttner et al., 1997; Missler et al., 1997) and peak levels were found between days 1-4 post ictus (Buttner et al., 1997; Missler et al., 1997). The peak serum S-100b concentration correlated with both infarct volume and clinical outcome (Abraha et al., 1997; Missler et al., 1997). Serum S-100b levels were also raised in patients with subarachnoid haemorrhage and correlate with neurological damage and with outcome assessed at six months (Abraha et al., 1997; Weismann et al., 1997).

Increased serum concentrations of S-100b have also been reported to occur in patients with head injury (Ingebrigtsen et al., 1996; Waterloo et al., 1997), Creutzfeldt-Jakob disease (Otto et al., 1998) and patients undergoing cardiopulmonary bypass (Blomquist et al., 1997).

Serum S-100b concentrations were increased immediately after head injury and declined hour by hour (Ingebrigtsen et al., 1996). In patients with head injury, those with raised serum S-100b concentrations had a greater incidence of post concussion symptoms and were hospitalised for longer when compared to those without detectable serum S-100b (Ingebrigtsen et al., 1995; Ingebrigtsen et al., 1997). A group of head injury patients with and without detectable serum S-100b

concentrations were subjected to neuropsychological assessment at twelve months post injury. Although there was no cognitive dysfunction in either group, those patients who had measurable serum S-100b at time of head injury had slower reaction times, poorer selective attention span and poorer sustained attention span as assessed by the Seashore rhythm test (Waterloo et al., 1997).

Serum S-100b concentrations have been proposed as a marker for cerebral injury after cardiac surgery (Westaby et al., 1996; Taggart et al., 1997). Studies have shown that patients who have undergone extracorporeal circulation during the course of cardiac surgery have increased post-operative concentrations of S-100b when compared to patients who have not (Westaby et al., 1996). Some reports have suggested that the concentration of S-100b was related to the length of perfusion (Westaby et al., 1996) and others have found no relationship (Taggart et al., 1997). Patients undergoing coronary artery bypass grafting (CABG) had lower serum S-100b concentrations than patients undergoing intracardiac surgery such as valve replacements (Taggart et al., 1997).

Serum S-100b concentrations have been reported to be elevated in patients who have had a cardiac arrest (Rosen et al., 1998). Peak concentrations occur between day 1-3 post event and are related to degree of coma and length of hypoxia. Those patients who died had higher peak serum S-100b concentrations than those patients who survived. (Rosen et al., 1998).

1.4 Tau protein

Tau protein is a microtubule-associated phosphoprotein found predominantly within the axons of the neurons of the central nervous system (Goedart et al., 1991a), although a form of tau, known as big tau, has been found within the peripheral nervous system (Goedart et al., 1992a). It promotes the assembly and stabilisation of neuronal microtubules (Goedert et al., 1991a). The tau gene is located on chromosome 17q21 and contains 15 exons, 11 of which are used to encode the major tau protein isoforms (Hardy et al., 1998). Six isoforms are found in the central nervous system of healthy adults (Lee et al., 1991), which range from 352-441 amino acids in length and differ from each other by the size of the N-terminal inserts and the presence of three or four tandem repeat regions of 31-32 amino acids in the carboxyl terminal end (Figure 1.1).

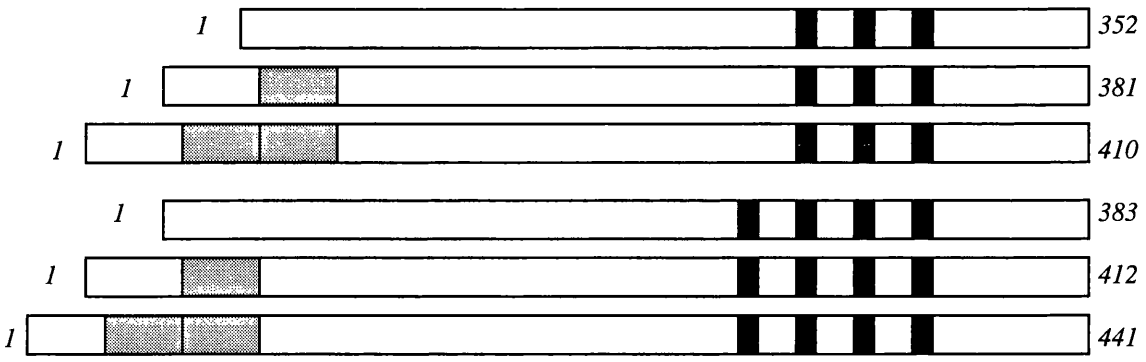


Figure 1.1 Schematic representation of the six adult tau isoforms. The region common to all isoforms is white, the stippled areas are the insertions and the black bars represent the tandem repeat regions

Studies with recombinant tau have shown that it is these tandem repeat regions in the carboxyl terminal regions which are the microtubule binding domains (Butner et al.,

1991) and each contain a characteristic Pro-Gly-Gly-Gly motif (Goedart et al., 1991b).

The expression of tau isoforms is developmentally controlled, in immature brain only the shortest isoform with three tandem repeat regions is expressed (Goedert et al., 1989). Tau protein is a phosphoprotein with two or three phosphorylation sites on all isoforms in the adult (Ksiezak-Reding et al., 1992). Foetal tau is phosphorylated at six to eight sites on the shortest isoform (Ksiezak-Reding et al., 1992). These phosphorylation sites are serine or threonine residues followed by proline, suggesting that protein kinases with a specificity for seryl-proline and threonyl-proline residues are responsible for phosphorylation. Protein kinases such as mitogen-activated protein (MAP) kinase, glycogen synthase kinase-3 and proline-directed protein kinase have been shown to phosphorylate recombinant tau on at least some of the residues seen in adult and foetal tau (Hangar et al., 1992). The phosphorylation status of a protein depends on a balance of phosphorylation and dephosphorylation, and tau phosphorylated by MAP kinase is only dephosphorylated by phosphatase 2A (Goedert et al., 1992b). The phosphorylation status of tau affects its ability to bind to microtubules, tau phosphorylated by MAP kinase has one tenth the ability of non-phosphorylated tau to bind to microtubules (Drechsel et al., 1992).

1.4.1 Degenerative diseases with abnormal tau pathology

There are a number of degenerative diseases of the central nervous system which are associated with either intracellular or extracellular inclusion bodies consisting of a hyperphosphorylated form of tau protein. These include Alzheimer's disease (AD),

Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD).

1.4.1.1 Alzheimer's disease

The most common of these disorders is Alzheimer's disease (AD) in which the hyperphosphorylated tau forms paired helical filaments (PHFs) which consist of two hyperphosphorylated tau molecules bound together in a helical fashion through the microtubule binding region to give a structure with a constriction every 80 nm and a width of 22 nm (Goedert, 1993). Straight filaments (SF) are found less frequently and consist of hyperphosphorylated tau bound together in a back-to-back arrangement, rather than a base-to-base arrangement as seen in PHF, to give a narrower width (Crowther, 1991). The phosphorylated tau seen in both of these structures is phosphorylated at many of the sites phosphorylated in foetal tau, with all six isoforms being phosphorylated (Goedert et al., 1991a). Mass spectrometry and use of phosphorylation dependent antibodies has identified many of these phosphorylation sites which include serine 202, threonine 231, and serines 235, 262 and 396 (Lee et al., 1991; Hasegawa et al., 1992; Goedert et al., 1994).

These PHF and SF together form two intraneuronal lesions: the most significant of which are the flame-shaped neurofibrillary tangles (NFTs) found in neuronal cell bodies and apical dendrites; the other being neuropil threads which are found in distal dendrites. Both lesions contain PHF as the major fibrous component with SF being the minor component. The appearance of NFTs and neuropil threads correlates with disease progression, with their presence starting in the medial temporal lobe and

gradually moving to the neocortex and allocortex as the disease progresses (Braak et al., 1991). A prospective study has shown that the density of NFTs correlated well with cognitive impairment (Dickson et al., 1995).

Along with the presence of β-amyloid plaques, the extracellular deposition of β-amyloid protein surrounded by dystrophic neuritic growth and reactive glia cells, the presence of NFTs are the histopathological hallmarks of Alzheimer's disease (Lantos et al., 1992). The hyperphosphorylated tau protein isolated from NFTs consists of a triplet of immunoreactive tau with molecular weights of 60, 64 and 68 kDa when subjected to SDS-PAGE and Western blotting (Goedert et al., 1992a).

1.4.1.2 Pick's disease

Pick's disease (PiD) is a progressive dementia associated with behavioural changes and language problems (Brun et al., 1994). It is associated with a marked severe lobular atrophy affecting both the frontal and temporal lobes. The histological hallmarks of the disease are large swollen cells called Pick cells and intraneuronal inclusions called Pick bodies (Lantos et al., 1992) which are found throughout the brain. Ultrastructurally these Pick bodies consist of bundles of disordered straight filaments with a few helical structures of 24 nm width and 160 nm periodicity (Kato et al., 1990; Murayama et al., 1990). These filaments demonstrate tau immunoreactivity and have been shown to have a similar phosphorylation pattern to that of Alzheimer's disease PHF, except for serine 262 which is phosphorylated in Alzheimer's disease but not in Pick's disease (Probst et al., 1996). The tau isolated from Pick bodies and subjected to SDS-PAGE showed a doublet of 60 and 64 kDa,

which corresponded to the smaller two bands of the three bands of PHF-tau isolated from the brains of patients with Alzheimer's disease (Delacourte et al., 1996).

1.4.1.3 Progressive supranuclear palsy

Progressive supranuclear palsy is a rare neurodegenerative disease the symptoms of which include a supranuclear down-gaze paresis and/or prominent early postural instability and progressive dementia (Collins et al., 1995). The neuropathological features are the presence of NFTs, and the absence of amyloid plaques and neuritic plaques. The NFTs are found in the subcortical nuclei of the brain stem, such as the basal ganglia and the brain stem (Hauw et al., 1994). In contrast to those seen in Alzheimer's disease, the NFTs seen in PSP are not flame shaped but globose and consist mainly of straight filaments. The hyperphosphorylated tau for PSP NFTs comprises of a doublet of proteins with molecular weights of 64 and 68 kDa.

1.4.1.4 Corticobasal degeneration

Corticobasal degeneration is a movement disorder with parkinsonian features associated with a degree of cognitive impairment. The clinical features are varied and can include memory loss, speech disturbance and behavioural changes (Schneider et al., 1997). The typical histopathological features include cortical and sub-cortical atrophy with gliosis and the presence of slightly basophilic subcortical neuronal inclusions, called corticobasal bodies (Feany et al., 1996a). Immunocytochemical and ultrastructural studies demonstrate that these bodies are similar in structure to the globose NFTs seen in PSP, and also comprise of straight

filaments of hyperphosphorylated tau made up of a doublet of 64 and 68 kDa (Ksiezak-Reding et al., 1994).

1.4.1.5 Tau-reactive glial cell inclusions

In addition to neuronal inclusions hyperphosphorylated tau has recently been demonstrated in astrocytic and oligodendroglial cells in Pick's disease, progressive supranuclear palsy and corticobasal degeneration (Ikeda et al., 1994; Ksiezak-Reding et al., 1994). The oligodendroglial cell inclusions, called coiled bodies, were more common in CBD than in PiD or PSP. The astrocytic inclusions were varied in structure. In PSP the abnormal tau was found in filaments in the proximal processes of astrocytes (Yamada et al., 1992) and in PiD the tau positive inclusions were located in the astrocyte cell body. When these astrocytes were present in the white matter the inclusion bodies resembled pale Pick bodies (Feany et al., 1996b). In CBD the abnormal tau accumulated in the distal cellular processes of reactive astrocytes, and these astrocytes formed an annular arrangement in the neuropil. The appearance was similar to a neuritic plaque except that the central core did not contain amyloid, but a central astrocyte with dilated distal processes demonstrating tau immunoreactivity (Feany et al., 1996b).

1.4.2 CSF tau in neurological disease

The development of antibodies to tau protein has enabled the concentrations of tau to be measured in CSF. Most of the studies investigating CSF tau concentrations in neurological disease have used ELISA technology, some using in-house assays

(Vandermeeren et al., 1993; Mori et al., 1995; Vigo-Pelfrey et al., 1995) and others using a commercial assay available from Innogenetics, Belgium (Arai et al., 1995; Jensen et al., 1995; Otto et al., 1997b). All of these assays measured both normal phosphorylated tau and hyperphosphorylated tau, and thus measured total tau.

The concentration of total tau in the CSF has been reported to be elevated in patients with Alzheimer's disease when compared to healthy non-demented controls (Vandermeeren et al., 1993; Arai et al., 1995; Jensen et al., 1995; Mori et al., 1995; Vigo-Pelfrey et al., 1995; Galasko et al., 1997). There was no relation between CSF total tau concentration and severity of cognitive impairment (Arai et al., 1995; Jensen et al., 1995; Vigo-Pelfrey et al., 1995) as marked elevations were seen early in the disease (Arai et al., 1995; Galasko et al., 1997). CSF total tau concentrations were not related to age at onset of disease or to the ApoE genotype (Arai et al., 1995). Increased concentrations were seen in patients with familial Alzheimer's disease and in carriers of 670/671 mutation in the amyloid precursor protein gene (Jensen et al., 1995). As CSF total tau concentrations were elevated early in Alzheimer's disease and were significantly elevated when compared to non-demented age-matched controls, it was suggested that CSF total tau could be used as an early premortem diagnostic test for Alzheimer's disease.

The problem with this idea is that elevated CSF total tau is not specific for Alzheimer's disease and elevated levels have been reported in other neurological diseases such as strokes, amyotrophic lateral sclerosis, normal pressure hydrocephalus, acquired immunodeficiency syndrome (AIDS), meningoencephalitis, multiple sclerosis, Guillian-Barre syndrome, olivopontocerebellar atrophy, hereditary

cerebellar atrophy, Herpes simplex encephalitis and Lyme disease (Vandermeeren et al., 1993; Arai et al., 1995; Jensen et al., 1995; Vigo-Pelfrey et al., 1995). Most of these conditions were distinguished from Alzheimer's disease on clinical grounds. More significantly, raised CSF total tau has also been reported to occur in other forms of dementia which were less easy to differentiate from Alzheimer's disease clinically. These forms of dementia included Creutzfeldt-Jakob disease, frontal lobe dementia, corticobasal degeneration and cortical Lewy body dementia (Arai et al., 1995; Vigo-Pelfrey et al., 1995; Arai et al., 1997; Otto et al., 1997b; Mitani et al, 1998).

The production of monoclonal antibodies specific for the hyperphosphorylated form of tau protein has enabled a specific ELISA to be developed which measured PHF-tau (Blennow et al., 1995). Using this ELISA it was found that PHF-tau was significantly raised in the CSF of patients with Alzheimer's disease when compared to age-matched healthy controls. PHF-tau was also increased in vascular dementia and frontal lobe dementia. There was a considerable overlap between the concentrations seen in Alzheimer's disease, vascular dementia and frontal lobe dementia. Thus measuring the abnormal forms of tau does not increase the specificity of CSF tau for the diagnosis of Alzheimer's disease.

1.5 14-3-3 proteins

14-3-3 proteins are a family of low molecular weight, acidic proteins having a molecular mass of 30 kDa (Toker et al., 1992). They were first described in the human brain by Boston et al., 1982a,b where they were found to constitute 1% of all

soluble brain protein. Originally these proteins were considered to be brain-specific but are now known to be distributed in other mammalian cells albeit in lower concentrations (Boston et al., 1982b; Aitken et al., 1992). There are seven brain-specific isoforms α-η, which are named after their respective elution profile using reverse phase high performance liquid chromatography (HPLC) (Ichimura 1988), and two other isoforms θ and τ located in epithelial cells and T-lymphocytes respectively (Martin et al., 1993). Of all of the brain-specific isoforms of 14-3-3 the γ isoform is thought to be the most brain-specific (Isobe et al., 1991). It has been recently shown that the isoforms α and δ are the phosphorylated forms of the β and ξ isoforms respectively (Aitken et al., 1995a). Size exclusion chromatography and crystallography have shown that 14-3-3 isoforms exist as dimers of 60 kDa (Toker et al., 1992; Jones et al., 1995).

A variety of techniques has been used to investigate the distribution of 14-3-3 proteins within the central nervous system. Early studies using immunohistochemistry demonstrated that 14-3-3 proteins were localised in neurones and autonomic ganglion cells (Boston et al., 1982a; Isobe et al., 1989). Other studies using *in situ* hybridisation with a cDNA probe showed that the η isoform gene was most strongly transcribed in Purkinje cells of the cerebellum (Watanabe et al., 1991).

The production of antisera with a high degree of specificity for each of the isoforms of 14-3-3 (Martin et al., 1993) enabled the subcellular localisation of these proteins to be investigated. The antisera were specific for the highly variable

N-terminal region of the proteins. This is the area which is involved in dimerisation and as such is hidden from view *in situ*. As a result, immunohistochemistry has not been able to reveal the localisation of individual isoforms (Martin et al., 1993). The localisation of 14-3-3 isoforms in rat brain was investigated by the production of subcellular fractions and subsequent SDS-PAGE and Western blotting with specific 14-3-3 antisera (Martin et al., 1994). This study found that 14-3-3 proteins were mainly cytosolic but a small amount, approximately 6% of that found in the cytoplasm, was membrane bound. The ε, η, γ, β and ζ isoforms were found in purified synaptic membranes but not found in myelin or mitochondria. The η, ε and γ isoforms but not β or ζ were associated with isolated synaptic junctions.

1.5.1 Physiological function of 14-3-3 proteins

The first function ascribed to 14-3-3 was as an activator of tyrosine and tryptophan hydroxylases, the rate limiting enzymes involved in the synthesis of catecholamines and serotonin respectively (Ichimura et al., 1988; Isobe et al., 1991). This activation also required the presence of calcium/calmodulin-dependent kinase II. The ε and ζ isoforms of the sheep pineal gland have been shown to co-purify with the enzyme serotonin N-acetyltransferase (NAT, EC 2.3.1.87) which is involved in melatonin synthesis. The 14-3-3 isoforms can be resolved from NAT, suggesting that the 14-3-3 proteins do not have NAT activity but may have a role in enzyme regulation and thus in conversion of serotonin to melatonin (Roseboom et al., 1994). Two proteins named Exo1 and Exo2 isolated from brain cytosol were found to stimulate Ca^{2+}- dependent exocytosis in permeabilized adrenal chromaffin cells, have been

identified as members of the 14-3-3 family (Morgan et al., 1992). There have been conflicting reports about the role of 14-3-3 in the regulation of Ca^{2+}- phospholipid dependent protein kinase C. Some reports have suggested that 14-3-3 inhibits this enzyme in a manner which does not involve competition with its substrates, ATP or the co-factors calcium or diacylglycerol (Toker et al., 1990). Other reports have found a stimulatory role with 14-3-3 activating protein kinase C activity in the presence or absence of diacylglycerol (Isobe et al., 1992). A more recently described role for 14-3-3 involves the activation of the protein kinase Raf via binding to phosphoserine. The isoforms β and ζ have been shown to interact with and possibly activate Raf protein kinase in the cytosol and membrane of mammalian cells (Freed et al., 1994). It has been suggested that 14-3-3 proteins may act as chaperone proteins to facilitate protein-protein interactions and thus may play a role in the protein cascades that are involved with intracellular signalling and cell cycle regulation (Xiao et al., 1995). The ability of 14-3-3 to act as a chaperone is possible due to the structure of the 14-3-3 dimers. The individual isoforms interact via the N-terminal regions to form a cup like structure (Aitken et al., 1995b) which contains a highly negatively charged centre which has two binding sites. It has been proposed that this centre could bind to a membrane associated protein and a series of other proteins bind to the outer surface of the protein. In this way 14-3-3 could bring proteins into close proximity and enable them to interact (Aitken et al., 1996).

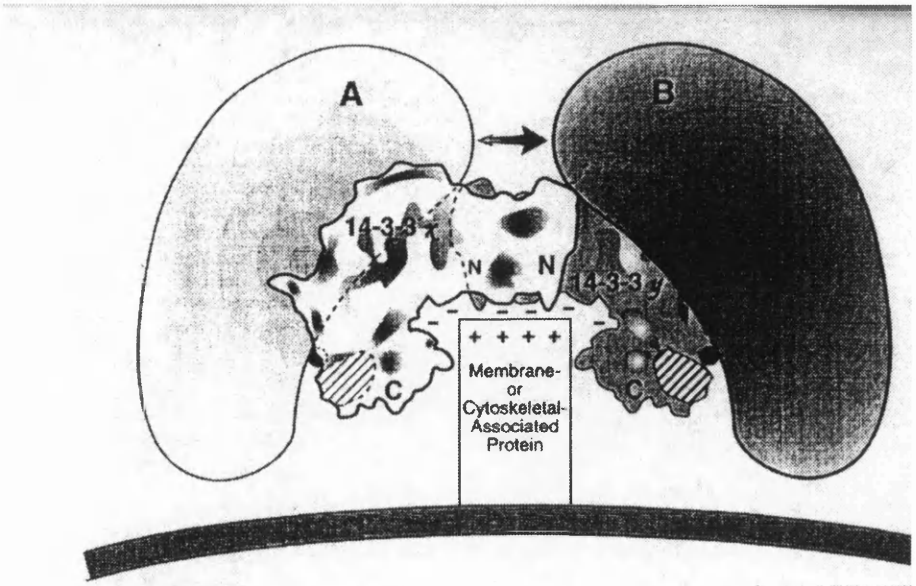


Figure 1.2 Diagram illustrating the proposed structure of the 14-3-3 dimer. Adapted from Jones et al., 1995

1.5.2 Clinical use of 14-3-3 measurement

Two proteins, p130 and p131, identified by two dimensional gel electrophoresis have been found in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease (CJD) (Harrington et al., 1986; Zerr et al., 1996). The protein p130 has a molecular weight of 26 kDa and a pI of 5.2 and p131 has a molecular weight of 29 kDa and a pI of 5.1 (Harrington et al., 1986). These proteins were found in a large percentage of patients with CJD, reports vary from 100% (Harrington et al., 1986) to 84% of histologically confirmed CJD (Zerr et al., 1996). Interestingly none of CSF samples from three patients with kuru demonstrated the presence of these proteins (Harrington et al., 1986) and only two out of six cases of familial CJD were positive for p130/p131 (Zerr et al., 1996). These proteins were not found in the cerebrospinal fluid of healthy volunteers, patients with Alzheimer's disease, acquired

immunodeficiency syndrome (AIDS) dementia, Parkinson-dementia complex of Guam, multi-infarct dementia, cerebral infarction, Parkinson's disease, progressive supranuclear palsy or encephalitis due to cytomegalovirus, mumps, and varicella viruses (Harrington et al., 1986). Some patients with Herpes simplex encephalitis, hypoxic brain damage, ischaemic stroke, subdural haematoma and brain tumours had detectable p130/p131 in the cerebrospinal fluid (Harrington et al., 1986; Zerr et al., 1996).

The protein p130 was isolated from normal brain tissue by elution from two dimensional electrophoresis gels and subsequently enzymatically digested to give a series of peptide fragments. These fragments were sequenced and the resulting amino acid sequences compared to those found on the Swiss-Prot data bank (Hsich et al., 1996). Three of the sequences obtained matched a part of the sequence of the human η isoform of 14-3-3 whilst the final sequence matched that of the bovine γ 14-3-3 isoform. Confirmation that p130/p131 proteins were members of the 14-3-3 family was undertaken by identifying the p130/p131 spots on two dimensional electrophoresis gels with specific 14-3-3 antisera (Hsich et al., 1996).

Subsequent studies using one dimensional SDS-PAGE with Western blotting have reported that 14-3-3 was detectable in 95% of patients with definite sporadic CJD and 92% of patients with probable sporadic CJD (Zerr et al., 1998). In contrast only 50% of patients with familial forms of CJD had detectable 14-3-3 (Zerr et al., 1998). A small study investigating the recently described new variant form of CJD which affects younger patients and is thought to have resulted from eating beef products from cattle with bovine spongiform encephalopathy (Will et al., 1996a; Bruce et al.,

1997; Hill et al., 1997), has found that 14-3-3 is not detected in all cases (Will et al., 1996b). 14-3-3 was absent from the cerebrospinal fluid of healthy volunteers and the majority of patients with Alzheimer's disease, but is present in some patients with Herpes simplex encephalitis, multi-infarct dementia with acute infarction, hypoxic brain damage, intracerebral metastases from a bronchial carcinoma, metabolic encephalopathy or progressive dementia of unknown cause (Hsich et al., 1996; Zerr et al., 1998).

1.6 Neurone-specific enolase (14-3-2)

After the isolation of bovine 14-3-2, specific antibodies were raised and used to establish that the protein was neuronal (Cicero et al., 1970). It was renamed neurone specific protein (Pickel et al., 1975) and finally neurone-specific enolase (NSE) when it was shown to have enolase (2-phospho-D-glycerate hydrolase EC 4.2.1.11) activity (Marangos and Zomzely-Neurath, 1976).

The enolases in mammalian tissues are dimers consisting of two of the following three immunologically distinct isoforms α, β and γ. The $\alpha\alpha$ enolase is found primarily in liver cells and the $\alpha\beta$ and $\beta\beta$ enolases are found in skeletal and cardiac muscle cells. The central nervous system contains three forms of enolase, $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$. The $\alpha\alpha$ form is found in glial cells and is identical to the enolase found in liver tissue. This form is known as non-neuronal enolase (NNE). The $\alpha\gamma$ and $\gamma\gamma$ forms of enolase are present in high concentrations in the neuronal cells of the central nervous system (Rider and Taylor 1975; Marangos et al., 1977; Marangos et

al., 1978b) and together are known as neurone-specific enolase (NSE). These enolases were initially thought to be found only within these cells but subsequent studies have demonstrated the presence of $\gamma\gamma$ enolase in neuroendocrine cells (Marangos and Schmechel, 1987) and the $\alpha\gamma$ form has been demonstrated in a number of non-neuronal tissues or cells including platelets, adrenal cells, large intestine and red blood cells (Hullin et al., 1980). Some studies have suggested that the hybrid enolase is an artifact of tissue homogenisation and does not exist *in vivo* (Schmechel and Marangos. 1983).

NNE is a larger and less acidic protein than NSE, is more prone to temperature inactivation, and is sensitive to inhibition with chloride and urea. Incubation of NNE at 50°C for 15 minutes will destroy practically all the enzymatic activity whereas NSE activity is unaffected even after 1 hour. NNE activity is completely inhibited by 0.5 M potassium chloride and by the presence of 3 M urea, whereas NSE is unaffected (Marangos et al., 1978a,b). Antisera to NNE shows no cross-reaction with NSE and suggests that these proteins are likely to be the product of two separate genes (Marangos and Schmechel, 1987). The different structural and chemical properties of NNE and NSE are summarised in Table 1.1.

Property	NNE (αα)	NSE (αγ)	NSE (γγ)
Molecular weight	87,000	82,500	78,000
Subunit molecular weight	43,500	43,500/ 39,000	39,000
Isoelectric point	7.2	/	4.7
Substrate K_m	1.3×10^{-4}	/	1.2×10^{-4}
K_a Mg	6.1×10^{-4}	/	2.4×10^{-4}
Chloride stability	no	/	yes
Urea stability (3M)	no	/	yes
Temperature stability	no	/	yes

Table 1.1 Structural and functional properties of NNE and NSE. Adapted from Marangos and Schmechel, 1987

The highest concentration of NSE was found the brain with concentrations varying from 4-21 μg/mg soluble protein. There was a wide range of staining intensities with some neurones staining up to five times more strongly than others. These differences did not appear to be related to cell type, cell size or neurotransmitter type, but maybe related to different metabolic needs within different regions of the brain (Marangos and Schmechel, 1987). Lower amounts were found in peripheral nervous tissue (0.2-1.2 μg/mg soluble protein) and in various neuroendocrine cells (0.9-8.5 μg/mg soluble protein) (Marangos and Schmechel, 1987). The neuroendocrine cells included the adrenal medulla, pineal gland, islet cells of the pancreas and other amine precursor uptake and decarboxylation (APUD) cells (Schmechel et al., 1978). Some cells which were neither neuronal nor neuroendocrine contained NSE, the most significant of these were platelets which

contained predominantly NNE, with smaller amounts of both αγ enolase and γγ enolase (Marangos et al., 1980). Other cells included erythrocytes, lymphocytes, smooth muscle cells of visceral organs, juxtaglomerular organ of kidney and smooth muscle of kidney (Brown et al., 1980; Haimoto et al., 1985). Immunohistochemical studies using monoclonal antibodies to the gamma subunit have demonstrated the presence of NSE in reactive astrocytes near tumours and in abnormal astrocytes within tumours of different grades of malignancy (Soler Federsppiel et al., 1987). NSE has been shown by immunocytochemical studies to be present in the cytoplasm, including the soma, axon and dendrites of neurones (Marangos and Schmechel, 1987), but not the nucleus or the golgi apparatus (Langley et al., 1980).

1.6.1 Methods of measurement of NSE

There are two types of analysis which have been devised to measure NSE in biological fluids, those based on immunological detection and those based on detection methods that exploit the enzymatic nature of NSE. The immunological methods include radioimmunoassay (Brown et al., 1980; Pahlman et al., 1984; Cunningham et al., 1990) and enzyme linked immunoassay (Scarna et al., 1982; Kato et al., 1983; Vermuyten, 1990). The enzymatic techniques are based on either protein separation by electrophoresis using agarose gels plus NADPH linked detection system (Wevers et al., 1983) or specific anti-NSE antisera linked to a solid phase plus an NADH linked detection system (Sorensen et al., 1988) or a bioluminescent detection system (Gerbitz et al., 1984).

1.6.2 Measurement of NSE in neurological disease

The fact that NSE is present in high concentrations in neurones and is relatively brain-specific has suggested that measurement of NSE concentrations in serum or cerebrospinal fluid (CSF) may give information about the extent of neuronal death.

Markedly raised concentrations of NSE in the CSF have been reported in patients with strokes (Scarna et al., 1982; Mokuno et al., 1983; Hay et al., 1984; Persson et al., 1987), brain tumours (Royds et al., 1981; Mokuno et al., 1983), head injury (Scarna et al., 1982, Dauberschmidt et al., 1983), bacterial meningitis (Vermuyten, 1990; Inoue et al., 1994), Creutzfeldt-Jakob disease (Wakayama et al., 1987; Jimi et al., 1992; Vermuyten, 1990; Zerr et al., 1995; Evers et al; 1998; Kropp et al., 1999) and neonatal hypoxic-ischaemic encephalopathy (Garcia-Alix et al., 1994; Thornberg et al., 1995). Smaller increases have been reported in Guillian-Barre syndrome (Vermuyten, 1990, Mokuno et al., 1994), Parkinson's disease (Mokuno et al., 1983), multiple sclerosis (Royds et al., 1983) and epilepsy (Vermuyten, 1990).
The relationship between CSF NSE concentrations and disease severity has been thoroughly investigated by studies of neurological damage such as that seen in strokes and head injury. A significant positive correlation was shown between the magnitude of CSF NSE concentration and the volume of cerebral infarct when measured by computed tomography (CT) scans in non-haemorrhagic cerebral infarctions (Hay et al., 1984). Higher CSF NSE concentrations were associated with a poorer prognosis for patients with cerebral infarction, subarachnoid haemorrhage or head injury (Scarna et al., 1982; Hay et al., 1984; Persson et al., 1987). The

appearance of NSE in the CSF was time dependent with maximal increases occuring between eighteen hours and four days post-ictus (Persson et al., 1987).

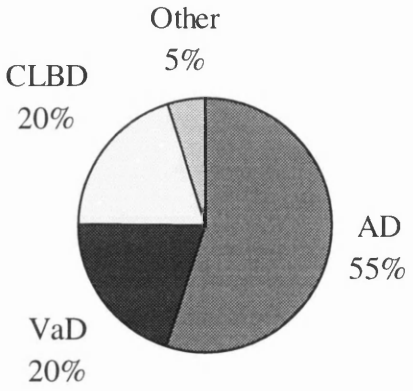
There was a close correlation between the NSE concentration in the CSF and that of the blood (Scarna et al., 1982). This has lead to a number of studies investigating the relationship between blood NSE and prognosis in patients with cerebral hypoxia, cerebral infarction, cerebral haemorrhage and head injury (Dauberschmidt et al., 1983; Schaarschmidt et al., 1993; Butterworth et al., 1996). In all groups investigated, higher blood NSE concentrations were associated with poorer outcomes (Dauberschmidt et al., 1983; Schaarschmidt et al., 1993). In cerebral hypoxia blood NSE concentrations were related to outcome, with those patients having blood NSE concentrations of greater than 120 ng/mL having a Glasgow Coma Score (GOS) of 1 (death) or 2 (vegetative state), patients with moderately raised NSE concentrations surviving with some neurological damage whilst those patients with no elevation in NSE concentrations having no permanent neurological deficit (Schaarschmidt et al., 1993). In contrast, patients with head injury, cerebral infarction or cerebral haemorrhage could have a poor outcome and show no elevation in blood NSE (Dauberschmidt et al., 1983; Schaarschmidt et al., 1993). This may be related to the extent and site of injury. The time at which blood concentrations of NSE reached their peak showed considerable variation: in a few patients there was an initial increase within twenty-four hours which rapidly declined, in the majority there was a second increase between twenty-four and seventy-two hours which may have been due to secondary neuronal death and in a final group there was a third peak which occurred after forty-eight hours and lasted for several days (Schaarschmidt et al., 1993). It was the increase in blood NSE which occurred after twenty-four hours

which often preceded the clinical deterioration, which was usually due to cerebral oedema.

1.7 Dementia syndromes

Dementia can be defined as a non-reversible cognitive impairment which may accompanied by behavioural and/or personality changes. The cognitive changes include impairment of memory, language, visuospatial skills, abstraction, calculation and judgement. The non-cognitive features may include personality changes, delusions, hallucinations, depression, mania, anxiety, aggression, purposeless hyperactivity and altered sexual activity (Absher and Cummings 1994). Dementia syndromes affect 3-5% of the population (Jorm et al., 1987) and with the mean age of the population rising this frequency is likely to increase over the next decade. The most common form of dementia is Alzheimer's disease, which accounts for over 50% of cases of dementia in patients over the age of 65 years, but only accounts for 34% of cases in patients younger than 65 years old (Figure 1.3).

a) over 65 years old



b) under 65 years old

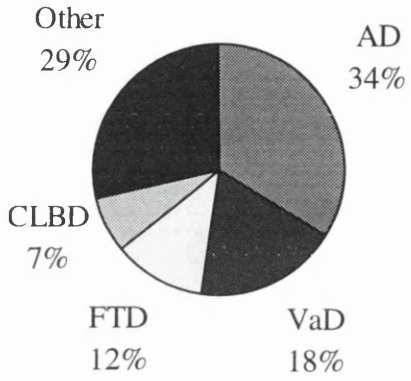


Figure 1.3 Relative frequencies of different dementia in patients a) over 65 years old and b) under 65 years old. AD: Alzheimer's disease; CLBD: cortical Lewy body disease; VaD: vascular dementia; FTD: frontotemporal dementia. Adapted from Galton and Hodges, 1999

1.7.1 Alzheimer's disease

The majority of cases of Alzheimer's disease occur sporadically but a small proportion are familial and these patients have an earlier age of onset. At present three genes have been identified, the amyloid precursor protein gene on chromosome 21, the presenillin 1 gene on chromosome 14 and the presenillin 2 gene on

chromosome 1. Mutations in these genes result in abnormal cleavage of amyloid precursor protein and the increased production of the amyloidogenic fragment $A\beta_{1-42}$. Alzheimer's disease is characterised by memory loss, with disorientation with regard time and place followed by aphasia and acalculia. The final stages of the disease may involve psychiatric disturbances such as aggression, psychosis, delusions and hallucinations. Death usually occurs between 5-10 years after the onset of symptoms. Several prospective studies which have followed patients to autopsy have shown that the accuracy of clinical diagnosis of Alzheimer's disease is over 80% (Morris et al., 1987; Tierney et al., 1988; Galasko et al., 1994; Klatka et al., 1996), but definitive diagnosis depends on the histological examination of the brain. The characteristic histological features are neuronal and synaptic loss together with the presence of senile plaques composed of β-amyloid protein, neuritic plaques and neurofibrillary tangles (NFTs). These NFTs consist of paired helical filaments (PHF) which are composed from hyperphosphorylated tau protein. Several studies have demonstrated that patients with Alzheimer's disease have elevated concentrations of tau protein and that these concentrations were raised early in the disease course (Vandermeeren et al., 1993; Arai et al., 1995; Blennow et al., 1995; Jensen et al., 1995; Tato et al., 1995; Vigo-Pelfrey et al., 1995; Galasko et al., 1997; Andreasen et al., 1998; Galasko et al., 1998; Kanai et al., 1998) . It has been suggested that elevated concentrations of tau protein may be used as a diagnostic marker for early Alzheimer's disease and enable accurate pre-mortem diagnosis to be made.

1.7.2 Frontotemporal dementia

It is increasing recognised that the incidence of non-Alzheimer's disease forms of dementia are more common than previously thought, and may make a significant contribution to all cases of dementia (Neary et al., 1988; Knopman et al., 1990). Frontotemporal dementia is one of these non-Alzheimer's diseases and presents initially with personality changes such as aggression, obsessive-compulsive behaviour and sexual disinhibition. There is preservation of memory, visuospatial and calculation skills but language disturbances such as decreased fluency make assessment difficult (Brown, 1992). There are two major forms of frontotemporal dementia, Pick's disease and frontal lobe degeneration. The histological features of Pick's disease include marked frontal or temporal lobe atrophy, intense astrogliosis, swollen ballooned neurones (Pick cells) with the presence of argentophilic neuronal inclusion bodies (Pick bodies). These Pick bodies demonstrate tau immunoreactivity and have been shown to be composed of hyperphosphorylated tau protein (see section 1.4.1.2). In the early stages Pick's disease may be difficult to distinguish from Alzheimer's disease (Litvan et al., 1997). Frontal lobe degeneration is associated with frontal or temporal lobe atrophy with neuronal loss and spongiform changes (microvacuolation) together with mild to moderate astrocytosis (Brun et al., 1994). Distinguishing between these two conditions on clinical grounds alone is difficult and for this reason the two diseases are considered together as frontotemporal dementia. A familial form of dementia known as frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) has recently been demonstrated to be caused by mutations in the tau gene. Four mutations have been

identified and all are associated with abnormal tau cell inclusion bodies (Spillantini et al., 1998).

1.7.3 Transmissible Spongiform Encephalopathies (TSEs)

Transmissible spongiform encephalopathies (TSEs) are a family of fatal neurological diseases which affect both humans and animals. The brains of patients and animals with these diseases have a typical histological appearance of microscopic holes giving the brain a sponge-like appearance, neuronal loss, reactive astrocytosis and the presence of amyloid plaques (Beck and Daniel, 1987). These amyloid plaques are distinct from plaques found in Alzheimer's disease, in that they contain a protein known as the prion protein, and as a result these diseases are often referred to as prion diseases. The prion protein is an abnormal form of a normal cellular glycoprotein (PrP^{c}) which is highly sensitive to digestion by proteinase K. The abnormal form of the prion protein (PrP^{sc}) is partially resistant to proteinase K digestion. PrP^{sc} has been shown to be able to transmit disease to experimental animals and is identical to the agent causing scrapie, a TSE affecting sheep. PrP^{sc} is resistant to many decontaminating procedures which would destroy viruses, such as ionizing and ultraviolet radiation, standard autoclaving procedures, alcohols, aldehydes such as formaldehyde, formalin and hydrogen peroxide (Advisory Committee on Dangerous Pathogens, HMSO, 1998).

1.7.3.1 Creutzfeldt-Jakob disease

Creutzfeldt-Jakob disease (CJD) was first described in 1922 (Spielmeyer, 1922) and is the most common of the human TSEs. It is characterised by a rapidly progressing dementia, myoclonus, cerebellar and/or extrapyramidal signs, and typical periodic sharp wave complexes on electroencephalogram (EEG). The onset usually occurs between the ages of 45-75 years with the peak incidence between 60-65 years. The majority (85%) of cases occur sporadically, with 10-15% of cases being familial due to mutations in the PRNP gene found on the short arm of chromosome 20 (Robakis et al., 1986). The remaining cases are iatrogenic, following the administration of contaminated growth hormone (Brown et al., 1985) or in the recipients of human dura mater (Thadani et al., 1988) or corneal grafts (Duffy et al., 1974). Gerstmann-Straussler-Scheinker (GSS) syndrome and familial fatal insomnia (FFI) are rare genetic variants of CJD and account for less than 1% of all the human TSEs.

The diagnosis of these diseases is made by the histological examination of brain tissue taken either at postmortem or from a brain biopsy. These procedures have several health and safety as well as cultural considerations. Firstly, the infective nature of the PrP^{sc} and its resistance to standard hospital sterilisation techniques means that surgical equipment used in these procedures cannot be re-used. There has been a case of CJD transmitted by the re-use of cortical electrodes (Bernoulli et al., 1977). This adds substantial costs to these investigations. There are also health and safety considerations for clinical and laboratory staff dealing with highly infectious brain material, and the problem of disposal of instruments and equipment used in

such investigations. Brain biopsy itself is not risk free and complications such as the formation of cerebral abscesses and haemorrhage may result. Finally patients may have cultural and religious objections to postmortem which precludes histological examination of brain tissue. All these reasons have spurred the search for an alternative diagnostic test for CJD.

Although CSF may contain a small amount of prion protein it is considered to be less infective than brain tissue (Advisory Committee on Dangerous Pathogens, HMSO, 1998). It is less hazardous to obtain than brain tissue, both for the patient and the clinician, and instruments used to collect the sample can be discarded at a lower cost than surgical instruments. Thus it would be advantageous if a diagnostic test could be found which used CSF rather than brain tissue.

1.7.3.2 Familial and iatrogenic CJD

Familial forms of CJD account for 15% of the total number of cases of CJD (Collinge and Palmer, 1997). These conditions are associated with either point mutations in the coding sequence of the PRNP gene or base pair repeat insertions. At present there are 12 point mutations and 9 insertions (Collinge, 1997). The discovery that the prion disease Gerstmann-Straussler-Scheinker syndrome (GSS) is also associated with a number of PRNP mutations (PrP Pro-Leu^{102}, PrP Ala-Val^{117}, PrP Phe-Ser^{198}) has led to it being considered an inherited prion disease (Hsiao et al., 1989; Doh ura et al., 1989; Dlouchy et al., 1992). Patients with GSS present with chronic cerebellar ataxia with dementia occurring much later in the disease course. The clinical course is much longer than sporadic CJD with the mean duration of

disease being five years. The presence of large numbers of PrP-amyloid plaques is a marked feature of GSS. As would be expected cerebellar pathology is quite marked with severe atrophy, neuronal loss and reactive astrocytosis.

The familial forms of CJD tend to have a longer duration of illness and an earlier age of onset than sporadic CJD. Some mutations, such as PrP Glu-Lys^{200}, may present with features indistinguishable from sporadic CJD, whilst others may present with cerebellar signs and symptoms (Collinge and Palmer, 1997). There is a wide range of clinical presentation even within a family, with some cases presenting with sporadic CJD and others having an atypical dementia of long duration (Collinge, 1997). Other genetic factors may play a part in the way the disease may manifest itself, for example patients with PrP Asp-Asn^{178} will present with sporadic CJD if codon 129 on the PRNP gene is homozygous for valine, but will present with fatal familial insomnia (FFI) if codon 129 is homozygous for methionine (Goldfarb et al., 1992).

Patients treated with human cadaveric pituitary-derived growth hormone for hypopituitary dwarfism have an increased risk of developing CJD (Brown et al., 1985). These patients present usually present with cerebellar ataxia with dementia appearing late in the disease course. (Johnson and Gibbs, 1998). The majority of these patients are homozygous for codon 129 (Johnson and Gibbs, 1998) but the disease can occur in patients who are heterozygous for codon 129, but in these cases the onset of disease is delayed (Deslys et al., 1998).

1.7.3.3 New variant CJD

In March 1996 a new form of CJD was described which affected younger patients, from age 16-39 years. These patients presented with psychiatric problems, such as anxiety, depression, withdrawal and behavioural problems; sensory symptoms such as pain in limbs and face; together with cerebellar signs and symptoms. Dementia, myoclonus, cortical blindness and akinetic mutism only occurred late in the disease, which had an unusually long course, with a median duration of 16 months (Will et al., 1996a; Zeidler et al., 1997a,b). Histological examination of the brains of these patients showed the characteristic changes associated with TSEs, namely spongiform change, neuronal loss and reactive astrocytosis. In addition these patients had kuru-like PrP amyloid plaques surrounded by a halo of spongiform change found throughout the cerebral cortex and cerebellum. These "florid" or "daisy-like" plaques are not seen in patients with sporadic CJD and are a consistent feature of the new variant form of CJD. Spongiform change was also found in the basal ganglia and the thalamus accompanied by severe thalamic astrocytosis.

The emergence of this form of CJD affecting young people and the temporal relationship to the bovine spongiform encephalopathy (BSE) epidemic, led to the possibility that this may be the human form of BSE. Studies involving the inoculation of brain tissue from patients with new variant CJD and cattle with BSE into transgenic mice (Bruce et al., 1997) and studies investigating the glycoform structure of the PrP^{sc} found in these diseases (Hill et al., 1997) have confirmed that new variant CJD and BSE are caused by the same PrP^{sc} strain.

1.7.3.4 Bovine Spongiform Encephalopathy (BSE)

In 1985 two diary cattle were noted to have become aggressive and nervous with progressive ataxia. Histological examination of the brains from these cows showed spongiform changes with astrocytosis which resembled scrapie (Wells et al., 1987). In the following years the numbers of cattle with these symptoms increased and a new TSE, called bovine spongiform encephalopathy (BSE), was described. Since 1985 170,000 cattle have become infected, with a peak incidence of 36,000 occurring in 1992 (Johnson and Gibbs, 1998). The clinical signs of BSE include aggression, increased nervousness with a wide eyed stare and a progressive ataxia (Bradley, 1997). The behavioural problems seen in these cattle has led to the disease being known as mad cow disease. Histological examination of brain tissue shows spongiform change, astrocytosis, neuronal cell loss and deposition of PrP^{sc} adjacent to and associated with areas of spongiform change (Wells et al., 1994). The consistency of the changes has allowed the diagnosis to be made from a single section of the medulla oblongata (Wells et al., 1989). Investigations into the strain of the infective agent made using transgenic mice have suggested that only one strain of agent has been involved (Bruce et al., 1994). The agent is thought to have arisen from the practice of preparing high protein concentrate feed containing meat and bone meal from waste sheep and cattle tissues. The rendering process was altered in the late 1970's to reduce the use of hydrocarbon solvent extraction of fat, and this is thought to have allowed a strain of scrapie to escape inactivation (Wilesmith et al., 1991). The epidemic was amplified by the allowing cattle infected with BSE to enter the rendering process and ultimately be fed to unaffected cattle.

1.7.4 AIDS Dementia Complex

The acquired immune-deficiency syndrome was first recognised in the late 1970's and is characterised by an acquired and profound depression of cell-mediated immunity. It is caused by infection with the human immunodeficiency virus (HIV) which is most commonly transmitted by sexual activity or the administration of contaminated blood or blood products. Infection with HIV produces a spectrum of disorders from the clinically silent seroconversion to widespread lymphadenopathy and benign systemic manifestations such as diarrhoea, weight loss and malaise (AIDS related complex). As the disease progresses the symptoms are related to the direct effects of the virus on all organ systems as well as effects of reduced cell-mediated immunity such as parasitic, fungal, viral and bacterial infections and a multiplicity of malignancies.

All parts of the central nervous system may be affected in HIV infection, this may be primary infection or secondary to immunosuppression (Adams et al., 1997). At autopsy nearly 90% of brains from patients with AIDS are abnormal (Navia et al., 1986), although only 30% of patients may present with clinical neurological abnormalities (Adams et al., 1997). The most common neurological complication of HIV infection is a dementing illness, called AIDS dementia complex (ADC). In the early stages it presents with lack of concentration and forgetfulness, then progresses to apathy, social withdrawal, inappropriate behaviour and acute mania or psychosis (Navia, 1994). The pathological changes associated with ADC are most frequent in the white matter and deep grey matter such as the basal ganglia, with the cortex being relatively spared. The common features are pallor of the white matter with reactive

astrocytosis accompanied by parenchymal and perivascular infiltrates of lymphocytes, macrophages and microglia. Similar changes are seen in the deep grey matter and severity reflects that of the white matter changes.

Other common neurological complications of HIV infection are opportunistic infections of the CNS, typical pathogens include toxoplasma, cytomegalovirus, cryptococcus, Herpes simplex and zoster; and primary cerebral lymphoma (Brew et al., 1988). In the early stages ADC complex can be difficult to distinguish between clinical depression and early signs of CNS infection or infiltration.

1.8 Problems with differential diagnosis of patients with dementia

Although many forms of dementia have characteristic clinical features, it may be very difficult to distinguish between them in the early stages of disease. Magnetic resonance scanning may be able to identify regions of brain atrophy, such as the hippocampus in Alzheimer's disease and the frontal and/or temporal lobes in frontotemporal dementia, but the degree of brain atrophy may be small in the early stages making accurate diagnosis difficult. At present definitive diagnosis is made by the histological examination of brain tissue, which is obtained either at autopsy or from a brain biopsy. The examination of brain tissue at postmortem provides much information about the relative frequencies of the different forms of dementia, but it does not give an early diagnosis. There are a number of complications which may result from taking a brain biopsy, such as the risk of intracranial haematoma or cerebral infection, which make this an unsatisfactory approach. In patients with suspected Creutzfeldt-Jakob disease there are the health and safety aspects to

consider. As the abnormal form of the prion protein PrP^{sc} is not destroyed by normal sterilisation techniques, the instruments used for obtaining the brain tissue cannot be reused. As these instruments are expensive this adds a financial burden to this investigation.

There are a number of neuropsychological tests which are used to evaluate patients with cognitive impairment. The most widely used of these is the mini mental state examination (MMSE) which is a series of tests designed to assess the patient's orientation, registration, attention and calculation ability, recall and language. It is marked on a scale from 0 to 30, with 30 representing a perfect score and less than 23 considered as evidence of sufficient cognitive impairment for the diagnosis of dementia (Cockrell and Folstein, 1988). This particular test is insensitive at detecting abnormalities of the frontal lobe, and patients with frontotemporal dementia may perform within the normal range despite widespread frontal and/or temporal atrophy.

The diagnosis of patients with dementia is complicated by the lack of a set of definitive diagnostic tests. The aim of this study is to see whether the measurement of brain-specific proteins in the cerebrospinal fluid of patients with dementia can help in the differential diagnosis of these patients.

Chapter 2 - Development of S-100b ELISA

2.1 Introduction

The measurement of brain-specific proteins has been hampered in the past by the quality of antisera available, which have either lacked specificity or high avidity. This has resulted in assays which suffered from poor sensitivity and/or cross reactivity with closely related proteins. As techniques for purifying proteins and producing antisera have improved so has the quality of the antisera available for these proteins.

In the past decade a number of commercially available assays have been produced for some brain-specific proteins. There are a number of disadvantages to using these assays for measuring CSF concentrations of brain-specific proteins. Firstly these assays are often designed to measure high concentrations of proteins which occur in some types of malignancy, for example high NSE in the serum of patients with neuroblastomas or oat cell carcinoma, and serum S-100b in melanoma. These assays are therefore not ideal for measuring the lower concentrations which are found in CSF. Secondly they are expensive and often require specialised equipment to use.

This part of the study was designed to develop a sensitive and specific assay for S-100b, which was robust, easy to use and relatively inexpensive, and where possible the results were compared with commercially available assays.

2.2 S-100b protein

S-100b belongs to a family of calcium binding proteins which include the calmodulins. The closely related protein, S-100a, shares 45-50% sequence homology with S-100b and many of the early assays for S-100 could not distinguish between these two forms (Sindic et al., 1982; Kato et al., 1982; Noppe et al., 1986; Persson et al., 1987). Recently monoclonal antisera have become available which are specific for either the alpha or the beta forms of S-100.

2.3 S-100b ELISA method

Recipes for buffers are given in Appendix A.

Wells of polystyrene 96 flat bottomed well microtitre plate (Nunc-Immuno™ Maxisorb, Product No: 4-30341A, Life Technologies, Paisley, UK) were coated with 100 µL of 0.67 M barbitone buffer, pH 8.6 containing 1 mM calcium and 1 µg monoclonal anti-bovine S-100b (Product No: S2532, Sigma-Aldrich Company Ltd, Poole, UK). The microtitre plates were incubated overnight at 4°C and then each well was washed with 4 x 250 µL of 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05% Tween 20 (wash solution). Unbound protein binding sites were blocked by incubating each well with 250 µL of 1% BSA in PBS for 30 minutes. The microtitre plate was washed as before and 50 µL of 0.1% BSA in 0.67 M barbitone buffer, pH 8.6 containing 1 mM calcium lactate (incubation buffer) was added to each well. Fifty microlitres of bovine S-100b standard (Product No: SP1467, Affiniti Research Products, Exeter, UK),

control samples, CSF or serum was added in duplicate to appropriate wells of the microtitre plate. The microtitre plate was incubated for 1 hour at 37°C before being washed as previously described. One hundred microlitres of horseradish peroxidase conjugated rabbit anti-bovine S-100b antiserum (Supplied on request, DAKO Ltd., Cambridge, UK) diluted 1:1000 with incubation buffer was added to each well and the plate was incubated for 1 hour at room temperature. The microtitre plate was washed and 100 µL of 1 mg/mL o-phenylenediamine in 0.02 M sodium acetate buffer, pH 5.0 containing 0.3% hydrogen peroxide was added to each well. The microtitre plate was incubated for 30 minutes in the dark and the enzymatic reaction was stopped by the addition of 50 µL of 1 M hydrochloric acid. The absorbance was read at 492 nm with blanking at 405 nm using an Anthos 2001 Plate Reader (Denley Instruments, Sussex, UK).

2.3.1 Calibration of S-100b ELISA

Bovine S-100b (Product No: SP1467, Affiniti Research Products, Exeter, UK) was diluted with 0.1% BSA in PBS to give concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 ng/mL. Aliquots of these were stored at -20°C. Each batch of standards was calibrated by analysing the new batch in parallel with the old batch on at least 20 separate occasions. Figure 2.1 shows a typical standard curve.

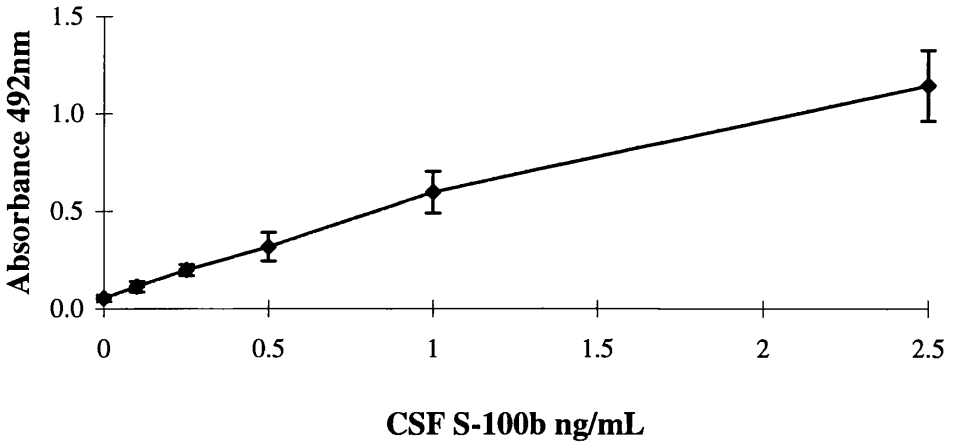


Figure 2.1 Typical S-100b standard curve. Each point represents the mean ± SD of ten separate investigations

2.3.2 The lower limit of detection

The lower limit of detection was calculated from the mean + 3SD of at least twenty separate replicate analyses of S-100b free sample (incubation buffer). The lower limit of detection was found to be 0.04 ng/mL.

2.3.3 Within and between batch precision of S-100b ELISA

The within batch precision (CV%) was calculated from the mean ± SD of at least twenty replicate analyses of pooled CSF spiked with S-100b, performed on a single microtitre plate. This was repeated three times and the mean CV% calculated. The within batch precision was found to be 9.3% and 5.6% at S-100b concentrations of 0.38 ng/mL and 0.8 ng/mL, respectively. The between batch precision was calculated from at least twenty replicates analyses of pooled CSF spiked with S-100b performed on separate microtitre plates. The between batch precision was found to

be 8.9% and 8.1% at S-100b concentrations of 0.12 ng/mL and 0.34 ng/mL, respectively.

2.3.4 The recovery of S-100b added to CSF

The ability of the ELISA to measure S-100b added to CSF was tested by analysing pooled CSF to which a known amount of S-100b had been added. Both the unspiked and spiked CSF samples were analysed and the difference in S-100b concentration calculated as a percentage of that added to the spiked sample. The recovery of 0.5 ng/mL S-100b was found to be 94%.

2.3.5 The linearity of the ELISA

Three samples with raised CSF S-100b concentrations were diluted with incubation buffer to give a set of serial dilutions. The S-100b concentrations in these samples were measured and found to give a linear relationship (Figure 2.2).

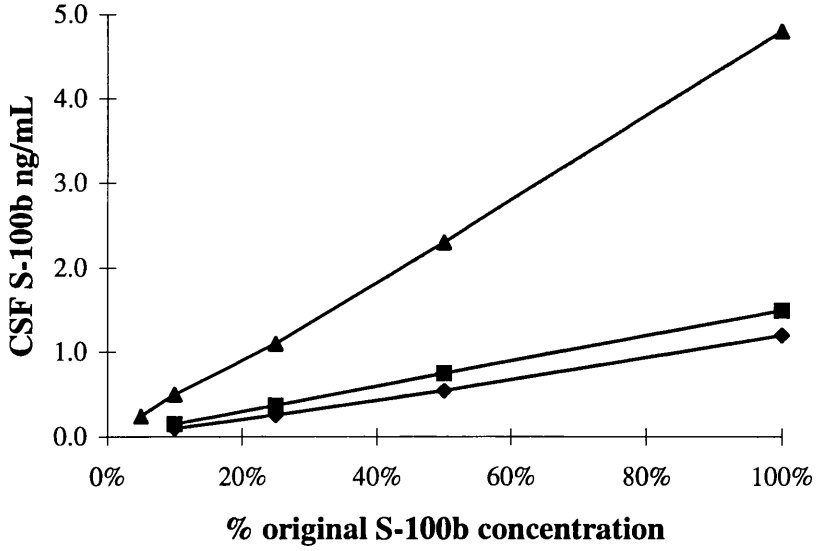


Figure 2.2 The effect of serial dilution of three CSF samples with elevated S-100b

2.3.6 Specificity of ELISA for S-100b

The specificity of the ELISA for the beta subunit of S-100 was investigated by analysing increasing concentrations of the closely related alpha subunit of S-100. S-100a (Product No: SP 1466, Affiniti Research Products, Exeter, UK) standards of 10, 50, 100, 250, 500 and 1000 ng/mL were prepared in PBS containing 0.1% BSA. These standards were analysed using the S-100b ELISA and only the 1000 ng/mL standard gave an appreciable result of 0.05 ng/mL. This related to a cross reactivity of 0.005% at a S-100a concentration of 1000 ng/mL. Thus the assay was highly specific for S-100b.

2.3.7 Effect of the calcium concentration of the barbitone buffer on assay performance

The effect of calcium on the structure of S-100b (Calissano et al, 1969) and on the performance of some assays has been well documented (Sindic et al., 1982). To find the calcium concentration which gave the optimal dose-response curve, incubation buffers containing no calcium, 0.5 mM, 1.0 mM, 2.5 mM and 5.0 mM calcium were prepared. The effect of calcium concentration on the S-100b standard dose-response is shown in Figure 2.3. The results shown are the mean of three separate investigations. A calcium concentration of 1.0 mM gave the most linear response and was used in all subsequent analyses.

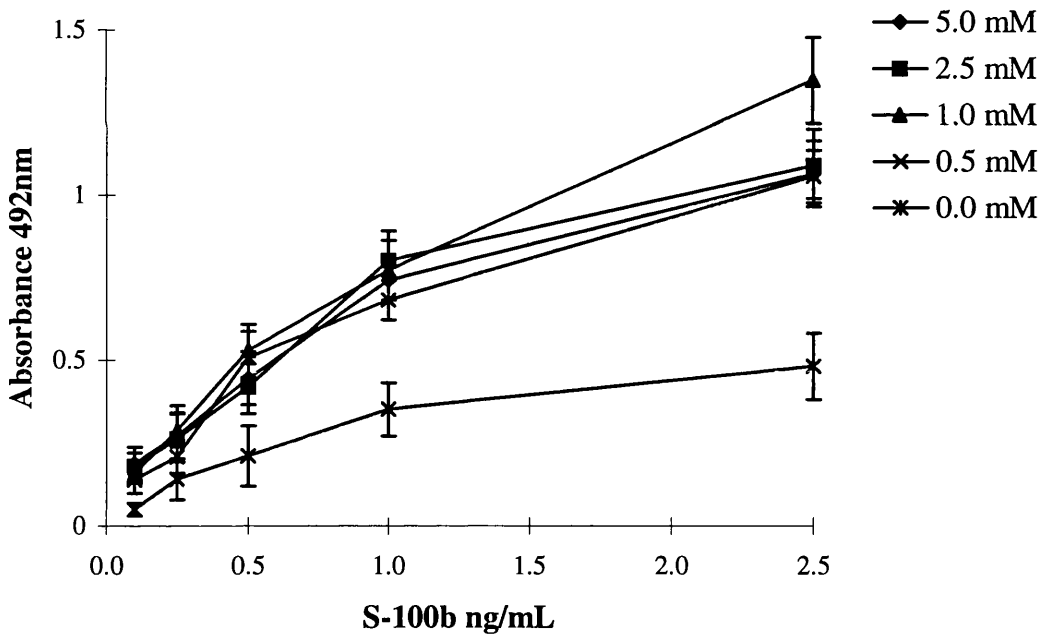


Figure 2.3 The effect of increasing calcium concentrations (0, 0.5, 1.0, 2.5 and 5.0 mM) on the absorbance of S-100b standards. Each point represents the mean ± SD of three separate investigations

2.3.8 Minimum sample volume required

Increasing volumes of S-100b standards were analysed to investigate the minimum volume of sample required. A set of standard curves was set up using 10 µL, 25 µL, 50 µL and 100 µL of sample. The maximal and most linear response was obtained using 50 µL of sample.

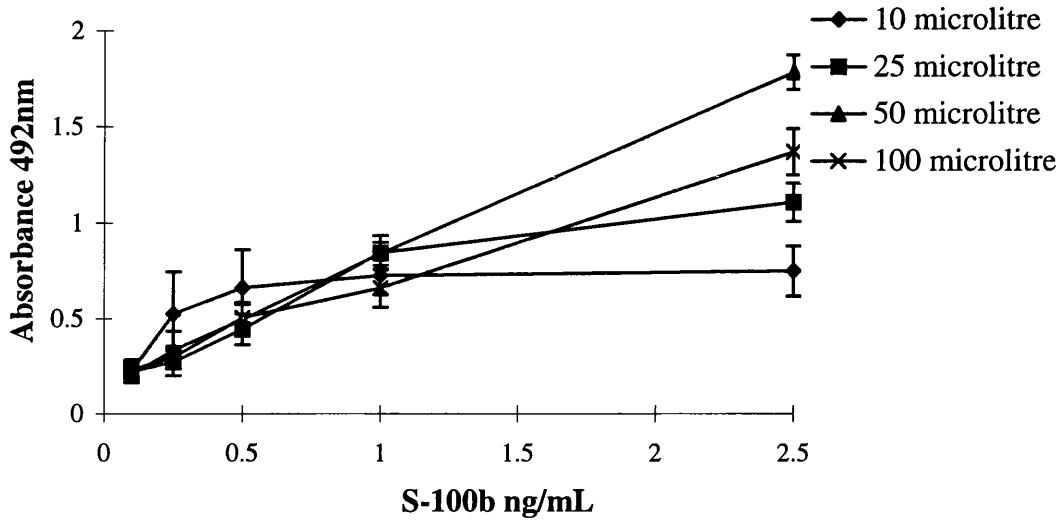


Figure 2.4 The effect of increasing sample volume on the shape of the standard curve. Each point represents the mean ± SD of three separate investigations

2.3.9 The effect of different sample incubation temperatures and times on the S-100b standard curve

The effect of different sample incubation temperatures and times on the standard curve was investigated by incubating S-100b standards at 4°C, room temperature and at 37°C for different lengths of time. Four different combinations of temperature and time were chosen; 4°C for 5 hours and overnight, room temperature for 5 hours

and 37°C for 1 hour. Although incubating the samples at 4°C overnight gave the highest absorbances, it also gave the highest background and gave a relatively flat dose response curve between 1.0-2.5 ng/mL. The lowest absorbances were obtained by incubating the samples at 4°C for 5 hours, but the best dose response curves were obtained by incubating the samples at either room temperature for 5 hours or 37°C for 1 hour. The latter combination was chosen as it required less time.

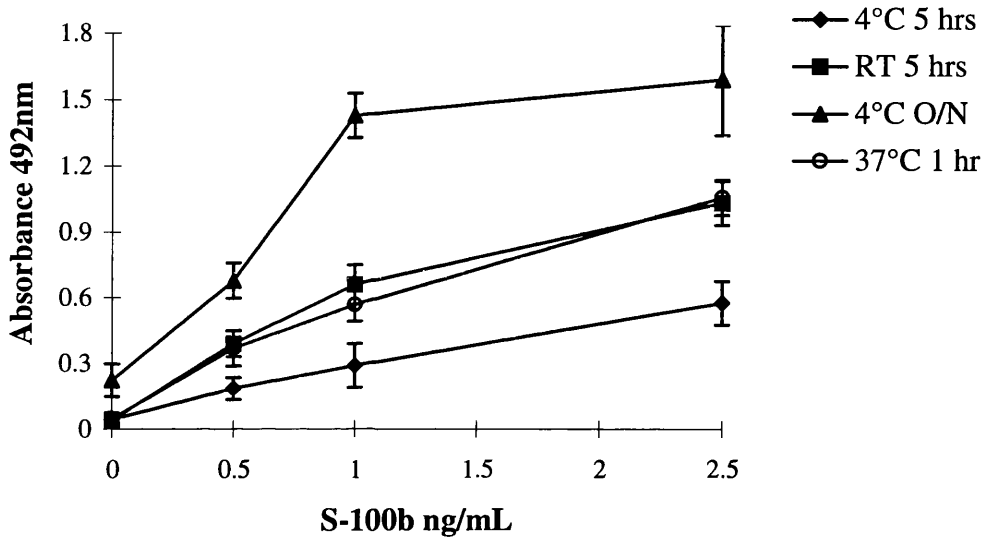


Figure 2.5 Effect of time and temperature of sample incubation on the S-100b dose-response curve. Results expressed as mean ± SD of three replicate analyses

2.3.10 Effect of storage temperature on CSF S-100b concentrations

Aliquots of three separate CSF samples were stored at both -20°C and at 4°C in polypropylene tubes. Each CSF sample was analysed on day 0 and at intervals over the next 55 days. The results were expressed as a percentage of the original value and are shown in Figure 2.6. CSF S-100b concentrations were stable in CSF

samples stored at -20°C and at 4°C for at least 30 days after which time the concentrations were seen to gradually decline.

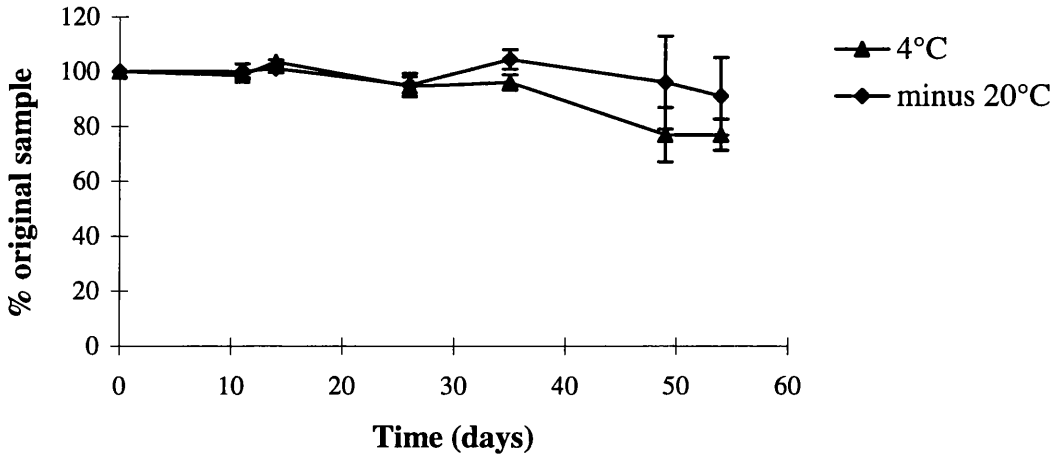


Figure 2.6 The effect of storage conditions on CSF S-100b concentrations. The results are expressed as the percentage of the original value. The results shown are the mean ± SD of three CSF samples

2.3.11 Effect of repeating freezing and thawing on CSF S-100b concentrations

CSF S-100b concentrations were measured in four CSF samples on the day they were received by the laboratory. These samples were subsequently stored at -20°C in polypropylene tubes and subjected to a number of freeze-thaw cycles over a four week period. S-100b concentrations were measured after each freeze-thaw cycle and were expressed as a percentage of the original S-100b concentration. The results are shown in Figure 2.7.

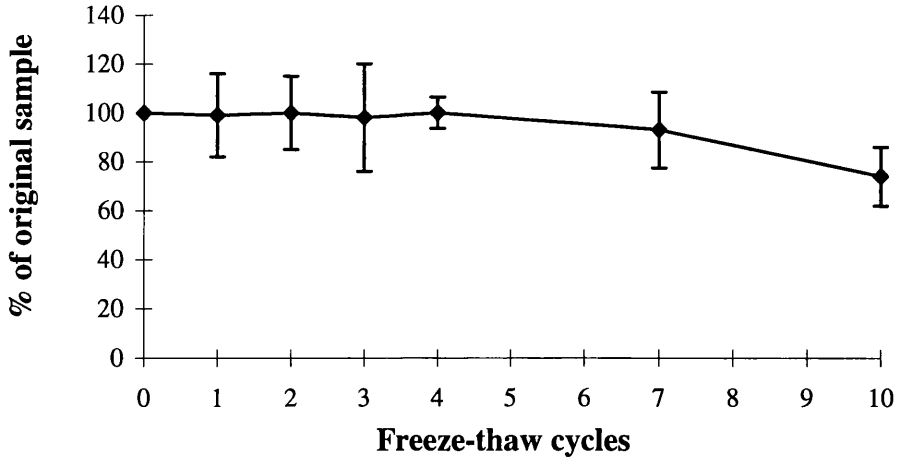


Figure 2.7 The effect of freeze-thaw cycles on CSF S-100b concentrations. The results are expressed as the percentage of the original value. The results shown are the mean ± SD of four CSF samples

CSF S-100b concentrations were able to withstand at least four freeze-thaw cycles, after which the concentrations may be affected.

2.4 Comparison of in-house S-100b ELISA with commercially available assays

2.4.1 S-100 micro-enzyme linked immunoassay

The calibration of the in-house S-100b ELISA was compared with a commercially available S-100 ELISA called S-100 micro-EIA (InRo BioMedTek AB/ Medisera AB) supplied by Affiniti Research Products. The S-100 micro-EIA is a double monoclonal-antibody enzyme immunoassay. Antibodies directed against S-100 are bound to the surface of the wells of a microtitre plate, and react with S-100 in the sample. After washing a second monoclonal antibody against S-100 which is

conjugated to β-galactosidase, is added. The conjugated anti S-100 antibody binds to the bound S-100 in a sandwich. The β-galactosidase is released by the addition of the reducing compound glutathione and reacts with the colourless substrate o-nitrophenol-β-galactoside to form yellow o-nitrophenol and colourless galactose. The absorbance at 410 nm is proportional to the amount of S-100 in the sample.

2.4.2 Comparison of the in-house S-100b ELISA and the S-100 micro-EIA calibration

The standards supplied with the S-100 micro-EIA assay were assayed using the in-house S-100b ELISA. The concentrations measured using the in-house S-100b ELISA were compared to those quoted in the literature accompanying the commercial assay, the results are shown in Table 2.1.

Micro-EIA S-100 standard concentrations (ng/mL)	Measured S-100b concentration using in-house ELISA (ng/mL)	Measured S-100b (in-house)/ quoted S-100 micro-EIA
0	0	0
0.15	0.08	0.53
0.5	0.09	0.18
1.5	0.16	0.11
5.0	0.48	0.10

Table 2.1 S-100b concentration of S-100 micro-EIA standards when assayed using the in-house S-100b ELISA

The in-house S-100b ELISA only detected an average of 23% of the S-100 micro-EIA standards. The information which accompanied the kit did not contain detailed information about the composition of the S-100 standards and it is possible that these standards contained a mixture of S-100b, which is detected by the in-house S-100b ELISA, and S-100a which is not. If this is true, the monoclonal antibodies in the S-100 micro-EIA will be able to measure both the α and β subunits of S-100 and as a result should be able to measure all of the S-100b in the in-house standards. To check this, the in-house standards were measured using the S-100 micro-EIA and the results are shown in Table 2.2.

In-house S-100b standard concentrations (ng/mL)	**Measured S-100 concentration using S-100 micro-EIA (ng/mL)**	**Measured S-100 (micro-EIA)/ S-100b in-house value**
0	0	0
0.5	0.8	1.6
1	1.05	1.05
2.5	2.0	0.8
5.0	4.8	0.96

Table 2.2 S-100 concentration of in-house S-100b standards when assayed using the S-100 micro-EIA

The S-100 micro-EIA measured nearly all the amount of S-100b in the in-house standards. This suggested that the S-100 micro-EIA measured both S-100a and S-100b and that the standards supplied in the S-100 micro-EIA assay contained both forms of S-100.

2.4.3 Comparison of the in-house S-100b ELISA and the Sangtec® S-100 IRMA calibration

The in-house S-100b ELISA was compared to a second commercially available assay, the Sangtec® S-100 IRMA, supplied by Cambridge Life Sciences, Cambridge, UK. This method uses two capture monoclonal antibodies against S-100 coated on to a solid phase bead and a third beta subunit specific monoclonal antibody labelled with I^{125} as a detector antibody. A direct comparison between the two S-100 methods was not possible as the National Hospital for Neurology and Neurosurgery did not have the necessary facilities to measure and handle radioisotopes. Nevertheless an investigation into the calibration of the two methods was possible by comparing the two sets of standards.

The standards provided in the Sangtec® S-100 IRMA kit were assayed using the in-house ELISA and the concentrations obtained compared to those assigned to the standards. The results are shown in Table 2.3.

Sangtec S-100® IRMA S-100 standard concentrations (ng/mL)	Measured S-100b concentration using in-house ELISA (ng/mL)	Measured S-100b (In-house ELISA)/ S-100 (Sangtec S-100® IRMA) value
0	0.07	0
0.1	0.08	0.8
0.5	0.18	0.36
1.0	0.32	0.32
5.0	2.54	0.5

Table 2.3 S-100b concentration of Sangtec® S-100 IRMA standards when assayed using the in-house S-100b ELISA

The in-house ELISA measured approximately 50% of the amount of the Sangtec® S-100 IRMA standards. The data sheet accompanying the Sangtec® S-100 IRMA contained little information as to the composition of the standards. To check whether these standards contained S-100a, they were measured using the S-100 micro-EIA which measures both S-100b and S-100a. The results are shown in Table 2.4.

Sangtec® S-100 IRMA S-100 standard concentrations (ng/mL)	Measured S-100 concentration using micro-EIA (ng/mL)	Measured S-100 (micro-EIA)/ S-100 (Sangtec® S-100 IRMA) value
0	0.6	
0.5	0.65	1.3
1	0.7	0.7
5	1.8	0.36
10	5.0	0.5
20	10.7	0.5
60	41.9	0.7

Table 2.4 S-100 concentration of Sangtec® S-100 IRMA standards when assayed using the S-100 micro-EIA

The micro-EIA S-100 measured on average 70% of the Sangtec® S-100 IRMA standards. This suggested that although there may be S-100a in the Sangtec® S-100 IRMA standards this did not entirely account for the difference between their values and those of the in-house standards.

2.4.4 Comparison of the in-house S-100b ELISA and the Sangtec® S-100 IRMA using patient samples

To investigate whether this difference in calibration was paralleled by a difference in S-100 results using patient samples, 28 serum samples from three patients with severe head injury were measured using the Sangtec® S-100 IRMA and the in-house S-100b ELISA. The serum samples were analysed using the Sangtec® S-100 IRMA

at the Clinical Biochemistry Department at The Royal London Hospitals NHS Trust. The results obtained using the in-house S-100b ELISA were lower, being approximately 40% of those obtained using the Sangtec® S-100 IRMA (Figure 2.8). The correlation between the two methods was good ($r^2 = 0.93$) when Sangtec® S-100 IRMA results of below 1 ng/mL were excluded (Figure 2.9). This probably reflects the insensitivity of the Sangtec® S-100 IRMA at this level where results were obtained by extrapolating below the bottom calibrant of the standard curve.

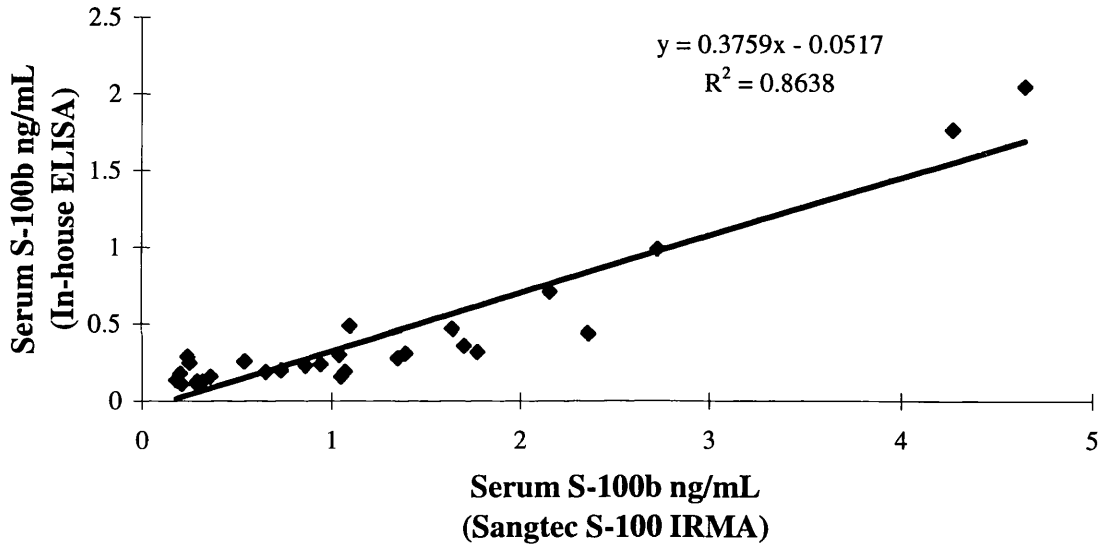


Figure 2.8 Correlation between all serum S-100 results from patients with head injury when measured using Sangtec® S-100 IRMA and in-house S-100b ELISA

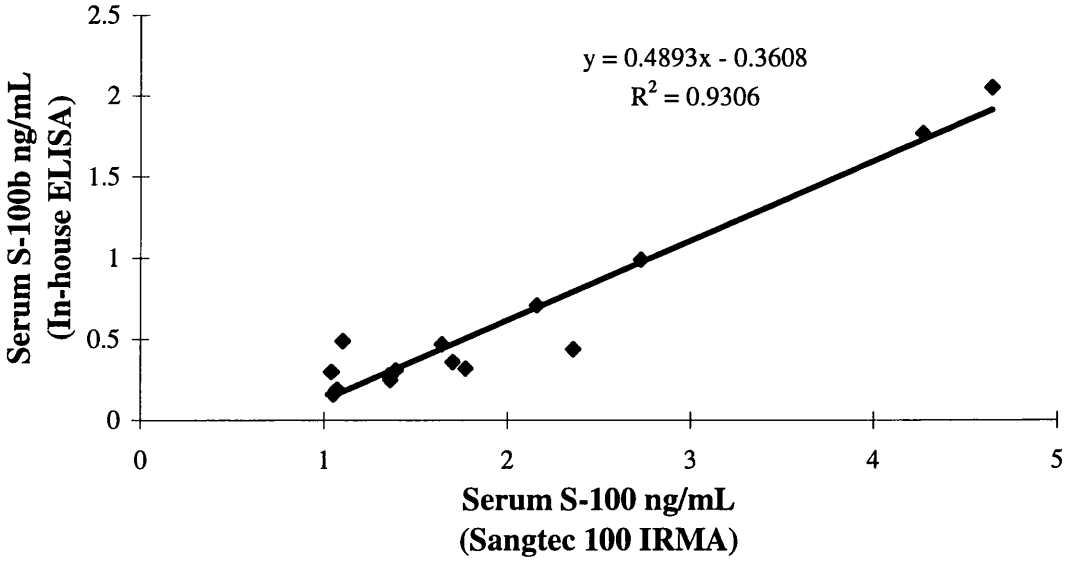


Figure 2.9 Correlation between serum S-100 results from patients with head injury when measured using Sangtec® S-100 IRMA and in-house S-100b ELISA. Sangtec® S-100 IRMA S-100b concentrations of less than 1 ng/mL excluded

The difference between the results obtained with these two assays can be explained by the difference in calibration. The calibrants used in the Sangtec® S-100 IRMA may possibly contain S-100a as well as S-100b, but in addition to this there was a difference in the assigned values. This difference could not be fully explained even after discussion with the company involved.

The reference ranges quoted in the literature for CSF concentrations of S-100b in patients with non-neurological disease vary considerably. Persson et al., 1987 quoted a value of <1 to 6.8 ng/mL, Noppe et al., 1986 a value of less than 0.8 ng/mL, Van Engelen et al., 1992 a range of 0.22-1.21 ng/mL and Mokuno et al., 1983 a value of 0.16-0.52 ng/mL and Missler et al., 1995 a range of 0.94 - 1.92 ng/mL. The reference range obtained using the method described in this section (0.18 ± 0.09 ng/mL) agreed closely with that obtained by Mokuno et al., 1983. The reasons for

the widely differing reference ranges obtained using these methods, are the different calibrants used and the specificities of the antibodies used. Until an international calibrant is produced, these differences in S-100b concentrations will persist and mean that direct comparison of results from different studies will not be possible. It also highlights the importance of obtaining method specific reference ranges.

In conclusion the in-house ELISA described in this section is sensitive, specific for S-100b, and cheaper than the commercially available assays.

Chapter 3 - Cerebrospinal fluid concentrations of S-100b

3.1 Introduction

Cerebrospinal fluid concentrations of S-100b were measured in 404 CSF samples from patients with a variety of neurological conditions, using the ELISA assay described in the Chapter 2. The majority of these samples were obtained from The National Hospital for Neurology, with a smaller number of samples being sent to the laboratory from hospitals within the United Kingdom or from within Europe. All CSF samples received by the laboratory were centrifuged on receipt and the supernatant frozen at -20°C within 24 hours. Wherever possible, CSF samples received from hospitals other than The National Hospital for Neurology and Neurosurgery, were centrifuged and stored at -20°C before being sent to the laboratory on dry-ice. The final clinical diagnosis was obtained from the discharge summary; if this was unclear the medical records were examined by a departmental neurologist. The diagnoses in patients with suspected Creutzfeldt-Jakob disease were confirmed by neurologists at The National CJD Surveillance Unit, Edinburgh.

Serum and CSF samples from cattle suspected of having bovine spongiform encephalopathy (BSE) were also investigated. These samples were provided by the Ministry of Agriculture, Food and Fisheries (MAFF) central veterinary laboratory in Weybridge, Surrey.

3.2 CSF S-100b in control subjects

One of the major problems in the investigation of CSF proteins is the lack of a suitable source of control material. As obtaining CSF by lumbar puncture can cause headache and is not without risk, it is hard to find healthy volunteers willing to undergo such a procedure. Consequently the control data in this study comes from patients with various diseases who have had a lumbar puncture taken as part of their diagnostic work up.

Twenty-one patients (mean age 43.3 ± 14.3 years, range 27-74 years; 7M :14F) with no evidence of organic brain disease at the time of discharge were used as controls, of these patients 9 had headaches, 8 had various neuralgia and 4 had psychological syndromes. The cerebrospinal fluid concentration of S-100b in this group of patients was 0.18 ± 0.09 ng/mL (mean ± SD). The CSF S-100b concentration gradually increased with age ($y = 0.0021x + 0.086$) (Figure 3.1). This is in agreement with Van Engelen et al., 1992. The reference range for patients greater than 50 years old was 0.22 ± 0.05 ng/mL. An upper limit of normal was taken to be 0.39 ng/mL. There was no affect of gender on the CSF S-100b concentrations with the concentration being 0.18 ± 0.09 ng/mL (mean ± SD) in females and 0.18 ± 0.04 ng/mL (mean ± SD) in males.

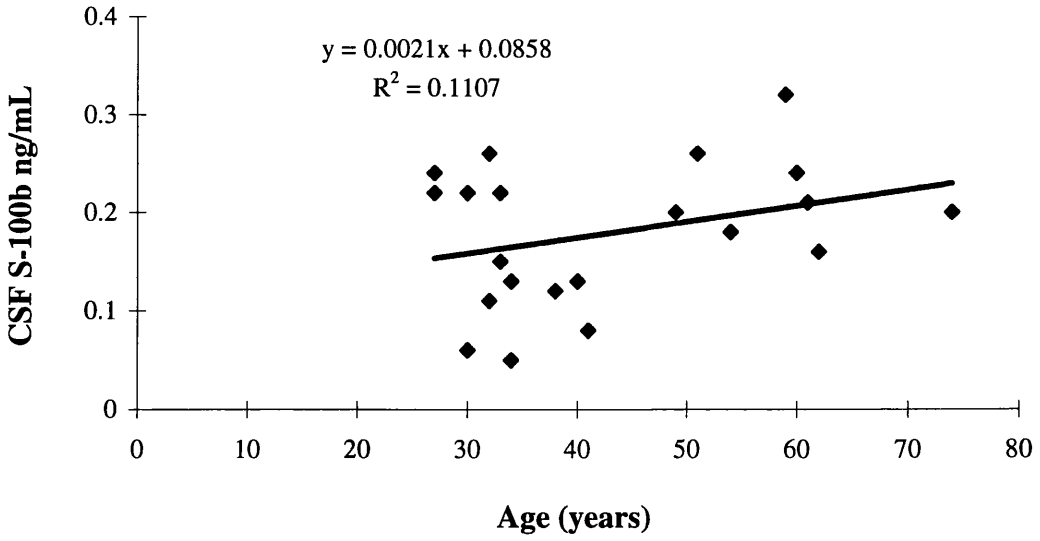


Figure 3.1 The effect of age on CSF concentrations of S-100b

3.3 CSF S-100b concentrations in patients with central nervous system (CNS) tissue damage and comparison with CNS inflammation

Cerebrospinal fluid concentrations of S-100b have been reported to increase in conditions associated with neuronal loss, such as brain tumours and strokes (Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986; Persson et al., 1987; Lamers et al., 1995), and in inflammatory diseases of the central nervous system, such as meningitis and Guillain-Barré syndrome (Sindic et al., 1982; Mokuno et al., 1983; Persson et al., 1987; Mokuno et al., 1994; Lamers et al., 1995). Much of the earlier work investigating CSF S-100 concentrations was done using methods which failed to distinguish between the alpha and beta subunits. The aim of this part of the study was to use the sensitive and beta subunit specific ELISA previously described to investigate CSF S-100b concentrations in a variety of neurological conditions.

The first group of patients studied was a group associated with acute damage to central nervous system (CNS) tissue due to diseases such as strokes, space occupying lesions such as brain tumours and finally patients with slowly progressive damage to the CNS due to either increased intracranial pressure or due to compression of the brain. These patients with CNS damage were compared to patients with inflammatory diseases of the central nervous system. The synthesis and release of S-100b has been shown to be increased by interleukin-1 (Wu et al., 1993) so CSF concentrations would be expected to be increased in inflammatory conditions of the central nervous system. CSF S-100b concentrations have been reported to be increased in Guillain-Barré syndrome (Sindic et al., 1982; Mokuno et al., 1994) but there have been conflicting reports about the CSF S-100b concentrations in patients with multiple sclerosis (Michetti et al., 1980; Massaro et al., 1985; Noppe et al., 1986; Lamers et al., 1995).

3.3.1 CSF S-100b concentrations in diseases with CNS tissue damage

CSF S-100b was measured in nineteen patients with cerebrovascular disease (mean age: 52.9 ± 13.8 years, range 17-77; 12M:7F). Nine of these patients had had a haemorrhagic stroke, 3 an ischaemic stroke and 7 an unspecified type of stroke. Fourteen patients with central nervous system malignancies were also investigated (mean age: 49.9 ± 19.8 years, range 20-69; 10M:4F). Of these patients 5 had benign tumours of the meninges, 2 had benign pituitary tumours, 5 had various malignant tumours and 2 had an unspecified type of malignancy. Fourteen patients with slowly progressing CNS damage were also investigated (mean age: 41.9 ± 13.6 years, range 24-67; 7M:7F). Of these patients 6 had hydrocephalus, 3 had dystonia, 2 had benign

intracranial hypertension, 1 had cervical cord compression, 1 had chondrodysplasia punctata and 1 had syringomyelia/Arnold Chiari malformation. Statistical analysis was performed using Mann-Whitney U test.

The CSF S-100b concentrations in these groups of patients are shown in Figure 3.2. The mean CSF S-100b concentrations were significantly raised in patients with cerebrovascular disease (7.90 ± 18.9 ng/mL, $p< 0.02$) and in patients with central nervous system malignancies (1.77 ± 1.86 ng/mL, $p<0.02$) when compared to control subjects. Four patients with cerebrovascular disease had CSF S-100b concentrations of greater than 10 ng/mL. All of these patients had a large intracerebral bleed and three of them subsequently died. There was no difference between the mean CSF S-100b concentrations seen in patients with benign tumours when compared to malignant tumors (1.30 ± 1.42 ng/mL vs 1.42 ± 1.48 ng/mL respectively). Although the mean S-100b concentration was raised in patients with cerebrovascular disease and in patients with brain tumours, not all patients had raised levels. Five patients with brain tumours and 8 patients with cerebrovascular disease had concentrations of S-100b that were considered to be normal. Two of these patients had benign pituitary tumours, two unspecified malignant tumours and one malignant tumour of the ethmoidal sinus. CSF S-100b concentrations were not significantly raised in patients with slowly progressing damage to the central nervous system (0.26 ± 0.20 ng/mL, p=ns) when compared to control subjects, although 3 patients with hydrocephalus did have concentrations that were considered abnormal. These results agree with those of Sindic et al., 1982; Mokuno et al., 1983; Persson et al., 1987; and Lamers et al., 1995 who found raised concentrations of S-100b in patients with

strokes and with the findings of Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986 who reported raised concentrations in patients with brain tumours. Therefore diseases associated with acute damage to the CNS or space occupying lesions may have raised CSF S-100b concentrations. In contrast, patients with slowly progressing damage to the CNS did not have significantly raised CSF S-100b concentrations, suggesting that S-100b may not be a useful marker of small amounts of CNS damage occurring over longer time.

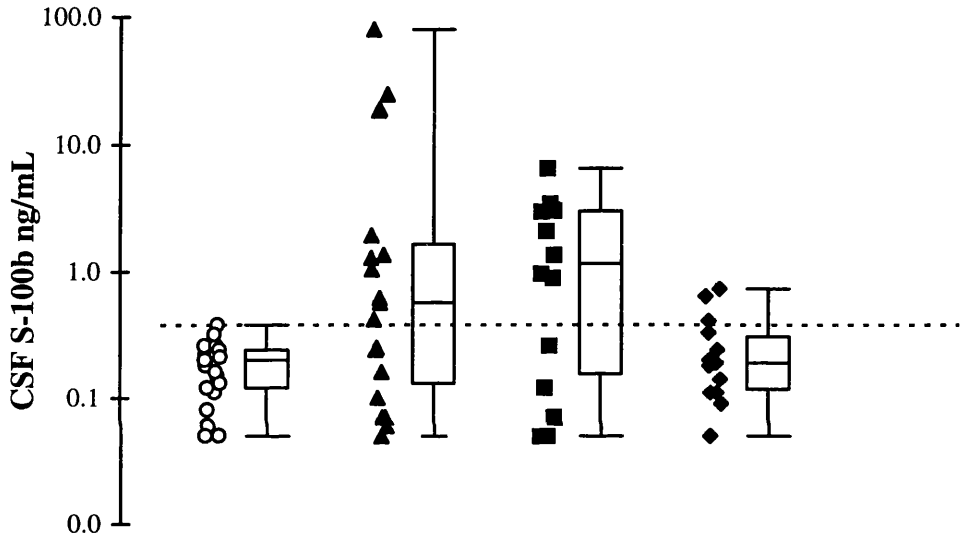


Figure 3.2 Combined scatter and box and whisker plots of CSF concentrations of S-100b in control subjects (○), *patients with cerebrovascular disease* (▲), *patients with malignancies of the central nervous system CNS* (■) *and patients with slowly progressing CNS damage* (◆). *The boxes represent the* 25^{th}-75^{th} *quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)*

3.2.2 CSF S-100b concentrations in inflammatory diseases of the central nervous system

CSF S-100b concentrations were measured in 38 patients with multiple sclerosis (MS) (mean age: 48.5 ± 13.8 years, range 31-70; 19M:18F). All of these patients had relapsing and remitting multiple sclerosis, but unfortunately no information was available concerning time between last relapse and the CSF sample being taken. Eighteen patients with various inflammatory diseases of the central nervous system (mean age 51.1 ± 18.3, range 28-55; 8M:10F) were also investigated. Of these patients 6 had an unspecified demyelinating disease of the central nervous system, 3 had autoimmune disease affecting the CNS, 3 had neuralgia, 2 had Guillain-Barré syndrome, 2 had sarcoidosis, 1 had intracranial phlebitis and 1 had transverse myelitis. Statistical analysis was performed using Mann-Whitney U test

The mean CSF S-100b concentrations in both of these groups are shown in Table 3.1. In neither group was the S-100b concentration significantly increased, although some samples did show elevated levels. Of the patients with multiple sclerosis 5% of patients had marginally elevated concentrations (0.41 ng/mL and 0.47 ng/mL); this agrees closely with other studies; Sindic et al., 1982 found 6% of patients with MS had raised CSF S-100b concentrations, whilst a more recent study found 7% of patients had elevated concentrations (Lamers et al., 1995). The latter study also found that a greater proportion of patients with chronic progressive MS had raised concentrations when compared to patients with relapsing and remitting MS. This suggests that CSF S-100b concentrations may depend on the clinical type of MS. An early study (Massaro et al., 1985) investigated the time course of S-100b release into

the CSF after acute exacerbation, and found that raised concentrations were detected 5 days after the first symptoms and reached maximal concentrations at three weeks. This suggests that CSF S-100b concentrations may be related to the type of MS and also to the time after relapse. The mean CSF S-100b concentration was not significantly raised in patients with inflammatory disease of the central nervous system (0.20 ± 0.06 ng/mL). Although the number of patients studied here is small, the results suggest that CSF S-100b may be increased in only a small proportion of patients with inflammatory diseases of the brain. The increases seen are also of a smaller magnitude than those found in patients with acute neurological damage such as that which is seen in brain tumours and cerebrovascular accidents.

Patient Group (n)	**S-100b ng/mL mean ± SD**	**Range**	**% samples S-100b > 0.39 ng/mL (n)**
Control subjects (21)	0.18 ± 0.07	0.05 - 0.39	0 (0)
Multiple Sclerosis (38)	0.18 ± 0.10	0.05 - 0.47	5 (2)
CNS Inflammatory disease (18)	0.20 ± 0.06	0.09 - 0.29	0 (0)

Table 3.1 Concentrations of S-100b in the cerebrospinal fluid of control subjects, patients with multiple sclerosis and patients with inflammatory diseases of the central nervous system

3.4 Serial CSF S-100b concentrations

Measurement of CSF S-100b concentration can be a useful marker of CNS damage if it reflects recent events. Serial CSF samples were obtained from two patients: patient 1 after the insertion of a ventricular-peritoneal drain and patient 2 after the

insertion of two external ventricular drains. In both cases the results obtained were related to the clinical course.

Patient 1, a 40 year old male with a frontal lobectomy for treatment of post traumatic generalised epilepsy, had 9 sequential CSF samples taken (Figure 3.3). This patient had had a lumbar peritoneal drain inserted for treatment of secondary hydrocephalus eight months prior to his admission. He was admitted following blockage of the lumbar peritoneal drain which was removed and replaced by a ventricular-peritoneal shunt on day 0. His post operative recovery was uneventful. The S-100b concentration was 4.67 ng/mL post operatively and declined to 0.51 ng/mL by day 5. By day 11 the concentration was within the reference range.

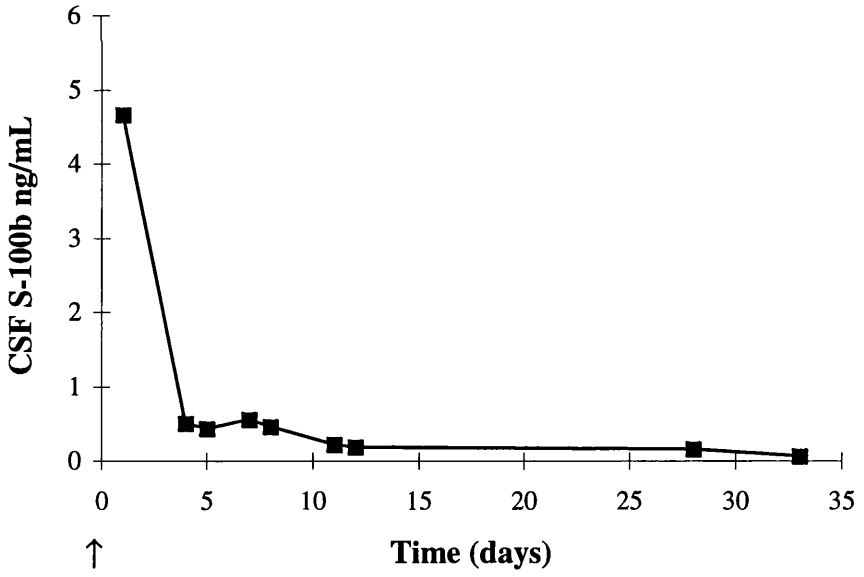


Figure 3.3 Serial CSF S-100b concentrations in Patient 1 (■)

In contrast to the uneventful outcome for patient 1, patient 2 had a complicated post-operative history which was reflected by the fluctuating CSF and serum S-100b concentrations. Patient 2, a 20 year old female with an optic nerve glioma, had 10 sequential CSF samples taken and 4 serum samples (Figure 3.4). This patient had hydrocephalus secondary to the glioma, which was previously treated by the insertion of a ventricular-peritoneal (VP) shunt. Prior to admission the patient complained of headaches and was diagnosed as having a blocked shunt. On day 0 the VP shunt was removed and replaced with an external ventricular drain. The following day she was noted to be pyrexial and during the next two days her condition did not improve. The external drain was reported as draining poorly during this time. The concentration of CSF S-100b in the drainage fluid was 2.1 ng/mL on day 1 and remained high at 2.5 ng/ml on day 3. During the evening of day 4 she had a seizure and it was decided to replace the external drain on day 6. Prior to surgery the CSF S-100b concentration had fallen to 0.47 ng/mL. The CSF S-100b post-operatively on day 6 was raised at 2.89 ng/mL, although the serum S-100b concentration was not detectable at this time. On day 7 the patient was pyrexial and coagulase negative staphylococcus was grown in culture from the drain CSF. The CSF S-100b was still raised at 1.13 ng/mL and S-100b was detectable in the serum at a concentration of 1.7 ng/mL. Over the next four days the patient did not improve despite the administration of intrathecal vancomycin and the CSF collected during this time was blood stained. The CSF S-100b concentrations declined from day 7 to day 10 after which they started to rise reflecting the presence of CNS infection and/or damage. On day 12 her condition deteriorated, she had another seizure and subsequently died. The serum S-100b concentrations mirrored those seen in the CSF.

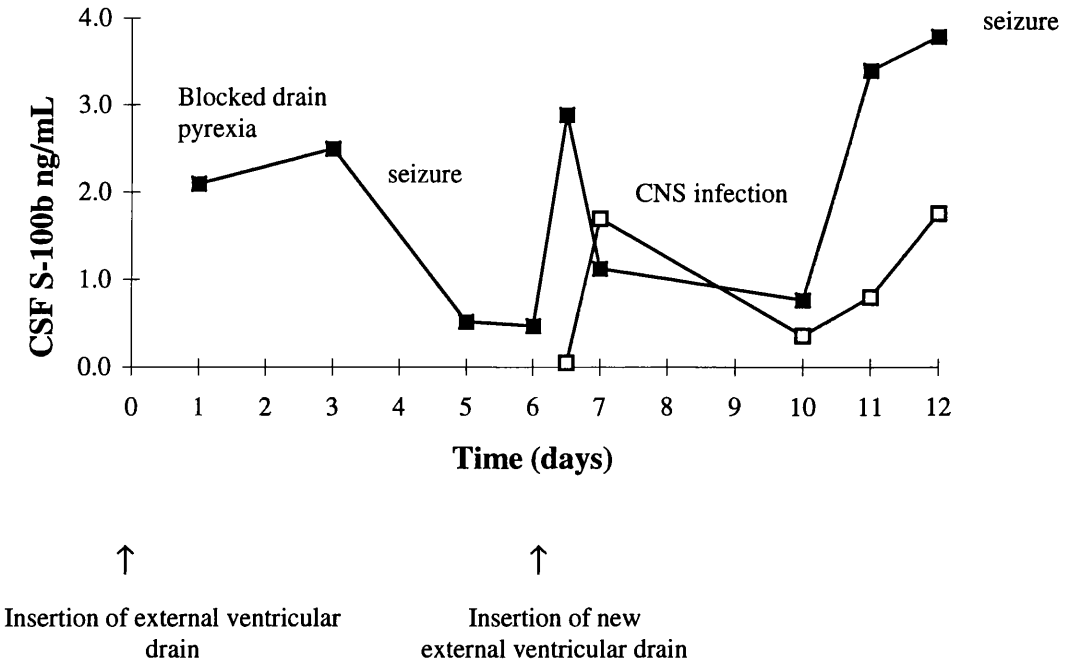


Figure 3.4 Serial CSF (■) and serum S-100b (□) concentrations in patient 2

The results from these two patients showed that CSF concentrations of S-100b declined steadily after an initial event when there were no complicating factors, but that a persistent increase in CSF S-100b concentrations suggested a continuing neurological insult. Hence CSF S-100b appears to be a marker of recent neurological damage.

3.5 CSF S-100b concentrations in sporadic Creutzfeldt-Jakob disease

CSF S-100b concentrations have been reported to be elevated in patients with sporadic CJD (Sindic et al., 1982; Noppe et al., 1986; Jimi et al., 1992; Otto et al., 1997a). The two earlier reports only investigated small numbers of patients and compared the results to either healthy controls or patients with other neurological diseases. A study investigating serial CSF samples taken in 3 patients found that

CSF S-100b remained elevated throughout the course of the illness in all patients (Jimi et al., 1992). A more recent study (Otto et al., 1997a) measured CSF S-100b in 135 patients suspected of having CJD and found that patients with histologically confirmed and probable sporadic CJD had significantly raised CSF S-100b concentrations when compared to patients found not to have the disease.

This part of the study was designed to repeat such work using the in-house S-100b ELISA and to include patients with the familial, iatrogenic and the new variant forms of CJD.

CSF S-100b concentrations were measured in 78 patients with suspected sporadic CJD. Of these patients, 29 were subsequently found not to have CJD (mean age: 65.1 ± 13.0 years, range 40-82; 18M:11F) and thus acted as appropriate disease controls. Of the remaining patients 31 had histologically confirmed sporadic CJD (mean age: 66.2 ± 9.7 years, range 49-79; 17M:14F), 10 had probable CJD (mean age: 65.0 ± 8.4 years, range 54-80; 6M: 4F) and 8 had possible CJD (mean age: 62.3 ± 8.8 years, range 51-75; 3M:5F). (see Appendix B for disease classification). The mean concentration of CSF S-100b in patients with histologically confirmed sporadic CJD was significantly raised when compared to control patients (1.73 ± 1.44 ng/mL vs 0.53 ± 0.25 ng/mL, $p < 0.001$). The mean concentration of CSF S-100b was raised in patients with probable CJD (1.90 ± 0.86 ng/mL) as well as in patients with possible CJD (2.30 ± 3.46 ng/mL). This work confirms the work by Otto et al., 1997a. Statistical analysis was performed using the Mann-Whitney U test.

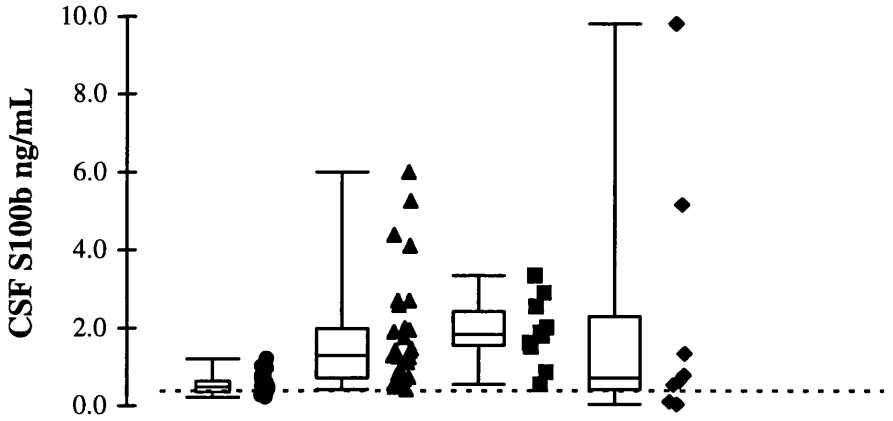


Figure 3.5 Combined scatter and box and whisker plots of CSF S-100b concentrations in patients initially suspected of having sporadic CJD but later proven not to have (●), patients with histologically confirmed sporadic CJD (▲), patients with probable CJD (■) and patients with possible CJD (◆). The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

The mean concentration of CSF S-100b was higher in patients initially suspected of having sporadic CJD but subsequently found not to have, when compared to healthy subjects (0.53 ± 0.25 ng/mL vs 0.18 ± 0.09 ng/mL). By definition these patients will have a dementing illness and the majority of these will have Alzheimer's disease (Otto et al., 1997a). CSF S-100b concentrations in patients with Alzheimer's disease and other non-CJD dementias will be discussed in section 3.8.

Most patients with CJD present with a rapidly progressing dementia and death usually occurs within 2-3 months (Collinge and Palmer, 1997). The acute nature of the disease is probably due to the rapid brain destruction which occurs in this

condition. This results in S-100b being released from damaged astrocytes causing an increase in the concentrations of this protein in the CSF. The reactive astrocytosis found in this condition may also contribute to the raised CSF concentrations of S-100b. As patients with CJD have significantly increased CSF S-100b concentrations, measurement of this protein may be useful for diagnosis. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of CSF S-100b for the diagnosis of histologically confirmed and probable sporadic CJD using four different cut-off concentrations is shown in Table 3.2. The greatest efficiency was obtained using a concentration of greater than 0.5 ng/mL.

	CSF S-100b >0.39 ng/mL	**CSF S-100b >0.5 ng/mL**	**CSF S-100b >0.8 ng/mL**	**CSF S-100b >1.0 ng/mL**
Sensitivity	100%	95%	71%	66%
Specificity	34%	66%	86%	93%
PPV	68%	80%	88%	93%
NPV	100%	90%	68%	68%
Efficiency	73%	83%	77%	77%

Table 3.2 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of CSF S-100b for the diagnosis of histologically confirmed and probable sporadic CJD using four different concentrations

3.6 CSF S-100b concentrations in patients with new variant Creutzfeldt-Jakob disease

As CSF S-100b concentrations were significantly increased in patients with sporadic CJD, it was decided to investigate whether they were raised in patients with the new variant form of this disease. CSF S-100b concentrations were measured in 59 patients suspected of having new variant CJD. Nineteen of these patients had histologically confirmed new variant CJD (mean age 29.0 ± 10.4 years, range 18-53; 9M:10F), 8 patients were classified as probable (mean age 25.4 ± 6.1 years, range 19-39; 4M:4F) and 1 patient was classified as possible new variant CJD (age 22 years, 1M). (see Appendix B for disease classification). Thirty-one patients (mean age 34.1 ± 13.1 years, range 15-56; 15M:16F) were found not to have the disease and acted as appropriate controls. The mean S-100b concentration was significantly raised in patients with histologically confirmed new variant CJD when compared to those patients without new variant CJD (1.03 ± 0.65 ng/mL vs 0.35 ± 0.21 ng/mL, $p<0.001$). The mean concentration of S-100b was 0.81 ± 0.51 ng/mL in patients with probable new variant CJD, and the patient with possible new variant CJD had a raised CSF S-100b of 0.83 ng/mL. Statistical analysis was performed using the Mann-Whitney U test.

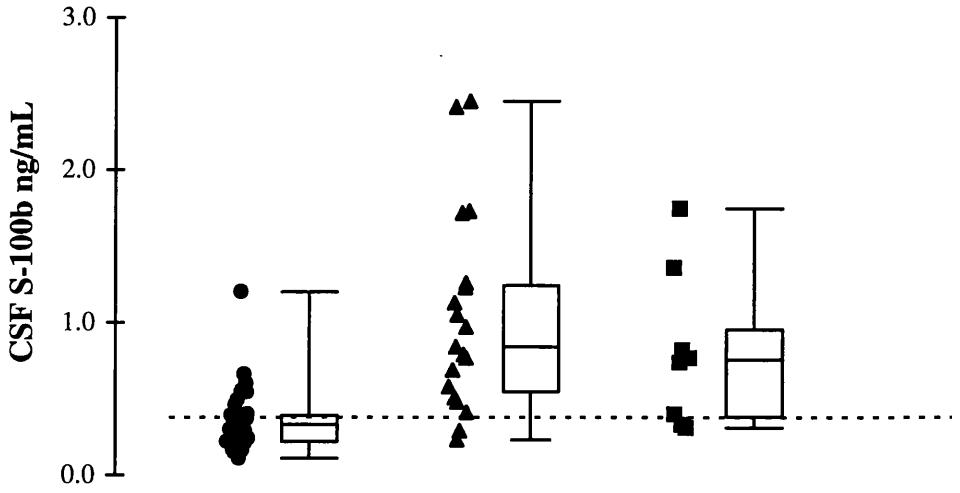


Figure 3.6 Combined scatter and box and whisker plots of CSF S-100b concentrations in patients initially suspected of having new variant CJD but subsequently proven to have not to have (●), patients with histologically confirmed new variant CJD (▲) and patients with probable new variant CJD (■). The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

The mean concentration of CSF S-100b was of a similar magnitude in patients with new variant CJD when compared to sporadic CJD (1.73 ± 1.44 vs 1.03 ± 0.65 ng/mL). The mean concentration of CSF S-100b in patients suspected of having new variant CJD but later proven not to have, was lower than that seen in patients suspected of having sporadic CJD but proven not to have (0.53 ± 0.25 vs 0.35 ± 0.21 ng/mL). This reflects the fact that different diseases may present as suspected cases of new variant when compared to sporadic CJD. The differential diagnosis for patients with sporadic CJD includes diseases like Alzheimer's disease and cortical Lewy body disease. As new variant CJD has only recently been described there is little information about diseases which may present with similar clinical features.

One patient with suspected new variant CJD who was found not to have the disease, had a raised CSF S-100b concentration of 1.2 ng/mL. The final diagnosis of this patient was not available at the time of writing.

One of the major differences between the clinical features of new variant when compared to sporadic CJD is the duration of illness. The median duration of illness for new variant is 16 months compared to 4.5 months for sporadic CJD. This suggests that new variant CJD may be a more slowly progressing disease. Despite the slower progression the mean CSF S-100b concentrations are of a similar magnitude to those seen in sporadic CJD.

Table 3.3. shows the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of CSF S-100b for the diagnosis of new variant CJD using four different cut-off concentrations. The best efficiency was obtained using a cut-off concentration of greater than 0.5 ng/mL.

	CSF S-100b >0.39 ng/mL	CSF S-100b >0.5 ng/mL	CSF S-100b >0.8 ng/mL	CSF S-100b >1.0 ng/mL
Sensitivity	89%	79%	53%	42%
Specificity	68%	84%	97%	97%
PPV	63%	75%	91%	89%
NPV	91%	87%	77%	73%
Efficiency	76%	82%	80%	76%

Table 3.3 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of CSF S-100b for the diagnosis of histologically confirmed and probable new variant CJD using four different concentrations

3.7 CSF S-100b concentrations in patients with mutations of the PRNP gene and patients with iatrogenic Creutzfeldt-Jakob disease

Table 3.4 illustrates the CSF S-100b concentrations found in 4 patients with iatrogenic CJD secondary to the administration of human cadaveric growth hormone, 3 patients with familial CJD and one patient with Gerstmann-Straussler-Scheinker syndrome (GSS). All patients were found to have elevated CSF S-100b concentrations. This supports the idea that CSF S-100b concentrations are elevated all forms of CJD. Patients with iatrogenic CJD secondary to human growth hormone administration usually present with cerebellar ataxia, with dementia occurring late in the disease. Patients with familial CJD may be indistinguishable clinically from patients with sporadic CJD, as in the case of patients with the PrP Glu-Lys^{200} mutation, or they may present with cerebellar signs and symptoms. Patients with

familial CJD tend to have an earlier age of onset of disease and a more protracted course. It is of interest that CSF S-100b concentrations were elevated in all patients with iatrogenic CJD and all mutations of the PRNP gene.

Type CJD	Patient Id	Mutation	Age (years)	Sex	S-100b ng/mL
Iatrogenic - GH	Case 1	n/a	34	M	0.63
Iatrogenic - GH	Case 2	n/a	37	M	1.74
Iatrogenic - GH	Case 3	n/a	28	M	6.54
Iatrogenic - GH	Case 4	n/a	27	F	1.23
Familial CJD	Case 1	Insert	67	F	3.80
Familial CJD	Case 2	Insert	66	M	0.90
Familial CJD	Case 3	PrP Glu-Lys^{200}	51	M	1.31
GSS	Case 1	PrP Ala-Val^{117}	44	F	0.46

Table 3.4 CSF S-100b concentrations in 4 patients with iatrogenic CJD secondary to growth hormone administration, 3 patients with familial CJD and 1 patient with Gerstmann-Straussler-Scheinker syndrome (GSS)

3.8 Serum and CSF S-100b concentrations in cattle with bovine spongiform encephalopathy (BSE)

As CSF S-100b concentrations were increased in patients with CJD a study was set up to investigate whether cattle with BSE had raised concentrations of serum and CSF S-100b.

3.8.1 Serum S-100b concentrations in cattle with and without bovine spongiform encephalopathy (BSE)

Blood samples were obtained from the jugular vein and were allowed to clot overnight. The serum was removed and stored at -20°C. Blood samples were obtained from 66 cattle with bovine spongiform encephalopathy (BSE), 14 cattle with clinical signs indistinguishable from BSE but histologically negative for BSE and 17 healthy cattle from New Zealand. The diagnosis of BSE was made by histological examination of the brain with immunohistochemical staining for the deposition of the disease associated abnormal prion protein (PrP^{sc}). The mean serum S-100b concentrations in healthy cattle and in cattle with non-BSE disease were similar, being 0.11 ± 0.07 ng/mL and 0.10 ± 0.08 ng/mL respectively. An upper limit of normal was calculated from the mean + 2 SD of the serum S-100b concentrations in healthy cattle and found to be 0.26 ng/mL. The mean serum S-100b concentration in cattle with BSE was not significantly increased, being 0.16 ± 0.20 ng/mL (Table 3.5). Only nine (14%) cattle with BSE had serum S-100b concentrations which would have been considered abnormal. Statistical analysis was performed using the Mann-Whitney U test.

Disease Group (n)	**S-100b ng/mL Mean ± SD**	**P value**
Healthy Cattle (17)	0.11 ± 0.07	
Non-BSE Cattle (14)	0.10 ± 0.08	ns
BSE Cattle (66)	0.16 ± 0.20	ns

Table 3.5 Concentrations of serum S-100b in healthy cattle, cattle with non-BSE disease and cattle with histologically confirmed BSE

3.8.2 CSF concentrations of S-100b in cattle with and without BSE

Cerebrospinal fluid samples were taken from the lumbar sac of 50 cattle with BSE (mean age 71.2 ± 16.3 months) and 16 cattle with clinical signs indistinguishable from BSE but histologically negative for BSE (mean age 69.3 ± 26.1 months). The samples, taken prior to slaughter, were centrifuged and the supernatant stored at -20°C prior to analysis. There was no significant difference between CSF S-100b concentrations taken prior to slaughter and those taken immediately after (4.71 ± 1.75 ng/mL vs 4.55 ± 1.55 ng/mL, p=ns). The mean CSF S-100b was significantly raised in cattle with BSE when compared to cattle without BSE (4.6 ± 3.8 ng/mL vs 2.0 ± 0.6 ng/mL, $p<0.006$) (Figure 3.7). Statistical analysis performed using Mann-Whitney U test.

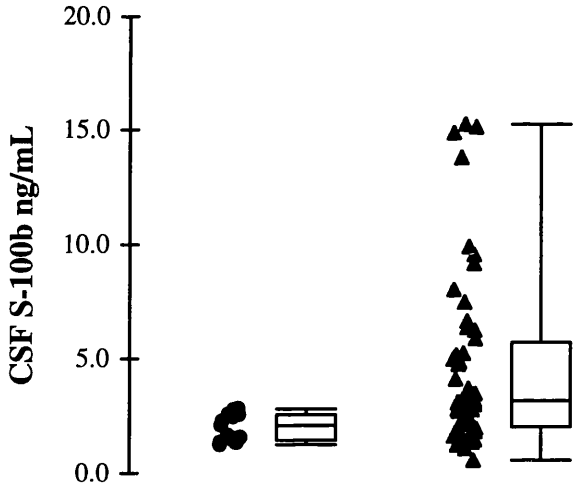


Figure 3.7 Combined scatter and box and whisker plots of CSF concentrations of S-100b in cattle suspected of having BSE but proven not to have (●) and cattle with histologically confirmed BSE (▲). The boxes represents the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represents the individual values from which the boxes and whiskers are derived

To obtain blood and CSF from cattle which could be guaranteed free from exposure to the BSE agent, it was necessary to import bovine samples from New Zealand, a country without a history of scrapie or BSE. Serum samples were obtained via this route but it was not possible to obtain CSF samples. Therefore a reference range for CSF S-100b in healthy cattle could not be established. Using a cut-off concentration of CSF S-100b of 3.18 ng/mL, calculated from the mean + 2 SD of S-100b concentrations in cattle without BSE, 50% of BSE affected cattle were found to have elevated CSF S-100b concentrations. This suggested that CSF S-100b concentrations are not sensitive enough to be used on their own as a diagnostic test. An elevated CSF S-100b may be indicative of BSE but a low concentration would not be able to exclude it. One reason for the poor sensitivity when compared to CJD may be the stage of illness at which the CSF sample is taken. Cattle with clinical signs of BSE are examined by veterinary officers, have clinical samples taken and are subsequently slaughtered. In contrast, patients with human TSEs are nursed and have a longer protracted illness. It is possible that the CSF samples are taken earlier in the disease course in BSE than in CJD. However, there was no correlation between CSF S-100b concentrations and duration of symptoms measured in days in the cattle with BSE in this study, $r^2 = 0.005$ (Figure 3.8). It is possible that the number of cattle investigated and the time window of symptoms is too small for a relationship between duration of illness and CSF S-100b concentrations to become evident. A study investigating the time course of CSF S-100b concentrations in cattle inoculated with the BSE agent is in progress in collaboration with Roy Jackman, Central Veterinary Laboratory, Weybridge, UK.

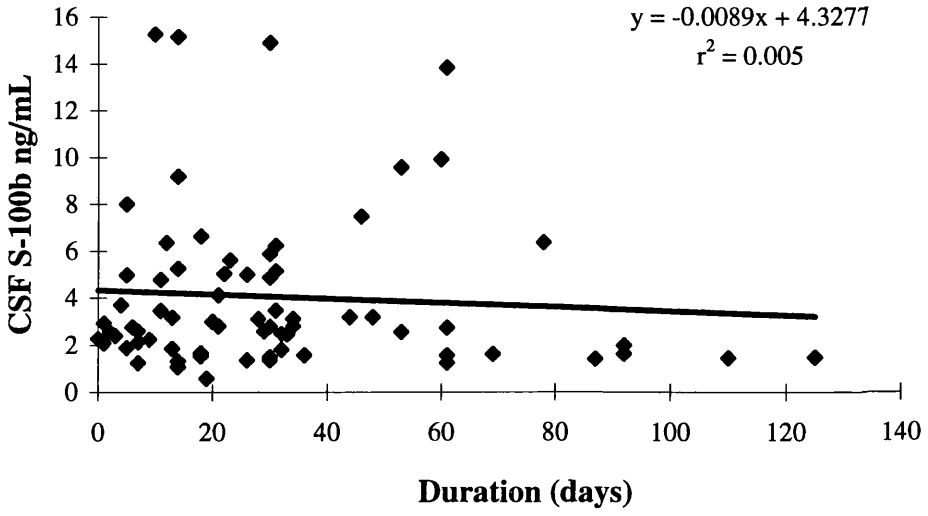


Figure 3.8 Relationship between the duration of symptoms prior to lumbar puncture and CSF S-100b concentrations in cattle with BSE

3.9 CSF S-100b concentrations in patients with Alzheimer's disease and other non-CJD dementias

In section 3.6 it was shown that patients who were initially suspected of having sporadic CJD but who were subsequently proven to have another diagnosis had marginally increased concentrations of CSF S-100b (0.53 ± 0.25 ng/mL). The majority of these patients would probably have had Alzheimer's disease, but obtaining a histologically confirmed diagnosis in this group patients is difficult as many would not have had post-mortems performed. It is well known that many forms of dementia such as Alzheimer's disease and frontotemporal dementia are associated with astrocytosis (Mann et al., 1993; Sheng et al., 1994; Brun et al., 1994). This study was established to investigate whether CSF S-100b concentrations are raised in patients with non-CJD dementia.

CSF S-100b concentrations were measured in 30 patients with Alzheimer's disease (mean age 59.3 ± 9.03, range 48-74 years; 17M:13F). Twenty-six of these patients had the sporadic form of the disease, and 3 had familial Alzheimer's disease, two patients had a mutation in the amyloid precursor protein gene on Chromosome 21 and 1 had familial Alzheimer's disease due to a mutation in the presenilin 1 gene on chromosome 14. Twenty six patients with frontotemporal dementia (mean age 61.6 ± 7.2, range 41-72 years; 18M:8F), 8 patients with cortical Lewy body disease (mean age 60.0 ± 12.1, range 47-71 years; 6M:2F), 7 patients with corticobasal degeneration (mean age 66.7 ± 12.5, range 44-78 years; 4M:3F) and 21 patients with unspecified dementia (mean age 54 ± 12.1, range 33-78 years; 10M:11F) were also investigated. CSF S-100b concentrations were also measured in a group of 19 patients with degenerative diseases of the central nervous system who had associated cognitive impairment (mean age 63.0 ± 8.9, range 37-76 years; 14M:5F). Five of these patients had progressive supranuclear palsy, 4 had motor neurone disease, 3 had multi-system atrophy, 3 had dementia associated with Parkinson's disease and 4 had dementia associated with an undefined degenerative process.

For each of the patients with Alzheimer's disease (AD) and frontotemporal dementia (FTD), the S-100b concentrations were related to the mini mental state examination (MMSE), age at onset of disease and duration of illness.

The mean concentrations of CSF S-100b in dementia are shown in Table 3.6. The mean S-100b concentration was significantly raised in patients with frontotemporal dementia. Although the mean CSF S-100b concentration was raised in patients with

Alzheimer's disease, this increase failed to reach significance. The magnitude of these increases was small with only 10% of patients having concentrations above 0.8 ng/mL. The mean concentration of CSF S-100b was also increased in patients with corticobasal degeneration (0.62 ± 0.69 ng/mL). Statistical analysis was performed using one way ANOVA with Bonferoni modification using SPSS.

For patients with Alzheimer's disease and frontotemporal dementia there was no correlation between the CSF S-100b concentrations and the MMSE at the time of CSF sampling ($r^2 = 0.004$), between CSF S-100b and age at onset ($r^2 = 0.006$) nor between CSF S-100b and duration of illness ($r^2 = 0.0004$). There is evidence to suggest that S-100b may play a role in the degenerating process in Alzheimer's disease (AD). Concentrations of S-100b are increased up to 20 fold in the brain tissue of patients with Alzheimer's disease when compared to age-matched controls without dementia (Pena et al., 1995) and the pattern of elevation across the different cerebral regions corresponds to those areas most affected with the disease (Van Eldik et al., 1994). Histological examination of brain tissue from AD patients has shown that reactive astrocytes containing elevated concentrations of S-100b are associated with neurofibrillary tangles (Yamaguchi et al., 1987). S-100b has been shown to stimulate neuritic outgrowth and increase intraneuronal calcium and has been proposed as a mediator of the cytokine induced degeneration seen in AD (Mrak et al., 1995). Severe astrocytosis is also seen in patients with frontotemporal dementia, particularly in patients with Pick's disease (Mann et al., 1993; Brun et al., 1994). The small elevation in mean CSF S-100b concentrations seen in patients with dementia, not associated with CJD, probably reflects the degree of astrocytosis which occurs in these diseases.

Disease Group (n)	S-100b ng/mL Mean ± SD	Range	% samples > 0.39 ng/mL (n)	% samples > 0.50 ng/mL (n)	% samples > 0.8 ng/mL (n)
Alzheimer's disease (30)	0.41 ± 0.25	0.10 - 1.08	47 (14)	37 (11)	10 (3)
Frontotemporal dementia (26)	0.48 ± 0.34*	0.13 - 1.70	50 (13)	35 (9)	11 (3)
Diffuse Lewy body disease (8)	0.35 ± 0.23	0.05 - 0.84	38 (3)	12 (1)	12 (1)
Corticobasal degeneration (7)	0.62 ± 0.69	0.24 - 2.15	43 (3)	28 (2)	14 (1)
Unspecified dementia (21)	0.49 ± 0.43	0.11 - 2.0	43 (9)	28 (6)	19 (4)
Degenerative disease plus dementia (19)	0.38 ± 0.27	0.16 - 1.13	31 (6)	21 (4)	<1 (1)

*Table 3.6 CSF S-100b concentrations in patients with dementia. Statistical analysis compared the CSF S-100b values obtained for each group of dementia patients with those obtained in age-matched controls. * $p < 0.03$*

3.10 CSF S-100b concentrations in patients with Human Immunodeficiency Virus (HIV) infection

CSF S-100b was measured in samples of CSF obtained from 73 consecutive HIV infected patients (mean age: 38.1 ± 7.9 years, range 27-62; 52M:21F) who were undergoing diagnostic lumbar puncture for assessment of possible neurological disease. Thirty-two patients had HIV associated dementia complex (ADC) and all had stage 2 or greater dementia according to the Memorial Slone Kettering Criteria (Price and Brew, 1988). Sixteen had systemic β-cell lymphoma and were being investigated for possible cerebral involvement, 4 had primary CNS lymphoma and 21 had CNS infections (7 had cryptococcal meningitis, 5 had cytomegalovirus (CMV) encephalitis, 6 had varicella zoster virus (VZV) meningoencephalitis, 3 had cerebral toxoplasmosis).

The mean CSF S-100b concentration was not significantly raised in patients with ADC when compared to control subjects (0.36 ± 0.28 ng/mL vs 0.18 ± 0.09 ng/mL, p=ns), although 31% of patients had S-100b concentrations which would have been considered abnormal. The mean concentration of CSF S-100b tended to be raised in patients with CNS lymphoma (0.57 ± 0.40 ng/mL) but this did not reach significance when compared to control subjects.

The CSF S-100b concentrations were significantly raised in patients with CNS infections when compared to control subjects (2.16 ± 3.4 ng/mL vs 0.18 ± 0.09 ng/mL, $p<0.02$). Patients with CNS infections had CSF S-100b concentrations

which were also significantly raised when compared to patients with ADC (2.16 ± 3.4 ng/mL vs 0.36 ± 0.28 ng/mL, $p<0.02$) and patients with CNS lymphoma (2.16 ± 3.4 ng/mL vs 0.57 ± 0.4 ng/mL, $p<0.05$).

Of the 6 HIV infected patients with infections who had CSF S-100b levels higher than those seen in the ADC group (Figure 3.9), 3 had VZV meningoencephalitis, 2 had CMV encephalitis and 1 had cerebral toxoplasmosis.

These results showed that whilst CSF S-100b concentrations were raised in some patients with ADC, there was considerable overlap with S-100b concentrations seen in patients with CNS infections and lymphoma. CSF S-100b concentrations greater than 2.0 ng/mL were associated with CNS infections rather than either ADC or CNS lymphoma.

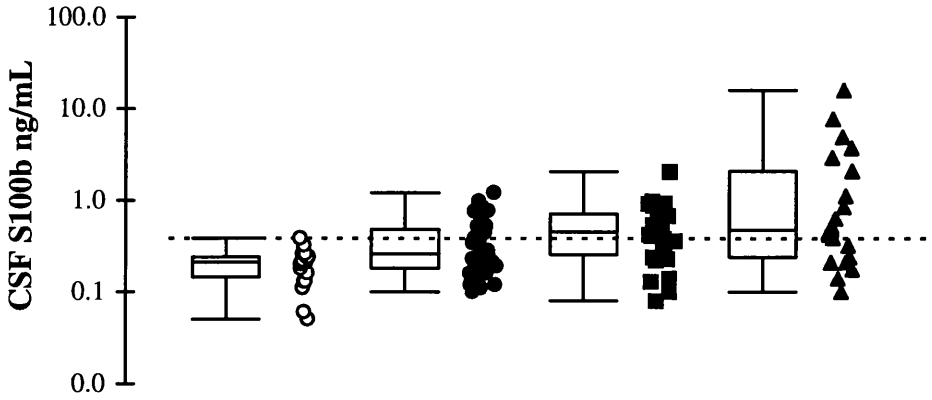


Figure 3.9 Combined scatter and box and whisker plots of cerebrospinal fluid concentrations of S-100b in controls subjects (○), *patients with ADC* (●), *systemic and CNS lymphoma* (■) *and CNS infections* (▲). *The boxes represents the* 25^{th}-75^{th} *quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line is represents the upper limit of normal (2.5 SD above the mean of the control subjects)*

There was no correlation between the CD4 count in patients with HIV infections and the CSF S-100b concentrations ($r^2 = 0.0066$).

3.11 General discussion of CSF S-100b results

Astrocytes are the most numerous cells within the CNS, outnumbering neurones by ten to one and make up one third of the brain volume (Norenberg et al., 1994). Traditionally astrocytes were considered functionally inert structural cells, this is no longer thought to be the case. Astrocytes are not isolated cells but are arranged in syncytium mediated through gap junctions (Kettenmann et al., 1983). They are involved in many processes within the CNS such as neuronal migration, neurite outgrowth, synaptogenesis, regulation of the blood brain barrier, maintenance of

water, ion and neurotransmitter metabolism, energy and nutritional support of neurones, modulation of immune responses and phagocytic function (Norenberg et al., 1994). In addition astrocytes possess many enzyme systems that enable them to metabolise glutamate, ammonia and free radicals (Norenberg et al., 1994), which may be released as a result of neurological injury.

Astrocytes swell in response to CNS trauma such as hypoxia and injury. This is mediated by an influx of water secondary to an increase in intracellular osmolality, caused by increased intracellular concentrations of Na^+, K^+ or glutamate. Other toxic agents such as free radicals or ammonia may also be involved. This swelling affects astrocyte integrity and function and as a result astrocytic proteins, such as S-100b, may be released. The results described in section 3.2.1 illustrate that elevated concentrations of CSF S-100b were found in hypoxic injury such as intracerebral haemorrhage and infarction. Increased concentrations were also seen in space occupying lesions such as brain tumours, which can be thought of CNS injury. The CSF S-100b concentrations in these cases could therefore be taken as a measure of astrocytic integrity.

Not all forms of CNS injury are associated with raised concentrations of CSF S-100b. Section 3.2.1 showed that CSF S-100b were not significantly raised in patients with diseases associated with slowly progressing damage to the central nervous system. In these cases it is possible that the amount and rate of brain damage was such that the astrocytic response could effectively cope with the released toxins without loosing any functional integrity.

Measurement of serial CSF S-100b concentrations in 2 patients with ventricular peritoneal drains, showed the close association between CSF S-100b concentrations and clinical outcome. It also demonstrated that CSF S-100b concentrations reflect recent events as the concentrations rapidly rose and fell in response to the clinical situation.

In addition to swelling, astrocytes can undergo other morphological changes, such cytoplasmic hypertrophy, production of long, thick cytoplasmic processes with the nuclear enlargement and increased amounts of chromatin. There are ultrastructural changes which include increased numbers of mitochondria, golgi complexes, endoplasmic reticulum and the appearance of bundles of intermediate filaments containing glial fibrillary acidic protein (GFAp) and vimentin. These astrocytes are known as reactive astrocytes and represent the most characteristic astrocytic response seen in the CNS. Reactive astrocytes respond to interleukin-1 (IL-1) by upregulating the expression of a number of cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), colony stimulating factor-1 (CSF-1), granulocyte-macrophage CSF, granulocyte CSF, tumour necrosis factor-α (TNF-α), S-100b and the production of astrocyte derived apolipoprotein E (Apo E) (Mrak et al., 1995).

Reactive astrocytosis is a common feature of Alzheimer's disease, frontotemporal dementia and AIDS dementia complex (ADC). In Alzheimer's disease reactive astrocytes are found surrounding neuritic plaques with their processes extending into the core of the plaques. S-100b has autocrine effects including astrocytic hypertrophy and proliferation and increasing astrocytic intracellular calcium concentrations. In addition, S-100b has been shown to be able to promote neuritic

outgrowth *in vitro*, increase intraneuronal free calcium and promote neuronal survival *in vitro*. The ability of S-100b to promote neuritic growth and the close proximity of reactive astrocytes to neuritic plaques has suggested a role for S-100b in plaque formation in Alzheimer's disease (Mrak et al., 1995). The neuronal cell loss in Alzheimer's disease has been linked to an increase in intraneuronal calcium, suggesting that S-100b may be involved in the cell loss seen in this disease (Griffin et al., 1993).

An increase in the number and size of S-100b expressing astrocytes has been demonstrated in brains from HIV infected patients (Stanley et al., 1994). Other pathological changes found in HIV brains include neuronal cell loss, thought to be mediated by an increase in intraneuronal calcium (Gibbons, 1990) and abnormal neuritic outgrowth.

The mean CSF S-100b concentrations seen in Alzheimer's disease and AIDS dementia complex are only marginally increased when compared to control subjects, although some patients have substantially raised concentrations. This suggests reactive astrocytosis per se is not necessarily associated with large increases in CSF S-100b.

Reactive astrocytosis can be present in diseases associated with acute neurological damage such as CJD and CNS infections secondary to AIDS. In both of these conditions CSF S-100b concentrations are significantly raised when compared to control subjects. It is probable that the acute neurological damage with its

subsequent astrocytic swelling and death is the major contributor to the elevation of CSF S-100b seen in these conditions.

CSF S-100b concentrations were elevated in all forms of CJD investigated: sporadic, new variant, familial and iatrogenic. The clinical presentation these types of CJD can vary, some forms presenting with ataxia, some with dementia and others with psychiatric symptoms. The duration of illness can also vary from a few months to several years. Although the underlying histopathology shows variations from type to type, which in the case of new variant CJD is very distinct, the characteristic spongiform change, neuronal loss, astrocytosis and PrP^{sc} deposition is common to all types. This suggests that CSF S-100b concentrations are more closely related to the common pathological changes seen in CJD than to the clinical presentation. This means that elevated CSF S-100b can be used as a marker of transmissible spongiform encephalopathies regardless of the type.

Inflammatory diseases of the CNS such as multiple sclerosis are not associated with increased CSF S-100b concentrations, despite the fact that S-100b can be induced by IL-1.

In summary, CSF S-100b concentrations may be elevated in diseases associated with acute damage to the central nervous system such as strokes and brain tumours; but are not necessarily increased in diseases associated with slowly progressing CNS damage or inflammatory diseases of the CNS. Elevated concentrations of S-100b are not found in all patients with acute damage to the CNS, so normal concentrations cannot exclude CNS damage.

The results described here suggest that CSF S-100b concentrations may be elevated in diseases associated with acute CNS damage, and that the concentrations reflect recent events. CSF S-100b concentrations may be increased in patients with dementia such as Alzheimer's disease, frontotemporal dementia and AIDS dementia complex. These increases are moderate and probably reflect the astrocytosis seen in these diseases. CSF S-100b concentrations are significantly raised in patients with CJD. This increase is seen in all forms of the disease, and therefore S-100b may play a role in the differential diagnosis of dementia where CJD is clinically suspected.

Chapter 4 - Innotest hTau Antigen ELISA for the measurement of CSF tau protein

The expense of using pure recombinant tau protein as a calibrant precluded the development of an in-house method for measuring CSF tau. As a result, all tau measurements were performed using the Innotest hTau Antigen, an enzyme-linked immunoassay supplied by Innogenetics, Belgium (UK suppliers: Autogen Bioclear, Wiltshire, UK). This assay measured both the normal phosphorylated tau protein and the abnormal hyperphosphorylated tau protein. A monoclonal antibody (AT120) which reacted with both the normal and hyperphosphorylated forms of tau protein (Vandermeeren et al., 1993) was used as a capture antibody. The monoclonal antibody was coated onto the wells of a microtitre plate and after blocking the unbound protein sites on the microtitre plate wells, standards and CSF samples were added in duplicate. Tau calibrants were prepared from recombinant tau protein and a set of calibrants 0, 75, 150, 300, 600 and 1200 pg/mL were used. A pair of biotinylated monoclonal antibodies (HT7 and BT2) were added to each well of the microtitre plate and the plate was incubated overnight at room temperature. The HT7 monoclonal antibody reacted with both normal and hyperphosphorylated tau protein, whilst the BT2 monoclonal antibody reacted preferentially with hyperphosphorylated tau protein (Goedart et al., 1994). After an overnight incubation the microtitre plate was washed and peroxidase conjugated streptavidin added. The streptavidin bound to the biotin on the monoclonal antibodies to form a complex, the quantity of which was proportional to the amount of tau protein in the standard or CSF sample. The microtitre plate was washed and the substrate

containing 3, 3', 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide was added. The peroxidase activity converted the colourless TMB into an insoluble blue precipitate. The peroxidase activity was stopped by the addition of sulphuric acid, which also converted the insoluble blue precipitate into a soluble yellow product. The absorbance of the yellow product was measured at 450 nm and was proportional to the amount of tau protein originally present in the sample. A typical standard curve is shown in Figure 4.1.

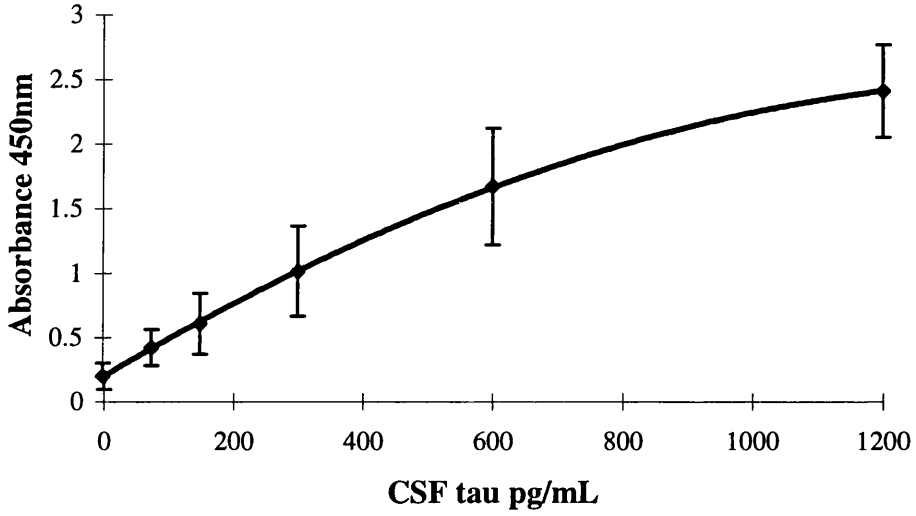


Figure 4.1 A typical standard curve for the Innotest hTau Antigen ELISA. Each point represents the mean ± SD of six separate investigations

Chapter 5 - Cerebrospinal fluid concentrations of tau

Cerebrospinal fluid concentrations of tau were measured in 389 CSF samples from patients with a variety of neurological conditions using the sandwich ELISA assay from Innogenetics (second generation), described in Chapter 4. This assay measured both normal phosphorylated tau protein and the abnormal hyperphosphorylated tau found in many dementing diseases. The CSF samples and final diagnoses were obtained as previously described (section 3.0).

5.1 CSF tau in control subjects

Twelve patients (mean age: 43.5 ± 12.3 years, range 29-66; 7M:5F), who had a lumbar puncture as part of their diagnostic investigations, and who were found to have no organic brain disease at the time of discharge were used as control subjects. Of these patients 7 had headache, 2 had psychological syndromes, 1 had a sebaceous cyst, 1 had strabismus and 1 had a disturbance of skin sensation. The cerebrospinal fluid concentration of tau was found to be 160 ± 61 pg/mL (mean ± SD). An upper limit of normal was taken to be 315 pg/mL (mean ± 2.5 SD). Gender did not affect CSF tau concentrations, the concentration being 160 ± 45 pg/mL in males and 151 ± 85 pg/mL in females. This agrees well with other studies using the second generation Innogenetics tau ELISA assay and the tau ELISA produced by Athena Neurosciences Inc, South San Francisco, California (Table 5.1). The reference ranges for CSF tau in healthy control populations obtained using the first generation Innogenetics tau ELISA is markedly different and must be borne in mind when comparing different studies.

Study	Source of ELISA	Population studied	CSF tau (pg/mL)
Arai et al (1995)	1st generation Innogenetics	Healthy Controls	9 ± 4.5
Arai et al (1997)	1st generation Innogenetics	Healthy Controls	12 ± 9
Arai et al (1998)	1st generation Innogenetics	Healthy Controls	20 ± 13
Tato et al (1995)	1st generation Innogenetics	Healthy Controls	26 ± 11
Blennow et al (1995)	2nd generation Innogenetics	Healthy Controls	185 ± 50
Andreasen et al (1997)	2nd generation Innogenetics	Healthy Controls	190 ± 57
Molina et al (1997)	2nd generation Innogenetics	Healthy Controls	160 ± 76
Kanai et al (1998)	2nd generation Innogenetics	Healthy Controls	267 ± 145
Mecocci et al (1998)	2nd generation Innogenetics	Healthy Controls	212 ± 42
Motter et al (1995)	Athena	Neurological Controls	212 ± 102
Vigo-Pelfrey et al (1995)	Athena	Neurological Controls	190 ± 80
Galasko et al (1997)	Athena	Neurological Controls	160 ± 69

Table 5.1 CSF tau reference ranges for healthy controls or neurological controls obtained using three different tau ELISAs

There was no effect of age on CSF tau concentration ($r^2 = 0.02$) (Figure 5.1). This confirms the findings of Blennow et al., 1995; Jensen et al., 1995; Tato et al., 1995; Vigo-Pelfrey et al., 1995; Andreasen et al., 1998; Galsako et al., 1998, who also found no correlation between age and CSF tau concentrations in patients with non-neurological disease. In contrast other reports have suggested that CSF tau concentrations increase with age (Arai et al., 1995; Kanai et al., 1998; Mecocci et al., 1998).

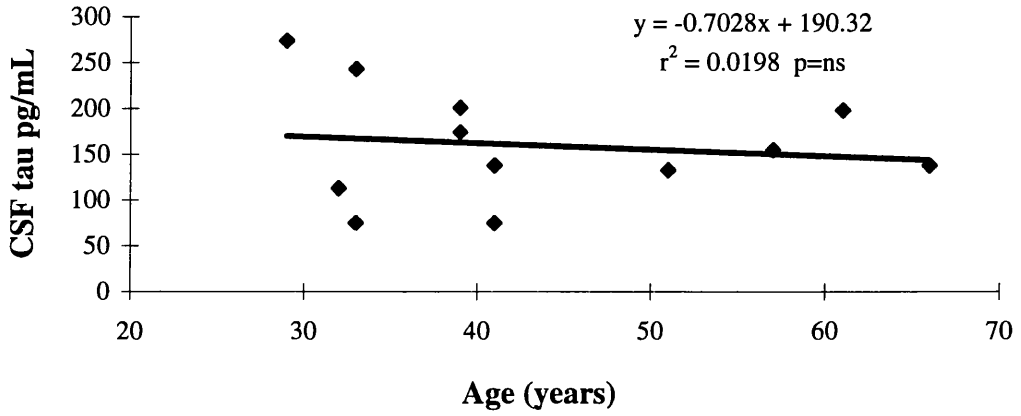


Figure 5.1 The effect of age on CSF tau concentrations.

5.2 CSF tau concentrations in patients with neurological diseases

CSF tau concentrations have been reported to increase in diseases associated with acute damage to the central nervous system (CNS) such as ischaemic infarction (Vandermeeren et al., 1993; Arai et al., 1995; Vigo-Pelfrey et al., 1995), Creutzfeldt-Jakob disease (Arai et al., 1995; Otto et al., 1997b) and Herpes simplex encephalitis (Vandermeeren et al., 1993). Other diseases with a more slowly progressing CNS damage such as amyotrophic lateral sclerosis and normal pressure hydrocephalus

may also be associated with elevated CSF tau concentrations (Vandermeeren et al., 1993; Vigo-Pelfrey et al., 1995). Some patients with inflammatory diseases such as multiple sclerosis and Guillain-Barré syndrome have been reported to have increased CSF tau concentrations (Vandermeeren et al., 1993).

The aim of this part of the study was to investigate whether CSF tau concentrations are elevated in patients with acute CNS damage and compare the concentrations to those found in patients with multiple sclerosis and miscellaneous neurological diseases.

CSF tau concentrations were measured in 51 patients (mean age: 50.6 ± 13.1 years, range 27-77; 27M:13F:11unknown) with various neurological diseases. Of these patients, 12 had diseases associated with CNS damage (8 had either a haemorrhagic or ischaemic stroke, 2 had a benign malignancy of the CNS, 1 had a malignant neoplasm and 1 had viral encephalitis); 14 patients had multiple sclerosis and 25 patients had miscellaneous neurological diseases (8 with unspecified degenerative disease of the CNS, 4 with encephalomyelitis, 4 with epilepsy, 3 with gait disorders, 2 with speech disorders, 1 with a dissociative disorder, 1 with a musculoskeletal disorder, 1 with a lumbar disc disorder and 1 with benign intracranial hypertension). Statistical analysis was performed using the Mann Whitney U test.

The CSF tau concentrations in these patients are shown in Figure 5.2. The mean concentration of CSF tau was significantly raised in patients with diseases associated with central nervous system damage when compared to control subjects

(3714 ± 5236 vs 160 ± 61 pg/mL, $p < 0.004$). Five patients had CSF tau concentrations greater than 4000 pg/mL: 2 had an intracranial haemorrhage, 1 had a malignant brain neoplasm, 1 had a benign brain neoplasm and 1 had viral encephalitis. The mean CSF tau concentration was not significantly raised in patients with multiple sclerosis (199 ± 103 pg/mL), although four patients did have marginally increased concentrations. This is in agreement with Vandermeeren et al., 1993 who found four out of twenty patients with multiple sclerosis had increased CSF tau concentrations. The mean CSF tau concentration was increased in patients with miscellaneous neurological diseases (340 ± 399 pg/mL) but this increase was not significantly different from control subjects. Six of the twenty-five patients with miscellaneous neurological disease had marginally increased CSF tau concentrations, 3 had unspecified neurodegenerative disorders, 1 had benign intracranial hypertension, 1 had a speech disorder and 1 had encephalomyelitis. The patient with encephalomyelitis had a CSF tau of greater than 700 pg/mL whilst 2 of the 3 patients with unspecified neurodegenerative disease had CSF tau concentrations of greater than 1000 pg/mL.

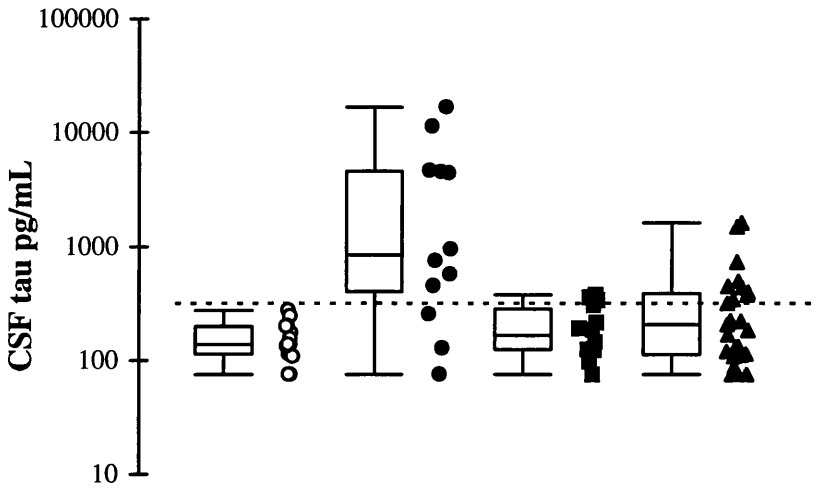


Figure 5.2 Combined scatter and box and whisker plots of CSF concentrations of tau in control subjects (○), *in patients with diseases associated with acute CNS damage* (●), *in patients with multiple sclerosis* (■) *and in patients with miscellaneous neurological diseases* (▲). *The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)*

These results suggest that CSF tau concentrations may be raised in some patients with intracranial haemorrhage, or pressure necrosis secondary to brain tumours, with smaller increases occurring in slowly progressing degenerative diseases. These results confirm the findings of Vandermeeren et al., 1993; Arai et al., 1995; Vigo-Pelfrey et al., 1995) who found raised concentrations in conditions such as stroke, cerebrovascular disease, amyotrophic lateral sclerosis, Parkinson's disease and hydrocephalus.

Tau protein is a microtubular protein found predominantly within the axons and dendritic processes of the neurones within the central nervous system (Goedart et al,

1991a). Diseases associated with damage to brain tissue either due to ischaemia following stroke or to pressure necrosis, may result in the release of large amounts of neuronal proteins such as tau protein into the CSF. This is supported by the observations that levels of CSF tau increase after acute ischaemic stroke, reaching a maximum within 1-2 weeks, after which the levels decline to normal (Andreasen et al., 1998; Arai et al., 1998). A transient increase in CSF tau was reported in a patient with asthma induced brain hypoxia (Ohrui et al., 1997). Diseases such as multiple sclerosis which are associated with a slowly progressing CNS degeneration, only small amount of neuronal proteins may be released into the CSF and thus increased concentrations CSF tau may not be detected.

5.3 CSF tau concentrations in patients with Creutzfeldt-Jakob disease (CJD)

Increased concentrations of CSF tau have been reported in patients with sporadic CJD (Arai et al., 1995, Otto et al., 1997b). This part of the study was designed to expand such work to include patients with familial, iatrogenic and new variant forms of the disease.

5.3.1 CSF tau concentrations in patients with sporadic CJD

CSF tau concentrations were measured in 30 patients with histologically confirmed sporadic CJD (mean age: 66.0 ± 9.1 years, range 49-79; 16M:14F), 10 patients with probable sporadic CJD (mean age: 65.0 ± 8.4 years, range 54-80; 6M:4F) and 8 patients with possible sporadic CJD (mean age 62.3 ± 8.8 years, range 51-75; 3M:5F). These results were compared with CSF tau concentrations in a control

group of 27 patients with suspected sporadic CJD but who were subsequently found to have other diagnoses (mean age 64.6 ± 13.4 years, range 40-85; 16M:11F). Using the Mann Whitney U test, the mean CSF tau concentration was significantly increased in patients with histologically confirmed sporadic CJD when compared to the controls (5791 ± 6499 vs 873 ± 2508 pg/mL, $p < 0.001$). The mean CSF tau concentration was also increased in patients with probable (10610 ± 8403 pg/mL) and possible sporadic CJD (4583 ± 5423 pg/mL). The results are shown in Figure 5.3.

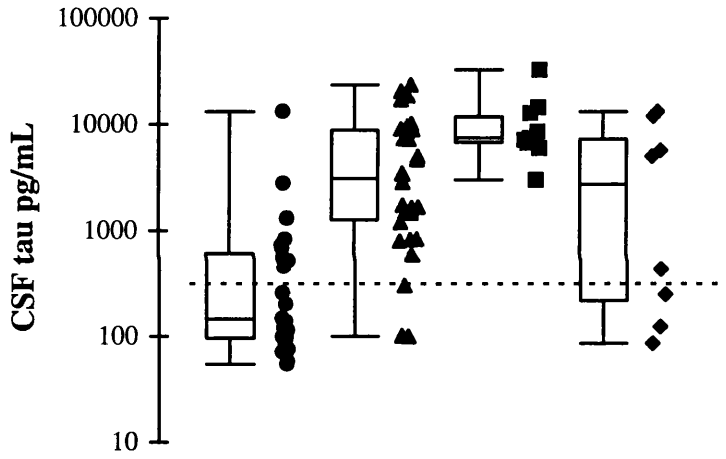


Figure 5.3 Combined scatter and box and whisker plots of CSF concentrations of tau in patients initially suspected of having sporadic CJD but later proven not to have (●), patients with histologically confirmed sporadic CJD (▲), patients with probable sporadic CJD (■), patients with possible sporadic CJD (◆). The box represents the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plot represents the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

The mean CSF tau concentration was raised in patients with suspected sporadic CJD but who were subsequently found to have another cause for their dementia, when compared to the control subjects (873 ± 2508 vs 160 ± 61 pg/mL). The 2 patients with the highest tau concentrations in the suspected CJD group had histologically confirmed Alzheimer's disease. This disease is known to be associated with elevated CSF tau concentrations and will be discussed further in section 5.4.1. The magnitude of CSF tau concentrations seen in patients with definite sporadic CJD was comparable to those previously described in the patients with strokes and brain tumours. This would be expected as the elevated CSF tau is thought to originate from neuronal damage and sporadic CJD is a rapidly progressing disease associated with large amounts of neuronal loss. There were 3 patients with histologically confirmed sporadic CJD who had CSF tau concentrations within the reference range. It is possible that these 3 patients had CSF samples taken either very early or very late in the disease course. It has been shown that the elevation of other brain-specific proteins such as S-100b and NSE in patients with sporadic CJD follow a bell shaped curve, with low CSF concentrations seen early in the disease, then rising as the disease progresses and subsequently falling prior to death (Jimi et al; 1992). The distribution of CSF tau concentrations in patients with possible sporadic CJD showed a bimodal distribution with 4 patients having concentrations of greater than 1000 pg/mL and 4 patients having concentrations of less than 1000 pg/mL. These patients are still being monitored and it is possible that those with high CSF tau concentrations are patients with sporadic CJD and those with low CSF tau concentrations do not have the disease.

The value of CSF tau in the diagnosis of sporadic CJD was assessed using three different cut-off concentrations of CSF tau. The sensitivity, specificity, negative and positive predictive values and efficiency at these three concentrations are shown in Table 5.2.

	CSF tau >315 pg/mL	**CSF tau > 1000 pg/mL**	**CSF tau >2000 pg/mL**
Sensitivity	92%	83%	65%
Specificity	63%	89%	93%
PPV	78%	92%	93%
NPV	85%	77%	64%
Efficiency	81%	85%	76%

Table 5.2 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of three different concentrations of CSF tau for the diagnosis of histologically confirmed sporadic and probable sporadic CJD

The best combination of positive predictive value (92%) and negative predictive value (77%) was obtained using a cut-off concentration of greater than 1000 pg/mL. This cut-off concentration also gave the greatest efficiency of 85%.

5.3.2 CSF tau concentrations in patients with new variant CJD

To investigate whether CSF tau measurement may have a role in the assessment of patients with suspected new variant CJD, CSF tau concentrations were measured in 19 patients with histologically confirmed new variant CJD (mean age: 29.0 ± 10.4

years, range 18-53; 9M:10F), in 8 patients with probable new variant CJD (mean age: 25.4 ± 6.1, range 19-39; 4M:4F) and in 1 patient with possible new variant CJD (age: 22 years, 1M). The CSF tau concentrations were compared to those found in 29 patients (mean age: 34.6 ± 12.5 years, range 15-56; 15M:14F) who were initially suspected of having new variant CJD, but subsequently proven not to have either by improvement in clinical course or by confirmation of another disease.

Although the mean concentration of CSF tau was significantly raised in patients with histologically confirmed new variant CJD when compared to control patients (1184 ± 1407 vs 450 ± 1178 pg/mL, $p<0.001$), there was considerable overlap between the two groups. Patients with histologically confirmed new variant CJD appeared to have a bimodal distribution of CSF tau concentrations but this was not evident in the patients with probable new variant CJD. Whether this bimodal distribution is an artefact of the small numbers investigated or is a true feature of this disease needs further investigation. The mean CSF tau concentration was also raised in patients with probable new variant CJD (978 ± 637 pg/mL) and in the patient with possible new variant CJD (1361 pg/mL). Statistical analysis was performed using the Mann Whitney U test. Results are shown in Figure 5.4.

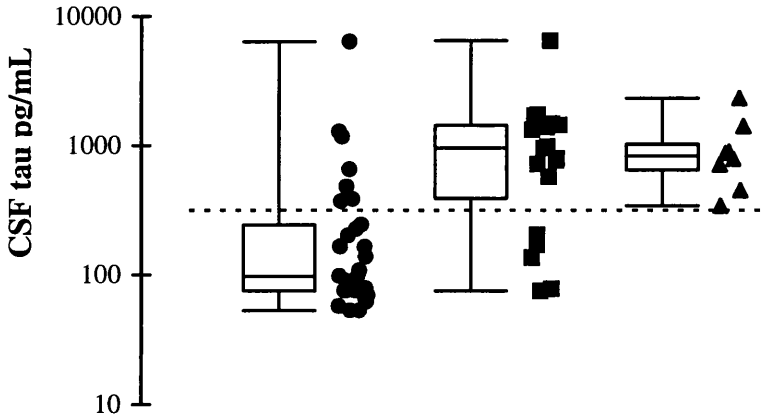


Figure 5.4 Combined scatter and box and whisker plots of CSF concentrations of tau in patients initially suspected of having new variant CJD but later proven not to have (●), patients with histologically confirmed new variant CJD (■) and patients with probable new variant CJD (▲). The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

The value of CSF tau in the diagnosis of new variant CJD was assessed using the sensitivity, specificity, positive and negative predictive value and the efficiency of the test at three different cut-off concentrations of CSF tau (Table 5.3).

	CSF tau > 315 pg/mL	CSF tau > 500 pg/mL	CSF tau > 1000 pg/mL
Sensitivity	74%	74%	42%
Specificity	79%	89%	93%
PPV	70%	82%	80%
NPV	82%	83%	71%
Efficiency	77%	83%	72%

Table 5.3 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of three different concentrations of CSF tau for the diagnosis of histologically confirmed new variant CJD

The best combination of positive predictive value (82%) and negative predicative value (83%) was obtained using a cut-off concentration of greater than 500 pg/mL. This concentration is lower than that which gives the best combination of predictive values in sporadic CJD.

The mean concentration of CSF tau found in new variant CJD is lower than that seen in sporadic CJD (5791 ± 6499 pg/mL vs 1184 ± 1407 pg/mL). The lower CSF tau concentration may be due to the more protracted clinical course seen in new variant CJD when compared to sporadic CJD. It is possible that with the longer disease duration the neuronal loss may occur at a slower rate leading to lower mean CSF tau concentrations.

The concentrations of CSF tau found in patients suspected of having new variant CJD were lower than in patients suspected of having sporadic CJD (450 ± 1178 pg/mL vs 873 ± 2508 pg/mL). This reflects the different diseases which may present with similar clinical features in the two diseases. The differential diagnosis for patients with sporadic CJD includes Alzheimer's disease and cortical Lewy body disease, which are conditions reported to have increased CSF tau concentrations. As new variant CJD has only recently been described, there is very little data about diseases which may present with similar clinical features, but it does appear that these diseases are less likely to be associated with raised CSF tau concentrations.

5.3.3 CSF tau concentrations in patients with mutations of the PRNP gene and iatrogenic CJD

To investigate whether CSF tau concentrations are elevated in patients with the iatrogenic and familial forms of the disease, CSF tau was measured in 4 patients with CJD secondary to the administration of cadaveric human growth hormone, in 3 patients with familial CJD and 1 patient with GSS. The results are shown in Table 5.4.

Type CJD	Patient Id	Mutation	Age (years)	Sex	CSF tau pg/mL
Iatrogenic - GH	Case 1	n/a	34	M	81
Iatrogenic - GH	Case 2	n/a	37	M	3539
Iatrogenic - GH	Case 3	n/a	28	M	8186
Iatrogenic - GH	Case 4	n/a	27	F	636
Familial CJD	Case 1	Insert	67	F	429
Familial CJD	Case 2	Insert	66	M	1656
Familial CJD	Case 3	PrP Glu-Lys^{200}	51	M	2107
GSS	Case 1	PrP Ala-Val^{117}	44	F	686

Table 5.4 CSF tau concentrations in 4 patients with iatrogenic CJD secondary to growth hormone administration, 3 patients with familial CJD and 1 patient with GSS syndrome.

CSF tau concentrations were elevated in 3 of the cases of iatrogenic CJD and in all of the cases of familial CJD and in the patient with GSS.

These results suggest that CSF tau concentrations are raised in nearly all patients with sporadic or familial CJD and in over 70% of patients with new variant CJD. The magnitude of the increases seen may be influenced by the type of CJD as patients with new variant CJD tend to have lower concentrations than those with sporadic CJD. It is possible that this reflects the rate of neuronal loss in these two diseases, with new variant CJD being associated with a longer disease duration, and a possible slower rate of neuronal loss, than sporadic CJD. It is also possible that the

amount of neuronal damage is less in new variant CJD than it is in the sporadic form of the disease.

5.4 CSF tau concentrations in patients with Alzheimer's disease and other forms of dementia

Many studies have shown that CSF tau concentrations are elevated in patients with Alzheimer's disease, and it has been suggested that CSF tau may be a useful diagnostic test for this disease (Vandermeeren et al., 1993; Arai et al., 1995; Blennow et al., 1995; Jensen et al., 1995; Tato et al., 1995; Vigo-Pelfrey et al., 1995; Galasko et al., 1997; Andreasen et al., 1998; Galasko et al., 1998; Kanai et al., 1998, Mecocci et al., 1998). Many of these studies did not compare the CSF tau concentrations seen in patients with Alzheimer's disease with other forms of dementia such as frontotemporal dementia or cortical Lewy body disease. These forms of dementia may be difficult to distinguish from Alzheimer's disease (Litvan et al., 1997) and are more prevalent than previously thought (Neary et al., 1988; Knopman et al., 1990). This part of the study was set up to investigate whether CSF tau concentrations could distinguish between these different forms of dementia.

CSF tau concentrations were measured in 26 patients (mean age: 62.2 ± 9.3 years, range 49-77; 18M:8F) with Alzheimer's disease diagnosed according to the NINCDS/ADRDA criteria (McKhann et al., 1984). Twenty-three of these patients had the sporadic form of the disease and 3 had autosomal dominant familial disease Alzheimer's disease, 2 patients had mutations of the amyloid precursor gene and 1

had a mutation of the presenillin 1 gene. The clinical diagnosis was confirmed by histological examination in 2 patients.

CSF tau concentrations were measured in 28 patients with frontotemporal dementia (mean age: 61.4 ± 9.1 years, range 41-74; 19M:9F) diagnosed according to the Lund and Manchester criteria (Brun et al., 1994). Of these patients 17 were clinically diagnosed with Pick's disease and 11 were diagnosed with frontal lobe degeneration. The clinical diagnosis was confirmed by pathological examination in 1 patient with Pick's disease and 1 patient with frontal lobe degeneration. CSF tau concentrations were also measured in 8 patients with cortical Lewy body disease (mean age: 59.3 ± 10.5 years, range 47-71; 6M:2F) who were diagnosed according to the criteria described by McKeith et al., 1992 and in 7 patients with corticobasal degeneration (mean age: 63.3 ± 13.1 years, range 46-78; 4M:3F). A group of 39 patients with miscellaneous causes of dementia were also investigated (mean age: 56.1 ± 14.2 years, range 17-85; 23M:16F). Of this group 26 had dementia of unknown cause, 3 had dementia associated with multi-system atrophy, 3 had progressive supranuclear palsy, 3 had a vascular cause for their dementia, 2 had Huntington's disease, 1 had primary progressive aphasia and 1 had motor neurone disease.

The CSF tau results are shown in Figure 5.5. The mean CSF tau concentrations were significantly raised in patients with Alzheimer's disease (823 ± 561 vs 160 ± 61 pg/mL, $p < 0.0005$) and frontotemporal dementia (617 ± 409 vs 160 ± 61 pg/mL, $p<0.03$) when compared to control subjects. There was no significant difference between the CSF tau concentrations in patients with Alzheimer's disease and patients

with frontotemporal dementia. Statistical analysis was performed using one way ANOVA with Bonferoni modification using SPSS.

The mean CSF tau concentration was raised corticobasal degeneration (613 ± 401 pg/mL). Three patients with cortical Lewy body disease had raised CSF tau concentrations. None of the patients with multi-system atrophy, progressive supranuclear palsy, primary progressive aphasia or motor neurone disease had raised CSF tau concentrations. One patient with Huntington's disease had marginally increased CSF tau concentrations and 2 patients with vascular dementia had raised CSF tau concentrations. Raised CSF tau concentrations were seen in 42% of the patients with unspecified dementia.

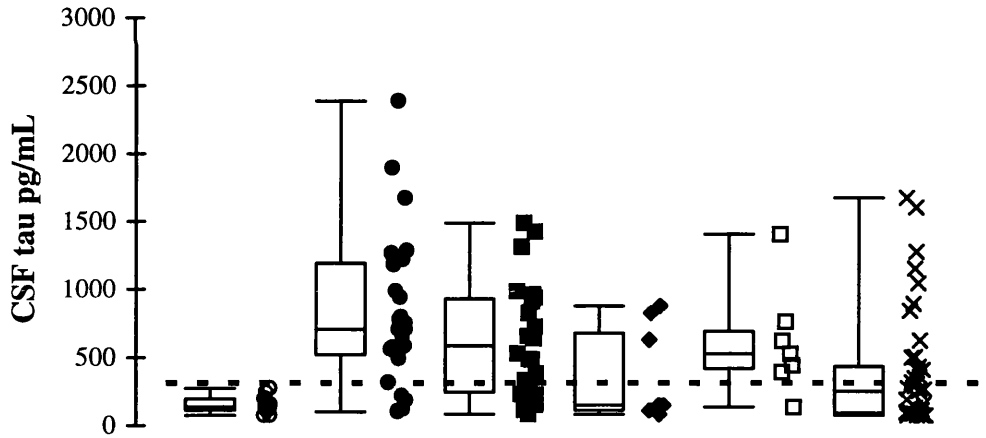


Figure 5.5 Combined scatter and box and whisker plots of CSF concentrations of tau in control subjects (○), *in patients with Alzheimer's disease* (●), *in patients with frontotemporal dementia* (■), *in patients with cortical Lewy body disease* (◆), *in patients with corticobasal degeneration* (□), *in patients with miscellaneous causes of dementia* (×). *The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the*

individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

5.4.1 CSF tau concentrations in Alzheimer's disease

The concentrations of CSF tau were significantly raised in patients with Alzheimer's disease when compared to control subjects (823 ± 561 vs 160 ± 61 pg/mL, $p < 0.0005$). This finding is in agreement with other studies (Vandermeeren et al., 1993; Arai et al., 1995; Blennow et al., 1995; Jensen et al., 1995; Tato et al., 1995; Vigo-Pelfrey et al., 1995; Galasko et al., 1997; Andreasen et al., 1998; Galasko et al., 1998; Kanai et al., 1998, Mecocci et al., 1998). Table 5.5 shows the mean CSF tau concentrations reported in Alzheimer's disease in four studies which used the same commercial ELISA (second generation, Innogenetics).

Study	**Method**	**CSF tau pg/mL (mean ± SD)**
Blennow et al (1995)	2nd generation Innogenetics	524 ± 280
Andreasen et al (1997)	2nd generation Innogenetics	796 ± 382
Kanai et al (1998)	2nd generation Innogenetics	489 ± 298
Mecocci et al (1998)	2nd generation Innogenetics	436 ± 67

Table 5.5 CSF tau concentrations (mean ± SD) in patients with Alzheimer's disease measured using the 2nd generation Innogenetics tau ELISA from four different studies

No relationship was seen between age and CSF tau in Alzheimer's disease ($r^2 = 0.038$). This is in agreement with other studies (Blennow et al., 1995; Andreasen et al, 1997). It has been reported that there is no difference between CSF tau concentrations in patients with early onset Alzheimer's disease (age less than 65 years) and late onset Alzheimer's disease (age greater than 65 years) (Arai et al., 1995), however other workers have found a difference between these two groups (Mecocci et al., 1998). It was not possible to obtain enough data concerning the age of onset of disease to investigate this in this study. There was no significant correlation between the mini mental state examination (MMSE) and CSF tau in patients with Alzheimer's disease ($r^2 = 0.17$, $p=0.18$). Although the numbers in this comparison are small it is consistent with the findings of Motter et al., 1995; Galasko et al., 1997 and Andreasen et al., 1998. Although Kanai et al., 1998 found a positive correlation between the MMSE and CSF tau, and Tato et al., 1995 found a positive correlation with the Folstein's mental test examination but not the Rosen score or the Blessed dementia scale. Five patients with clinically diagnosed Alzheimer's disease had CSF tau concentrations that would be considered normal. A number of other studies have found a small number of patients with clinically diagnosed Alzheimer's disease who had normal CSF tau concentrations (Vandermeeren et al., 1993; Blennow et al., 1995; Motter et al., 1995; Vigo-Pelfrey et al., 1995; Galasko et al., 1997 and Andreasen et al, 1998).

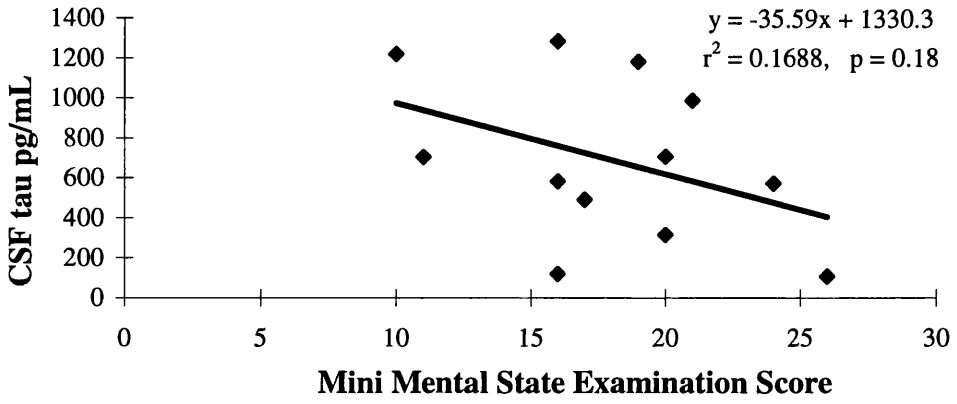


Figure 5.6 Relationship between CSF tau concentrations and the MMSE in patients with Alzheimer's disease

The origin of the increased CSF tau in patients with Alzheimer's disease may be due in part to release from dying or degenerating neurones, and in part due to the increased amount of tau protein found within the neurones, in the form of neurofibrillary tangles. It is known that tau protein is found in higher concentrations in brain tissue from patients with Alzheimer's disease than in control brain tissue (Khatoon et al., 1992).

5.4.2 CSF tau concentrations in patients with frontotemporal dementia

The concentrations of CSF tau are significantly raised in patients with frontotemporal dementia when compared to control patients (617 ± 409 vs 160 ± 61 pg/mL, $p < 0.03$). CSF tau concentrations have been reported to be increased in patients with frontotemporal dementia (Arai et al., 1997) and patients with frontal lobe degeneration (Blennow et al., 1995). In contrast, a recent study did not find an increase in CSF tau in patients with frontal lobe degeneration (Mecocci et al., 1998). Although the mean concentration of CSF tau is lower in patients with frontotemporal

dementia than patients with Alzheimer's disease, there was no statistical difference between the two groups.

The relationship between CSF tau and the severity of disease as assessed by the MMSE was examined in 13 patients with frontotemporal dementia (Figure 5.7). Although the correlation did not reach significance, a relationship may exist between CSF tau and the MMSE ($r^2 = 0.27$, p=0.07) (Figure 5.7); as those patients with more severe dementia, as assessed by a lower MMSE score, had higher CSF tau concentrations. This will require further investigation using a larger number of patients.

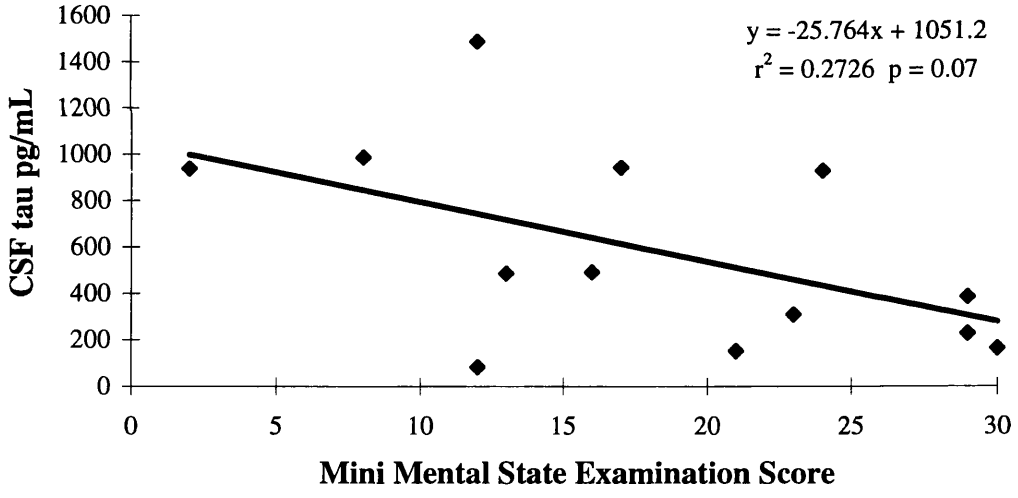


Figure 5.7 The relationship between CSF tau concentrations and the MMSE in thirteen patients with frontotemporal dementia

The mean CSF tau concentrations in those patients thought clinically to have Pick's disease and those thought to have frontal lobe degeneration were 775 ± 397 pg/mL and 374 ± 304 pg/mL, respectively (Figure 5.8). These diseases are difficult to

distinguish clinically and the final diagnosis can only be obtained by histological examination of the brain. Patients with Pick's disease are characterised by the presence of intraneuronal inclusion bodies called Pick bodies which have been shown to contain abnormal hyperphosphorylated tau. It is possible that the brain tissue from patients with Pick's disease may contain elevated tissue concentrations of tau protein, in a similar manner to patients with Alzheimer's disease. In contrast, patients with frontal lobe degeneration do not have tau containing intraneuronal inclusion bodies. Thus the difference between the mean CSF tau concentrations between these two groups may be due to the amount of tau protein released by dying or degenerating neurones. It may be that CSF tau concentrations may be able to help distinguish these two groups, but this needs further investigation with a larger number of patients and histological verification of the clinical diagnosis.

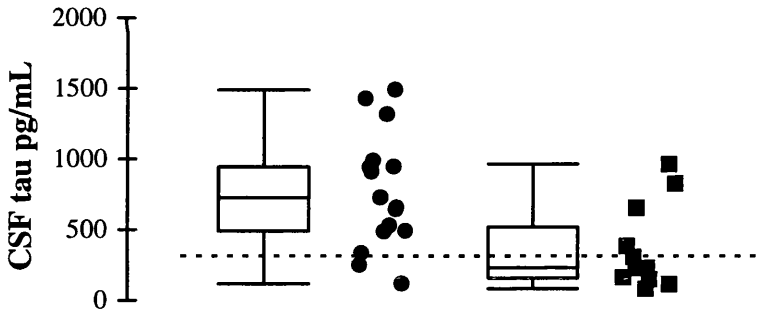


Figure 5.8 Combined scatter and box and whisker plots of CSF concentrations of tau in clinically diagnosed Pick's disease patients (●) and in clinically diagnosed patients with frontal lobe degeneration (■). The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 SD above the mean of the control subjects)

5.4.3 CSF tau concentrations in other forms of dementia

The mean CSF tau concentration was increased in patients with corticobasal degeneration and supports the findings of Mitani et al., 1998 who found raised concentrations of CSF tau in 4 of 9 patients with corticobasal degeneration.
Of the 8 patients with cortical Lewy body disease, 3 had raised concentrations of CSF tau. This is in contrast to a previous study which found CSF tau concentrations were significantly raised in all 6 patients with cortical Lewy body disease investigated (Arai et al, 1997). Cortical Lewy body disease can exist in a pure form or in conjunction with Alzheimer's disease (Lennox, 1992). It is possible that the group with normal tau concentrations may be patients with pure cortical Lewy body disease and the group with elevated CSF tau could be patients with a mixed pathology. With the small numbers investigated it could be that these two groups of CSF tau concentrations reflect the extremes seen in this disease. Further investigation using larger numbers of patients with a histological diagnosis is required to examine these possibilities. None of the patients with progressive supranuclear palsy had raised CSF tau concentrations.

5.5 CSF tau concentrations in patients with Human Immunodeficiency Virus (HIV) infection

The most common neurological complication of HIV infection is a dementing illness called AIDS dementia complex (ADC). In the early stages it presents with lack of concentration and forgetfulness, then progresses to apathy, social withdrawal, inappropriate behaviour and acute mania or psychosis (Navia, 1994).

Other common neurological complication of HIV infection are opportunistic infections of the CNS, particularly with toxoplasma, cytomegalovirus, cryptococcus, Herpes simplex and zoster; and primary cerebral lymphoma (Brew et al., 1988). In the early stages ADC can be difficult to distinguish from clinical depression and early signs of CNS infection or infiltration. This study was set up to investigate whether CSF tau concentrations are raised in patients with ADC, and whether measurement of CSF tau can help distinguish between ADC and other CNS complications of HIV infection.

CSF tau was measured in samples of CSF obtained from 79 consecutive HIV infected patients (mean age: 38.3 ± 8.4 years, range 28-62; 63M:16F) undergoing diagnostic lumbar puncture for assessment of possible neurological disease. Of these, 26 patients (mean age: 37.4 ± 8.4 years, 22M:4F) had ADC with stage 2 or greater dementia according to the Memorial Slone Kettering Criteria (Price and Brew, 1988), 6 had systemic B-cell lymphoma and were being investigated for possible cerebral involvement, 4 had primary CNS lymphoma, 18 had CNS infections (7 had cryptococcal meningitis, 5 had cytomegalovirus (CMV) encephalitis, 3 had varicella zoster virus (VZV) meningoencephalitis, 3 had cerebral toxoplasmosis) and 25 patients had a miscellaneous collection of abnormalities (8 had peripheral neuropathy, 8 had headache, 4 had confusion, 2 patients had had fits, 1 had hysterical paraparesis, 1 suffered from alcoholism, 1 had strabismus).

The mean CSF tau concentration was not significantly raised in ADC (212 ± 180 pg/mL), lymphoma (135 ± 84 pg/mL), CNS infections (265 ± 200 pg/mL) or miscellaneous disorders (143 ± 72 pg/mL) when compared to control subjects (160 ± 61 pg/mL) (Figure 5.9). Five patients with ADC had increased CSF tau concentrations as did 1 patient with CNS lymphoma and 6 patients with CNS infections. None of the patients with a range of miscellaneous disorders had raised CSF tau concentrations. In a recent study Ellis et al., 1998 reported that CSF tau concentrations were not raised in any of the patients with ADC investigated. Both of these studies have involved small numbers of patients and need to be expanded. Of the 6 patients with CNS infections with elevated tau, 2 had cerebral toxoplasmosis, 2 had CMV encephalitis, 1 had varicella zoster encephalitis and 1 had cryptococcal encephalitis. Statistical analysis was performed using one way ANOVA with Bonferoni modification using SPSS.

Of the 5 patients with ADC who had elevated CSF tau concentrations, 4 died within two months of the CSF sample being taken. There did not appear to be any relation to the severity of dementia, although this was difficult to assess in retrospect. The patient with CNS lymphoma who had an elevated CSF tau, also died shortly after the CSF sample was taken and histopathology showed a very necrotic CNS pathology. These findings suggest that the elevated CSF tau may be related to active brain damage occurring in these patients prior to death.

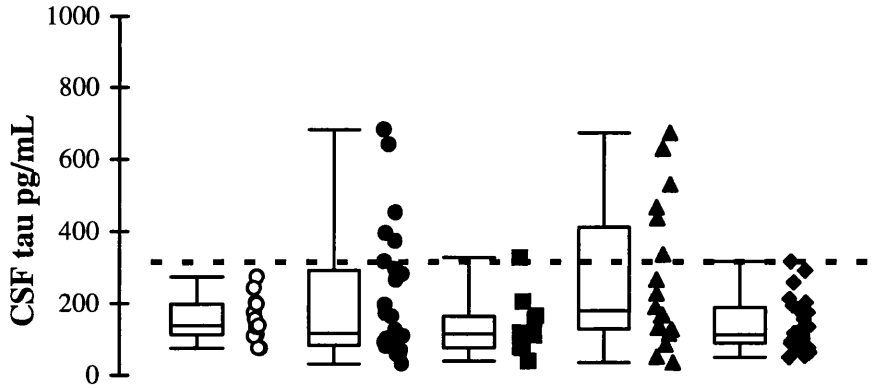


Figure 5.9 Combined scatter and box and whisker plots of CSF concentrations of tau in control subjects (○), *patients with AIDS associated dementia complex* (●), *patients with lymphoma* (■), *patients with CNS infections* (▲) *and patients with a miscellaneous range of disorders* (◆). *The boxes represents the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2.5 standard deviations above the mean of the control subjects)*

There was no significant correlation between the CD4 count in all patients with HIV and CSF tau concentrations ($r^2 = 0.033$, $p = 0.11$). This is in agreement with Ellis et al., 1998 who also failed to find a correlation between CSF tau concentrations and CD4 count in patients with HIV infection.

5.6 General discussion of CSF tau in neurological disease

CSF tau is one of the most abundant microtubule-associated proteins found in the vertebrate nervous system. It has been shown by immunohistochemistry to be located mainly within the axons and is thought to be important in maintaining microtubule stability (Goedert et al., 1991a). There are six isoforms of tau protein

which are produced from a single gene by alternative mRNA splicing (Lee et al., 1991), each of which is present in the adult human brain. Tau protein is phosphorylated at two or three sites on all isoforms in the adult (Ksiezak-Reding et al., 1992). The tau protein in some types of dementia is hyperphosphorylated and forms cell inclusion bodies such as neurofibrillary tangles in Alzheimer's disease and supranuclear palsy, Pick bodies in Pick's disease and corticobasal bodies in corticobasal degeneration. The main component of these inclusion bodies is hyperphosphorylated tau protein and this has stimulated interest in the measurement of CSF tau concentrations in these conditions.

As tau protein is a major constituent of the axonal microtubular system, any damage to the axons would be expected to release tau protein into the extracellular fluid and ultimately into the CSF. The results described in section 5.2 show that elevated concentrations of tau protein may be found in diseases associated with ischaemia such as intracerebral haemorrhage or infarction; and in necrosis seen in viral encephalitis or brain tumours. These disease processes may be associated with either an acute brain injury or severe necrosis, and as a result release significant amount of axonal proteins into the CSF. This is reflected by the high concentrations of tau protein seen in the CSF in these conditions. In contrast diseases such as multiple sclerosis, where smaller amounts of axonal damage occur over a longer period of time, are not found to be associated with increased CSF tau concentrations.

Creutzfeldt-Jakob disease (CJD) is a fatal neurodegenerative disease characterised by extensive neuronal loss, astrocytosis and deposition of an abnormal form of the prion protein. The sporadic form of the disease presents as a rapidly progressing dementia

with death occurring typically within six months. The acute nature of the illness and its severity suggests that neuronal loss is rapid and extensive. This would result in a large amount of axonal proteins being released into the CSF and results in section 5.3.1 show that high concentrations of CSF tau are found in patients with this disease. Patients with the new variant form of the disease have a longer duration of illness and the mean CSF tau concentration is lower that that found in sporadic CJD. The amounts of brain atrophy and spongiform changes are similar in both diseases, suggesting that the difference in mean CSF concentrations in these diseases may be the rate of neuronal loss, rather than the amount of neuronal loss.

These results demonstrate that CSF tau concentrations may be elevated in diseases associated with acute and/or severe neuronal damage, but are less likely to be increased in slowly progressive neuronal damage such as that found in multiple sclerosis. In view of this finding, it is interesting that patients with Alzheimer's disease and frontotemporal dementia have elevated CSF tau concentrations. These patients have a slowly progressing cognitive impairment with brain atrophy due neuronal loss. The CSF tau concentrations would not be expected to be increased due to the slow nature of the neuronal loss, especially as other markers of neuronal damage such as 14-3-3 and neurone-specific enolase are not elevated (section 7.8 and section 9.5 respectively). The reason for the elevated CSF tau concentrations found in these dementia syndromes, maybe due to increased brain concentrations of tau protein (Khatoon et al., 1992). Both Alzheimer's disease and Pick's disease are associated with intraneuronal inclusion bodies whose main constituent is hyperphosphorylated tau protein. It is possible that the presence of the inclusion bodies increases the total amount of tau present within the brain, so that for a given

degree of axonal loss the amount of tau protein is higher than would be expected. The finding that mutations in the tau gene are responsible for the frontotemporal dementia with Parkinsonism linked to Chromosome 17 (Spillantini et al., 1998), has increased the speculation that abnormal tau can cause neuronal damage directly. This could mean that neurones containing large amounts of hyperphosphorylated tau inclusion bodies are more likely to degenerate than those without tau inclusion bodies. This suggests that CSF tau may be a very sensitive marker for selective neuronal death in these conditions. This is currently being studied by comparing MRI imaging as a measure of brain atrophy with CSF tau concentrations. Another possible explanation for the high CSF tau concentrations is that hyperphosphorylated tau may not be cleared from the CSF as rapidly as normally phosphorylated tau. So the elevated CSF tau concentrations seen in Alzheimer's disease and frontotemporal dementia could be due to increased amounts being released from dying neurones or from decreased clearance from the CSF.

The use of CSF tau measurement in the assessment of patients with dementia is probably limited to distinguishing sporadic CJD from Alzheimer's disease or cortical Lewy body disease, provided an appropriately high cut-off concentration is used as described in section 5.3.1. Its role in the differential diagnosis of patients with new variant form of CJD is unclear at present due to the small number of patients investigated. CSF tau concentrations cannot distinguish Alzheimer's disease from other dementia syndromes such as frontotemporal dementia. Future studies may discover whether CSF tau concentrations can distinguish between Pick's disease and frontal lobe degeneration. Further work is also needed to investigate whether those patients with cortical Lewy body disease who have high CSF tau, have a dual

pathology including Alzheimer's disease. The significance of the elevated CSF tau concentrations seen in some patients with AIDS dementia complex (ADC) is also unclear and needs further investigation.

In summary, CSF tau concentrations are raised in patients with sporadic CJD and in some patients with new variant CJD. Concentrations of CSF tau may also be elevated in conditions associated with acute and/or extensive neuronal loss; dementia syndromes associated with tau inclusion bodies and in some patients with ADC. The role of CSF tau measurement is at present limited to distinguishing sporadic CJD from other forms of dementia.

Chapter 6 - SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting for the detection of CSF 14-3-3

6.1 SDS-Polyacrylamide gel electrophoresis

In SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis proteins are not separated by their intrinsic electrical charge but by their molecular weight. Prior to electrophoresis the sample is diluted with a buffer containing sodium dodecyl sulphate (SDS) and a reducing agent, and heated to 100°C. The reducing agent breaks any inter and intra subunit disulphide bonds and SDS, an anionic detergent, denatures the protein by wrapping around the polypeptide backbone. As a result the proteins in the sample become rods of negative charge with equal charge per unit length. The migration of the protein in PAGE is a function of its size, and after visualisation the intensity of the band can give an indication of the amount of protein present in the sample.

There are two types of buffer systems used in this type of electrophoresis, continuous and discontinuous. In the continuous system the same buffer is used in the gel and in the tank, and although this system is easy to set up, it cannot concentrate the protein bands and results in poor resolution. The discontinuous system, a large pore non-restrictive gel called the stacking gel is layered on top of a separating (running) gel. Each gel layer is made with a different buffer and the tank buffer is different to the gel buffers. The mobility of the protein is intermediate between the buffer ion in the stacking gel (leading ion) and the buffer ion in the upper tank (trailing ion).

When the electrophoresis is started the buffer ions and proteins move into the stacking gel, and the differences in mobility of the buffer ions and proteins cause the proteins to be concentrated into a very thin zone called the stack. The proteins are then separated on the basis of size once they migrate into the separating gel.

Acrylamide is used for preparing electrophoretic gels for separating proteins by size. A mixture of acrylamide and bis-acrylamide polymerises to form a cross-linked network with a pore size that is dependent on the concentration of acrylamide used. The higher the concentration of acrylamide, the smaller the pore size. The polymerisation process is initiated by the addition of ammonium persulphate and TEMED (N, N, N', N' - tetramethylenediamine). TEMED catalyses the production of free radicals from ammonium persulphate which in turn starts the polymerisation process. The presence of oxygen will inhibit polymerisation so the gel mixtures are often de-gassed prior to use.

6.2 Western blotting

The process of transferring the separated proteins from the gel onto a support matrix, usually nitrocellulose to which they bind and become immobilised, using electrophoretic transfer is known as either Western blotting or electroblotting. The most important advantage of Western blotting is that it moves the proteins from the gel matrix which can hinder the access of immunological probes, especially true in gradient gels where the gradation in pore size will result in variable access to antibodies, to a medium which offers easy and uniform access. Incubation times are also reduced as the proteins are bound on the surface of the support matrix. The

support matrix is less fragile than the polyacrylamide gel and this eases subsequent visualisation. The most common support matrix is nitrocellulose which is easy to handle and has a good capacity for binding protein (80-100 $\mu g/cm^2$). Even with this high capacity some proteins, especially low molecular weight proteins, may bind only weakly or not at all. The mechanism of binding is poorly understood but is thought to involve hydrophobic interactions.

In a wet blotting system the gel and nitrocellulose are placed between two porous pads soaked in transfer buffer, in a plastic holder. The plastic holder is placed in a tank between two platinum electrodes, and the tank filled with transfer buffer. Typical buffers used are Tris-glycine buffers, Tris-borate buffers and sodium phosphate buffers, the selection of which depends on the gel and detection system used. Methanol (20%) is often included to minimise the swelling of the gel which occurs during blotting and to increase the protein binding capacity of nitrocellulose. The disadvantage of including methanol in the transfer buffer is that it reduces the efficiency of transfer so that it needs to be carried out for longer. The blotting occurs overnight using a constant current of around 200mA. If constant voltage is used, the current will increase as the buffer components of the gel elute out, and this increase in current may exceed 1A.

A semi-dry blotting system can be used as an alternative, in this case the gel and nitrocellulose are sandwiched between filter paper soaked in transfer buffer and placed directly between two platinum electrode plates. The transfer times are shorter, but some high molecular weight proteins may not transfer.

6.3 CSF 14-3-3 detection using SDS-PAGE and Western blotting with colorimetric detection

The methods described in this section are based on modifications of the method described by Hsich et al., 1996.

6.3.1 Preparation of SDS-PAGE reagents

Running Gel buffer (1.5 M Tris-Cl, pH 8.8)

Dissolve 36.3 g Tris in 150 mL deionised water, adjust pH to 8.8 with HCl. Make up to 200 mL.

Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)

Dissolve 6.0 g Tris in 80 mL deionised water, adjust pH to 6.8 with HCl. Make up to 100 mL.

10% SDS

Dissolve 10 g SDS in 100 mL deionised water

10% ammonium persulphate

Dissolve 0.1 g ammonium persulphate in 1 mL deionised water. Prepare immediately before use.

10% acrylamide running gel

30% Acrylamide/bis-acrylamide solution (mix ratio 37.5: 1)	20 mL
(Product Code:A-3699, Sigma-Aldrich Chemicals, Dorset, UK)	
Running gel buffer	15 mL
10% SDS	0.6 mL
Deionised water	24.1 mL
10% ammonium persulphate	300 µL
TEMED	20 µL

4% acrylamide stacking gel

30% Acrylamide/bis-acrylamide solution (mix ratio 37.5: 1)	2.66 mL
Stacking gel buffer	5.0 mL
10% SDS	0.2 mL
Deionised water	12 mL
10% ammonium persulphate	100 µL
TEMED	10 µL

Water saturated 2-methyl-1-propanol

Add 20 mL deionised water to 40 mL 2-methyl-1-propanol in a glass bottle. Shake and allow to separate

Gel overlay solution (0.375 M Tris-Cl, 0.1% SDS, pH 5.8)

Running gel buffer	25 mL
10% SDS	1.0 mL

Make up to 100 mL with deionised water.

Sample diluent (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8)

Stacking gel buffer	2.5 mL
10% SDS	4.0 mL
glycerol	2.0 mL
bromophenol blue	2.0 mg
dithiothreitol	0.31 g

The above was dissolved in 10 mL deionised water and stored in 0.5 mL aliquots at -20°C

Electrophoresis buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3)

Tris	30.3 g
glycine	144.1 g
SDS	10 g

Dissolve the above in 10 L of deionised water.

6.3.2 Preparation of SDS-PAGE gel (10% running gel and 4% stacking gel)

A SE 600 vertical slab gel unit (Amersham Pharmacia Biotech, Herts, UK) was assembled in the dual gel casting stand according to the manufacturer's instructions, using a set of 1.5 mm spacers. The reagents for the running gel, omitting the ammonium persulphate and TEMED, were mixed in a 150 mL glass beaker and degassed by bubbling helium through. After addition of the ammonium persulphate and TEMED the solution was poured between the two 180 mm x 160 mm glass plates, assembled in the casting stand. A gap of 4 cm was left at the top of the plates and a layer of water saturated 2-methyl-1-propanol was carefully pipetted on top of the gel. The gel was allowed to polymerise. Once the gel had polymerised the

saturated 2-methyl-1-propanol and unpolymerised acrylamide was poured off and 1 mL of gel overlay solution was added.

In a 50 mL glass beaker all the reagents for the stacking gel, apart from the ammonium persulphate and the TEMED, were added and degassed in the same manner as the running gel. The gel overlay solution was poured away and after rinsing the surface of the gel with 1-2 mL of the stacking gel solution, the remaining stacking gel was added. A 10 space comb was added to the stacking gel, being careful not to trap any bubbles, and the gel was left to polymerise.

6.3.3 Sample Preparation for SDS-PAGE

For each CSF sample 60 µL was diluted with an equal volume of sample diluent, and placed in a boiling water bath for 4 minutes.

6.3.4 Running the SDS-PAGE gel

The comb was carefully removed from the stacking gel and the wells were rinsed with electrophoresis buffer. After fitting the gaskets to the upper tank chamber it was placed onto the gel and secured using the cams supplied. The lower tank chamber was filled with 3 L of electrophoresis buffer and the gel plus upper chamber were lowered into the lower tank chamber being careful to avoid trapping any bubbles at the bottom of the gel. The upper chamber was filled with electrophoresis buffer, making sure that there was no leakage of buffer into the lower chamber. To each well 100 µL of diluted CSF sample was added. A positive control

(cerebrospinal fluid from a histopathologically confirmed case of sporadic Creutzfeldt-Jakob disease), a negative control (cerebrospinal fluid from a patient without histopathological evidence of sporadic CJD) and molecular weight markers were included on each run. The gel was run at 250 V, 50 mA for 3 hours using an EPS 200 power supply (Amersham Pharmacia Biotech, Herts, UK).

6.3.5 Transfer of 14-3-3 onto nitrocellulose using Western blotting

6.3.5.1 Preparation of transfer buffer

Transfer buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 containing 20% methanol)

Tris	9.1 g
glycine	43.2 g
SDS	3 g

Dissolve the above in 2 L of deionised water, add 600 mL methanol and make up to 3 L with deionised water.

Coomassie Brilliant Blue

Dissolve 100 mg Coomssie Brilliant Blue in 100 mL destain solution (70 mL water: 10 mL glacial acetic acid: 20 mL ethanol). Filter prior to use. Store at room temperature.

6.3.5.2 Conditions of Western blotting

At the end of the electrophoresis, the gel was removed from the electrophoresis system and the lane containing the molecular weight markers was removed and placed in Coomassie Brilliant Blue stain for 1 hour and then destained using deionised water. The remaining gel was placed into pre-chilled transfer buffer for 30 minutes. Two pieces of filter paper and a piece of nitrocellulose were cut to the same dimensions as the gel and placed into transfer buffer, along with the two fibre pads supplied with the Trans-blot® Electrophoretic Transfer cell (Bio-Rad, Herts, UK). After 30 minutes one fibre pad was placed onto the plastic gel holder and onto this was placed a piece of wet filter paper ensuring that there were no trapped air bubbles. The gel was removed and placed on top of the filter paper. A piece of pre-wetted nitrocellulose was placed on top of the gel, being careful not to trap any bubbles. The remaining filter paper and fibre pad were placed on top of the gel, all bubbles removed and the gel holder closed. The transfer cell was filled with pre-chilled transfer buffer and the cooling coil placed inside the cell and attached to a free flowing water system. The gel holder placed into the Trans-blot® Electrophoretic Transfer cell with the gel facing the cathode. The transfer of proteins was carried out using a constant current of 400 mA and a starting voltage of 50 V for 16 hours.

6.3.6 Detection of 14-3-3 using immunoblotting

6.3.6.1 Preparation of immunoblotting reagents

Blocking solution (2% milk in phosphate buffered saline (PBS))

Dissolve 1 g non-fat milk powder (Marvel) in 50 mL PBS

Antibody diluent (0.1% milk in PBS)

Dissolve 0.05 g non-fat milk powder (Marvel) in 50 mL PBS

Wash solution (PBS containing 0.05% Tween 20)

500 μL Tween 20 added to 1 L PBS

Rabbit polyclonal anti-human 14-3-3γ gamma subunit (Product Code sc-731, Santa Cruz Ltd, Germany, supplied by Autogen Bioclear, Wilts, UK)

50 μL of the above antibody was diluted in 50 mL PBS containing 0.1% non-fat milk powder

Swine polyclonal anti-rabbit Immunoglobulin HRP conjugate (Product Code: P0217, Dako Ltd, Cambs, UK)

50 μL of the above antibody was diluted in 50 mL PBS containing 0.1% non-fat milk powder

Amino-ethylcarbazole colour reagent

Dissolve 50 mg of 3-amino-9-ethylcarbazole in 20 mL ethanol, add 100 mL acetate buffer, pH 5.2 and 100 μL hydrogen peroxide

6.3.6.2 14-3-3 immunoblotting technique

After transferring the 14-3-3 onto nitrocellulose, the unbound protein binding sites were blocked by incubating the membrane in 2% non-fat milk in PBS for 30 minutes at room temperature. The nitrocellulose membrane was rinsed with running water and then washed with PBS containing 0.05% Tween 20 (wash solution), and placed in 50 mL PBS/milk containing rabbit anti-human 14-3-3γ. After incubating the nitrocellulose membrane overnight at 4°C, the membrane was rinsed with running water and washed as described previously, and placed in 50 mL PBS/milk containing swine anti-rabbit immunoglobulin HRP conjugate. After incubating the nitrocellulose membrane for 1 hour at room temperature, the membrane was rinsed with running water and washed with several changes of wash solution over 1 hour. The membrane was developed with aminocarbazole colour reagent for 20-30 minutes at room temperature, then rinsed under running water and then washed for 30 minutes before being dried. A 14-3-3 immunoblot prepared using this method is shown in Figure 6.1.

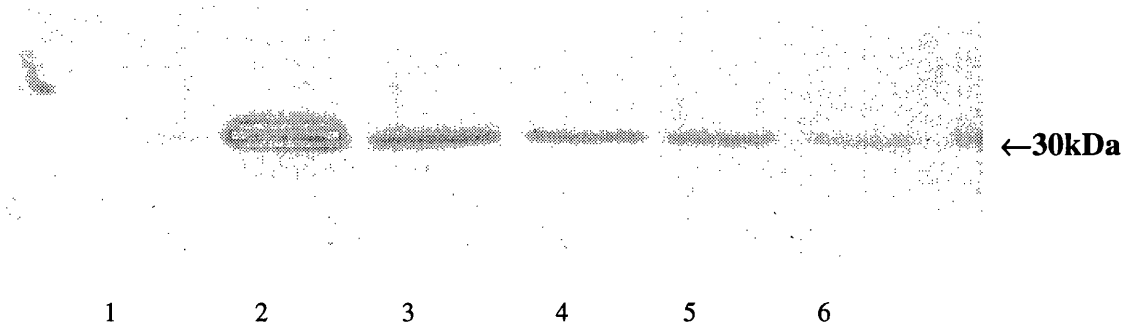


Figure 6.1 14-3-3 immunoblot using colorimetric detection, lane 1: negative control; lane 2: positive control; lanes 3-6: samples from 4 patients with sporadic CJD. The immunoblot illustrates the range of 14-3-3 which can be detected in the CSF of patients with sporadic CJD

6.4 CSF 14-3-3 detection using SDS-PAGE and Western blotting with enhanced chemiluminescence detection

The method previously described for detecting 14-3-3 in the CSF took three days to complete and the immunoblots could be difficult to interpret as the14-3-3 bands were very faint. To improve the sensitivity, enhanced chemiluminescence was used to detect the 14-3-3 bands. The improved sensitivity of this method had two other advantages, firstly the volume of CSF sample used could be reduced and secondly a mini-gel system could be used which reduced the analysis time. The SDS-PAGE used the same reagents as previously reported and only significant changes will be described.

6.4.1 Preparation of SDS-PAGE reagents

10% acrylamide running gel

30% Acrylamide/bis-acrylamide solution (mix ratio 37.5: 1)	5.0 mL
Running gel buffer	3.8 mL
10% SDS	150 µL
Deionised water	6.0 mL
10% ammonium persulphate	150 µL
TEMED	10 µL

4% acrylamide stacking gel

30% Acrylamide/bis-acrylamide solution (mix ratio 37.5: 1)	0.5 mL
Stacking gel buffer	1.0 mL
10% SDS	45 µL
Deionised water	2.5 mL
10% ammonium persulphate	75 µL
TEMED	10 µL

6.4.2 Preparation of SDS-PAGE gel (10% running gel and 4% stacking gel)

A SE 250 Mighty Small™ II vertical slab gel unit (Amersham Pharmacia Biotech, Herts, UK) was assembled in a SE 245 Mighty Small dual gel casting stand according to the manufacturer's instructions, using a set of 0.75 mm spacers. The reagents for the running gel, omitting the ammonium persulphate and TEMED, were mixed in a 50 mL glass beaker and degassed by bubbling helium through. After addition of the ammonium persulphate and TEMED the solution was poured between the two 100 mm x 80 mm glass plates, assembled in the casting stand. A gap of 2 cm was left at the top of the plates and a layer of water saturated 2-methyl-

1-propanol was carefully pipetted on top of the gel. The gel was allowed to polymerise. Once the gel had polymerised the saturated 2-methyl-1-propanol and unpolymerised acrylamide was poured off and 1 mL of gel overlay solution was added.

In a 50 mL glass beaker all the reagents for the stacking gel, apart from the ammonium persulphate and the TEMED, were added and degassed in the same manner as the running gel. The gel overlay solution was poured away and after rinsing the surface of the gel with 0.5 mL of the stacking gel solution, the remaining stacking gel was added. A 10 space comb was added to the stacking gel, being careful not to trap any bubbles, and the gel was left to polymerise.

6.4.3 Sample Preparation for SDS-PAGE

For each CSF sample 15 µL was diluted with an equal volume of sample diluent, and placed in a boiling water bath for 4 minutes.

6.4.4 Running the SDS-PAGE gel

The comb was carefully removed from the stacking gel and the wells were rinsed with electrophoresis buffer. The gel was placed into the SE 250 Mighty Small II electrophoresis tank and secured using the clips provided. The 'upper' tank buffer chamber, which in this design is actually behind the gel, was filled with 75 mL electrophoresis buffer, and the lower tank buffer chamber was filled with 125 mL electrophoresis buffer. To each well 20 µL of diluted CSF sample was added, and as

before a positive and negative control was included on each run. The gel was run using a 120 V, 40 mA for approximately 1 hour using an EPS 200 power supply (Amersham Pharmacia Biotech, Herts, UK).

6.4.5 Transfer of 14-3-3 onto nitrocellulose using Western blotting

6.4.5.1 Conditions used for Western blotting

The proteins from the gel were transferred onto nitrocellulose using a SemiPhor™ Semi-Dry Transfer unit (Amersham Pharmacia Biotech, Herts, UK). Six pieces of filter paper and a piece of nitrocellulose were cut to the same dimensions as the gel and placed into transfer buffer. A mylar mask which had a hole slightly larger than the gel was placed onto the base of the transfer unit, which housed the anode. The filter papers, nitrocellulose and gel were assembled as shown in Figure 6.2. All bubbles were carefully removed between each layer by rolling a glass rod over the surface of the filter papers and gel. The transfer of proteins was carried out using a constant current of 64 mA and a starting voltage of 8 V for 2 hours using an EPS 200 power supply (Amersham Pharmacia Biotech, Herts, UK).

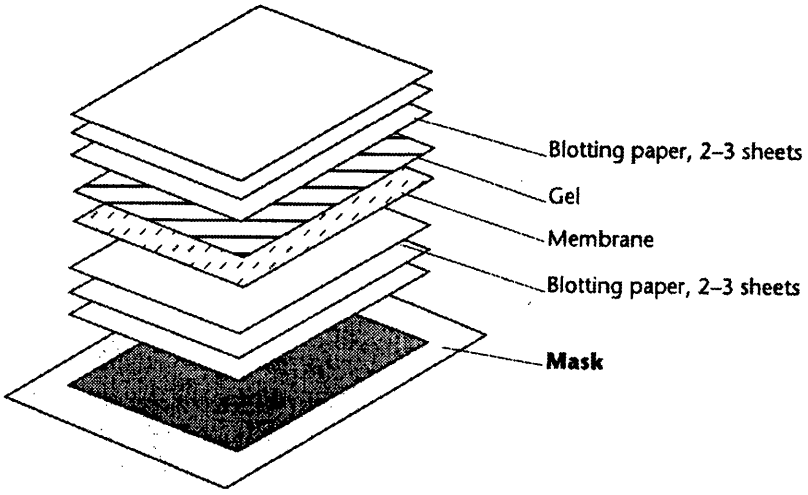


Figure 6.2 Schematic representation of assembly of filter paper, nitrocellulose and gel for protein transfer using the SemiPhor™ Semi-Dry Transfer unit

6.4.6 Detection of 14-3-3 using immunoblotting

After transferring the 14-3-3 from the gel the nitrocellulose membrane was blocked and incubated with antibodies as previously described.

6.4.6.1 Enhanced chemiluminescence

Enhanced chemiluminescence exploits the ability of oxygen radicals to react with luminol to produce light (Figure 6.3). The production of oxygen radicals is catalysed by the action of horseradish peroxidase on hydrogen peroxide. This light is very short lived, merely a few nanoseconds, without the presence of enhancers which prolong the emission of light to minutes. This enables the light to be measured either in a luminometer for immunoassays or by light sensitive photographic film for Western blots.

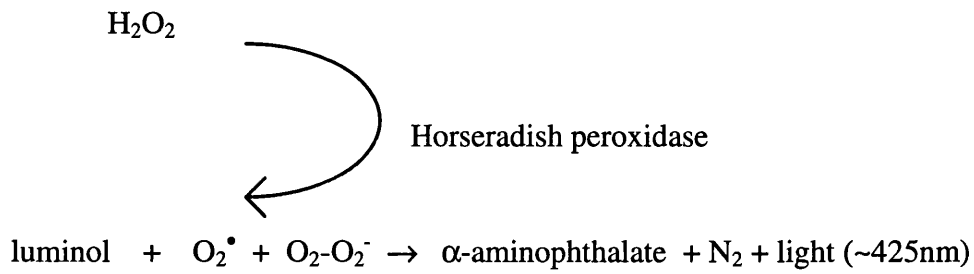


Figure 6.3 Horseradish peroxidase catalysed oxidation of luminol

6.4.6.2 Preparation of enhanced chemiluminescent reagents

SuperSignal® Chemiluminescent Substrate Stable Peroxide Solution

(Product Code: 1856135, Pierce & Warriner, Chester, UK).

SuperSignal® Chemiluminescent Substrate Luminol/Enhancer

(Product Code: 1856136, Pierce & Warriner, Chester, UK).

Working ECL reagent

Mix equal volumes of the above solutions, immediately prior to use.

6.4.6.3 Detection of 14-3-3 using enhanced chemiluminescence

After washing the membrane was drained of excess fluid, placed on a level surface and covered with 3 mL of working ECL reagent ensuring that the whole surface was covered. The membrane was left for 2 minutes before the ECL reagent was poured away and the membrane wrapped in clingfilm. The membrane was placed into a hypercassette™ (Amersham International plc, Bucks, UK) and covered with a piece

of Hyperfilm™ ECL™ photographic film (product code: RPN2103H, Amersham International plc, Bucks, UK). After 5 minutes the film was removed and developed using a X-ograph compact x4 imaging system (Tetenal, Wilts, UK). A 14-3-3 immunoblot using this method is shown in Figure 6.4.

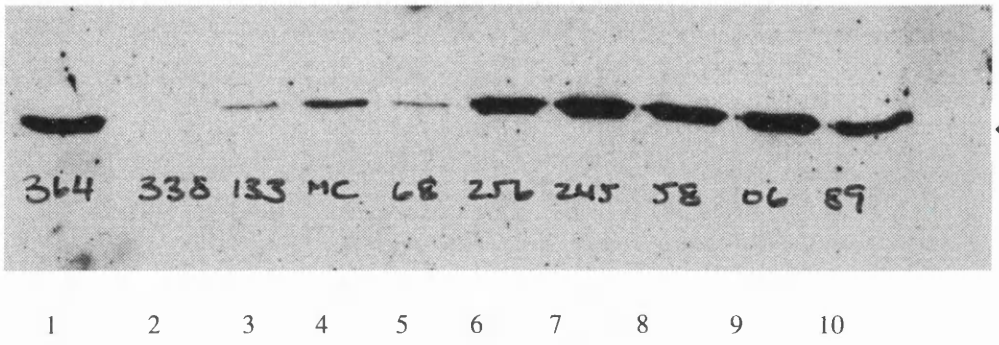


Figure 6.4 A 14-3-3 immunoblot developed using enhanced chemiluminescence. Lane 1: positive control; lane 2: negative control, lanes 3-10: eight CSF samples from patients with sporadic CJD.

6.5 Investigation into the presence of 14-3-3 in blood cells

Many CSF samples that were received for analysis were blood stained and this part of the study was designed to investigate whether the haemolysis would interfere with the analysis of 14-3-3. In addition the presence of 14-3-3 in white blood cells was investigated.

Four healthy volunteers (mean age 36.2 ± 6.9 years, 2M:2F) donated 10 mL blood which was collected into lithium heparin. Each of the samples was mixed with an equal volume of Hanks' balanced salt solution (modified) (Product code: H 4385 Sigma-Aldrich Company Ltd, Dorset, UK), and layered carefully onto 5 mL cold

Lymphoprep™ (Product Code: 1002705, Nycomed Pharma AS, Norway, supplied by Sigma-Aldrich Company Ltd, Dorset, UK) in a 30 mL plastic universal bottle. The blood and Lymphoprep™ was centrifuged at 3600 g for 25 minutes. The layer of white blood cells was removed and placed into a glass tube. The remaining Lymphoprep™ was removed and discarded. The red blood cell pellet was washed twice with saline. The Lymphoprep™ containing the white blood cells was centrifuged at 3600 g for 15 minutes. The resulting white blood cell pellet was washed twice with saline. The red blood cells and the white blood cell pellets were stored overnight at -20°C.

The red blood cells had haemolysed on storage at -20°C and each of the four haemolysates were diluted 1:10 with PBS. Two of the white blood cell pellets obtained were diluted 1:2 with PBS. To 30 µL of the red blood cell haemolysates and 30 µL of the diluted white blood cells, an equal volume of SDS-PAGE sample buffer was added. These samples were boiled for 4 minutes and 20 µL of each of the samples was added to two SDS-PAGE gels. In addition, three CSF samples were run, one from a histologically confirmed case of sporadic CJD (positive control), one from a patient with who was found not to have CJD (negative control) and a third sample which came from a traumatic lumbar puncture (test sample). The SDS-PAGE gels were subject to the electrophoresis conditions described above and the proteins were blotted onto nitrocellulose using the SemiPhor™ Semi-Dry Transfer unit. After protein transfer the nitrocellulose membranes were blocked with 2% non-fat milk in PBS for 30 minutes at room temperature. After rinsing the membranes with running water, the membranes were washed with PBS containing 0.05% Tween

20 and placed in either 0.1% non-fat milk in PBS without antibody or 1:1000 dilution of 14-3-3 γ in 0.1% non-fat milk in PBS. The nitrocellulose membranes were incubated overnight at 4°C. The nitrocellulose membranes were rinsed and washed as described. The membrane which had been incubated with 14-3-3 antiserum, was placed in a 1:1000 dilution of swine anti-rabbit immunoglobulin HRP in 0.1% non-fat milk in PBS plus 0.05% Tween 20; and the other membrane was incubated in 0.1% non-fat milk in PBS plus 0.05% Tween 20. Both membranes were incubated for an hour at room temperature before being washed for 2 hours with PBS containing 0.05% Tween 20. The nitrocellulose membranes were developed using ECL detection as previously described.

The resultant immunoblots are shown in Figure 6.5. The 14-3-3 immunoblot shows the presence of 14-3-3 in the positive control, test sample and the two white blood cell extracts. T-cell lymphocytes have been reported to contain a specific isoform of 14-3-3, known as tau 14-3-3 (Nielsen, 1991), which is also in low concentrations in spleen cells and in mammalian brains (Martin et al, 1993). These results suggest that in addition to the tau isoform white blood cells also contain a substantial amount of gamma 14-3-3.

A large amount of reactivity is seen in the red blood cell haemolysate, including a band with a similar molecular weight to 14-3-3. Examination of the blot which was obtained without incubation with 14-3-3 antisera showed no reactivity with the positive control, test sample or white blood cell extracts. The red blood cell haemolysates showed a similar reactivity in both blots, including the presence of the

band with the similar molecular weight to 14-3-3. This suggested that the reactivity seen in the haemolysates was due, in part, to endogenous peroxidase activity, which is likely to be caused by the haem group present in haemoglobin. There is a band present in the 14-3-3 immunoblot which is not present in the other blot, which maybe due to cross reactivity of either the 14-3-3 antiserum or the swine anti-rabbit immunoglobulin HRP antiserum with other protein(s) containing 14-3-3 like sequences. The presence of 14-3-3 immunoreactivity has previously been reported in red blood cells (Boston et al., 1982b). The test sample only showed reactivity in the blot incubated with 14-3-3 antisera, suggesting that the band seen is due to the presence of 14-3-3 from either haemolysed red blood cells or from the CSF and not due to endogenous peroxidase activity.

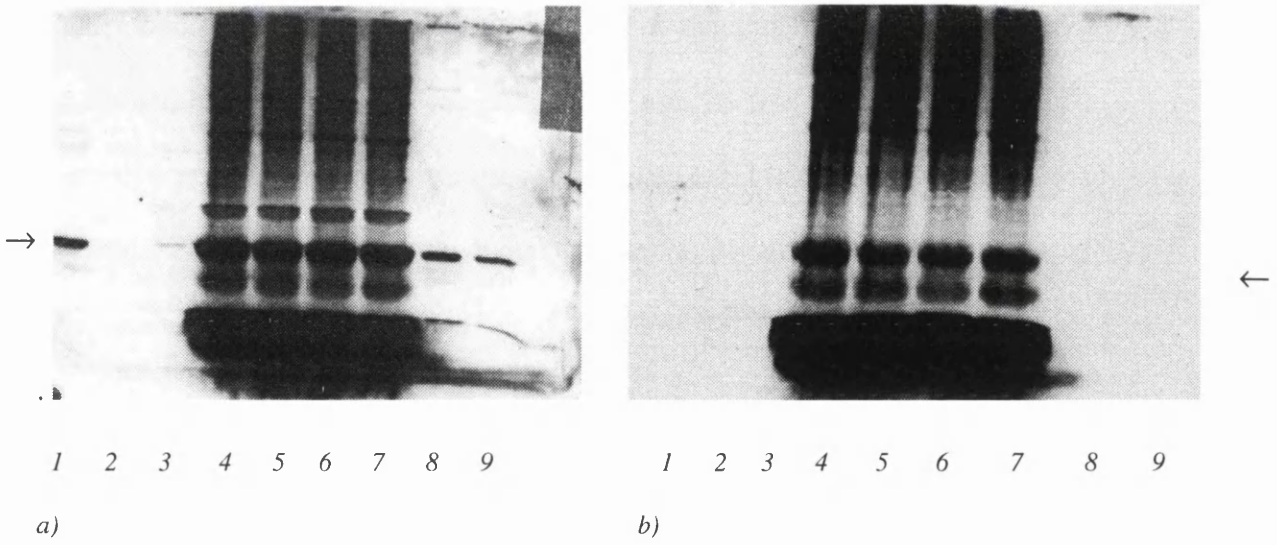


Figure 6.5 Immunoblots of CSF samples, red blood cell haemolysates and white blood cell preparations (a) with and (b) without 14-3-3 antisera. Lane 1, positive control; lane 2, negative control; lane 3, test CSF; lanes 4-7 red blood cell haemolysates; lane 8,9 white blood cell preparations. Arrows mark the position of the 14-3-3 band

6.5.1 Titration of the 14-3-3-like reactivity of red blood cell haemolysates

Another red blood cell haemolysate was prepared as described and was diluted in PBS to give a range of dilutions of 1:40-1:5000. These samples were analysed as above to investigate the degree of haemolysis which would produce a positive result on the 14-3-3 immunoblot. The haemoglobin concentration was measured in the 1:40 dilution using a Max M Beckman Coulter analyser (Beckman Coulter UK Ltd, High Wycombe, UK) and found to be 7.2 g/L. Samples containing concentrations of haemoglobin of 0.4 g/L or greater had significant endogenous peroxidase activity (Figure 6.6). This peroxidase activity was present in a number of bands including one which was in the 30kDa position, which could be confused with 14-3-3. This band was only detectable in the samples having haemoglobin concentration of greater than 1.8 g/L. The corresponding bands on the immunoblot developed using 14-3-3 antisera were stronger, suggesting there may be 14-3-3 in the red blood cell haemolysate. This may be due to the presence of 14-3-3 in red blood cells or due to contaminating cells such as white blood cells and/or platelets which have not been adequately removed by the Lymphoprep.

T-cell lymphocytes are known to contain a specific form of 14-3-3, the tau isoform, and the gamma isoform. Platelets have been shown to contain beta, gamma and zeta 14-3-3 isoforms (Wheeler-Jones et al., 1996). It is also possible that 14-3-3, including the gamma form, is present in red blood cells.

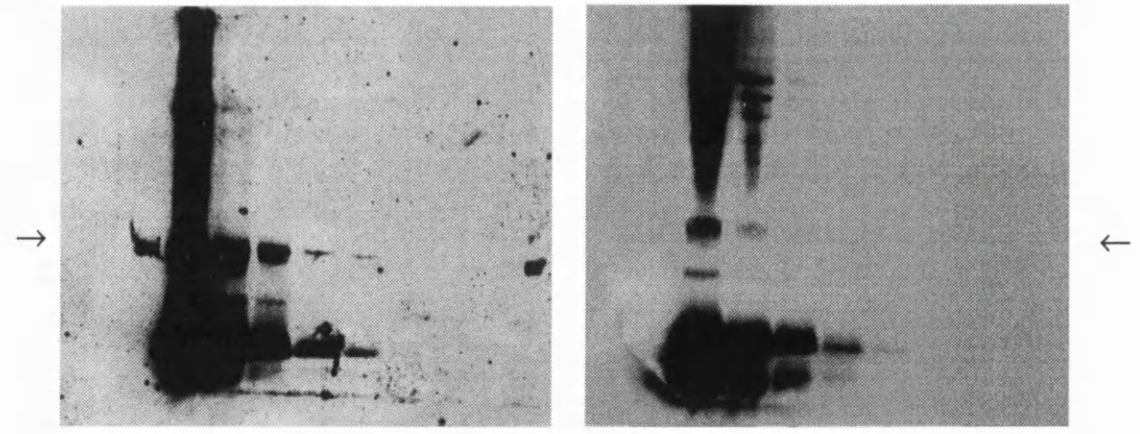


Figure 6.6 Immunoblots of CSF sample and red blood cell haemolysates with (a) and without (b) 14-3-3 antisera. Lane 1: positive control; lane 2-9: red blood cell haemolysates; lane 10: test CSF. Arrows mark the position of the 14-3-3 band

Chapter 7 - Detection of 14-3-3 in cerebrospinal fluid

The presence of 14-3-3 in cerebrospinal fluid was investigated in 296 CSF samples from patients with a variety of neurological conditions, using polyacrylamide electrophoresis and enhanced chemiluminescence which is described in section 6.4. The CSF samples and diagnoses were obtained as previously described in section 3.0.

7.1 14-3-3 in patients with sporadic Creutzfeldt-Jakob disease (CJD)

The presence of 14-3-3 was examined in 79 CSF samples from patients suspected of having sporadic CJD. Of these patients, 31 were found to have histologically confirmed sporadic CJD (mean age: 66.2 ± 9.7 years, range 49-79; 17M:14F), 10 had probable sporadic CJD (mean age: 65.0 ± 8.4 years, range 54-80; 6M: 4F) and 8 had possible sporadic CJD (mean age: 62.3 ± 8.8 years, range 51-75; 3M:5F). The remaining 30 patients were initially suspected of having sporadic CJD but this diagnosis was subsequently excluded (mean age: 60.4 ± 17.9 years, range 40-82; 19M:11F). This group of patients acted as appropriate controls.

The samples were analysed without prior knowledge of the clinical findings and wherever possible histological confirmation of the clinical diagnosis was obtained. The 14-3-3 results, including the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency are shown in Table 7.1.

Patient Group	CSF 14-3-3 (number positive/ total number)
Histologically confirmed sporadic CJD	24/31
Probable sporadic CJD	10/10
Possible sporadic CJD	4/8
Not sporadic CJD	4/30
Sensitivity	83%
Specificity	87%
PPV	89%
NPV	79%
Efficiency	85%

Table 7.1 The incidence of detectable 14-3-3 in the CSF of patients with histologically confirmed, probable and possible sporadic CJD, and in patients initially suspected of having sporadic CJD but subsequently proven not to have. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of a positive CSF 14-3-3 were calculated using the results obtained from the patients with histologically confirmed and probable sporadic CJD

The sensitivity of detection of 14-3-3 for the diagnosis of histologically proven and probable sporadic CJD has been reported to be between 90-96% and the specificity to be between 94-100% (Hsich et al., 1996; Zerr et al., 1998; Beaudry et al., 1999). The sensitivity found in this study was lower at 83%. The reason for this is probably the high proportion of atypical cases with long duration of illness which were found to be negative for 14-3-3 (Table 7.2). The number of CSF samples received from patients with histologically confirmed and probable sporadic CJD from November

1996 - January 1999 was 41. This is much lower than the total number of cases confirmed, which was 108 for 1997-1998. This means that just over a third of patients with sporadic CJD were investigated for the presence of 14-3-3, and this suggests that the data is skewed towards those patients with an atypical presentation. Differences in methodology are unlikely to be the cause of the lower sensitivity, as an exchange of CSF samples with Dr. I. Zerr at the German CJD Surveillance Unit, showed that there was total concordance of the results produced by the two laboratories.

Of the 7 patients with histologically proven sporadic CJD who did not have detectable CSF 14-3-3, 5 had either a more protracted disease course or were heterozygous for valine:methionine at codon 129 on the PRNP gene (Table 7.2).

Case number	**Age at onset disease (years)**	**Duration of disease (months)**	**Codon 129 polymorphism**
Case 1	61	8	MV
Case 2	68	4	MM
Case 3	61	5	MM
Case 4	79	17	Unknown
Case 5	58	19	Unknown
Case 6	44	17	MM
Case 7	63	>21	MV

Table 7.2 The age at onset, disease duration and codon 129 polymorphism in patients with histologically confirmed sporadic CJD who did not have detectable CSF 14-3-3 at the time of lumbar puncture

It is known that the codon 129 polymorphisms on the PRNP gene can alter the clinical manifestation of familial CJD, and it has been reported that patients with sporadic CJD who are heterozygous at codon 129 are more likely to have a long duration of illness and are atypical in presentation (Palmer and Collinge, 1992). Only 2 of the negative 14-3-3 cases had what would be considered a typical disease duration or were homozygous at codon 129. Unfortunately there is no information available concerning the timing of the lumbar puncture in relation to the disease duration. It is possible that the appearance of 14-3-3 in the CSF follows a bell-shaped curve as has been shown for CSF S-100b and neurone-specific enolase (NSE) (Jimi et al., 1992), and if the CSF sample was taken either very early or very late in the disease, 14-3-3 may not be elevated at that time. The specificity of the 14-3-3 is less than that previously reported and the reason for this is unclear.

Of the 4 false positive cases, 1 patient had Alzheimer's disease, 1 had carcinomatous meningitis secondary to small cell lung carcinoma, 1 had cerebral B-cell lymphoma and the final patient had a non-viral encephalitis. The presence of 14-3-3 in the CSF of these patients could reflect the neuronal cell damage occurring in these diseases, although it is very unusual to have a positive CSF 14-3-3 in patients with Alzheimer's disease (see section 7.8).

7.2 14-3-3 in patients with new variant Creutzfeldt-Jakob disease (CJD)

The presence of 14-3-3 was examined in 59 CSF samples from patients suspected of having new variant CJD. Of these patients 19 were shown to have histologically confirmed new variant CJD (mean age: 29.0 ± 10.4 years, range 18-53; 9M:10F),

8 had probable new variant CJD (mean age: 25.4 ± 6.1 years, range 19-39; 3M:5F) and 1 patient had possible new variant CJD (age 22 years, 1M). The remaining 31 patients were initially suspected of having new variant CJD but this diagnosis was subsequently excluded (mean age: 35.1 ± 13.9 years, range 14-59; 15M:16F). These patients acted as appropriate controls. The samples were analysed without prior knowledge of the clinical findings and wherever possible histological confirmation of the clinical diagnosis was obtained. The 14-3-3 results obtained are shown in Table 7.3.

Patient Group	**CSF 14-3-3 (number positive/ total number)**
Histologically confirmed new variant CJD	11/19
Probable new variant CJD	5/8
Possible new variant CJD	1/1
Not new variant CJD (control patients)	2/31
Sensitivity	58%
Specificity	94%
PPV	85%
NPV	78%
Efficiency	80%

Table 7.3 The incidence of detectable 14-3-3 in the CSF of patients with histologically confirmed, probable and possible new variant CJD, and in patients initially suspected of having new variant CJD but subsequently proven not to have. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of a positive CSF 14-3-3 were calculated using the results obtained from the patients with histologically confirmed new variant CJD

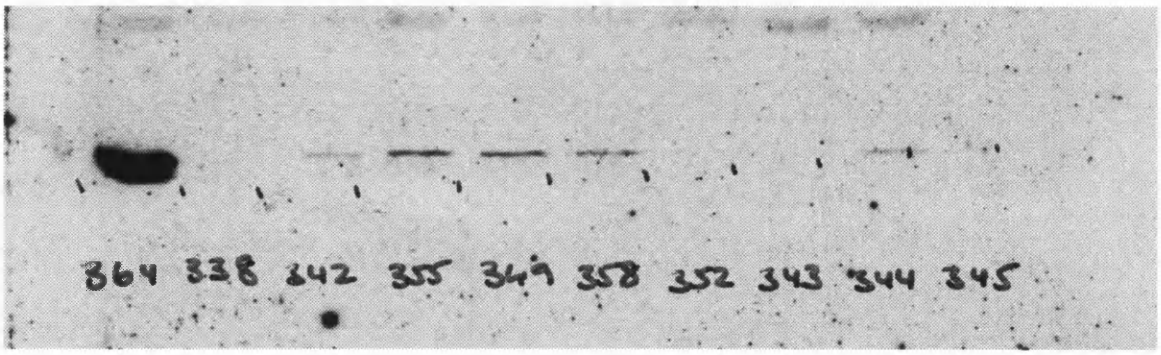
The clinical criteria for probable new variant CJD are not as well established as for the sporadic form of the disease and therefore it has not been included in the assessment of the diagnostic value of 14-3-3. The efficiency of 14-3-3 for the diagnosis of new variant CJD is somewhat less than that for the diagnosis of sporadic CJD (80% vs 85%). This reflects the higher percentage of patients with new variant CJD who do not have detectable 14-3-3 results. There is little difference between those patients who are negative for 14-3-3 and those who are positive in terms of age at onset of disease, disease duration and the timing of the lumbar puncture in terms of disease duration (Table 7.4). Of the 2 patients with false positive results, 1 patient had a stroke-like illness and subsequently recovered and the other was diagnosed as having multiple sclerosis.

Patient group (n)	**Age at onset of disease in years**	**Duration of illness in months**	**Time of lumbar puncture as % of disease duration**
14-3-3 positive (10)	31 (19-52)	15 (7-29)	64% (31-83%)
14-3-3 negative (8)	27 (18-51)	14 (8-30)	72% (23-88%)

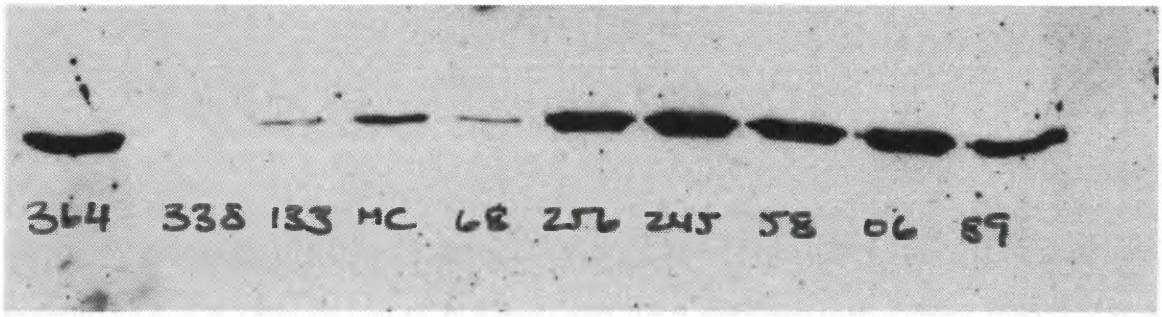
Table 7.4 Comparison of the age at onset of disease, duration of illness and time of lumbar puncture as percentage of illness between those new variant patients positive for 14-3-3 and those new variant patients who are negative for 14-3-3. All results expressed as mean (range)

7.3 Comparison of the CSF 14-3-3 results in patients with sporadic CJD and the new variant CJD

The percentage of patients with histologically confirmed new variant CJD who have detectable CSF 14-3-3 is 58%, this compares to 77% of patients with histologically confirmed sporadic CJD. The reason for the lower incidence in new variant CJD, is possibly the longer duration of disease seen in this form of CJD, when compared to the sporadic form (median duration of 16 months vs 4.5 months, respectively). In addition to this difference, the magnitude of the 14-3-3 band peaks seen in sporadic CJD is much higher than that seen in new variant. This is illustrated in Figure 7.1 which shows the range of 14-3-3 band peaks seen in new variant CJD (a) and in sporadic CJD (b). The positive control sample (364) and the negative control sample (338) are common to both immunoblots, demonstrating that the difference is not due to between-assay variation.



a) 1 2 3 4 5 6 7 8 9 10



b) 1 2 3 4 5 6 7 8 9 10

Figure 7.1 Comparison of the 14-3-3 immunoblots obtained in patients with (a) new variant CJD and (b) sporadic CJD

Immunoblot (a) lane 1: positive control; lane 2: negative control; lanes 3,4,5,6 and 9: positive CSF 14-3-3 samples from 5 patients with histologically confirmed new variant CJD; lanes 7, 8 and 10: negative CSF 14-3-3 samples from 3 patients with histologically confirmed new variant CJD

Immunoblot (b) lane 1, positive control; lane 2, negative control; lanes 3-10 positive 14-3-3 CSF samples from 8 patients with histologically confirmed sporadic CJD

The previous illustration also demonstrates the range of positivity that can be found in these immunoblots. The interpretation can be difficult when smaller 14-3-3 band peaks are present, as seen in Figure 7.1a. To see if densitometric scanning of the immunoblots would help interpretation, the developed photographic films were scanned at 600 dpi using a Bio-Rad Model GS-690 Imaging Densitometer (Bio-Rad, Herts, UK), using transmitted light and a red filter. The peak height and peak area were calculated for each of the 14-3-3 bands and expressed as a percentage of the positive control (364). The results are shown in Table 7.5. It was not possible to distinguish the two CSF samples shown in Figure 7.1a with small positive 14-3-3 bands (342 and 344) from the background using densitometric scanning. It appears that the eye is better for discriminating these weakly positive results rather than densitometric scanning. The majority of the patients with sporadic CJD have large amounts of CSF 14-3-3, although some patients have much lower amounts (133 and 68). In general patients with new variant CJD have smaller amounts of CSF 14-3-3 than the majority of sporadic CJD patients.

Patient Group (n)	**Peak height % of positive control (Mean ± SD)**	**Peak area % of positive control (Mean ± SD)**
Sporadic CJD (8)	72 ± 34	67 ± 48
New variant CJD (5)	21 ± 5	3.9 ± 0.7

Table 7.5 Semi-quantitative measurement of CSF 14-3-3 in 5 patients with histologically confirmed new variant CJD and 8 patients with histologically confirmed sporadic CJD

7.4 The precision of densitometric scanning as a means of quantifying 14-3-3

To investigate the reproducibility of densitometric scanning as a means of quantifying 14-3-3, a CSF sample was run seven times on a single gel. The centre of each band was scanned to assess within gel precision, and one band was scanned eight times at equally spaced positions throughout the band to assess within band precision. The peak height and area was obtained and expressed as a percentage of the positive control (364). The results are shown in Table 7.6.

Precision (n)	Peak height % of positive control Mean ± SD (CV%)	Peak area % of positive control Mean ± SD (CV%)
Within gel (7)	70.5 ± 12.4 (17.5%)	17.1 ± 6.2 (36.2%)
Within band (8)	72.1 ± 7.0 (9.7%)	16.3 ± 3.4 (20.7%)

Table 7.6 The within gel and within band precision of densitometric scanning of 14-3-3 as assessed by peak height and peak area

The best within gel and within band precision was obtained using the peak height rather than the peak area as a measure of the amount of 14-3-3. The within gel precision for peak height was 17.5% which suggests that densitometric scanning can only be used as a semi-quantitative estimation of the concentration of 14-3-3.

7.5 Investigation of CSF 14-3-3 isoforms in patients with sporadic and new variant CJD

Although the gamma isoform of 14-3-3 is thought of as the most brain-specific isoform, it is not the only one present within the central nervous system. The composition of the 14-3-3 isoforms in the CSF was investigated using antiserum raised against each of the isoforms (Courtsey of Dr. A. Aitken, Institute of Medical Research, Mill Hill, London). Briefly, 500 µL of CSF was diluted with SDS-PAGE sample buffer, boiled and loaded into a 9 cm well on a SDS-PAGE gel (4% stacking gel and 10% running gel). A set of molecular weight markers was also run. The electrophoresis and protein transfer were performed as described in section 6.3. The molecular weight markers were stained with Coomassie Brilliant Blue. After transferring the CSF proteins to nitrocellulose, the unbound protein sites on the nitrocellulose were blocked using 2% non-fat milk powder in PBS for 30 minutes at room temperature. After which the membrane was washed with running water and cut into 8 strips. Each strip was placed into 20 mL of 0.1% non-fat milk powder in PBS containing 20 µL of either an isoform specific antiserum or one raised against all isoforms. The antisera used are described in Table 7.7.

Specificity	Source	Product No	Code
All isoforms	Santa Cruz	sc-629-G	All
All isoforms	Dr. A. Aitken	1006	All-a
Gamma isoform	Dr. A. Aitken	1106	Gamma
Epsilon	Dr. A. Aitken	2048	Epsilon
Epsilon - carboxy terminal	Dr. A. Aitken	1116	ECT
Eta	Dr. A. Aitken	2058	Eta
Zeta	Dr. A. Aitken	1002	Zeta
Tau	Dr. A. Aitken	199	Tau
Sigma	Dr. A. Aitken	789	Sigma

Table 7.7 Description of the source and specificity of the 14-3-3 antisera used in the investigation of 14-3-3 isoforms present in the CSF of a patient with sporadic CJD and a patient with new variant CJD

The strips were incubated at 4°C overnight. They were then rinsed with running water, washed with 0.1% non-fat milk in PBS containing 0.05% Tween 20, and placed in 20 mL of 0.1% non-fat milk in PBS containing 20 µL swine anti-rabbit immunoglobulin conjugated with horseradish peroxidase. The strips were incubated for 1 hour at room temperature, rinsed under running water and washed with PBS containing 0.05% Tween 20 for 2 hours. The strips were developed using ethylaminocarbazole as described in section 6.3.6.2.

The CSF 14-3-3 isoforms detected in the CSF of a patient with sporadic CJD and in a patient with new variant CJD can be seen in Figure 7.2. The gamma and the epsilon isoforms were detected in both forms of CJD, but a third isoform, zeta, could

only be confirmed in sporadic CJD. A third isoform which could be zeta was detected in the new variant CSF using the All antisera, but unfortunately the zeta specific antiserum was not sensitive enough to detect the zeta isoform in this patient, so the identity of the third isoform seen in the new variant CSF cannot be confirmed but is likely to be the zeta isoform. Unfortunately the All-a antiserum donated by Dr. Aitken did not appear to have any 14-3-3 reactivity. It was not possible to obtain quantitative scans of the immunoblots to ascertain the relative amounts of these isoforms, because of the small size of the bands seen and the very high background staining obtained.

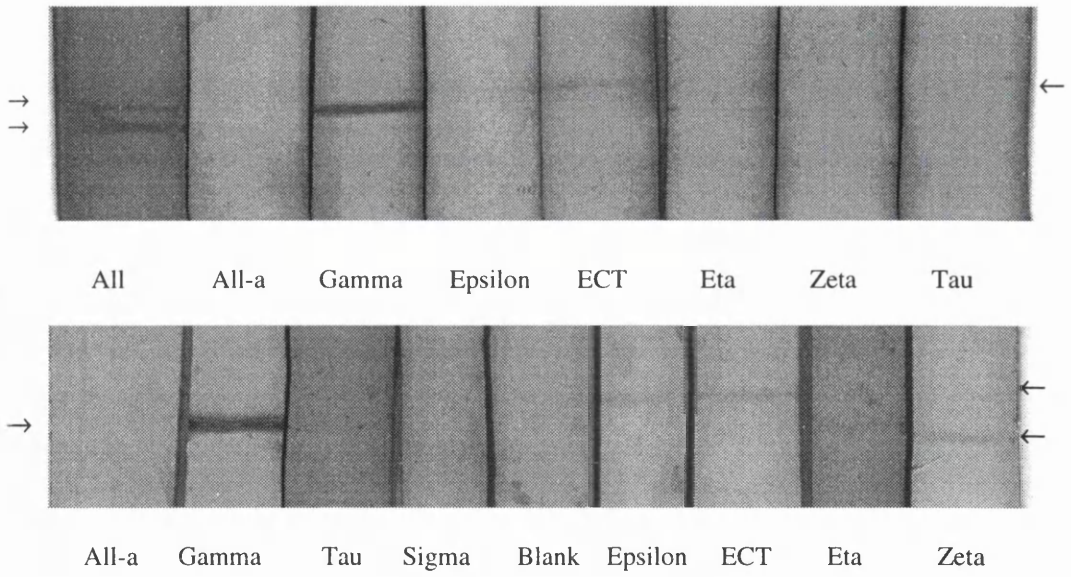


Figure 7.2 Immunoblots showing the isoforms of 14-3-3 present in the CSF of a) a patient with new variant CJD and b) a patient with sporadic CJD. For the source and specificity of the individual antisera see Table 7.7

The molecular weights of the 14-3-3 isoforms seen were calculated and are shown in Table 7.8.

14-3-3 Isoform	Sporadic CJD (kDa)	New variant CJD (kDa)	Reported molecular weights**
Epsilon	30.312	30.564	29.219
Gamma	30.105	30.345	28.218
Zeta	29.768	29.950*	27.790

*Table 7.8 The calculated molecular weights for the 14-3-3 isoforms detected in the CSF of a patient with sporadic CJD and a patient with new variant CJD. *14-3-3 isoform detected using the All 14-3-3 antiserum and thought to be the zeta isoform. ** Molecular weights obtained from human tissue using mass spectroscopy*

The immunoblots in Figure 7.2 showed that the same 14-3-3 isoforms are seen in sporadic CJD and in new variant CJD. This suggests that the source of 14-3-3 is the same in both forms of the disease.

7.6 CSF 14-3-3 in patients with familial and iatrogenic CJD

The presence of CSF 14-3-3 was investigated in 4 patients with iatrogenic CJD secondary to the administration of human cadaveric growth hormone, 3 patients with familial CJD and 1 patient with Gerstmann-Straussler-Scheinker syndrome. The results are shown in Table 7.9. Two of the cases of iatrogenic CJD had detectable CSF 14-3-3, as did 1 patient with an insert mutation, however the patient with Gerstmann-Straussler-Scheinker (GSS) syndrome did not have detectable CSF 14-3-3. Those patients with positive CSF 14-3-3 had concentrations which appeared

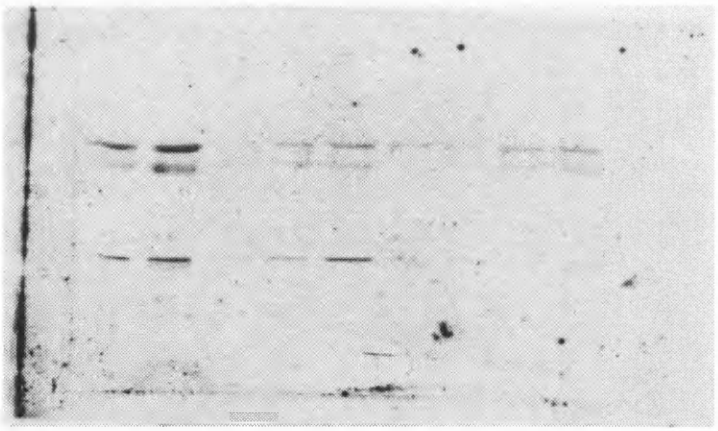
to be indistinguishable from those found in patients with sporadic CJD. Patients with iatrogenic CJD secondary to human cadaveric growth hormone administration usually present with cerebellar ataxia rather than dementia and have a more protracted disease than sporadic CJD. The lower incidence of CSF 14-3-3 in these patients may be related to the longer duration of disease or the different clinical presentation. Further studies are needed to investigate the incidence of detectable CSF 14-3-3 in these conditions. Patients with the PrP Glu-Lys^{200} mutation have a very similar clinical presentation and disease course to patients with sporadic CJD, and 14-3-3 has been detected in a large number of patients who present with this disease, but not in healthy carriers of the mutation (Rosenmann et al., 1997; Zerr et al., 1998). CSF 14-3-3 was detected in one of two patients with GSS associated with the PrP Pro-Leu^{102} mutation (Zerr et al., 1998). There is little information about the incidence of 14-3-3 in patients with PRNP insert mutations, but the results described above suggest that 14-3-3 may only be detected in 50% of cases.

Type CJD	Patient Id	Mutation	Age (years)	Sex	CSF 14-3-3
Iatrogenic - GH	Case 1	n/a	34	M	Negative
Iatrogenic - GH	Case 2	n/a	37	M	Positive
Iatrogenic - GH	Case 3	n/a	28	M	Positive
Iatrogenic - GH	Case 4	n/a	27	F	Negative
Familial CJD	Case 1	Insert	67	F	Positive
Familial CJD	Case 2	Insert	66	M	Negative
Familial CJD	Case 3	PrP Glu-Lys^{200}	51	M	Positive
GSS	Case 1	PrP Ala-Val^{117}	44	F	Negative

Table 7.9 The incidence of 14-3-3 in 4 patients with iatrogenic CJD secondary to growth hormone administration, 3 patients with familial CJD and 1 patient with Gerstmann-Straussler-Scheinker syndrome (GSS)

7.7 CSF 14-3-3 in cattle with and without Bovine Spongiform Encephalopathy (BSE)

Initial experiments showed that the SDS-PAGE with Western blotting and ECL detection method described in section 6.4 was not sensitive enough to detect the 14-3-3 in bovine CSF. The sensitivity was improved using an Ultra Super Sensitive ECL reagent (Product Code 1856157(8), Pierce and Warriner, Chester, UK). A typical immunoblot using this detection system is shown in Figure 7.3.



Lane 1 2 3 4 5 6 7 8 9

Figure 7.3 CSF 14-3-3 Immunoblot developed using Ultra SuperSensitive ECL reagent. Lanes 1-5, CSF from cattle with BSE; lanes 6-9, CSF from cattle without BSE

Using this more sensitive ECL detection system the presence of CSF 14-3-3 was investigated in 21 cattle with histologically confirmed BSE and 11 cattle suspected of having BSE but with no histological changes consistent with this diagnosis. The results are shown in Table 7.10. CSF 14-3-3 was detected in 71% of the cattle with BSE and in 18% of the cattle without BSE. These results gave the detection of CSF 14-3-3 a positive predictive value of 88% and a negative predictive value of 60% for the diagnosis of BSE. These results are based on a very small population and need to be expanded.

Cattle Group (n)	CSF 14-3-3 (number positive/ total number)
BSE (21)	15/21
Non BSE disease (11)	2/11
Sensitivity	71%
Specificity	82%
PPV	88%
NPV	60%
Efficiency	75%

Table 7.10 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of the detection of CSF 14-3-3 for the diagnosis of BSE

The amount of 14-3-3 detected in the CSF of cattle with BSE is small. This may be due to the poorer reactivity of the anti-14-3-3 antiserum used, for the bovine form of the protein when compared to the human form. This will limit the use of this test unless more sensitive methods of detection can be found. Due to the problems of obtaining bovine brain material, it is unlikely that purified bovine 14-3-3 will become available in the immediate future, limiting the chance of producing antisera specific for the bovine form of the protein.

7.8 CSF 14-3-3 in patients with non-CJD dementia and other neurological diseases

The presence of CSF 14-3-3 was investigated in 150 patients with either non-CJD forms of dementia or neurological/psychological disorders. Of these patients, 19 had Alzheimer's disease (mean age: 60.6 ± 9.3 years, range 47-75; 12M:7F), 17 patients had the sporadic form of the disease and 2 had autosomal dominant familial disease. Twenty-four patients had frontotemporal dementia (mean age: 60.3 ± 12.6 years, range 39-94; 18M:6F), of whom 15 had clinically diagnosed Pick's disease, 1 had histologically confirmed Pick's disease and 8 had clinically diagnosed frontal lobe degeneration. Six patients had cortical Lewy body disease (mean age: 59.3 ± 12.7 years, range 45-69; 4M:2F), 6 patients had corticobasal degeneration (mean age: 64.8 ± 12.0 years, range 46-76; 4M:2F), 4 patients had vascular dementia (mean age: 61.0 ± 11.6 years, range 59-77; 3M:1F) and 10 patients (mean age: 33.3 ± 3.97 years, range 27-40; 10M) had HIV associated dementia complex (ADC) with stage 2 or greater dementia according to the Memorial Slone Kettering Criteria (Price and Brew, 1988). A group of 19 patients with slowly progressive neurodegenerative diseases was also investigated (mean age: 60.9 ± 10.1 years, range 38-77; 14M:5F), 5 had dementia in association with Parkinson's disease, 4 had unspecified neurodegenerative disorders, 4 had progressive supranuclear palsy, 3 had multi-system atrophy, 2 had Huntington's disease and 1 had motor neurone disease. A group of 40 patients with a dementia of unknown aetiology (mean age: 56.3 ± 11.3 years, range 34-79; 21M:19F) and a group of 22 patients with miscellaneous neurological or psychological disorders (mean age: 44.5 ± 15.2 years, range 28-66;

8M:14F) were also investigated. Within the miscellaneous group of disorders, 3 patients had brain tumours, 1 had a stroke, 5 had encephalopathy, 6 had epilepsy, 2 had multiple sclerosis, 2 had psychological syndromes, 2 had cerebral Whipples's disease and 1 had dystonia. The results are shown in Table 7.11.

Disease Group	**CSF 14-3-3 (number positive/ total number)**
Alzheimer's disease	0/19
Frontotemporal dementia	1/24
Cortical Lewy body disease	0/6
Corticobasal degeneration	1/6
Vascular dementia	0/4
AIDS related dementia complex	0/10
Unspecified dementia	0/40
Degenerative disease	1/19
Miscellaneous neurological and psychological disease	4/22
Total	7/150

Table 7.11 CSF 14-3-3 in patients with different forms of dementia, patients with neurodegenerative diseases associated with dementia and miscellaneous neurological and psychological diseases

None of the patients with Alzheimer's disease, cortical Lewy body disease, vascular dementia or unspecified dementia had detectable 14-3-3 in the CSF. One patient with histologically confirmed Pick's disease and 1 patient with corticobasal degeneration had detectable CSF 14-3-3. None of the patients with progressive

supranuclear palsy, Huntington's disease or Parkinson's disease with dementia had detectable CSF 14-3-3, although 1 patient with multi-system atrophy had a positive CSF 14-3-3. All 3 patients with brain tumours and the patient with suffering from a stroke had detectable 14-3-3, but none of the other neurological or psychological disorders investigated was positive.

7.9 Discussion of CSF 14-3-3 results

In 1996, the first report of the use of 14-3-3 in CSF as a diagnostic marker for CJD was published (Hsich et al., 1996). It was reported to have a high sensitivity and specificity for sporadic CJD. Earlier that year a new form of CJD which affects younger patients and has a longer disease duration was described (Will et al., 1996a). It was later proved that this disease was related to the ingestion of BSE-infected meat products (Bruce et al., 1997; Hill et al., 1997). This stimulated interest in evaluating CSF 14-3-3 as a marker of CJD. The need for a premorbid diagnostic test for CJD which does not involve the examination of brain tissue has been prompted by the finding that instruments used to obtain brain tissue can transmit the disease to other patients, even after standard sterilizing procedures.

CSF 14-3-3 has been proven to be of value in the investigation of patients with suspected sporadic CJD (Hsich et al., 1996; Zerr et al., 1998; Beaudry et al., 1999). This study has shown that there is a population of patients with sporadic CJD who do not have detectable 14-3-3 in the CSF. This may be dependent on the duration of the

illness, the codon 129 phenotype and/or when during the disease course the CSF sample was taken.

Patients with other forms of dementia which may be confused with CJD, such as Alzheimer's disease and frontotemporal dementia, have a very low incidence of 14-3-3; which accounts for the high specificity of this investigation. The 3 patients with cerebral tumours and the 1 patient who had a stroke had a positive CSF 14-3-3. This would be expected as the majority of 14-3-3 within the CNS is found in the cytoplasm of neuronal cells with a smaller proportion being membrane bound (Martin et al., 1994), therefore any disease which causes neuronal damage or loss would be expected to be associated with a detectable CSF 14-3-3. Other studies have shown that patients with multi-infarct dementia, strokes, Herpes simplex encephalitis and brain tumours have detectable 14-3-3 (Hsich et al., 1996; Zerr et al., 1998).

Patients with the familial, iatrogenic and new variant forms of the disease have a lower incidence of CSF 14-3-3, than sporadic CJD. In familial and iatrogenic CJD this may be related to the disease duration and the clinical manifestation of the disease. When 14-3-3 is present in the CSF of patients with these forms of CJD it is of the same magnitude as patients with the sporadic form of the disease. In contrast, the amount of 14-3-3 present in the CSF of patients with new variant CJD is generally less than that seen in the sporadic form of the disease. This suggests that there are factors other than disease duration which affect the amount of 14-3-3 released in new variant CJD. As 14-3-3 is a neuronal protein, it is possible that there is less neuronal damage in patients with new variant CJD, although from the

histopathological examination of the brain tissue from patients with new variant CJD, this does not appear to be the case.

In conclusion, CSF 14-3-3 is a useful diagnostic test for the investigation of patients with sporadic CJD. It is occasionally detected in patients with non-CJD dementia. However, it is likely to be of less value in the investigation of patients with suspected new variant CJD, or in the investigation of familial or iatrogenic forms of the disease.

Chapter 8 - Enzyme linked immunoassay for measurement of neurone-specific enolase (NSE) in cerebrospinal fluid

Roche Diagnostics Ltd (Welwyn Garden City, UK) kindly donated Cobas®-Core NSE Enzyme immunoassay kits. The majority of the CSF NSE results described in this thesis were obtained using these kits. The remaining results were obtained using kits purchased by the Department of Neuroimmunology.

8.1 Cobas®-Core NSE Enzyme Immunoassay

The Cobas®-Core NSE Enzyme Immunoassay is a solid phase sandwich enzyme immunoassay which measures both the $\alpha\gamma$ and $\gamma\gamma$ isoforms. A polystyrene beads coated with a monoclonal antibody specific for the gamma subunit of NSE were incubated with standards containing known amounts of NSE, controls samples or patient samples. A rabbit polyclonal anti NSE antibody was added to each bead, which were then incubated at 37°C for 15 minutes. The NSE in the standards, controls or patient samples reacted with both the monoclonal and the polyclonal antisera to form a sandwich. After incubation the beads were washed to remove any unbound rabbit polyclonal antiserum, and incubated with a goat anti rabbit immunoglobulin antiserum conjugated with horseradich peroxidase. The goat antiserum reacted with any rabbit anti NSE antiserum bound to the beads.

After washing the amount of NSE bound to the bead was proportional to the amount of horseradish peroxidase activity. This activity was measured by adding the

chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide. The horseradish peroxidase activity converted the colourless TMB into an insoluble blue precipitate. The horseradish peroxidase activity was stopped by the addition of sulphuric acid, which also converted the insoluble blue precipitate into a soluble yellow product. The absorbance of the yellow product was measured at 450 nm and was proportional to the amount of NSE protein originally present in the sample. A typical standard curve is shown in Figure 8.1.

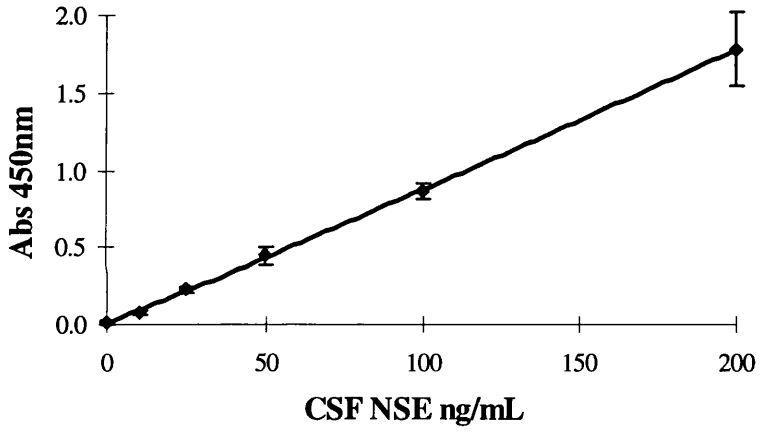


Figure 8.1 Typical standard curve for the Cobas®-Core NSE Enzyme Immunoassay. Results shown are the mean and SD of six replicate analyses

8.2 Stability of CSF NSE

Preliminary studies showed that CSF samples which had been stored at -20°C had very low concentrations of NSE. The following study was set up to investigate the stability of CSF NSE at different storage temperatures and the ability of CSF NSE to withstand repeated freeze-thaw cycles.

8.2.1 Storage of CSF NSE at -20°C and at 4°C

Three CSF samples were pipetted into 100 µL aliquots and stored at both -20°C and at 4°C. CSF NSE was measured in each sample on day 0 and throughout the next 30 days. The results are shown in Figure 8.2.

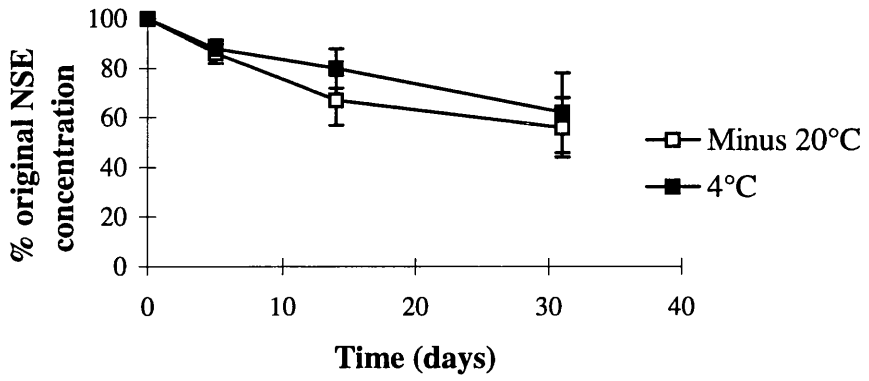


Figure 8.2 Effect of storage at -20°C and at 4°C on CSF NSE concentration. Values shown are the mean and SD of three CSF samples

There is a gradual loss of NSE concentrations in CSF samples stored at both -20°C and at 4°C. The NSE concentrations appeared to be more stable at 4°C than at -20°C, but overall there was little difference between the temperatures.

8.22 The effect of freezing and thawing CSF samples

NSE concentrations were measured in three CSF samples within four hours of being taken. Each CSF sample had a 1 mL aliquot stored at -20°C in a polypropylene tube. Each sample was thawed and refrozen four times and the NSE concentration measured on each occasion. All analyses were performed within seven days to limit the amount of NSE decay due to storage. The results are shown in Figure 8.3.

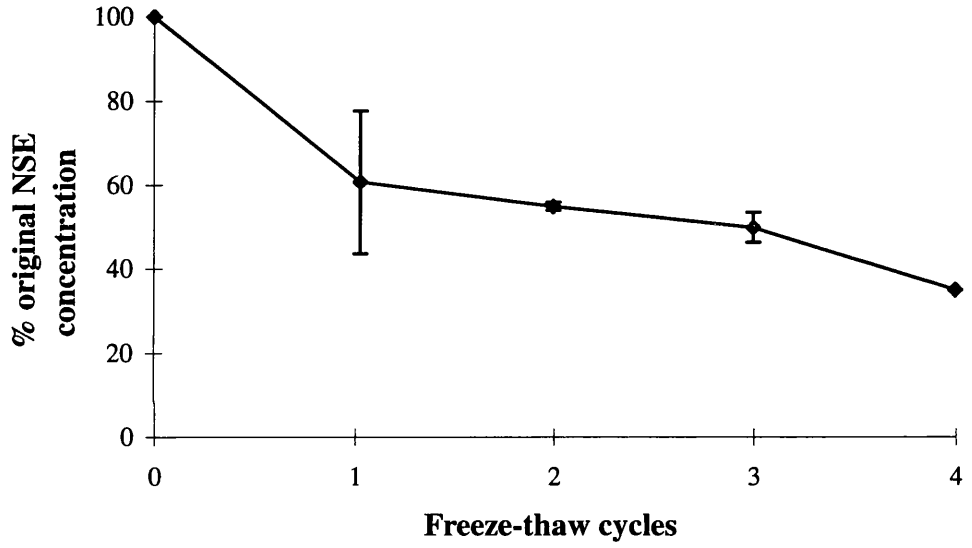


Figure 8.3 The effect of repeated freezing and thawing on CSF NSE concentration. Results shown are the mean of three CSF samples

There was significant loss of NSE activity after 1 freeze-thaw cycle and this decay continued with further freezing and thawing. A large percentage of the samples analysed for NSE were sent to the laboratory from hospitals other than The National Hospital for Neurology and Neurosurgery. To standardise the amount of loss of NSE, all CSF samples were frozen at -20°C before being sent to the laboratory on dry-ice by courier. The samples were frozen within 2-3 hours of being taken and

transported to the laboratory within 1-2 days of being taken. It was hoped that this would ensure that all samples were kept at the same temperature during transport. Ideally the samples would not be frozen but kept at 4°C but it is difficult to ensure that this temperature is maintained during transport. The couriers used in this study took two days to deliver the samples and by storing the samples on wet-ice, it is possible that some samples would have arrived at 4°C and some at ambient temperature. The majority of samples received from outside hospitals arrived frozen. For uniformity, all CSF samples from The National Hospital for Neurology were also frozen once before NSE concentrations were measured. The problem of stability was discussed with Roche Diagnostics Ltd who recognised the problem which was also evident in plasma but to a lesser extent. Some studies have stored CSF samples at -20°C after the addition of 50% glycerol (Mokuno et al, 1983; Wakayama et al., 1987), but it would have been difficult to arrange for the appropriate amounts of glycerol to be added to the CSF samples sent to the laboratory from outside hospitals.

Chapter 9 - Cerebrospinal fluid concentrations of Neurone-specific enolase (NSE)

Cerebrospinal fluid concentrations of neurone-specific enolase (NSE) were measured in 305 CSF samples from patients with a variety of neurological conditions, using the Cobas®-Core NSE Enzyme Immunoassay from Roche Diagnostics (Welwyn Garden City, UK), described in Chapter 8. This assay measures both the $\alpha\gamma$ and $\gamma\gamma$ forms enolase which together constitute neurone-specific enolase. The CSF samples and final diagnoses were obtained as described in section 3.0.

9.1 CSF NSE in control subjects

It was not possible to obtain a reference range for patients with non-neurological disease due to the lack of stability of NSE in CSF and the expense of the Cobas®-Core NSE Enzyme Immunoassay. The reference ranges obtained for CSF S-100b and CSF tau protein were obtained by collecting over 300 CSF samples and storing them at -20°C until analysis. Those samples from patients who had no evidence of organic brain disease at the time of the lumbar puncture were subsequently analysed and a reference range obtained. As the concentrations of NSE are not stable in the CSF, a large number of CSF samples would have to be measured prospectively before adequate numbers of samples from patients with no evidence of organic brain disease were obtained. This was prohibitively expensive. The CSF NSE concentrations in patients with sporadic and new variant CJD were compared to patients suspected of having CJD but subsequently proven not to have the disease. These patients acted as appropriate controls for these conditions.

9.2 CSF NSE concentrations in patients with sporadic CJD

Sporadic CJD is a disease associated with acute neuronal loss, astrocytosis and spongiform changes within the brain. Elevated concentrations of CSF NSE have been reported in this condition (Wakayama et al., 1987; Vermuyten, 1990; Jimi et al., 1992; Zerr et al., 1995; Evers et al; 1998; Kropp et al., 1999). This part of the study was designed to expand this work and to include other forms of CJD such as the new variant and familial forms of the disease.

CSF NSE concentrations were measured in 29 patients with histologically confirmed sporadic CJD (mean age: 65.6 ± 9.8 years, range 49-79; 17M:12F), 10 patients with probable sporadic CJD (mean age: 65.0 ± 8.4 years, range 54-80; 6M:4F) and 8 patients with possible sporadic CJD (mean age 62.3 ± 8.8 years, range 51-75; 3M:5F). These results were compared with CSF NSE concentrations in a control group of 29 patients with suspected sporadic CJD but who were subsequently found to have other diagnoses (mean age 63.6 ± 12.7 years, range 40-85; 18M:11F). Using the Mann Whitney U test, the mean CSF NSE concentration was significantly increased in patients with histologically confirmed sporadic CJD when compared to the controls (58.6 ± 55.9 vs 12.3 ± 7.5 ng/mL, $p < 0.001$). The mean CSF NSE concentration was also increased in patients with probable (157.0 ± 164.7 ng/mL) and possible sporadic CJD (76.8 ± 96.1 ng/mL). The results are shown in Figure 9.1.

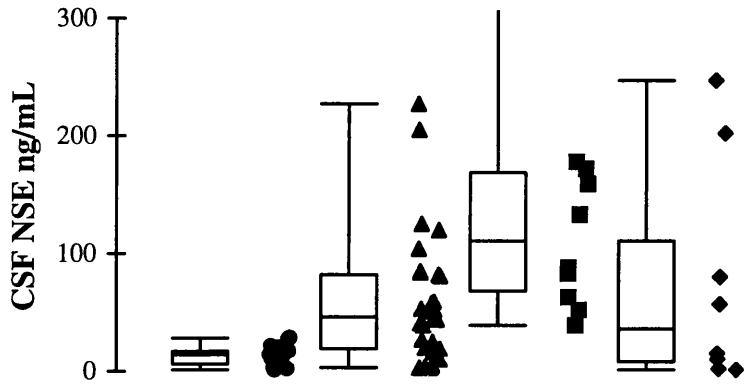


Figure 9.1 Combined scatter and box and whisker plots of CSF concentrations of NSE in control patients (●), patients with histologically confirmed sporadic CJD (▲), patients with probable sporadic CJD (■), patients with possible sporadic CJD (◆). The boxes represent the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers were derived

One patient with probable CJD had a markedly raised CSF NSE concentration of 603 ng/mL. The NSE concentrations in patients with possible sporadic CJD showed a wide scatter of results. It is possible that those patients with lower NSE concentrations may have pathology other than sporadic CJD. At the time of writing the final diagnoses for these patients were not available.

The sensitivity, specificity, positive predictive value and negative predictive value of NSE for the diagnosis of histologically confirmed and probable sporadic CJD at three different cut-off concentrations are shown in Table 9.1.

	CSF NSE >25 ng/mL	CSF NSE >35 ng/mL	CSF NSE >50 ng/mL
Sensitivity	74%	72%	59%
Specificity	93%	100%	100%
PPV	94%	100%	100%
NPV	73%	73%	64%
Efficiency	82%	84%	76%

Table 9.1 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of three different cut-off concentrations of CSF NSE for the diagnosis of histologically confirmed or probable sporadic CJD

The specificity values for the cut-off concentrations of 25 ng/mL and 35 ng/mL agree well with other studies. Beaudry et al., 1999 found that CSF NSE had a specificity of 91.5% using a cut-off concentration of 25 ng/mL and Zerr et al., 1995 found that CSF NSE had a specificity of 92% using a cut-off concentration of 35 ng/mL. The sensitivities found in this study are slightly lower than those of the above studies, as Beaudry et al., 1999 found a sensitivity of 79.7% using a cut-off of 25 ng/mL and Zerr et al., 1995 found a sensitivity of 80% using a cut-off concentration of 35 ng/mL. The reason for this lower sensitivity could be the higher incidence of long duration cases of sporadic CJD found in this study (see section 7.1).

9.3 CSF NSE in new variant CJD

CSF NSE concentrations were measured in 19 patients with histologically confirmed new variant CJD (mean age: 29.0 ± 10.4 years, range 18-53; 9M:10F), in 8 patients

with probable new variant CJD (mean age: 25.4 ± 6.1, range 19-39; 4M:4F) and in 1 patient with possible new variant CJD (age: 22 years, 1M). The CSF NSE concentrations were compared to those found in 30 patients (mean age: 34.1 ± 13.5 years, range 15-59; 14M:16F) who were initially suspected of having new variant CJD but subsequently proven not to have, either by improvement in clinical course or by confirmation of other disease.

The mean concentrations of CSF NSE were significantly raised in patients with histologically confirmed new variant CJD when compared to control patients (22.5 ± 14.7 vs 14.6 ± 14.2 ng/mL, $p<0.02$), but there was a considerable overlap with the control patients. The mean concentration of NSE was also raised in patients with probable new variant CJD (27.8 ± 21.9 ng/mL). The patient with possible new variant CJD had a CSF NSE concentration of 21.0 ng/mL. Statistical analysis was performed using the Mann-Whitney U test. The results are shown in Figure 9.2.

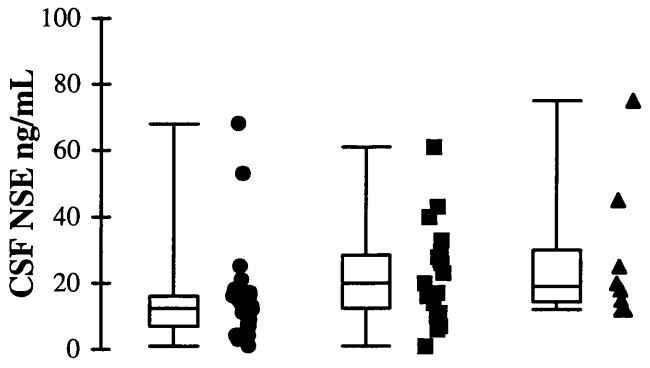


Figure 9.2 Combined scatter and box and whisker plots of CSF concentrations of NSE in control patients (●), patients with histologically confirmed new variant CJD (■) and patients with probable new variant CJD (▲). The boxes represents the 25th-75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plots represent the individual values from which the boxes and whiskers were derived

Two of the control patients had high CSF NSE concentrations. One of these patients had a stroke-like illness from which she subsequently recovered. It is likely that the raised NSE concentration was due to acute neuronal damage as a result of the stroke. It is known that patients with both haemorrhagic and ischaemic strokes have raised CSF NSE concentrations (Scarna et al., 1982; Mokuno et al., 1983; Hay et al., 1984; Persson et al., 1987), and that the level of NSE is related to the extent of the infarction or haemorrhage (Hay et al., 1984). The diagnosis in the second patient is not known.

The sensitivity, specificity, positive predictive value and negative predictive value of NSE for the diagnosis of histologically confirmed new variant CJD at three different cut-off concentrations are shown in Table 9.2.

	CSF NSE >25 ng/mL	CSF NSE >35 ng/mL	CSF NSE >50 ng/mL
Sensitivity	42%	16%	5%
Specificity	93%	93%	93%
PPV	80%	60%	33%
NPV	72%	64%	61%
Efficiency	73%	63%	59%

Table 9.2 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of three different cut-off concentrations of CSF NSE for the diagnosis of histologically confirmed new variant CJD

The sensitivity of CSF NSE is much lower for the diagnosis of new variant CJD than it is for the sporadic form of the disease, although the specificity is comparable. The reason for this is the much lower mean CSF NSE concentration seen in new variant CJD when compared to sporadic CJD (22.5 ± 14.7 vs 58.6 ± 55.9 ng/mL). This may be due to the longer disease duration seen in new variant CJD. The mean CSF NSE concentrations are similar in the patients suspected of having either new variant CJD or sporadic CJD (14.6 ± 14.2 vs 12.3 ± 7.5 ng/mL, respectively).

9.4 CSF NSE concentrations in patients with mutations of the PRNP gene and iatrogenic CJD

CSF NSE was measured in 4 patients with iatrogenic CJD secondary to the administration of human cadaveric growth hormone, 3 patients with familial CJD and 1 patient with Gerstmann-Straussler-Scheinker syndrome (GSS).

Type CJD	Patient Id	Mutation	Age (years)	Sex	CSF NSE ng/mL
Iatrogenic - GH	Case 1	n/a	34	M	25.6
Iatrogenic - GH	Case 2	n/a	37	M	41.3
Iatrogenic - GH	Case 3	n/a	28	M	112.1
Iatrogenic - GH	Case 4	n/a	27	F	15.3
Familial CJD	Case 1	Insert	67	F	96.8
Familial CJD	Case 2	Insert	66	M	9.7
Familial CJD	Case 3	PrP Glu-Lys^{200}	85	M	85.6
GSS	Case 1	PrP Ala-Val^{117}	44	F	28.7

Table 9.3 CSF NSE concentrations in patients with PRNP mutations and iatrogenic CJD secondary to growth hormone administration

Three out of 4 patients with iatrogenic CJD secondary to cadaveric human growth hormone administration had CSF NSE concentrations of greater than 25 ng/mL. Three out of 4 patients with mutations of the PRNP gene had CSF NSE concentrations of greater than 25 ng/mL. The magnitude of the increases seen in both the iatrogenic and genetic forms of the disease suggests that the increases seen may be of the same magnitude as those seen in sporadic CJD.

9.5 CSF NSE in patients with non-CJD dementia and other neurological diseases

CSF NSE was measured in 119 patients with either non-CJD forms of dementia or other neurological diseases. Of these patients, 12 had Alzheimer's disease (mean age: 59.7 ± 8.1 years, range 48-74; 7M:5F), 11 patients had the sporadic form of the disease and 1 had autosomal dominant familial disease; 9 patients had frontotemporal dementia (mean age: 60.1 ± 6.7 years, range 53-72; 7M:2F), 4 of these had clinically diagnosed Pick's disease, and 5 had clinically diagnosed frontal lobe degeneration; 1 patient had disease corticobasal degeneration (age:46 years, 1M), 3 patients had cortical Lewy body disease (mean age: 59.3 ± 5.0 years, range 55-64; 3M) and 3 patients had vascular dementia (mean age: 67.0 ± 9.2 years, range 59-77; 3M). A group of 25 patients with slowly progressive neurodegenerative diseases was investigated (mean age: 59.6 ± 10.4 years, range 37-77; 19M:6F), 8 of these patients had unspecified neurodegenerative disorders, 5 had dementia in association with Parkinson's disease, 4 had progressive supranuclear palsy, 4 had multi-system atrophy, 2 had Huntington's disease and 2 had motor neurone disease. A group of 45 patients with a dementia of unknown aetiology (mean age: 57.6 ± 12.2 years, range 34-79; 23M:22F) and a group of 21 patients with miscellaneous neurological or psychological disorders (mean age: 46.1 ± 15.0 years, range 27-71; 8M:13F) were investigated. Within the miscellaneous group of disorders, 7 had encephalopathy, 6 had epilepsy, 2 had multiple sclerosis, 2 had psychological syndromes, 2 had cerebral Whipples's disease, 1 had meningitis and 1 had dystonia. The mean CSF NSE concentrations in each of these groups are shown in Table 9.4.

Patient Group (n)	NSE ng/mL (mean ± SD)	Range	% samples NSE > 25 ng/mL (n)
Alzheimer's disease (12)	13.8 ± 11.1	1.5 - 32.1	17 (2)
Frontotemporal dementia (9)	8.4 ± 7.6	2.2 -26.0	11 (1)
Corticobasal degeneration (1)	16		0
Cortical Lewy body disease (3)	8.0 ± 8.0	2.1 - 17.6	0 (0)
Vascular dementia (3)	9.9 ± 7.4	1.7 - 16.5	0 (0)
Neurodegenerative disease (25)	10.7 ± 7.8	1.4 - 35.2	4 (1)
Unspecified dementia (45)	15.4 ± 9.6	1.0 - 40.2	20 (9)
Miscellaneous neurological and psychological disorders (21)	12.1 ± 8.7	1.8 - 41.4	5 (1)

Table 9.4 CSF NSE concentrations in patients with different forms of dementia, patients with neurodegenerative diseases associated with dementia and miscellaneous neurological and psychological diseases

There was no significant difference between the CSF NSE concentrations in any of the non-CJD dementia and degenerative disease patient groups studied. Some studies have found that CSF NSE concentrations are not raised in patients with Alzhemier's disease when compared to controls (Vermuyten et al., 1990; Parnetti et al., 1995), some have found increased concentrations (Blennow et al., 1994) and some have found reduced concentrations (Cutler et al., 1986). An early study investigating CSF NSE concentrations found that a number of patients with senile dementia had raised concentrations (Royds et al., 1983). The results described here suggest that there are no differences between the CSF NSE concentrations found in different forms of dementia, and that the increases seen in individual cases are of small magnitude. This suggests that CSF NSE measurement is probably of limited value in the investigation of patients with dementia.

9.6 CSF NSE in patients with HIV infection

CSF NSE concentrations were measured in 45 patients with HIV infections (mean age: 38.6 ± 8.4 years, range 27-62; 36M:9F) undergoing diagnostic lumbar puncture for assessment of possible neurological disease. Of these, 11 patients (mean age: 39.5 ± 10.9 years, range 27-62; 9M:2F) had AIDS associated dementia complex (ADC) with stage 2 or greater dementia according to the Memorial Slone Kettering Criteria (Price and Brew, 1988), 3 had systemic B-cell lymphoma and were being investigated for possible cerebral involvement, 3 had primary CNS lymphoma, 13 had CNS infections (5 had cryptococcal meningitis, 3 had cytomegalovirus (CMV) encephalitis, 2 had varicella zoster virus (VZV) meningoencephalitis, 3 had cerebral toxoplasmosis) and 15 patients had a miscellaneous collection of

abnormalities (5 patients had headache, 3 had peripheral neuropathy, 2 had confusion, 2 had had fits, 1 had hysterical paraparesis, 1 suffered from alcoholism, 1 had strabismus). The results are shown in Table 9.5. There was no significant difference between the CSF NSE concentrations found in any of the groups of patients investigated, using one-way ANOVA with Bonferroni modification using SPSS.

Group	NSE ng/mL (mean ± SD)	NSE Range	% samples NSE > 25 ng/mL (n)
AIDS dementia complex (11)	11.2 ± 10.0	2.2 - 37.4	9 (1)
Lymphoma (6)	10.8 ± 6.2	5.6 - 20.8	0 (0)
Opportunistic infections (13)	14.3 ± 12.2	1.1 - 49.7	8 (1)
Miscellaneous symptoms (15)	9.3 ± 7.5	1.1 - 24.2	0 (0)

Table 9.5 CSF NSE concentrations in patients with HIV infection.

CSF NSE was increased in one patient with CNS lymphoma and in one patient with cryptococcal meningitis. These conditions are associated with neuronal damage secondary to necrosis and as such CSF NSE concentrations would be expected to be increased.

9.7 General discussion of CSF NSE concentrations

Neurone-specific enolase is a glycolytic enzyme found in high concentration within the neurones of the central nervous system, where it has been reported to constitute 1.5% of all soluble brain protein. Although it was initially thought to be found only

within the neuronal cells, NSE has been shown to be present in neuroendocrine cells (Schmechel et al., 1978), erythrocytes (Hullin et al., 1980), platelets (Marangos et al., 1980a) and lymphocytes (Haimoto et al., 1985).

CSF concentrations are increased in diseases associated with CNS damage and/or cell loss such as strokes (Scarna et al., 1982; Mokuno et al., 1983; Hay et al., 1984; Persson et al., 1987), brain tumours (Royds et al., 1981; Mokuno et al., 1983), head injury (Scarna et al., 1982, Dauberschmidt et al., 1983), bacterial meningitis (Vermuyten, 1990; Inoue et al., 1994) and neonatal hypoxic-ischaemic encephalopathy (Garcia-Alix et al., 1994; Thornberg et al., 1995). The concentrations have been shown to be related to the extent of neuronal damage in intracerebral haemorrhage and ischaemia (Hay et al., 1984; Persson et al., 1987) and in head injury (Scarna et al., 1982). Thus CSF NSE concentrations may be an indicator of the presence of neuronal damage and the levels a measure of the extent of damage.

The mean concentrations of CSF NSE were significantly increased in patients with either sporadic or new variant CJD, when compared to controls. As CJD is a disease associated with neuronal loss, the concentrations of NSE would be expected to increase. In sporadic CJD the disease duration is short, with a median duration of 4.5 months, and the loss of neuronal tissue is rapid, and consequently the concentrations of NSE can be markedly raised. In contrast, in new variant CJD the disease duration is longer, median duration being 16 months, and the mean concentration of CSF NSE is lower than in sporadic CJD. This suggests that a more slowly progressing disease, with a smaller rate of neuronal loss will result in lower CSF NSE concentrations.

This slow rate of disease progression may be one of the reasons why CSF NSE concentrations do not appear to be increased in other causes of dementia such as Alzheimer's disease, frontotemporal dementia and HIV related dementia complex. In each of these conditions, the disease duration is much longer than in that found in CJD and the rate of neuronal loss is likely to be much slower. The half life of NSE from the CSF has been reported to be 3.9 hours in rats (Steinberg et al., 1983), this suggests that NSE is rapidly cleared. It is therefore unlikely that NSE accumulates during slowly progressing diseases.

The lack of stability of NSE in the CSF when measured using the Cobas®-Core NSE Enzyme Immunoassay limits the usefulness of this assay for diagnosis. However if CSF samples are analysed shortly after being taken, the measurement of CSF NSE is of value in the investigation of patients with suspected CJD.

Chapter 10 - General Discussion

10.1 CSF brain-specific protein results in different diseases

In this section the results for all of the brain-specific proteins investigated in this study for each of five diseases are considered together, and the overall value of the CSF proteins discussed. The pattern of results will be compared between the different diseases and assessed in context of the underlying pathology.

10.2 Sporadic CJD

Sporadic CJD is a rapidly progressing dementia with a median disease duration of 4.5 months. The characteristic pathological features are neuronal loss, astrocytosis and spongiform changes. There is also widespread deposition of the abnormal PrP^{sc} protein. The rapid loss of neuronal cells would account for the elevated mean concentrations of CSF NSE and CSF tau, and the high incidence of CSF 14-3-3 in these patients (Table 10.1).

CSF protein	Sporadic CJD Concentration Mean ± SD	Control patients Concentration Mean ± SD
NSE	58.6 ± 55.9 ng/mL	12.3 ± 7.5 ng/mL
14-3-3	24/31 cases positive	4/30 cases positive
tau	5791 ± 6499 pg/mL	873 ± 2508 pg/mL
S-100b	1.73 ± 1.44 ng/mL	0.53 ± 0.25 ng/mL

Table 10.1 The mean concentrations of brain-specific proteins in patients with histologically confirmed sporadic CJD and control patients

The increased mean CSF S-100b is a result of the rapid loss of brain tissue, which would also include the astrocytes, but in addition a proportion of this increase is due to the reactive astrocytosis seen in this condition. These brain-specific proteins were significantly raised when compared to patients who were initially suspected of having sporadic CJD but later proved to have another diagnosis. As a result these markers can be used in the diagnosis of patients with suspected sporadic CJD. The predictive values and the efficiency of each of these proteins in the diagnosis of sporadic CJD is given in Table 10.2.

	NSE > 35 ng/mL	14-3-3	S-100b > 0.5 ng/mL	tau > 1000 pg/mL
PPV	100%	89%	80%	92%
NPV	73%	79%	90%	77%
Efficiency	84%	85%	83%	85%

Table 10.2 Positive predictive value (PPV) and negative predictive value (NPV) for CSF NSE, 14-3-3, S-100b and tau protein for the diagnosis of sporadic CJD

The efficiencies of these proteins were comparable, but each has a different set of positive and negative predictive values. To investigate whether combinations of tests would improve the diagnostic power of these proteins, the predictive values and efficiencies of pairs of proteins were calculated and are shown in Table 10.3. Neurone-specific enolase was not included in these calculations as it had a low efficiency in the diagnosis of new variant CJD. A diagnostic test for CJD would have to be able to diagnose both forms of the disease, as although new variant CJD has a different clinical presentation in the early stages, in the latter stages the patients can resemble sporadic CJD. As can be seen from Table 10.3 combining the tests did not significantly increase the diagnostic power. The numbers of patients investigated were small so these studies need to be continued to clarify the situation.

	14-3-3 or CSF S-100b	**14-3-3 and CSF S-100b**	**14-3-3 or CSF tau**	**14-3-3 and CSF tau**	**CSF S-100b or CSF tau**	**CSF S-100b and CSF tau**
Sensitivity	94%	77%	86%	69%	93%	76%
Specificity	59%	93%	80%	95%	50%	100%
PPV	71%	92%	86%	95%	73%	100%
NPV	89%	79%	80%	68%	83%	74%
Efficiency	77%	85%	84%	80%	76%	86%

Table 10.3 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of different combinations of tests in the diagnosis of sporadic CJD. 14-3-3 = detectable 14-3-3 in CSF; CSF S-100b = greater than 0.5 ng/mL; CSF tau = greater than 1000 pg/mL

In contrast to other studies (Hsich et al., 1996; Zerr et al., 1998) this study found that a significant proportion of patients with histologically confirmed sporadic CJD did not have detectable 14-3-3 in the CSF. This high proportion may reflect the skewed population investigated, as was explained in section 7.1. These 7 cases which did not have detectable 14-3-3 are of interest, and information concerning the duration of illness and the codon 129 phenotype was obtained where possible. This information is shown in Table 10.4.

Sex	Age at onset	Codon 129 phenotype	Disease duration (months)	tau pg/mL	NSE ng/mL	S-100b ng/mL	14-3-3
F	61	MV	8	1731	16.0	0.57	Negative
M	71	MM	4	nd	3.0	0.59	Negative
M	64	MV	> 21	835	5.0	0.92	Negative
M	63	nd	17	102	4.4	0.41	Negative
M	79	MM	17	101	10.0	0.47	Negative
M	61	MM	5	598	46.0	1.28	Negative
M	58	nd	19	1560	59.0	0.73	Negative

Table 10.4 The sex, codon 129 phenotype, disease duration and CSF S-100b, CSF tau and CSF NSE concentrations in 7 patients with histologically confirmed sporadic CJD who did not have detectable 14-3-3, nd = not done

Two of these 7 patients had essentially normal concentrations of CSF NSE and CSF tau with marginal increases in CSF S-100b. The normal concentrations of all three neuronal proteins suggest that when the CSF sample was taken there was little neuronal damage occurring. The marginall increases in CSF S-100b may be due to

the presence of astrocytosis. These 2 patients had a long disease duration and it is possible that these samples were taken late in the course and that the brain loss had already occurred by the time the samples were taken. Alternatively the long disease duration may reflect the rate of pathological change occuring within the central nervous system and the slower rate of neuronal loss resulted in smaller amounts of these proteins being released per unit time and the resulting concentration increase could not be detected. Two patients had increases in the other CSF proteins and had the 14-3-3 checked because of this, and the negative 14-3-3 was confirmed. The remaining 3 patients had low concentrations of CSF NSE but elevated concentrations of CSF tau and/or CSF S-100b. This suggests that these proteins may not be released at the same time in the disease process. Two of these 3 patients were heterozygous at codon 129, which may have an influence on the pathological changes seen in CJD. A number of studies have shown that patients who are heterozygous at codon 129 have a higher incidence of plaque formation (de Silva et al., 1994; Parchi et al., 1996; Tranchant et al., 1999). It is known that patients who are heterozygous at codon 129 have a longer disease duration, which is seen in 1 patient in this study who has a disease duration of at least 21 months. Codon 129 genotype also has an influence on the disease incubation time in patients who have received contaminated growth hormone preparations, patients who are heterozygous have a longer incubation than patients who are homozygous (Deslys et al., 1998). Patients who are heterozygous at codon 129 are less likely to have EEG changes than patients who are homozygous (M Zeidler, personal communication). The influence codon 129 phenotype on the pattern of CSF brain-specific proteins needs further investigation and these studies are underway.

10.3 New variant CJD

In contrast to sporadic CJD, new variant CJD presents with behavioural changes, psychiatric signs and sensory symptoms (Zeidler et al., 1997a,b). As the disease progresses cerebellar ataxia develops and dementia, myoclonus and akinetic mutism occur late in the disease. The disease affects a younger population and the median duration of disease is 16 months. The mean concentrations of the CSF proteins measured are shown in Table 10.5.

CSF protein	New variant CJD Concentration mean ± SD	Control patients Concentration mean ± SD	Concentrations in new variant CJD expressed as % of sporadic CJD
NSE	22.5 ± 14.7 ng/mL	14.6 ± 14.2 ng/mL	38%
14-3-3	11/19 cases positive	2/31 cases positive	
tau	1184 ± 1407 pg/mL	450 ± 1178 pg/mL	20%
S-100b	1.03 ± 0.65 ng/mL	0.35 ± 0.21 ng/mL	60%

Table 10.5 The mean concentrations of CSF proteins in patients with histologically confirmed new variant CJD

The mean concentrations of CSF NSE, CSF tau and CSF S-100b were significantly raised when compared to the controls. The mean concentration of each of these proteins was less than that found in sporadic CJD, and the incidence of 14-3-3 was less than that seen in sporadic CJD. This suggests that the either the amount of neuronal loss which occurs in new variant CJD is less than that found in sporadic CJD or that the rate of neuronal loss is slower. There is no evidence from the

histological examination of the brain that patients with new variant CJD have less neuronal loss than patients with sporadic CJD. It is established that the disease duration is longer in patients with new variant CJD, so this could account for the smaller increase in the concentrations of these proteins. This is unlikely to be the only reason as there is no difference between the length of disease duration in patients who are negative for 14-3-3 and those which are positive for 14-3-3 (Table 7.4). The mean CSF S-100b concentration in patients with new variant CJD is 60% of that seen in patients with sporadic CJD, suggesting that astrocytosis is a more consistent feature in these two forms of CJD than is neuronal loss. Indeed elevated CSF S-100b concentrations are found in iatrogenic and familial forms of the disease.

As the mean concentrations of these proteins were significantly raised when compared to control patients, they may be of value in the investigation of patients suspected of having new variant CJD. The value of each of these proteins in the diagnosis of new variant CJD is shown in Table 10.6.

	NSE > 25 ng/mL	**14-3-3**	**S-100b > 0.5 ng/mL**	**tau > 500 pg/mL**
PPV	80%	85%	75%	82%
NPV	72%	78%	87%	83%
Efficiency	73%	80%	82%	83%

Table 10.6 Positive predictive value (PPV) and negative predictive value (NPV) for CSF NSE, 14-3-3, S-100b and tau protein for the diagnosis of new variant CJD

The efficiency of CSF NSE was less than the other three proteins and is likely to be of less value in the assessment of patients with new variant CJD. In addition, the concentrations of NSE decline on storage and are reduced by freezing and thawing (section 8.2), and as such will limit its usefulness. The efficiencies of CSF 14-3-3, CSF tau and CSF S-100b were comparable but were slightly less than those seen for sporadic CJD. This may be due to the smaller concentrations seen in this condition. To investigate whether the efficiency of these markers was improved by measuring combinations of proteins, the efficiency of using pairs of proteins was calculated and shown in Table 10.7. The efficiency of each of the markers was not significantly improved by the addition of another protein.

	14-3-3 or CSF S-100b	14-3-3 and CSF S-100b	14-3-3 or CSF tau	14-3-3 and CSF tau	CSF S-100b or CSF tau	CSF S-100b and CSF tau
Sensitivity	84%	53%	71%	59%	88%	71%
Specificity	80%	97%	95%	95%	76%	95%
PPV	73%	91%	92%	91%	75%	92%
NPV	89%	76%	80%	74%	89%	80%
Efficiency	82%	79%	84%	79%	82%	84%

Table 10.7 The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and efficiency of different combinations of tests in the diagnosis of new variant CJD. 14-3-3 = detectable 14-3-3 in CSF; CSF S-100b = greater than 0.5 ng/mL; CSF tau = greater than 500 pg/mL

The percentage of patients with histologically confirmed new variant CJD with detectable 14-3-3 was less than that seen in sporadic CJD. There was no significant difference between the duration of illness, the time in the disease course the CSF sample was taken and the age of the patient, between those patients who were negative for 14-3-3 and those that were positive (Table 7.4). The CSF NSE, CSF tau and CSF S-100b concentrations in the 14-3-3 negative patients are shown in Table 10.8.

Sex	Age	tau pg/mL	NSE ng/mL	S-100b ng/mL	14-3-3
F	18	203	15.7	0.23	Negative
M	24	<75	<1	0.29	Negative
F	51	nd	6	0.41	Negative
F	27	78	6.6	0.51	Negative
F	20	136	26	0.79	Negative
F	33	170	10.1	0.84	Negative
M	20	961	11	0.97	Negative
F	23	1327	14	0.77	Negative

Table 10.8 The sex, age, CSF tau, CSF NSE and CSF S-100b concentrations in the eight patients with histologically confirmed new variant CJD who were negative for 14-3-3, nd = not done

The results shown in Table 10.8 show that 5 of the 8 patients who were negative for 14-3-3 had normal concentrations of CSF tau and/or CSF NSE, suggesting that in these patients neuronal damage was too small to be detected by these markers, or was occurring too slowly for the concentration of the markers to increase in

concentration. Of the remaining 3 patients, 1 had a marginally increased CSF NSE with a normal CSF tau protein, and the other 2 patients had increased CSF tau but low CSF NSE concentrations. These results suggest that CSF tau protein may be either a more sensitive marker of neuronal damage in these patients, or that the release of CSF tau occurs at a different time to the release of CSF NSE and 14-3-3. The latter two proteins are soluble and found mainly in the cytoplasm of the neuronal cells, with a smaller amount being associated with the cell membrane in the case of 14-3-3. In contrast tau protein is a structural protein located in the microtubular system of the neuronal cells. It is possible that the release of cytoplasmic proteins occurs more rapidly than the release of the structural proteins such as tau. The only means of investigating this, is to take serial CSF samples from these patients and measure each of the proteins over time, however this is not feasible due to the ethical considerations involved. Of these 8 patients, 5 patients have CSF S-100b concentrations above the cut-off concentration of 0.5 ng/mL, with a further patient having a small increase in CSF S-100b concentration. This suggests that astrocytosis is a prominent feature of new variant CJD, and one of the pathological hallmarks of this disease is a severe thalamic astrocytosis (Will et al., 1996a).

10.4 Alzheimer's disease and frontotemporal dementia

These diseases are associated with a slowly progressing cognitive decline, and although each disease has a different set of clinical signs and symptoms, they can be difficult to distinguish clinically in the early stages. Both of these dementia syndromes are associated with neuronal loss and astrocytosis. Alzheimer's disease is characterised by the presence of amyloid plaques which consist of β-amyloid protein,

and neurofibrillary tangles. These tangles are formed from paired helical filament of an abnormal hyperphosphorylated tau protein. There are two forms of frontotemporal dementia, Pick's disease and frontal lobe degeneration. Pick's disease is characterised by the presence of Pick bodies which are intraneuronal inclusion bodies consisting of abnormal hyperphosphorylated tau protein, in contrast frontal lobe degeneration does not have any tau containing inclusion bodies. The mean concentrations of the CSF proteins measured are shown in Table 10.9. There was no increase in the concentrations of CSF NSE nor was there a substantial number of cases with detectable 14-3-3 in patients with either Alzheimer's disease or frontotemporal dementia. As both of these conditions are associated with a prolonged disease duration of several years, the rate of neuronal loss is likely to be too slow for these proteins to be significantly raised. In contrast the CSF concentrations of tau were significantly raised in both Alzheimer's disease and frontotemporal dementia. It is known that the brain concentrations of hyperphosphorylated tau protein are increased in patients with Alzheimer's disease (Wischik et al., 1992), and it is possible that they are also increased in patients with frontotemporal dementia. This, together with the fact that hyperphosphorylated tau is thought to be neurotoxic, could mean that the neurones containing large amounts of tau inclusion bodies are more likely to die and as a result will release large amounts of tau protein. This suggests that CSF tau concentrations may be used a sensitive marker of neuronal damage and possibly disease severity, although the lack of relationship between the MMSE and CSF tau in patients with Alzheimer's disease found in this study would not support this. If this hypothesis is true patients with dementia not associated with abnormal tau inclusions would be expected to have low concentrations of CSF tau. The results given in Table 10.9 show that patients with

frontal lobe degeneration have lower concentrations of CSF tau than patients with Pick's disease, although they have very similar rates of neuronal loss. There is an overlap between the two groups as illustrated in Figure 5.8 in section 5.4.2, but there were only 3 patients with Pick's disease with essentially normal CSF tau concentrations and only 3 patients with frontal lobe degeneration with elevated CSF tau concentrations. As these conditions are difficult to distinguish clinically, it is possible that these patients may have been mis-diagnosed. Further studies will be needed to investigate this.

The CSF S-100b concentrations were moderately increased in patients with Alzheimer's disease and in patients with frontotemporal dementia. There was no difference between the concentrations seen in patients with Pick's disease and the patients with frontal lobe degeneration. Astrocytosis is a common feature of all these forms of dementia.

Patient Group	NSE ng/mL mean ± SD (n)	14-3-3 positive	tau pg/mL mean ± SD (n)	S-100b ng/mL mean ± SD (n)
Alzheimer's disease	13.8 ± 11.1 (12)	0/19	823 ± 561 (26)	0.41 ± 0.25 (30)
Frontotemporal dementia	8.4 ± 7.6 (9)	1/24	617 ± 409 (28)	0.48 ± 0.34 (26)
Pick's disease	8.7 ± 11.6 (4)	1/16	775 ± 397 (17)	0.47 ± 0.39 (17)
Frontal lobe degeneration	8.2 ± 4.1 (5)	0/8	374 ± 304 (11)	0.50 ± 0.25 (9)
Control subjects	nd	nd	160 ± 61 (12)	0.18 ± 0.09 (21)

Table 10.9 The mean CSF protein concentrations in patients with Alzheimer's disease, frontotemporal dementia and control subjects, nd = not done

10.5 CSF 'brain-specific proteins' in patients with AIDS dementia complex (ADC)

AIDS dementia complex is the most common neurological complication of HIV infection. Pathological features include neuronal loss, particularly in the basal ganglia, reactive astrocytosis and inflammatory cell infiltration in the white matter. The early clinical features can be difficult to distinguish from opportunistic infections affecting the central nervous system. The mean CSF protein concentrations for patients with ADC and opportunistic infections are shown in Table 10.10.

Patient Group	**NSE ng/mL mean ± SD (n)**	**tau pg/mL mean ± SD (n)**	**S-100b ng/mL mean ± SD (n)**
AIDS dementia complex	11.2 ± 10.0 (11)	212 ± 180 (26)	0.36 ± 0.28
Opportunistic infections	14.3 ± 12.2 (13)	265 ± 200 (18)	2.16 ± 3.4 (21)
Control subjects	nd	160 ± 61 (12)	0.18 ± 0.09

Table 10.10 The mean concentrations of CSF NSE, tau and S-100b in patients with AIDS dementia complex and HIV infected patients with opportunistic infections of the central nervous system

The mean concentrations of the neuronal proteins NSE and tau were not significantly elevated in patients with ADC, suggesting that the neuronal loss in this condition was either too small or occurring too slowly to be detected by these markers. It is interesting to note that the mean tau concentration in these patients was essentially normal, in contrast to the tau inclusion body forms of dementia which were

associated with high mean concentrations, despite the fact that they both are slowly progressing diseases. The mean CSF S-100b concentration was slightly increased in patients with ADC, and reflects the reactive astrocytosis seen in this condition.

The mean concentrations of NSE and tau were not elevated in patients with opportunistic infections, in contrast to the marked increase in CSF S-100b. This suggests that the cause of the elevated S-100b is not due to acute brain damage affecting both neurones and astrocytes, but due to a specific astrocytic response. Astrocytes are known to respond to a number of factors released within the brain in response to injury, by proliferating and swelling in size. An opportunistic infection can be thought of as a form of brain injury and it is possible that factors such as interleukin-1, interleukin-6, tumour necrosis factor and interferon gamma from microglial cells; and fibroblast growth factor from the neurones will cause the astrocytes to proliferate release S-100b. S-100b itself can induce further astrocytic proliferation and hypertrophy. These stimulated astrocytic release other factors such as growth factors, protease inhibitors, amyloid precursor protein, apolipoprotein E and transforming growth factor-β, which are thought to play a role in repair.

Chapter 11 - Conclusions

The aim of this study was to investigate whether measurement of brain-specific proteins have a role in the differential diagnosis of patients with dementia. The results described here show that the CSF concentrations of S-100b, tau and NSE were significantly raised in patients with sporadic CJD when compared to patients with other forms of dementia. In addition, the presence of detectable CSF 14-3-3 was much more common in patients with sporadic CJD than in other forms of dementia. These results demonstrate that measurement of CSF proteins has a role in the diagnosis of sporadic CJD. Indeed, since this study was started the detection of CSF 14-3-3 has been recommended as a diagnostic criterion for the classification of probable sporadic CJD (Global Surveillance, Diagnosis and Therapy of Human Transmissible spongiform Encephalopathies: Report of a WHO Consultation, 1998). One of the advantages 14-3-3 has over the other proteins investigated in this study is the relatively low incidence of detectable 14-3-3 in other forms of dementia. In contrast, CSF S-100b and tau are increased in Alzheimer's disease and frontotemporal dementia, but not to the same degree, so each laboratory would have to establish its own cut-off concentration. This may lead to difficulties in defining a 'positive' test. The lack of stability of CSF NSE makes it unsuitable as a diagnostic test, as CSF samples are often transported to laboratories elsewhere for analysis.

There was a group of patients with sporadic CJD who did not have detectable 14-3-3. These patients had either a longer duration of illness and/or were heterozygous at codon 129. This needs further investigation to clarify whether these patients

represent a sub-group of patients with sporadic CJD who have different clinical and pathological findings; or whether the absence of 14-3-3 is due to analytical considerations, such as the sensitivity of the assay or timing of CSF sampling in relation to the disease course.

The value of measurement of these proteins in other forms of CJD has yet to be established. It appears that these proteins may not be as valuable in the assessment of patients with the iatrogenic or familial forms of the disease. The numbers investigated in this study were small, and need to be increased before firm conclusions can be drawn. From the numbers of patients investigated with new variant CJD, it appears that measurement of 'brain-specific' proteins may not be as useful as it is in sporadic CJD.

Although the mean CSF S-100b concentrations were significantly raised in cattle with BSE when compared to cattle with non-BSE diseases, only 50% of the BSE cattle had elevated concentrations. This limits its value in diagnosis, in addition the practical problem of obtaining CSF samples from these cattle make it unsuitable for diagnosis.

Creutzfeldt-Jakob disease is a rare form of dementia, and it would be helpful if measurement of these proteins could help in the diagnosis of the more common forms of dementia such as Alzheimer's disease and frontotemporal dementia. Initial studies found elevated concentrations of CSF tau in patients with Alzheimer's disease and it was suggested that CSF tau may be an early and sensitive test for this disease. The results described in Chapter 5, showed that CSF tau was elevated not

only in patients with Alzheimer's disease but also in patients with frontotemporal dementia and in patients with Creutzfeldt-Jakob disease. Indeed, there was no significant difference between mean concentrations of CSF tau in patients with Alzheimer's disease and frontotemporal dementia, suggesting that measurement of CSF tau cannot help distinguish between these two forms of dementia. The other markers of neuronal damage such as CSF NSE and 14-3-3 were not increased in patients with non-CJD dementia and as such, are of little value in the differential diagnosis of these patients. The mean CSF S-100b concentrations were significantly increased in patients with frontotemporal dementia, but there was considerable overlap with control subjects and patients with other forms of dementia, such as Alzheimer's disease, thereby limiting its diagnostic use. CSF S-100b concentrations were also raised in a third of patients with AIDS dementia complex, but again there was considerable overlap with concentrations from control subjects, which limits its diagnostic use.

The measurement of a range of different brain-specific proteins can give some insight into the pathology underlying different diseases. In patients with CJD the most consistent finding in each of the different forms of CJD, whether it be sporadic, iatrogenic, familial or new variant, was an increase in CSF S-100b concentration. This suggests that astrocytosis may be a more consistent pathological finding than neuronal loss or damage, in terms of disease type and/or disease duration. This may be because astrocytosis occurs throughout the disease course whereas neuronal loss is likely to occur later in the disease. The rate of neuronal loss may vary between the forms of CJD and this may be why markers of neuronal damage are high in some patients and not in others. It was not possible in this study to investigate the

relationship between disease duration, time of CSF sampling and CSF protein concentrations, but this will need to be studied in detail to answer these questions.

The finding that patients with Alzheimer's disease and frontotemporal dementia have elevated concentrations of CSF tau but normal concentrations of other neuronal markers, suggest that there must be an additional cause for the increase in CSF tau in these patients. It is possible that the abnormal tau found in these diseases accumulates in the neurones and is responsible for the neuronal death. As a result CSF tau would be a very sensitive marker for selective neuronal loss in these patients. A study investigating the relationship between CSF tau concentrations and measures of brain atrophy such as MRI imaging is currently underway in patients with Alzheimer's disease.

In summary, CSF brain-specific proteins, in particular 14-3-3, have a role in the diagnosis of sporadic CJD. The value of these proteins in the assessment of patients with new variant CJD is unclear at present, but it is unlikely that they will be have the same diagnostic potential. At present the measurement of these proteins does not have a role in the assessment of patients with non-CJD dementia.

References

Abraha HD, Butterworth RJ, Bath PMW, Wassif WS, Garthwaite J, Sherwood RA. Serum S-100 protein, relationship to clinical outcome in acute stroke. *Ann Clin Biochem* 1997; 34:546-550

Absher JR, Cummings JL. Cognitive and non-cognitive aspects of dementia syndromes: an overview. In: Burns A, Levy R eds. *Dementia.* London: Chapman and Hall Medical, 1994: 59-76

Adams RD. Viral infections of the nervous system. In: Adams, Victor, Ropper (eds) *Principles of Neurology.* McGraw-Hill, New York 1997: 759-776

Advisory Committee on Dangerous Pathogens, Spongiform Encephalopathy Advisory Committee. *Transmissible Spongiform Encephalopathy Agents: Safe working and the prevention of infection.* HMSO,1998

Aitken A, Amess B, Howell S, Jones D, Martin H, Patel Y, Robinson K, Toker A. The role of specific isoforms of 14-3-3 protein in regulating protein kinase activity in the brain. *Biochem Soc Trans* 1992; 20:607-11

Aitken A, Howell S, Jones D, Madrazo J, Patel Y. 14-3-3 α and δ are the phosphorylated forms of Raf-activating 14-3-3 β and ζ. *J Biol Chem* 1995a; 270:5706-9

Aitken A, Jones D, Soneji Y, Howell S. 14-3-3 proteins: biological function and domain structure. Biochem Soc Trans 1995b; 23:605-11

Aitken A. 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends in Cell Biol 1996; 6:341-7

Andreasen N, Vanmechelen E, Van de Voorde A, Davidsson P, Hesse C, Tarvonen, Raiha I, Sourander L, Winblad B, Blennow K. Cerebrospinal fluid tau protein as a biochemical marker for Alzheimer's disease: a community based follow up study. J *Neurol Neurosurg Pyschiatry* 1998; 64:298-305.

Arai H, Terajima M, Miura M, Higuchi S, Muramatsu T, Machida N, Seiki H, Takase S, Clark CM, Lee VMY, Trojanowski JQ, Sasaki H. Tau in cerebrospinal fluid: a potential diagnostic marker in Alzheimer's disease. *Ann Neurol* 1995; 38:649-52

Arai H, Morikawa Y, Higuchi M, Matsui T, Clark CM, Miura M, Machida N, Lee VMY, Trojanowski JQ, Sasaki H. Cerebrospinal fluid tau levels in neurodegenerative diseases with distinct tau related pathology. *Biochem Biophys Res Commun* 1997; 236:262-64

Arai H, Satoh-Nakagawa T, Higuchi M, Morikawa Y, Miura M, Kawakami H, Seki H, Takase S, Sasaki H. No increase in cerebrospinal fluid tau protein levels in patients with vascular dementia. *Neurosci Lett* 1998; 256:174-76

Aurell A, Rosengren LE, Wikkelso C, Nordberg G, Haglid KG. The S-100 protein in cerebrospinal fluid: a simple ELISA method. *J Neurol Sci* 1989; 89:157-64

Azmitia EC, Dolan K, Whitaker-Azmitia PM. S-100b but not NGF, EGF, insulin or calmodulin is a CNS serotonergic growth factor. *Brain Res* 1990; 516:354-6

Barger SW, Van Eldik LJ. S-100b stimulates calcium fluxes in glial and neuronal cells. *J Biol Chem* 1992; 267:9689-94

Baudier J, Cole RD. Reinvestigation of the sulfydryl reactivity in bovine brain S-100 (bb) protein and the microtubule-associated tau proteins. Ca^{2+} stimulates disulfide cross-linking between the S100b beta subunit and the microtubule-associated tau (2) protein. *Biochemistry* 1988a; 27:2728-2736

Baudier J, Cole RD. Interactions between the microtubule-associated tau proteins and S-100b regulate tau phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase II. *J Biol Chem* 1988b; 263:5876-83

Beaudry P, Cohen P, Brandel JP, Delasneriel Laupretre N, Richard S, Launay JM, Laplanche JL. 14-3-3 protein, neuron-specific enolase and S-100 protein in cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. *Dementia and geriatric cognitive disorders* 1999; 10:40-46

Beck E, Daniel PM. Neuropathology of transmissible spongiform encephalopathies. In: Prusiner SB, McKindley MP eds. *Prions: novel infectious pathogens causing scrapie and Creutzfeldt-Jakob disease*. San Diego: Academic Press, 1987:331-85

Bernoulli C, Siegfried J, Baumgartner G, Regli F, Rabinowicz T, Gajdusek DC, Gibbs CJ. Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. *The Lancet* 1977; 1(8009):478-79

Bianchi R, Giambanco I, Donato R. S-100 protein, but not calmodulin, binds to the glial fibrillary acidic protein and inhibits its polymerisation in a Ca^{2+}-dependent manner. *J Biol Chem* 1993; 268:12669-74

Blennow K, Wallin A, Ekman R. Neuron-specific enolase in cerebrospinal fluid: a marker for neuronal degeneration in dementia disorders? *J Neural Transmission - Parkinsons disease and dementia section.* 1994; 8:183-91

Blennow K, Wallin A, Agren H, Spenger C, Siegfried J, Vanmechelen E. Tau protein in cerebrospinal fluid. A biochemical marker for axonal degeneration in Alzheimer disease. *Mol Chem Neuropathol* 1995; 26:231-45

Blomquist S, Johnsson P, Luhrs C, Malmkvist G, Solem JO, Alling C, Stahl E. The appearance of S-100 protein in serum during and immediately after cardiopulmonary bypass surgery: a possible marker for cerebral injury. *J Cardiothorac Vasc Anaes* 1997; 11(6):699-703

Boston PF, Jackson P, Kynoch PA, Thompson RJ. Purification, properties and immunohistochemical localisation of human brain 14-3-3 protein. *J Neurochem* 1982a; 38:1466-74

Boston PF, Jackson P, Thompson RJ. Human 14-3-3 protein: Radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. J Neurochem 1982b; 38:1475-1482

Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol (Berl)* 1991; 82:239-259

Bradley R. Animal Prion diseases. In: Collinge J, Palmer MS eds. *Prion diseases.* Oxford: Oxford University Press, 1997:89-129

Brew B, Sidtis J, Petito CK, Price RW: The Neurologic complications of AIDS and human immunodeficiency virus infection. Plum F (ed): *Advances in contemporary Neurology.* Philadelphia, Davis, 1988: chapter 1

Brown J. Pick's disease. In: Rossor, MN ed. *Unusual dementias.* London: Bailliere Tindall, London 1992; 535-57

Brown KW, Kynoch PA, Thompson RJ. Immunoreactive nervous system specific enolase (14-3-2 protein) in human serum and cerebrospinal fluid. *Clin Chim Acta* 1980; 101:257-64

Brown P, Gajdusek DC, Gibbs CJ, Asher DM. Potential epidemic of Creutzfeldt-Jakob disease from human growth hormone therapy. *New Engl J Med* 1985; 313:728-31

Bruce ME, Chree A, McConnell I, Foster J, Pearson G, Fraser H. Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos Tran. R Soc London* Ser B 1994; 343:405-11

Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, McCardle L, Chree A, Hope J, Birkett C, Cousens S, Fraser H, Bostock CJ. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. *Nature* 1997; 389:498-501

Brun A, Englund B, Gustafson L, Passant U, Mann DMA, Neary D, Snowden JS. Clinical and neuropathological criteria for frontotemporal dementia. *J Neurol Neurosurg Psychiatry* 1994; 57:416-8

Butner KA, Kirschner MW. Tau protein binds to microtubules through a flexible array of distributed weak sites. *J Cell Biol* 1991; 115:717-30

Buttner T, Weyers S, Postert T, Sprengelmeyer R, Kuhn W. S-100 protein: serum marker of focal brain damage after ischaemic territorial MCA infarction. *Stroke* 1997; 28(10):1961-5

Butterworth RJ, Wassif WS, Sherwood RA, Gerges A, Poyser KH, Garthwaite J, Peters TJ, Bath PMW. Serum neuron-specific enolase, carnosinase, and their ratio in acute stroke - An enzymatic test for predicting outcome? *Stroke* 1996; 2064-2068

Cicero TJ, Cowan WM, Moore BW, Suntzeff V. The cellular localization of the two brain specific proteins, S-100 and 14-3-2. *Brain Res* 1970; 18:25-34

Cockrell JR, Folstein MF. Mini-mental state examination (MMSE). *Psycho Pharmacol Bull* 1988;24:689-92

Collinge J. Human prion diseases nad bovine spongiform encephalopathy (BSE). Human Molecular genetics 1997; 6:1699-1705

Collinge J, Palmer MS. Human Prion diseases. In: Collinge J, Palmer MS, eds. *Prion diseases.* Oxford: Oxford University Press, 1997:18-56

Collins SJ, Ahlskog JE, Parisi JE, Maraganore DM. Progressive supranuclear palsy: neuropathologically based diagnostic clinical criteria. *J Neurol Neurosurg Psychiatry* 1995; 58:167-73

Crowther RA. Straight and paired helical filaments in Alzheimer disease have a common structural unit. *Proc Natl Acad Sci USA* 1991; 88:2288-92

Cunningham RT, Johnstone CFIGB, McIlrath EM, McNeill A, Buchanan KD. Development of a radioimmunoassay for neurone specific enolase (NSE) and its application in the study of patients receiving intra-hepatic arterial streptozotocin and floxuridine. *Clin Chim Acta* 1990; 189:275-286

Cutler NR, Kay AD, Marangos PJ, Burg C. Cerebrospinal fluid neuron-specific enolase is reduced in Alzheimer's disease. *Arch Neurol* 1986; 43:153-54

Dauberschmidt R, Marangos PJ, Zinsmeyer J, Bender V, Klages G, Gross J. Severe head trauma and the changes of concentration of neuron-specific enolase in plasma and in cerebrospinal fluid. *Clin Chim Acta* 1983; 131:165-70

Delacourte A, Robitaille Y, Sergeant N, Buee L, Hof PR, Wattez A, Laroche-Cholette A, Mathieu J, Chagnon P, Gavreau D. Specific pathological tau protein variants characterize Pick's disease. *J Neuropathol Exp Neurol* 1996; 55(2):159-68

de Silva R, Ironside JW, McCardle L, Esmonde T, Bell J, Will RG, Windl O, Dempster M, Estibeiro P, Lathe R. Neuropathological phenotype and 'prion protein' genotype correlation in sporadic Creutzfeldt-Jakob disease. *Neurosci Lett* 1994; 179(1-2):50-52

Deslys JP, Jaegly A, d'Aignaux H, Mouthon F, de Villemeur TB, Dormont D. Genotype at codon 129 and susceptibility to Creutzfeldt-Jakob disease. *The Lancet* 1998; 351:1251

Dickson DW, Crystal HA, Bevona C, Honer W, Vincent I, Davies P. Correlations of synaptic and pathological markers with cognition of the elderly. *Neurobiol Aging* 1995; 16:285-98

Dlouchy SR, Hsiao K, Farlow MR, Foroud T, Conneally PM, Johnson P, Prusiner SB, Hodes ME, Ghetti B. Linkage of the Indiana kindred of to Gerstmann-Straussler-Scheinker disease to the prion protein gene. *Nature Genetics* 1992; 1:64-67

Doh ura K, Tateishi J, Sasaki H, Kitamoto T, Sakaki Y. Pro-Leu change at position 102 of prion protein is the most common but not the sole mutation related to Gerstmann-Straussler-Scheinker syndrome. *Biochem Biophy Res Commun* 1989; 163: 974-79

Donato R. Perspectives in S-100 protein biology. *Cell Calcium* 1991; 12:713-26

Dreschsel DN, Hyman AA, Cobb MH, Kirschner MW. Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. *Mol Biol Cell* 1992; 3:1141-1154

Duffy P, Wolf J, Collins G, DeVoe AG, Streeten B, Cowen D. Possible person-to-person transmission of Creutzfeldt-Jakob disease. *New Engl J Med* 1974; 290:692

Ellis RJ, Seubert P, Motter R, Galasko D, Deutsch R, Heaton RK, Heyes MP, McCutchan JA, Atkinson JH, Grant I. Cerebrospinal fluid tau protein is not elevated in HIV-associated neurologic disease in humans. *Neurosci Lett* 1998; 254:1-4

Evers S, Droste DW, Ludemann P, Oberwittler C. Early elevation of cerebrospinal fluid neuron-specific enolase in Creutzfeldt-Jakob disease. *Neurology* 1998; 245:52-53

Fano G, Mariggio MA, Angelella P, Nicoletti I, Antonica A, Fulle S, Calissano P. The S-100 protein causes an increase of intracellular calcium and death of PC12 cells. *Neuroscience* 1993; 53:919-25

Fano G, Biocca S, Fulle S, Mariggio MA, Belia S, Calissano P. The S-100: a protein family in search of a function. *Progress in Neurology* 1995; 46:71-82

Feany MB, Dickson DW. Neurodegenerative disorders with extensive tau pathology: A comparative study and review. *Ann Neurol* 1996a; 40:139-148

Feany MB, Mattiace LA, Dickson DW. Neuropathologic overlap of progressive supranuclear palsy, Pick's disease and corticobasal degeneration. *J Neuropathol Exp Neurol* 1996b; 55:53-67

Freed E, Symons M, MacDonald SG, McCormick F, Ruggieri R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* 1994; 265:1713-16

Fulle S, Mariggio MA, Fano G, Calissano P. Effect of S-100 protein on adenylate cyclase and phospholipase C of rat cerebral cortex membranes. *Proc XXXI Int Congr Physiol Sci* 1989; (Helsinki 9-14 July):P5455

Fulle S, Mariggio MA, Fano G, Salvatore AM, Mercanti D, Petrelli C, Calissano P. Activation of brain adenylate cyclase by S-100 protein via a possible interaction with G-protein(s). *Neurosci Res Comm* 1992; 10(1):35-43

Galasko D, Hansen LA, Katzman R, Wiederholt W, MasLiah E, Terry R, Hill R, Lessin P, Thal LJ. Clinical-neuropathological correlations in Alzheimer's disease and related dementias. *Arch Neurol* 1994; 51:888-95

Galasko D, Clark L, Chang L, Miller B, Green RC, Motter R, Seubert P. Assessment of CSF levels of tau protein in mildly demented patients with Alzheimer's disease. *Neurology* 1997; 48:632-35

Galasko D, Chang L, Motter R, Clark CM, Kaye J, Knopman D, Thomas R, Kholodenko D, Schenk D, Lieberburg I, Miller B, Green R, Basherad R, Kertiles L, Boss MA, Seubert P. High cerebrospinal fluid tau and low amyloid β42 levels in the clinical diagnosis of Alzheimer disease and relation to apolipoprotein E genotype. *Arch Neurol* 1998; 55:937-945

Galton CJ, Hodges JR. The spectrum of dementia and its treatment. *Journal of the Royal College of Physicians of London* CME Neurology 1999; 33:234-239

Garcia-Alix A, Cabanas F, Pellicer A, Hernanz A, Stiris TA, Quero J. Neuron-specific enolase and myelin basic protein: relationship of cerebrospinal fluid concentrations to the neurologic condition of asphyxiated full-term infants. *Pediatrics* 1994; 93(2):234-40

Gerbitz KD, Summer J, Thallemer J. Brain-specific proteins: solid-phase immunobioluminescence assay for neuron-specific enolase in human plasma. *Clin Chem* 1984; 30(3):382-6

Gibbons A. Is AIDS dementia due to increases in calcium? *Science* 1990; 248:303

Global Surveillance, Diagnosis and Therapy of Human Transmissible spongiform Encephalopathies: Report of a WHO Consultation, 1998

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA. Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* 1989; 3:519-26

Goedert M, Spillantini MG, Crowther RA. Tau proteins and neurofibrillary degeneration. *Brain Pathology* 1991a; 1:279-286

Goedert M, Crowther RA, Garner CC. Molecular characterization of microtubule-associated protein tau and MAP2. *Trends Neurosci* 1991b; 14:193-199

Goedart M, Spillantini MG, Crowther RA. Cloning of a big tau-microtubule associated protein characteristic of the peripheral nervous system. *Proc Natl Acad Sci USA* 1992a; 89:1983-87

Goedert M, Cohen ES, Jakes R, Cohen P. p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1. Implication for Alzheimer's disease. *FEBS Lett* 1992b; 312:95-99

Goedert M. Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Trends Neurosci* 1993; 16(11):460-5

Goedart M, Jakes R, Crowther A, Cohen P, Vandermeeren E, Vandermeeren M, Cras P. Epitope mapping of monoclonal antibodies to the paired helical filaments of Alzheimer's disease: identification of phosphorylation sites in tau protein. *Biochem J* 1994; 301:871-877

Goldfarb LG, Petersen RB, Tabaton M, Brown P, LeBlanc AC, Montagna P et al. Fatal Familial Insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science* 1992; 258:806-8

Griffin WST, Stanley LC, Yeralan O, Rovnaghi CR, Marshak DR. Cytokines in human neurodegenerative disease. *Meth Neurosci* 1993; 17:267-87

Haimoto HTY, Takahashi Y, Koshikawa T, Nagura H, Kato K. Immunohistochemical localisation of γ enolase in normal human tissues other than nervous and neuroendocrine tissues. *Lab Invest* 1985; 52:257-63

Hanger DP, Hughes K, Woodgett JR, Brion JP, Anderton BH. Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci Lett* 1992;147:58-62.

Hardy J, Duff K, Hardy KG, Perez-Tur J, Hutton M. Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. *Nature Neuroscience* 1998; 1:355-58

Harrington MG, Merril CR, Asher DM, Gadjusek DC. Abnormal proteins in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. *N Engl J Med* 1986; 315:279-283

Hasegawa M, Morishima-Kawashima M, Takio K, Suzuki M, Titani K, Ihara Y. Protein sequence and mass spectrometric analyses of tau in the Alzheimer's disease brain. *J Biol Chem* 1992; 267:17047-54

Hauw JJ, Daniel SE, Dickson D, Horoupian DS, Jellinger K, Lantos PL, McKee A, Tabaton M, Litvan I. Preliminary NINDS neuropathological criteria for Steel-Richardson-Olszewski syndrome (progressive supranuclear palsy). *Neurology* 1994; 44:2015-9

Hay E, Royds JA, Davies-Jones GA, Lewtas NA, Timperley WR, Taylor CB. Cerebrospinal fluid enolase in stroke. *J Neurol Neurosurg Psychiatry* 1984; 47:724-9

Heizmann CW, Hunziker W. Intracellular calcium binding protein: more sites than insights. *Trends Biochem Sci* 1991; 16:98-103

Hidaka H, Endo T, Kawamoto S, Yamada E, Umekawa H, Tanabe K, Hara K. Purification and characterisation of adipose tissue S-100b protein. *J Biol Chem* 1983; 258:2705-9

Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J, Doey LJ, Lantos PL. The same prion strain causes nvCJD and BSE. *Nature* 1997; 389:448-450

Hsiao K, Baker, HF, Crow TJ, Poulter M, Owen F, Terwilliger JD, Westaway D, Ott J, Prusiner SB. Linkage of a prion protein missense variant to Gerstmann-Straussler-Scheinker syndrome. *Nature* 1989; 338:342-45

Hsich G, Kenney K, Gibbs CJ, Lee KH, Harrington MG. The 14-3-3 brain protein in cerebrospinal fluid as a marker for transmissible spongiform encephalopathies. *N Engl J Med* 1996; 335:924-30

Hu J, Castets F, Guevara JL, Van-Eldik LJ. S-100b stimulates inducible nitric oxide synthase activity and mRNA levels in rat cortical astrocytes. *J Biol Chem* 1996; 271:2543-7

Hu J, Ferreira A, Van-Eldik LJ. S-100b induces neuronal cell death through nitric oxide release from astrocytes. *J Neurochem* 1997; 69:2294-301

Hullin DA, Brown K, Kynoch PA, Smith C, Thompson RJ. Purification, radioimmunoassay and distribution of human brain 14-3-2 protein (nervous-system specific enolase) in human tissues. *Biochem Biophys Acta* 1980; 628:98-108

Ichimura T, Isobe T, Okuyama T, Takahashi H, Araki K, Kuwano R, Takahashi Y. Molecular cloning of cDNA coding for brain specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *Proc Natl Acad Sci USA* 1988; 85:7084-8

Ikeda K, Akiyama H, Haga C, Kondo H, Arima K, Oda T. Argyrophilic thread-like structure in corticobasal degeneration and supranuclear palsy. *Neurosci Lett* 1994; 174:157-9

Ingebrigtsen T, Romner B, Kongstad P, Langbakk B. Increased serum concentrations of protein S-100 after minor head injury: a biochemical serum marker with prognostic value? *J Neurol Neurosurg Psychiatry* 1995; 59:103-4

Ingebrigtsen T, Romner B. Serial S-100 protein measurements related to early magnetic resonance imaging after minor head injury. *J Neurosurg* 1996; 85:945-8

Ingebrigtsen T, Romner B, Trumpy JH. Management of minor head injury: the value of early computed tomography and serum protein S-100 measurements. *J Clin Neurosci* 1997; 4:29-33

Inoue S, Takahashi H, Kaneko K. The fluctuations of neuron-specific enolase (NSE) levels of cerebrospinal fluid during bacterial meningitis: the relationship between the fluctuations of NSE levels and neurological complications or outcome. *Acta Paediatr Jpn* 1994; 36(5):485-8

Isobe T, Ichimura T, Okuyama T. Chemistry and cell biology of neuron and glia-specific proteins. *Arch Histol Cytol* 1989; 52(Suppl):25-32

Isobe T, Ichimura T, Sunaya T, Okuyama T, Takahashi N, Kuwano R, Takahashi Y. Distinct forms of the protein kinase dependent activator of tyrosine and tryptophan hydroxylases. *J Mol Biol* 1991; 217:125-32

Isobe T, Hiyane Y, Ichimura T, Okuyama T, Takahashi N, Nakajo S, Nakaya K. Activation of protein kinase C by the 14-3-3 proteins homologous with Exo 1 protein that stimulates calcium-dependent exocytosis. *FEBS Lett* 1992; 308:121-4

Jennett B, Bond M. Assessment of outcome after severe brain damage. *The Lancet* 1975; 1:480-4

Jensen M, Basun H, Lannfelt L. Increased cerebrospinal fluid tau in patients with Alzheimer's disease. *Neurosci Lett* 1995; 186:189-91

Jimi T, Wakayama Y, Shibuya S, Nakata H, Tomaru T, Takahashi Y, Kosaka K, Asano T, Kato K. High levels of nervous system-specific proteins in cerebrospinal fluid in patients with early stage Creutzfeldt-Jakob disease. *Clin Chim Acta* 1992; 211:37-46

Johnson R, Gibbs CJ. Creutzfeldt-Jakob disease and related transmissible spongiform encephalopathies. *New Engl J Med* 1998; 339:1994-2004

Jones DH, Ley S, Aitken A. Isoforms of 14-3-3 protein can form homodimers and heterodimers in vivo and in vitro: implications for function as adapter proteins. *FEBS Lett* 1995; 368:55-58

Jongren PJH, Lamers KJB, Doesburg WH, Lemmens WAJG, Hommes OR. Cerebrospinal fluid analysis differentiates between relapsing remitting and secondary progressive multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1997; 63:446-51.

Jorm AF, Korten AE, Henderson AS. The prevalence of dementia: a quantitative integration of the literature. *Acta Psychiatr Scand* 1987; 76:465-79

Kahn HJ, Baumal R, Van Eldik LJ, Dunn RJ, Marks A. Immunoreactivity of S-100b in heart, skeletal muscle and kidney in chronic lung disease: Possible induction by cAMP. *Mod Pathol* 1991; 4:698-701

Kanai M, Matsubara E, Isoe K, Urakami K, Nakashima K, Arai H, Sasaki H, Abe K et al. Longitudinal study of cerebrospinal fluid levels of tau, Aβ1-40, and Aβ1-42(43) in Alzheimer's disease: a study in Japan. *Ann Neurol* 1998; 44:17-26

Kato K, Nakajima T, Ishiguro Y, Tenhoshimaru M. Sensitive enzyme immunoassay for S-100 protein: determination in human cerebrospinal fluid. *Biomed Res Toyko* 1982; 3(1):24-8

Kato K, Asai R, Shimizu A, Suzuki F, Ariyoshi Y. Immunoassay of three enolase isozymes in human serum and in blood cells. *Clin Chim Acta* 1983; 127: 353-363

Kato K, Kimura S. S-100ao (alpha alpha) protein is mainly located in the heart and striated muscles. *Biochim Biophys Acta* 1985; 842:146-50

Kato K, Kimura S, Haimoto H, Suzuki F. S-100 ao (aa) protein: distribution in muscle tissues of various animals and purification from human pectoral muscle. *J Neurochem* 1986; 46:1555-60

Kato K, Nakamura H. Presence of two different fibril subtypes in the Pick body: an immunoelectron microscopic study. *Ann Neuropathol* 1990; 81:125-9

Kettenmann H, Orkand RK, Schachner M. Coupling among identified cells in mammalian nervous system cultures. *J Neurosci* 1983; 3:506-16

Khatoon S, Grundke-Iqbal I, Iqbal K. Brain levels of microtubule-associated protein tau are elevated in Alzheimer's disease: a radioimmuno-slot-blot assay for nanograms of the protein. *J Neurochem* 1992; 59:750-53

Kindblom LG, Lodding P, Rosengren L, Baudier J, Haglid K. S-100 protein in melanocytic tumours. An immunohistochemical investigation of benign and malignant melanocytic tumors and metastases of malignant melanoma and a characterization of the antigen in comparison to human brain. *Acta Pathol Microbiol Immunol Scand A* 1984; 92:219-30

Klatka LA, Schiffer RB, Powers JM, Kazee AM. Incorrect diagnosis of Alzheimer's disease: a clinicopathologic study. *Arch Neurol* 1996; 53:35-42

Kligman D, Marshak DR. Purification and characterisation of a neurite extension factor from bovine brain. *Proc Natl Acad Sci USA* 1985; 82:7136-9

Knopman DS, Mastri AR, Frey WH, Sung JH, Rustan T. Dementia lacking distinctive histologic features: a common non-Alzheimer degenerative dementia. *Neurology* 1990; 40:251-66

Kropp S, Zerr I, Schultz-Schaeffer WJ, Riedemann C, Bodemer M, Laske C, Kretzschmar HA, Poser S. Increase of neuron-specific enolase in patients with Creutzfeldt-Jakob disease *Neurosci Lett* 1999; 261 124-126

Ksiezak-Reding H, Liu WK, Yen SH. Phosphate analysis and dephosphorylation of modified tau associated with paired helical filaments. *Brain Res* 1992; 597:209-19

Ksiezak-Reding H, Morgan K, Mattiace LA, Davies P, Liu WK, Yen SH, Weidenheim K, Dickson DW. Ultrastructure and biochemical composition of paired helical filaments in corticobasal degeneration. *Am J Pathol* 1994; 145:1496-508

Lamers KJB, van Engelen BGM, Gabreels FJM, Hommes OR, Borm GF, Wevers RA. Cerebrospinal neuron-specific enolase, S-100 and myelin basic protein in neurological disorders. *Acta Neurol Scand* 1995; 92:247-51

Langley OK, Ghandour MS, Vincendon G, Gombos G. An ultrastructural immunocytochemical study of nerve-specific protein in rat cerebellum. *J Neurocytol* 1980; 9:783-98

Lantos PL. Neuropathology of unusual dementias; an overview. In: Rossor, MN ed. *Unusual dementias*. London: Baillière Tindall, London 1992: 485-516

Lee VMY, Balin BJ, Otvos LJr, Trojanowski JQ. A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* 1991; 251:675-78

Lennox G. Lewy body dementia. In: Rossor, MN ed. *Unusual dementias*. London: Baillière Tindall, London 1992: 653-76

Litvan I, Agid Y, Sastrj N, Jankovic J, Wenning GK, Goetz CG, Verny M, Brandel JP, Jellinger K, Chaudhuri KR, McKee A, Lai EC, Pearce RKB, Bartko JJ. What are the obstacles for an accurate clinical diagnosis of Pick's disease. A clinicopathologic study. *Neurology* 1997; 49:62-69

Mahadik SP, Graf L, Korenovsky A, Rapport MM. Immunochemical properties of S-100 proteins and their subunits. *J Neurochem* 1979; 33:763-71

Mann DMA, South PW, Snowden JS, Neary D. Dementia of frontal lobe type: neuropathology and immunohistochemistry. *J Neurol Neurosurg Psychiatry* 1993; 56:605-14

Marangos PJ, Zomzely-Neurath C. Determination and characterisation of neuron specific protein (NSP) associated enolase activity. *Biochem Biophys Res Commun* 1976; 1309-16

Marangos PJ, Zomzely-Neurath C, Goodwin FK. Structural and immunological properties of neuron specific protein (NSP) from rat, cat and human brain. *J Neurochem* 1977; 28:1097-107

Marangos PJ, Parma AM, Goodwin FK. Functional properties of neuronal and glial isoenzymes of brain enolase. *J Neurochem* 1978a; 31:727-32

Marangos PJ, Zis AP, Clark RL, Goodwin FK. Neuronal, non-neuronal and hybrid forms of enolase in brain: structural, immunological and functional comparisons. *Brain Res* 1978b; 150:117-33

Marangos PJ, Campbell IC, Schmechel DE, Murphy DL, Goodwin FK. Blood platelets contain a neuron specific enolase subunit. *J Neurochem* 1980; 34:1254-8

Marangos PJ, Schmechel DE. Neuron specific enolase, a clinically useful marker for neurons and neuroendocrine cells. *Annu Rev Neurosci* 1987; 10:269-95

Martin H, Patel Y, Jones D, Howell S, Robinson K, Aitken A. Antibodies against the major brain isoforms of 14-3-3 protein. An antibody specific for the N-acetylated amino terminus of a protein. *FEBS Lett* 1993; 331:296-303

Martin H, Rostas J, Patel Y, Aitken A. Subcellular localisation of 14-3-3 isoforms in rat brain using specific antibodies. *J Neurochem* 1994; 63:2259-65

Massaro AR, Michetti F, Laudisio A, Bergonzi P. Myelin basic protein and S-100 antigen in cerebrospinal fluid of patients with multiple sclerosis in the acute phase. *It J Neurosci* 1985; 6:53-6

McKeith IG, Perry RH, Fairbairn AF, Jabeen S, Perry EK. Operational criteria for senile dementia of Lewy body type (SDLT). *Psychol Med* 1992; 22:911-22

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan E M. Clinical diagnosis of Alzheimer's Disease: Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984; 34:939-944

Mecocci P, Cherubini A, Bregnocchi M, Chionne F, Cecchetti R, Lowenthal DT, Senin U. Tau protein in cerebrospinal fluid: a new diagnostic and prognostic marker in Alzheimer's disease? *Alzheimer's disease & associated disorders* 1998; 12:211-14

Michetti F, Massaro A, Russo G, Rigon G. S-100 antigen in CSF as a possible index of cell injury in the nervous system. *J Neurol Sci* 1980; 44:259-63

Missler U, Wiesmann M. Measurement of S-100 protein in human blood and cerebrospinal fluid: analytical method and preliminary clinical results. *Eur J Clin Chem Biochem* 1995; 33(10):743-8

Missler U, Wiesmann M, Friedrich C, Kaps M. S-100 protein and neuron-specific enolase concentrations in blood as indicators of infarction volume and prognosis in acute ischaemic stroke. *Stroke* 1997; 28(10):1956-60

Mitani K, Furiya Y, Uchihara T, Ishii K, Yamanouchi H, Mizusawa H, Mori H. Increased CSF tau in corticobasal degeneration. *J Neurol* 1998; 245:44-46

Mokuno K, Kato K, Kawai K, Matsuoka Y, Yanagi T, Sobue I. Neuron-specific enolase and S-100 protein levels in cerebrospinal fluid in patients with various neurological diseases. *J Neurol Sci* 1983; 60:443-51

Mokuno K, Kiyosawa K, Sugimura K, Yasuda T, Riku S, Murayama T, Yanagi T, Takahashi H, Kato K. Prognostic value of cerebrospinal fluid neuron-specific enolase and S-100b protein in Guillain-Barre syndrome. *Acta Neurol Scand* 1994; 89:27-30

Molina JA, Benito-Leon ., Jimenez-Jimenez FJ, Orti-Pareja M, Berbel A, Tallon-Barranco A, de Bustos F, Hernanz A. Tau protein concentrations in cerebrospinal fluid of non-demented Parkinson's disease patients. *Neurosci Lett* 1997; 238:139-41

Moore and McGregor. Chromatographic and electrophoretic fractionation of soluble proteins of brain and liver. *J Biol Chem* 1965; 240:1647-1653

Moore BW, Perez VJ. Complement fixation for antigens at a picogram level. *J Immunol* 1966; 96:1000-5

Moore BW, Perez VJ. Cicero TJ, editors. New York: Prentice-Hall; 1968; *Physiological and biochemical aspects of nervous integration.* p.343-59

Moore BW. Moore BW, Kligman D, editors. *Neuronal and Glial Proteins: Structure, Function and Clinical Application.* New York: Academic Press; 1988; The S100 protein. p. 137-67

Morgan A, Burgoyne RD. Exo 1 and Exo 2 proteins stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. *Nature* 1992; 355:833-6

Mori H, Hosoda K, Matsubara E, Nakamoto T, Furiya Y, Endoh R, Usami M, Shoji M, Maruyama S, Hirai S. Tau in cerebrospinal fluids: establishment of the sandwich ELISA with antibody specific to the repeat sequence in tau. *Neurosci Lett* 1995; 186:181-3

Morris JC, Berg L, Fulling K, Torack RM, McKeel DW. Validation of clinical diagnostic criteria for senile dementia of the Alzheimer type. *Ann Neurol* 1987; 22:122

Motter R, Vigo-Pelfrey C, Kholodenko D, Barbour BS, Johnson-Wood K, Galasko D, Chang L, Miller B et al. Reduction of β-amyloid peptide$_{42}$ in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann Neurol* 1995; 38:643-48

Mrak RE, Sheng JG, Griffin WST. Glial cytokines in Alzheimer's disease: review and pathogenic implications. *Human Pathology* 1995; 26:816-23

Murayama S, Mori H, Ihara Y, Tomonaga M. Immunocytochemical and ultrastructural studies of Pick's disease. *Ann Neurol* 1990; 27:394-405

Navia BA, Cho ES, Petito CK, Price RW. The AIDS dementia complex: II Neuropathology *Ann Neurol* 1986; 19:525-35

Navia BA. Acquired immunodeficiency Syndrome In: Burns A, Levy R eds. *Dementia.* London: Chapman and Hall Medical, 1994:763-80

Neary D, Snowden JS, Northen B, Goulding P. Dementia of frontal type. *J Neurol Neurosurg Psychiatry* 1988;51:353-61

Nielsen PJ. Primary structure of a human protein kinase regulator protein. *Biochim Biophys Acta* 1991; 1088: 425-428

Noppe M, Crols R, Andries D, Lowenthal A. Determination in human cerebrospinal fluid of glial fibrillary acidic protein, S-100 and myelin basic protein as indices of non-specific or specific central nervous tissue pathology . *Clin Chim Acta* 1986; 155:143-50

Norenberg MD. Astrocyte responses to CNS injury *J Neuropath and Exp Neurol* 1994; 53:213-20

Ohrui T, Yamaya M, Arai H, Sekizawa K, Sasaki H. Disturbed consciousness and asthma. *The Lancet* 1997; 349:652

Otto M, Stein H, Szudra A, Zerr I, Bodemer M, Gefeller O, Poser S, Kretzschmar HA, Mader M, Weber T. S-100 protein concentration in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. *J Neurol* 1997a; 244:566-70

Otto M, Wiltfang J, Tumani H, Zerr I, Lantsch M, Kornhuber J, Weber T, Kretzschmar HA, Poser S. Elevated levels of tau-protein in cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. *Neurosci Lett* 1997b; 225:210-2

Otto M, Wiltfang J, Schutz E, Zerr I, Otto A, Pfahlberg A, Gefeller O, Uhr M, Giese A, Weber T, Kretzschmar HA, Poser S. Diagnosis of Creutzfeldt-Jakob disease by measurement of S100 protein in serum: prospective case-control study. *Brit Med J* 1998; 316:577-82

Pahlman S, Esscher T, Bergvall P, Odelstad L. Purification and characterisation of human neuron-specific enolase: radioimmunoassay development. *Tumour Biol* 1984; 5:127-139

Palmer MS, Collinge J. Human prion diseases. In: Rossor, MN ed. *Unusual dementias*. London: Baillière Tindall, London 1992: 627-51

Parchi P, Castellani R, Capellari S, Ghetti B, Young K, Chen SG, Farlow M, Dickson DW, Sima AAF, Trojanowski JQ, Petersen RB, Gambetti P. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Ann Neurol* 1996; 39(6):767-778

Parnetti L, Palumbo B, Cardinali L, Loreti F, Chionne F, Cecchetti R, Senin U. Cerebrospinal fluid neuron-specific enolase in Alzheimer's disease and vascular dementia. *Neurosci Lett* 1995; 183:43-45

Pena LA, Brecher CW, Marshak DR. Beta-amyloid regulates gene-expression of glial trophic substance S-100b in C6 glioma and primary astrocyte cultures. *Molecular Brain Res* 1995; 34:118-26

Persson L, Hardemark HG, Gustafsson J, Rundstrom G, Mendel-Hartvig I, Esscher T, Pahlman S. S-100 protein and neuronal-specific enolase in cerebrospinal fluid and serum: markers of cell damage in human central nervous system. *Stroke* 1987; 18:911-8

Pickel VM, Reis DJ, Marangos PJ, Zomzely-Neurath C. Immunocytochemical localization of nervous system specific protein (NSP-R) in rat brain. *Brain Res* 1975; 105:184-7

Price RW, Brew CJ. The AIDS dementia complex. *J Infect Dis* 1988; 158:1079-83

Probst A, Tolnay M, Langui D, Goedert M, Spillantini MG. Pick's disease - hyperphosphorylated tau protein segregates to the somatoaxonal compartment. *Acta Neuropathol (Berl)* 1996; 92(6):588-96

Rider CC, Taylor CB. Evidence for a new form of enolase in rat brain. *Biochem Biophys Res Commun* 1975; 66:814-20

Robakis NK, Devine Gage EA, Jenkins EC, Kascsak RJ, Brown WT, Krawczun MS, Silverman WP. Localisation of a human gene homologous to the PrP gene on the p arm of chromosome 20 and detection of PrP-related antigens in normal human brain. *Biochem Biophys Res Commun* 1986; 140:758-65

Roseboom PH, Weller JL, Babila T, Aitken A, Sellers LA, Moffett JR, Namboodiri MA, Klein DC. Cloning and characterisation of the ε and ζ isoforms of the 14-3-3 proteins. *DNA Cell Biol* 1994; 13:629-39

Rosen H, Rosengren L, Herlitz J, Blomstrand C. Increased serum levels of the S-100 protein are associated with hypoxic brain damage after cardiac surgery. *Stroke* 1998; 29(2):473-7

Rosenmann H, Meiner Z, Kahana E, Halimi M, Lenetsky E, Abramsky O, Gabizon R. Detection of 14-3-3 protein in the CSF of genetic Creutzfeldt-Jakob disease. *Neurology* 1997; 49:593-595

Royds JA, Timperley WR, Taylor CB. Levels of enolase and other enzymes in the cerebrospinal fluid as indices of pathological change. *J Neurol Neurosurg Psychiatry* 1981; 44:1129-35

Royds JA, Davies-Jones GAB, Lewtas NA, Timperley WR, Taylor CB. Enolase isoenzymes in the cerebrospinal fluid of patients with diseases of the nervous system. *J Neurol Neurosurg Psychiatry* 1983; 46:1031-6

Rusca H, Calissano P, Alema S. Identification of a membrane bound fraction of the S-100 protein. *Brain Res* 1972; 49:223-7

Scarna H, Delafosse B, Steinberg R, Debilly G, Mandrand B, Keller A, Pujol JF. Neuron-specific enolase as a marker of neuronal lesions during various comas in man. *Neurochem Int* 1982; 4 (5):405-411

Schaarschmidt HE, Prange HW, Reiber H. Serial plasma analyses of neuron-specific enolase: a diagnostic and prognostic parameter in cerebral damage. In: Felgenhauer K, Holzgraefe M, Prange H, eds. *CNS barriers and modern CSF diagnostics*. Verlag Chernie, Weinheim, 1993: 434-432

Schmechel D, Marangos PJ, Brightman M. Neuron-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature* 1978; 276:834-6

Schneider JA, Watts RL, Gearing M, Brewer RP, Mirra SS. Corticobasal degeneration: Neuropathologic and clinical heterogeneity. *Neurology* 1997; 48:959-69

Selinfreund RH, Barger SW, Pledger WJ, Van Eldik LJ. Neurotrophic protein S-100b stimulates glial cell proliferation. *Proc Natl Acad Sci USA* 1991; 88:3554-8

Sheng JG, Mrak RE, Griffin WST. S-100b protein expression in Alzheimer disease: potential role in the pathogenesis of neuritic plaques. *J Neurosci Res* 1994; 39:398-404

Sindic CJM, Chalon MP, Cambiaso CL, Laterre EC, Masson PL. Assessment of damage to the central nervous system by determination of S-100 protein in the cerebrospinal fluid. *J Neurol Neurosurg Psychiatry* 1982; 45:1130-5

Soler Federsppiel BSS, Cras P, Gheuens J, Andries D, Lowenthal A. Human γγ-enolase: two-site immunoradiometric assay with a single monoclonal antibody. *J Neurochem* 1987; 48(1):22-8

Sorensen K, Brodbeck U, Norgaard-Pedersen B. An enzyme antigen immunoassay for the determination of neuron-specific enolase in serum samples. *Clin Chim Acta* 1988; 175:337-344

Spielmeyer W. Die histopathologische Forschung in der Psychiatrie. *Klin Wochenschr* 1922; 2:1817-19

Spillantini MG, Bird TB, Ghetti B. Frontotemporal dementia and Parkinsonism linked to chromosome 17: A new group of tauopathies. *Brain Pathology* 1998; 8:387-402

Stanley LC, Mrak RE, Woody RC, Perrot LJ, Zhang S, Marshak DR, Nelson SJ, Griffin WST. Glial cytokines as neuropathogenic factors in HIV infection: pathogenic similarities to Alzheimer's disease. *J Neuropath and Exp Neurol* 1994; 53:231-238

Steinberg R, Scarna H, Keller A, Pujol JF. Release of neuron-specific enolase (NSE) in cerebrospinal fluid following experimental lesions of the rat brain. *Neurochem Int* 1983; 5(1):145-151

Taggart DP, Mazel JW, Bhattacharya K, Meston N, Standing SJ, Kay JD, Pillai R, Johnsson P, Westaby S. Comparison of serum S-100 beta levels during CABG and intracardiac operations. *Ann Thorac Surg* 1997; 63(2):492-6

Takahashi K, Isobe T, Ohtsuki Y, Sonobe H, Yamaguchi H, Akagi T. S-100 protein positive human T-lymphocytes. *Am J Clin Pathol* 1985; 83:69-72

Takashi M, Haimoto H, Murase T, Mitsuya H, Kato K. An immunochemical and immunohistochemical study of S-100 protein in renal cell carcinoma. *Cancer* 1988; 61(5):889-95

Tato RE, Frank A, Hernanz A. Tau protein concentrations in cerebrospinal fluid of patients with dementia of the Alzheimer's type. *J Neurol Neurosurg Pyschiatry* 1995; 59:280-83

Thadani V, Pena PL, Partington J, Kalb R, Janssen R, Schonberge, LB, Rabkin CS, Prichard JW. Creutzfeldt-Jakob disease probably acquired from a cadaveric dura mater graft. Case report. *J Neurosurg* 1988; 69:766-69

Thornberg E, Thiringer K, Hagberg H, Kjellmer I. Neuron specific enolase in asphyxiated newborns: association with encephalopathy and cerebral function monitor trace. *Arch Dis Child Fetal - Neonatal Ed* 1995; 72:F39-F42

Tierney MC, Fisher RH, Lewis AJ, Zorzitto ML, Snow WG, Reid DW, Nieuwstraten P. The NINCDS-ADRDA Work Group criteria for the clinical diagnosis of probable Alzheimer's disease. *Neurology* 1988; 38:359-64

Toker A, Ellis CA, Sellers LA, Aitken A. Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur J Biochem* 1990; 191:421-9

Toker A, Sellers LA, Amess B, Patel Y, Harris A, Aitken A. Multiple isoforms of a protein kinase C inhibitor (KCIP-1.14-3-3) from sheep brain. Amino acid sequence of phosphorylated forms. *Eur J Biochem* 1992; 206:453-461

Tranchant C, Geranton L, Guiraud-Chaumeil C, Mohr M, Warter JM. Basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Neurology* 1999; 52(6): 1244-1249

Van Eldik LJ, Griffin WST. S-100b expression in Alzheimer's disease: relation to neuropathology in brain regions. *Biochim Biophys Acta* 1994; 1223:398-403

Van Engelen BGM, Lamers KJB, Gabreels FJM, Wevers RA, Van Geel WJA, Born GF. Age-related changes of neuron-specific enolase, S-100 protein and myelin basic protein concentrations in cerebrospinal fluid. *Clin Chem* 1992; 38:813-816

Vandermeeren M, Mercken M, Vanmechelen E, Six J, van de Voorde A, Martin JJ, Cras P. Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay. *J Neurochem* 1993; 61:1828-34

Vermuyten K, Lowenthal A, Karcher D. Detection of neuron-specific enolase concentrations in cerebrospinal fluid from patients with neurological disorders by means of a sensitive enzyme immunoassay. *Clin Chim Acta* 1990; 187:69-78

Vigo-Pelfrey C, Seubert P, Barbour R, Blomquist C, Lee M, Lee D, Coria F, Chang L, Miller B, Lieberburg I, Schenk D. Elevation of microtubule-associated protein tau in the cerebrospinal fluid of patients with Alzheimer's disease. *Neurology* 1995; 45(4):788-93

Wakayama Y, Shibuya S, Kawase J, Sagawa F, Hashizume Y. High neuron-specific enolase level of cerebrospinal fluid in the early stage of Creutzfeldt-Jakob disease. *Klin Wochenschr* 1987; 65:798-801

Watanabe M, Isobe T, Okuyama T, Ichimura T, Kuwano R, Takahashi Y, Kondo H. Molecular cloning of cDNA to rat 14-3-3 η chain polypeptide and the neuronal expression of the mRNA in the central nervous system. *Mol Brain Res* 1991; 10:151-8

Waterloo K, Ingebrigtsen T, Romner B. Neuropsychological function in patients with increased serum levels of protein S-100 after minor head injury. *Acta Neurochir (Wien)* 1997; 139:26-31

Wells GAH, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. *Vet Rec* 1987; 121:419-20

Wells GAH, Hancock RD, Cooley WA, Richards MS, Higgins RJ, David GP. Bovine spongiform encephalopathy: diagnostic significance of vacuolar changes in selected nuclei of the medulla oblongata. *Vet Rec* 1989; 125:521-24

Wells GAH, Spencer YI, Haritani M Configurations and topographic distribution of PrP in the central nervous system in bovine spongiform encephalopathy: an immunohistochemical study. *Annals of the New York Academy of Sciences.* 1994; 724:350-52

Westaby S, Johnsson P, Parry AJ, Blomquist S, Solem JO, Alling C, Pillai R, Taggart DP, Grebenik C, Stahl E. Serum S-100 protein - a potential marker for cerebral events during cardiopulmonary bypass. *Ann Thorac Surg* 1996; 61(1):88-92

Wevers RA, Jacobs AAC, Hommes OR. A bioluminescent assay for enolase (EC 4.2.1.11) activity in human serum and cerebrospinal fluid. *Clin Chim Acta* 1983; 135:159-68

Wheeler-Jones CPD, Learmonth MP, Martin H, Aitken A. Identification of 14-3-3 proteins in human platelets: effects of synthetic peptides on protein kinase C activation. *Biochem J* 1996; 315:41-47

Whitaker-Azmitia PM, Murphy R, Azmitia EC. Stimulation of astroglial 5-HT1A receptors releases the serotonergic growth factor, protein S-100 and alters astroglial morphology. *Brain Res* 1990; 528:155-8

Wiesmann M, Missler U, Hagenstrom H, Gottmann D. S-100 protein plasma levels after aneurysmal subarachnoid haemorrhage. *Acta Neurol Chir* 1997; 139(12):1155-60

Wilesmith JW, Ryan JBM, Atkinson MJ. Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec* 1991;128:199-203

Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK. *The Lancet* 1996a; 347:921-5

Will RG, Zeidler M, Brown P, Harrington MG, Lee KH, Kenney KL. Cerebrospinal fluid test for new variant Creutzfeldt-Jakob disease. *The Lancet* 1996b; 348:955

Wischik CM, Harrington CR, Mukaetova-Ladinska EB et al. Molecular characterization and measurement of Alzheimer's disease pathology: implications for genetic and environmental aetiology. In: *Aluminium in Biology and Medicine* (Ciba Foundation Symposium No 169), Chichester: John Wiley & Sons, 1992:268-302

Wu JH, Sheng JG, Skinner RA et al. Interleukin-1 (IL-1) induced expression of Alzheimer disease-related proteins in rat brain. *Soc Neurosci Abstr* 1993; 19:1037

Xiao B, Smerdon SJ, Jones DH, Dodson GG, Soneji Y, Aitken A, Gamblin SJ. Structure of a 14-3-3 protein and implications for co-ordination of multiple signalling pathways. *Nature* 1995; 376:188-91

Yamada T, McGeer PL, McGeer EG. Appearance of paired nucleated, tau-positive glia in patients with progressive supranuclear palsy brain tissue. *Neurosci Lett* 1992; 135:99-102

Yamaguchi H, Morimatsu M, Hirai S, Takahashi K. Alzheimer's disease neurofibrillary tangles are penetrated by astroglial processes and appear eosinophilic in their final stages. *Acta Neuropathol Berl* 1987; 72:214-17

Zeidler M, Stewart GE, Barraclough CR, Bateman DE, Bates D, Burn DJ, Colchester AC, Durward W, Fletcher NA, Hawkins SA, MacKenzie JM, Will RG. New variant Creutzfeldt-Jakob disease: neurological features and diagnostic tests. *Lancet* 1997a; 350:903-907

Zeidler M, Johnstone EC, Bamber RWK, Dickens CM, Fisher CJ, Francis AF, Goldbeck R, Higgo R, Johnson Sabine EC, Lodge GJ, McGarry P, Mitcell S, Tarlo L, Turner M, Ryley P, Will RG. New variant Creutzfeldt-Jakob disease: psychiatric features. *Lancet* 1997b; 350:908-910

Zerr I, Bodemer M, Racker S, Grosche S, Poser S, Kretzschmar HA, Weber T. Cerebrospinal fluid concentration of neuron-specific enolase in diagnosis of Creutzfeldt-Jacob disease. *The Lancet* 1995; 345:1609-10

Zerr I, Bodemer M, Otto M, Poser S, Windl O, Kretzschmar HA, Gefeller O, Weber T. Diagnosis of Creutzfeldt-Jakob disease by two dimensional gel electrophoresis of cerebrospinal fluid. *The Lancet* 1996; 348:846-9

Zerr I, Bodemer M, Gefeller O, Otto M, Poser S, Wiltfang J, Windl O, Kretzschmar HA, Weber T. Detection of 14-3-3 protein in the cerebrospinal fluid supports the diagnosis of Creutzfeldt-Jakob disease. *Ann Neurol* 1998; 43:32-40

Zimmer DB, Van Eldik LJ. Identification of a molecular target for the calcium-modulated protein S-100. Fructose-a,6-biphosphate aldolase. *J Biol Chem* 1986; 261:1424-8

Zimmer DB, Van Eldik LJ. Analysis of calcium-modulated proteins, S-100 and calmodulin, and their target proteins during C6 glioma cell differentiation. *J Cell Biol* 1989; 108:141-51

Zimmer DB, Cornwall EH, Landar A, Song W. The S-100 protein family - history, function and expression. *Brain Res Bull* 1995; 37:417-29

Appendix A

Recipes for commonly used buffers

- Phosphate buffered saline (10x concentrated stock)

  23 g sodium phosphate

  4 g potassium phosphate

  160 g sodium chloride

  4 g potassium chloride

Dissolve the above in 1500 mL deionised water. Make up to 2 L. Dilute 1 + 9 with deionised water before use.

- 0.67 M Barbitone buffer, pH 8.6 containing 1 mM calcium (S-100b ELISA)

  13.1 g sodium barbitone

  2.1 g barbitone

  290 mg calcium lactate

Dissolve the above in 750 mL deionised water. Check the pH, adjust if necessary. Make up to 1 L.

- 0.2 M Sodium acetate buffer, pH 5.0 (10x Stock concentration)

  54.4 g sodium acetate

  9 mL glacial acetic acid

Dissolve the above in 1500 mL deionised water. Check the pH and adjust to pH 5.1 with more glacial acetic acid if necessary. Dilute 1 + 9 with deionised water before use.

Appendix B Disease classification for Creutzfeldt-Jakob Disease

Sporadic CJD Disease Classification Criteria.

a) Definite (histologically confirmed) sporadic CJD:

Diagnosed by standard neuropathological techniques; and/or
Immunocytochemically and/or Western blot confirmed protease resistant PrP and/or presence of scrapie-associated fibrils.

b) Probable sporadic CJD:

Progressive dementia of less than two years plus at least two out of the following four clinical features:

Myoclonus
Visual or cerebellar disturbance
Pyramidal/extrapyramidal dysfunction
Akinetic mutism;

and a typical EEG during an illness of any duration and/or
A positive 14-3-3 CSF assay and a clinical duration to death <2 years;
Routine investigations should not suggest an alternative diagnosis.

c) Possible sporadic CJD:

Progressive dementia of less than two years plus at least two out of the following four clinical features:

Myoclonus
Visual or cerebellar disturbance
Pyramidal/extrapyramidal dysfunction
Akinetic mutism;

but without an abnormal EEG pattern

New variant CJD Disease Classification Criteria.

a) Definite (histologically confirmed) new variant CJD

Progressive neuropsychiatric syndrome and neuropathological examination of brain tissue showing following features:

Spongiform encephalopathy with abundant PrP deposition, in particular multiple fibrillary PrP plaques surrounded by a halo of spongiform vacuoles ('florid' plaques, 'daisy-like' plaques) and other PrP plaques, and amorphous pericellular and perivascular PrP deposits especially prominent in the cerebellar molecular layer.

b) Probable new variant CJD

i) patients present with all of the following features:

progressive neuropsychiatric disorder

duration of illness > 6 months

routine investigations do not suggest an alternative diagnosis

no history of potential iatrogenic exposure

plus both of the following:

EEG pattern not typical of sporadic CJD* (or EEG not performed)

Posterior thalamic high signal on MRI scan*

ii) patients present with 4 of 5 of the following features:

early psychiatric symptoms

persistent painful sensory symptoms

ataxia

myoclonus or chorea or dystonia

dementia

plus both of the following:

EEG pattern not typical of sporadic CJD* (or EEG not performed)

Posterior thalamic high signal on MRI scan*

c) possible new variant CJD

Either a) or b) without the EEG and MRI investigation

* After review by the National CJD Surveillance Unit in Edinburgh

matic procedure, is a feasible option because of the economic and sentimental value of South American camelids. Finally, because of the limited gene pool in this country and the relative lack of knowledge of camelid genetics, veterinary surgeons and owners should be aware of potential hereditary defects, and ensure that affected animals are excluded from breeding programmes.

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References

BLOOD, D. C. & STUDDERT, V. P. (1990) Bailliere's Comprehensive Veterinary Dictionary, Bailliere Tindall. p 458

FERNANDEZ BACA, S. (1971) La Alpaca Reproduccione y Crianza. *Boletin de Divulgacion* **7**, Centro de Investigacion, Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA), 12-17

FOWLER, M. E. (1989a) Surgery. In Medicine and Surgery of South American Camelids. 1st edn. Iowa State University Press. pp 64-101

FOWLER, M. E. (1989b) Digestive System. In Medicine and Surgery of South American Camelids. 1st edn. Iowa State University Press. pp 224-262

FOWLER, M. E. (1989c) Congenital/Hereditary Conditions. In Medicine and Surgery of South American Camelids. 1st edn. Iowa State University Press. pp 337-365

FOWLER, M. E. (1990) Obstruction of the Urinary Tract. *Llamas* **4**, 108-109

HOFMEYER, C. B. (1987) The Urinary System: Kidneys, Ureters and Bladder. In Ruminant Urogenital Surgery. 1st edn. Iowa State University Press. pp 6-27

JONES, T. C., HUNT, R. D. & KING, N. W. (1996) The Urinary System. In Veterinary Pathology. 6th edn. Philadelphia, Williams & Wilkins. pp 1111-1147

KOCK, M. D. & FOWLER, M. E. (1982) Urolithiasis in a 3 month old llama. *Journal of the American Veterinary Medical Association* **181**, 1411

LEIB, M. S., ALLEN, T. A., KONDE, L. J. & JOKINEN, M. P. (1988) Bilateral hydronephrosis attributable to bilateral ureteral fibrosis in a cat. *Journal of the American Veterinary Medical Association* **192**, 795-797

MAXIE, M. G. (1991) The Lower Urinary Tract. In Pathology of Domestic Animals. Vol 2. Eds K. V. F. Jubb, P. C. Kennedy, N. Palmer. 4th edn. London, Academic Press. pp 522-538

MITCHELL, G. E. & BERNE, A. S. (1973) Radiologic evaluation of ureteral duplication. *Medical Radiography and Photography* **49**, 30-57

NORTH, D. C. (1978) Hydronephrosis and Hydroureter in a kitten – a case report. *Journal of Small Animal Practice* **19**, 237-240

O'HANDLEY, P., CARRIG, C. B. & WALSHAW, R. (1979) Renal and ureteral duplication in a dog. *Journal of the American Veterinary Medical Association* **174**, 484-487

USON, A. C., COX, L. A. & LATIMER, J. K. (1968) Hydronephrosis in infants and children: some clinical and pathological aspects. *Journal of the American Medical Association* **205**, 71-74

VAN HOOGMOED, L., SNYDER, J. R., ROBERTS, G. & HARMON, F. A. (1997) Unilateral nephrectomy in a juvenile llama. *Veterinary Surgery* **26**, 497-501

WILLIAMS, D. I. & JOHNSTON, J. H. (1982) Ureteral Duplications and Ectopia. In Paediatric Urology. 2nd edn. London, Butterworth. pp 167-187

SHORT COMMUNICATIONS

Increased S-100b in the cerebrospinal fluid of some cattle with bovine spongiform encephalopathy

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S-100 belongs to a family of acidic, low molecular weight, calcium binding proteins which are well conserved throughout the vertebrate species (Donato 1991). There are three isoforms of S-100 consisting of two subunits α and β. S-100b (ββ) is found in high concentrations in the central nervous system (CNS) where it is located in the cytoplasm of astrocytes; smaller amounts are found outside the CNS in Schwann cells (Moore 1988), adipocytes (Hidaka and others 1983) and melanocytes (Kindblom and others 1984).

Increased concentrations of S-100b have been reported in the cerebrospinal fluid (CSF) of human patients with Creutzfeldt-Jakob disease (CJD) (Jimi and others 1992, Otto and others 1997). The rise in S-100b occurs early in CJD and remains high even when other biochemical markers of brain damage such as neurone specific enolase return to normal (Jimi and others 1992). Increased concentrations of S-100b have also been reported in the serum of patients with CJD (Otto and others 1998).

CJD belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs) which are associated with characteristic spongiform changes in the brain, neuronal loss, astrocytosis and deposition within the central nervous system of an abnormal form of the prion protein PrP^{Sc} (Beck and Daniel 1987). In 1986, a form of TSE was described in cattle and named bovine spongiform encephalopathy (BSE) (Wells and others 1987). Since it was first described over 170,000 cattle have been affected. At present, diagnosis of BSE depends on the histological examination of brain tissue and demonstration of the presence of PrP^{Sc} in CNS tissue. Other procedures such as polyacrylamide gel electrophoresis and Western blotting for PrP^{Sc}, and the demonstration of scrapie-associated fibrils in CNS tissue by electron microscopy have also been used diagnostically. These tests are expensive, time consuming and can only be performed postmortem. There is a need for a rapid diagnostic test which can be performed while the animal is still alive using blood, urine or CSF. This study investigates whether cattle with BSE have elevated concentrations of serum and CSF S-100b and whether the measurement of S-100b could be useful in the assessment of cattle with suspected BSE.

Blood samples were obtained from the jugular vein and the samples were allowed to clot overnight. The serum was removed and stored at –20°C. Blood samples were obtained

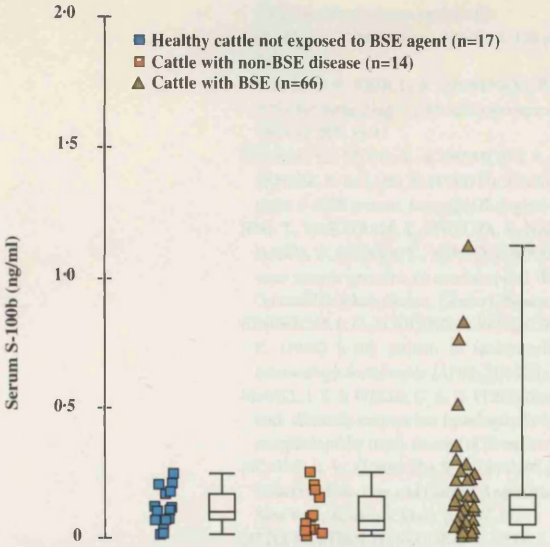

FIG 1: Combined scatter and box and whisker plot of individual serum S-100b concentrations. The box represents the 25th to 75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plot represents the individual values from which the box and whiskers are derived

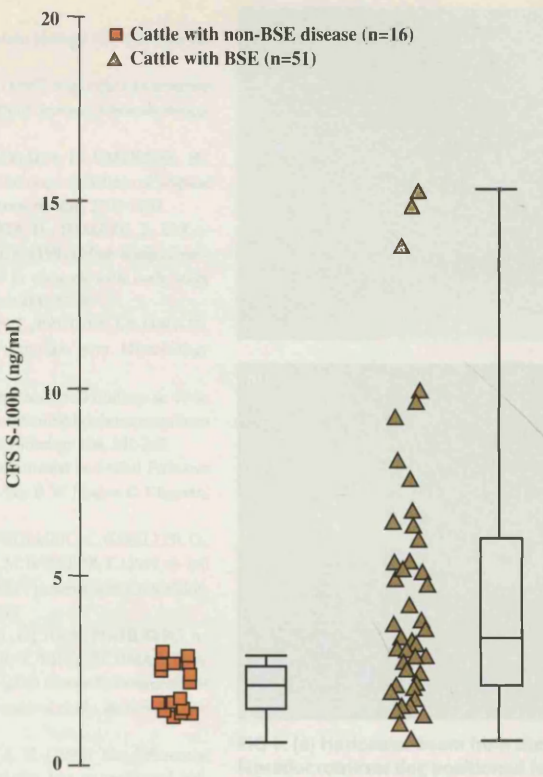

FIG 2: Combined scatter and box and whisker plot of individual cerebrospinal fluid S-100b concentrations. The box represents the 25th to 75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plot represents the individual values from which the box and whiskers are derived

from 66 cattle clinically affected with BSE, 14 cattle with clinical signs indistinguishable from BSE, but which were histologically negative for BSE and 17 healthy cattle obtained from New Zealand. Diagnosis of BSE was made postmortem by histological examination of the brain sections for vacuolation and immunohistochemical staining for the deposition of the disease associated isoform of the prion protein PrP^{Sc}. Cattle with non-BSE disease had no histological evidence of vacuolation or PrP^{Sc} deposition.

CSF samples were taken from the lumbar sac from a separate group of 50 cattle with BSE, age 71·2 (16·3) months, and a separate group of 16 cattle with clinical signs indistinguishable from BSE but which were histologically negative for BSE, age 69·3 (26·1) months. Samples were centrifuged and the supernatant stored at –20°C before analysis. CSF samples were taken before slaughter.

S-100b was measured using a two site enzyme-linked immunosorbant assay which has previously been reported (Green and others 1997). The assay uses bovine S-100b as a calibrant and mouse monoclonal anti-bovine S-100b (Sigma) and horseradish peroxidase conjugated rabbit polyclonal anti-bovine S-100b (Dako). The within-batch and between-batch precision as measured by the coefficient of variance was less than 6 per cent.

Statistical analysis was performed using one-way ANOVA with Bonferroni analysis.

The mean serum S-100b in cattle with BSE was slightly raised (0·16 [0·20] ng/ml) when compared to healthy cattle (0·11 [0·07] ng/ml) and cattle with non-BSE disease (0·10 [0·08] ng/ml), but this increase was not statistically significant (Fig 1). There was no significant difference between the mean S-100b concentration in healthy cattle and cattle with non-BSE disease.

The mean CSF S-100b was significantly raised in cattle with BSE when compared to cattle without BSE (4·6 [3·8] ng/ml vs 2·0 [0·6] ng/ml, $P<0·006$) (Fig 2). There was no correlation between CSF S-100b concentrations and duration of symptoms measured in days ($r^2=0·005$). Using a cut-off of 3·18 ng/ml calculated from the mean ±2SD of the non-BSE disease group, 50 per cent of the cattle with BSE would have CSF S-100b concentrations that would be considered abnormal.

This study is the first to report that CSF S-100b concentrations are significantly raised in some cattle with BSE when compared to cattle with clinical signs indistinguishable from BSE but which are histologically negative for BSE. CSF samples from healthy cattle were not investigated due to the difficulty in obtaining samples from cattle that could be guaranteed free from exposure to the BSE agent within the UK. Agreement was not obtained to get CSF samples from cattle in New Zealand as part of this project. In addition, the relevance of these cattle as a control group may be open to question due to differences in breed and husbandry. Cattle with clinical signs indistinguishable from BSE but proven histopathologically not to be BSE, were considered to be a more appropriate control group. If a diagnostic test is to be useful it has to be able to distinguish between these two groups.

The cause of the elevated CSF S-100b in cattle with BSE is probably due in part to the astrocytosis and in part to neuronal and astrocytic cell death. None of the cattle without BSE had histological abnormalities including astrocytosis on examination of brain tissue. No alternative diagnoses were obtained in any of these cases. This supports studies which found that astrocytosis was rare in cattle suspected of having BSE but which were histologically negative for the disease (Pollin and others 1992, McGill and Wells 1993). Although the mean concentrations of CSF S-100b are raised in cattle with BSE, only 50 per cent of the cattle with BSE have elevated levels when compared to non-BSE diseased cattle. The average time interval between first clinical signs and the CSF sample being taken was one month. CSF S-100b concentrations may not be elevated in the very early stages of BSE, and studies are underway to investigate the time course of S-100b release. The low sensitivity of CSF S-100b in detecting cattle with BSE may preclude its use as a screening test when used alone, but studies are underway to investigate whether the inclusion of other tests such as CSF 14-3-3 may improve the detection rate.

References

BECK, E. & DANIEL, P. M. (1987) Neuropathology of transmissible spongiform encephalopathies. In Prions: Novel Infectious Pathogens Causing Scrapie and Creutzfeldt-Jakob Disease. Eds S. B. Prusiner, M. P. McKinley. San

Diego, Academic Press. pp 331-385
DONATO, R. (1991) Perspectives in S-100 protein biology. *Cell Calcium* **12**, 713-726
GREEN, A. J. E., KEIR, G. & THOMPSON, E. J. (1997) A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid. *Journal of Immunological Methods* **205**, 35-41
HIDAKA, H., ENDO, T., KAWAMOTO, S., YAMADA, E., UMEKAWA, H., TANABE, K. & HARA, K. (1983) Purification and characterisation of adipose tissue S-100b protein. *Journal of Biological Chemistry* **258**, 2705-2709
JIMI, T., WAKAYAMA, Y., SHIBUYA, S., NAKATA, H., TOMARU, T., TAKAHASHI, Y., KOSAKA, K., ASANO, T. & KATO, K. (1992) High levels of nervous system proteins in cerebrospinal fluid in patients with early stage Creutzfeldt-Jakob disease. *Clinica Chimica Acta* **211**, 37-46
KINDBLOM, L. G., LODDING, P., ROSENGREN, L., BAUDIER, J. & HAGLID, K. (1984) S-100 protein in melanocytic tumours. *Acta Microbiology Immunology Scandinavia [A]* **92**, 219-230
McGILL, I. S. & WELLS, G. A. H. (1993) Neuropathological findings in cattle with clinically suspect but histologically unconfirmed bovine spongiform encephalopathy (BSE). *Journal of Comparative Pathology* **108**, 241-260
MOORE, B. W. (1988) The S-100 protein. In Neuronal and Glial Proteins: Structure, Function and Clinical Application. Eds B. W. Moore, D. Kligman. New York, Academic Press. pp 137-167
OTTO, M., STEIN, H., SZUDRA, A., ZERR, I., BODEMER, M., GEFELLER, O., POSER, S., KRETZSCHMAR, H. A., MADER, M. & WEBER, T. (1997) S-100 protein concentration in the cerebrospinal fluid of patients with Creutzfeldt-Jakob disease. *Journal of Neurology* **244**, 566-570
OTTO, M., WILTFANG, J., SCHUTZ, E., ZERR, I., OTTO, A., PFAHLBERG, A., GEFELLER, Q., UHR, M., GIESE, A., WEBER, T., KRETZSCHMAR, H. A. & POSER, S. (1998) Diagnosis of Creutzfeldt-Jakob disease by measurement of S-100 protein in serum: prospective case-control study. *British Medical Journal* **316**, 577-582
POLLIN, M. M., McGILL, I. S. & WELLS, G. A. H. (1992) The differential neurohistological diagnoses of clinically suspect but unconfirmed BSE. *Neuropathology and Applied Neurobiology* **18**, 633
WELLS, G. A. H., SCOTT, A. C., JOHNSON, C. T., GUNNING, R. F., HANCOCK, R. D., JEFFREY, M., DAWSON, M. & BRADLEY, R. (1987) A novel progressive spongiform encephalopathy in cattle. *Veterinary Record* **121**, 419-420

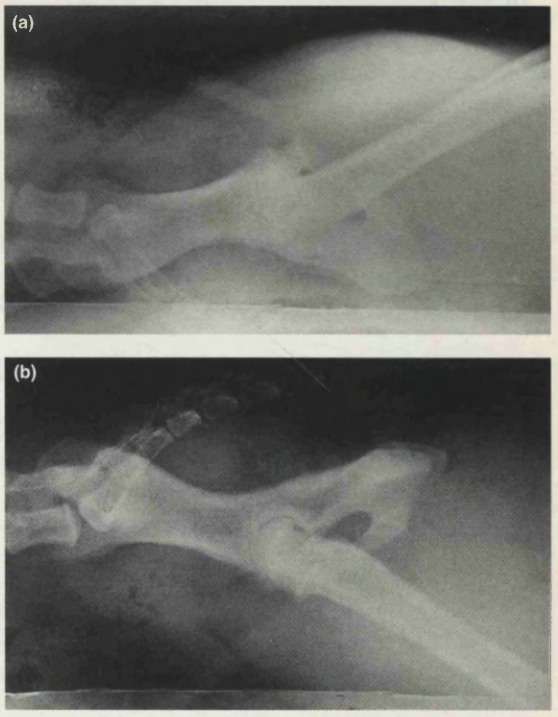

FIG 1: (a) Horizontal beam from the pelvis and femurs of a labrador retriever dog positioned for a hip in extended ventrodorsal (VD) projection. The os ilium and os ischium are close to the tabletop, and the femurs make an angle of 25° with the tabletop, (b) Horizontal beam from the pelvis and femurs of the same dog positioned for a hip in extended dorsoventral (DV) projection. The os ilium and femur are in the same axis forming an angle of 25° with the tabletop. Height of the coxofemoral joint relative to the tabletop does not vary between the two positionings

Comparison of ventrodorsal and dorsoventral radiographic projections for hip dysplasia diagnosis

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CANINE hip dysplasia is a common developmental musculoskeletal disorder. At birth, the hips are generally normal, but histological changes compatible with hip dysplasia begin 60 days after birth (Riser 1973). Radiography is still the most common method for confirmation of hip dysplasia, and the radiographic signs are: subluxation of the coxofemoral joints, remodelling of the edges of the acetabulum, the cranial effective acetabular rim and the acetabular fossa, remodelling of the femoral head and/or neck, and signs of degenerative joint disease (Gibbs 1997). In 1961, the American Veterinary Medical Association recommended a standard projection for diagnosis of hip dysplasia (AVMA 1961), in which the ventrodorsal (VD) position was preferred. The hindlimbs were recommended to be straight and fully extended caudally, and the femurs were to be parallel with the patellas superimposed over their midline. Since then, this positioning has been accepted worldwide (Ackerman and Nyland 1977, Rendano and Ryan 1985).

Positioning of the pelvis and femurs relative to the film differs between the VD and dorsoventral (DV) projections (Fig 1). The pelvic angle and the pelvis-film angle, which are both thought to affect the standardisation of hip scoring, are different between the two projections (Gibbs 1997). The aim of this study was to determine if there would be any differences in the diagnosis of hip dysplasia between radiographs made in VD and DV positions on sedated dogs.

Thirty dogs representing eight breeds (eight German shepherd dogs, seven labrador retrievers, three golden retrievers, three rottweilers, three Bernese mountain dogs, two Newfoundland dogs, two Irish setters and two bearded collies) and with a mean age of 17·6 months (from eight to 36 months) were radiographed for evaluation of hip dysplasia. All of the dogs were sedated with an intramuscular injection of 40 µg/kg medetomidine (Domitor; Pfizer). Two radiographs of the pelvis, a VD projection and a DV projection, were taken of each dog (Fig 2), with positioning of the dogs always undertaken by the same technician. The VD projection was performed according to previously described techniques (AVMA 1961, Rendano and Ryan 1985) and the same technique was performed for the DV projection, except that the dogs were placed in sternal recumbency. One half of the dogs had the VD projection performed first, while for the other half, the DV projection was first.

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A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid

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Abstract

A sensitive, simple and specific sandwich ELISA for S-100b is described. This method involves the binding of a monoclonal anti-S-100b antibody to the wall of a microtitre plate. This capture antibody is subsequently incubated with S-100b standard, control or patient sample in the form of cerebrospinal fluid (CSF). After incubation, the microtitre plate is washed and horseradish peroxidase-labelled polyclonal anti-S-100b is added (detector antibody). The amount of detector antibody bound to the microtitre plate is proportional to the amount of S-100b in the sample. The assay has a lower limit of detection of 0.04 ng/ml and shows < 0.006% reactivity with the closely related polypeptide S-100a. The assay has a mean within-batch precision of 9.3 and 5.6% at S-100b concentrations of 0.38 and 0.8 ng/ml, respectively. The between batch precision is 8.9 and 8.1% at S-100b concentrations of 0.12 and 0.34 ng/ml, respectively. The recovery of S-100b from CSF spiked with 0.5 ng/ml was 94% with a CV of 8.5%. The assay may be completed in less than 5 h using precoated microtitre plates, thus lending itself to routine use in clinical laboratories. Using this ELISA, 154 CSF samples were analysed and 19% of samples were found to have elevated levels. The highest levels were found in patients with cerebral haemorrhage or central nervous system malignancy. S-100b concentrations from individuals without evidence of neurological disease were found to be less than 0.4 ng/ml. Only 5% of patients with multiple sclerosis were found to have elevated CSF S-100b concentrations. Serial CSF samples taken from a patient with an infected in-dwelling shunt showed a dramatic decline, suggesting that S-100b is rapidly cleared. © 1997 Elsevier Science B.V.

Keywords: S-100b; ELISA; Cerebrospinal fluid; Central nervous system

1. Introduction

The S-100 family of proteins is defined as a group of acidic low molecular weight calcium binding proteins which show a highly conserved amino acid sequence within the vertebrate species. S-100 proteins are dimers of two subunits called α and β (Fano et al., 1995) and there are three possible variants, namely S-100b (ββ), S-100a (αβ) and S-100ao (αα). S-100b and S-100a are found primarily within the central nervous system (CNS), where S-100b constitutes 90% of the total S-100 (Fano et al., 1995; Zimmer et al., 1995). S-100b and S-100a have different locations within the central nervous system (CNS), with S-100b being an astrocytic protein, whilst S-100a is found in neurones, particularly

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; PBS, phosphate-buffered saline; CV, coefficient of variation; SD, standard deviation

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hippocampal neurones. S-100ao is found in high concentrations in heart, striated muscle and renal cells (Kato and Kimura, 1985; Kato et al., 1985).

A number of studies have reported increased S-100 levels in the cerebrospinal fluid of patients with acute neurological damage, such as cerebrovascular disorders, CNS tumours, viral meningitis and encephalitis (Michetti et al., 1980; Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986). Some reports have suggested that S-100 concentrations may be related to the extent of damage in cerebral haemorrhage and infarction, and could be used as a prognostic marker (Persson et al., 1987).

These studies have used a variety of methods including a microcomplement conversion assay (Michetti et al., 1980), a particle-counting immunoassay (Sindic et al., 1982), a radioimmunoassay (Noppe et al., 1986; Persson et al., 1987) and an enzyme immunoassay (Kato et al., 1982; Aurell et al., 1989; Griffin et al., 1993). These assays show considerable variation in specificity, sensitivity and ease of use. Many of the early methods used antibodies which were not shown to distinguish between the α- and β-subunits of S-100. These methods also had widely differing sensitivities, with one assay able to detect 0.04 ng/ml (Griffin et al., 1993) and another only 6 ng/ml (Michetti et al., 1980). As a result of these variations, studies investigating S-100 concentrations in CSF have reached no consensus for the concentrations found in patients without evidence of neurological disease, although all studies have demonstrated raised levels in patients with acute brain damage.

In the light of these limitations a specific and sensitive ELISA was devised and used to investigate S-100b concentrations in patients admitted to this hospital for diagnostic assessment of suspected neurological disease.

2. Materials and methods

2.1. Materials

Mouse monoclonal anti-bovine S-100b was purchased from Sigma (Sigma Chemical Company, Poole, UK). Specificity for the β-subunit was confirmed using isoelectric focusing and immunoblotting against both the α- and β-subunits of S-100 (Affiniti Research Products, Exeter, UK).

Horseradish peroxidase-conjugated rabbit anti-bovine S-100 was purchased from Dako (Copenhagen, Denmark). Isoelectric focusing and immunoblotting demonstrated that this antibody reacted primarily against the β-subunit of S-100, with only a minor reactivity against the α-subunit.

S-100b is a well conserved protein within species and both the monoclonal and polyclonal antibodies react with S-100 from a wide variety of mammalian species. Sodium barbitone, barbitone, *o*-phenylenediamine, 30% hydrogen peroxide, and bovine serum albumin were of analytical grade (Sigma Chemical Co., Poole, UK). Calcium lactate, acetic acid, sodium acetate, hydrochloric acid, sodium phosphate, potassium phosphate, potassium chloride and sodium chloride were obtained from Merck, FRG.

Nunc Maxisorb Microtitre plates were obtained from Life Technologies (Paisley, Scotland).

2.2. S-100 standards

Bovine S-100b and S-100a were obtained from Affiniti Research Products, Exeter. Isoelectric focusing of S-100b produced a single band with a pI of approximately 3.0. A range of standards from 0.1 to 2.5 ng/ml were prepared by diluting bovine S-100b in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and stored in aliquots at −20°C.

2.3. CSF recovery and precision

Pooled CSF samples spiked with S-100b were used to calculate the within- and between-batch precision and recovery. The within-batch precision is expressed as CV%, and was calculated from the mean and SD of at least 20 replicate analyses of pooled CSF spiked with S-100b, performed on a single microtitre plate.

The between-batch precision, expressed as CV%, was calculated from the mean and SD from at least 20 replicates of pooled CSF spiked with S-100b, each analysis was performed on a separate occasion.

The recovery of S-100b was obtained by measuring S-100b in pooled CSF before and after the addition of a known amount of S-100b. At least 20

replicates of unspiked and spiked pooled CSF were measured.

2.4. CSF samples

CSF samples from 154 neurological patients (20–78 years, 66 females, 88 males) undergoing a routine diagnostic lumbar puncture were obtained. The CSF samples from 19 patients (21–68 years, 11 females, 8 males) with no evidence of organic brain disease were used as controls. Of these 19 patients, 8 patients had headache, 8 patients had various neuralgia and 3 had psychological syndromes. The remaining CSF samples were from 7 patients with cerebral haemorrhage, 8 patients with cerebrovascular disease, 13 patients with intracranial neoplasm, 40 patients with multiple sclerosis and 67 patients with other neurological diseases. The latter category included patients with treated meningitis and encephalitis, polyneuropathy, Arnold Chiari malformation, status epilepticus, Stiff man syndrome, motor neurone disease, psychosis and benign intracranial hypertension.

The CSF samples were centrifuged on receipt and the supernatant frozen at −20°C within 24 h. Analysis was performed within 3 months.

2.5. Analytical procedure

The microtitre plates were coated overnight with monoclonal anti-S-100b at a concentration of 9.3 μg/ml in 0.05 M carbonate buffer, pH 9.5. The plate was washed with 0.1% bovine serum albumin in PBS containing 0.05% Tween 20 (wash solution), and the unbound sites were blocked with 1% bovine serum in PBS for 30 min.

50 μl of standard, control or CSF sample were added in duplicate to the plate, and 50 μl of 0.06 M barbitone buffer, pH 8.6 containing 1 mM calcium lactate were then added to each well (incubation buffer). The plate was incubated at 37°C for 3 h. After washing, rabbit anti-S-100 antibody conjugated with horseradish peroxidase diluted 1/1000 with incubation buffer was added. The microtitre plate was incubated for 1 h at room temperature. The microtitre plate was washed with wash solution and 100 μl of enzyme substrate (1 mg/ml *o*-phenylenediamine in 0.05 M acetate buffer containing 0.01% hydrogen peroxide) was added. After incubating the plate in the dark for 30 min, the reaction was stopped by adding 1 M HCl. The absorbance was read at 492 nm with 405 nm as the reference wavelength, using an Anthos 2001 Plate Reader (Denley Instruments, Sussex, UK).

3. Results

3.1. ELISA standard curve

A typical standard curve is shown in Fig. 1. The lower limit of detection was calculated from the mean plus 3 SD of at least 20 replicate analyses of S-100b-free sample (incubation buffer) and was found to be 0.04 ng/ml. A range of working standards from 0.05 to 2.5 ng/ml were used. The specificity of this assay was assessed by measuring the apparent S-100b concentration in a range of S-100a standards. The only measurable response was seen with 1000 ng/ml S-100a which gave an apparent S-100b concentration of 0.051 ng/ml. This corresponds to a cross reactivity of less than 0.006%.

3.2. Precision of ELISA

The mean within-batch precision was 9.3% (0.38 ng/ml) and 5.6% (0.8 ng/ml) and the between-batch precision was 8.9% (0.12 ng/ml) and 8.1% (0.34 ng/ml). The recovery of S-100b was 94% (0.5 ng/ml) with a CV of 8.5%.

3.3. Storage of CSF samples

10 CSF samples were divided into two and one aliquot was stored at −20°C and the other at −70°C.

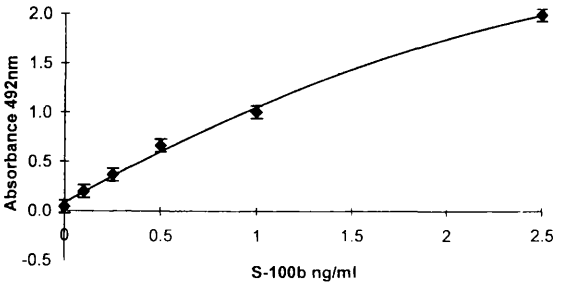

Fig. 1. A typical ELISA standard curve for S-100b: 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 ng/ml standards were prepared using bovine S-100b. Standard deviations are shown.

No statistical difference was seen between the CSF S-100b concentrations of samples stored at −20°C and those stored at −70°C when analysed within 3 months.

3.4. Linearity

Serial dilution of a CSF sample containing 4.7 ng/ml S-100b gave results which demonstrated a linear relationship (Fig. 2). This suggested that there

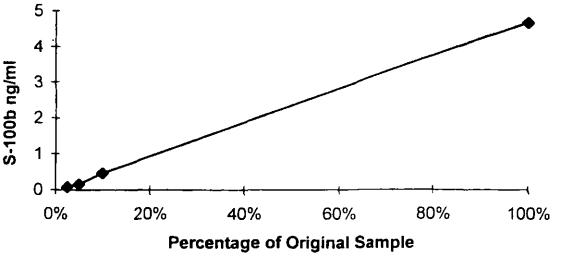

Fig. 2. Effect of dilution on CSF S-100b concentrations from a patient with subarachnoid haemorrhage demonstrating the lack of endogenous binding of S-100b and a linear dose–response.

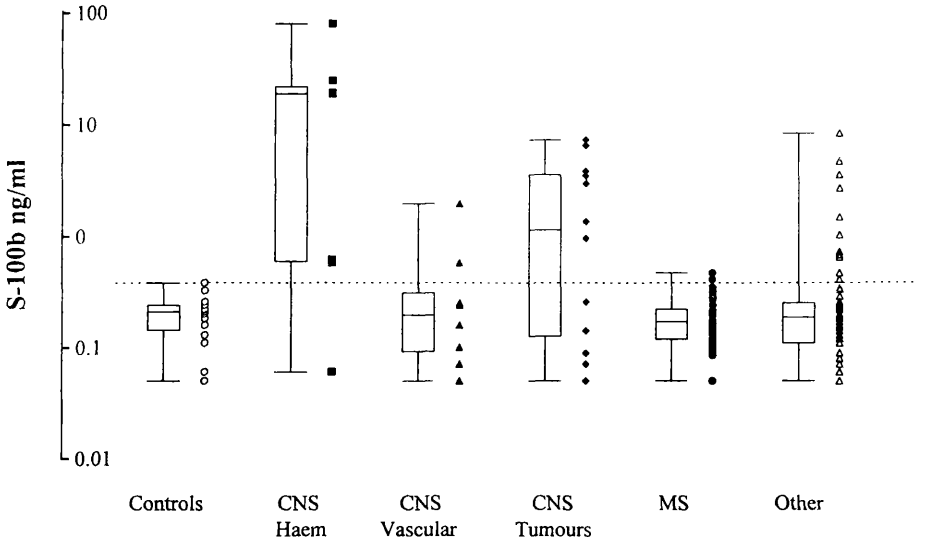

Fig. 3. Box and whisker plot of CSF S-100b ng/ml (log_{10} scale) in various neurological diseases. Plot shows 0, 25th, 50th, 75th and 100th centiles. CNS Haem, cerebral haemorrhage; CNS vascular, cerebral vascular pathology excluding haemorrhage; MS, multiple sclerosis; Other, see text. Upper limit of normal (—).

Table 1
Distribution of CSF S-100b concentrations in various neurological disorders

Diagnosis	% Samples with S-100b values > 0.4 ng/ml	Mean S-100b ± SD (ng/ml)	Range
Controls	0	0.20 ± 0.08	0.05–0.38
Cerebral haemorrhage	86 (6) [a]	20.45 ± 28.07	0.06–79.6 [b]
Cerebral vascular disease	25 (2)	0.42 ± 0.63	0.05–1.93
CNS neoplasm	62 (8)	2.13 ± 2.51	0.05–7.23 [b]
Multiple sclerosis	5 (2)	0.18 ± 0.09	0.05–0.47
Other neurological diseases	18 (12)	0.52 ± 1.24	0.05–8.25

[a] Numbers in parentheses indicate number of samples.
[b] Results show a significant difference when compared to controls ($p < 0.05$).

Table 2
Diagnosis of patients from the 'other neurological diseases' group who have elevated CSF S-100b concentrations

Diagnosis	Number of patients	CSF S-100b (ng/ml)
Hydrocephalus	1	0.41
Meningococcal meningitis	1	0.47
Mechanical problem of shunt	4	0.64, 0.73, 1.47, 4.67
Polyneuropathy	1	0.68
Alcoholic neuropathy	1	1.02
Ménières disease	1	1.49
Schizophrenia	1	2.7
Spinal cord disease excluding myelitis	1	3.56
Neurosarcoidosis	1	8.25

was no endogenous binding of S-100b uncovered by dilution.

3.5. CSF S-100b concentrations in neurological patients

The CSF S-100b concentrations in 154 CSF samples are illustrated in Fig. 3, and Tables 1 and 2. The mean ± SD S-100b concentration in CSF samples obtained from individuals with no evidence of neurological disease was 0.20 ± 0.08 ng/ml. Thus a reference range of less than 0.4 ng/ml was used for all comparisons. Patients with cerebral haemorrhage had significantly elevated CSF S-100b concentrations with the mean ± SD being 20.5 ± 28.07 ng/ml and the highest concentration being 79.6 ng/ml. In contrast, patients with cerebral vascular disease had marginally elevated CSF S-100b concentrations with a mean ± SD of 0.42 ± 0.63 ng/ml and a maximum value of 1.93 ng/ml. This slight elevation failed to reach significance.

Patients with various malignancies of the central nervous system had significantly raised CSF S-100b concentrations with a mean ± SD of 2.13 ± 2.51 ng/ml and a maximum value of 7.23 ng/ml. Only 2 out of 40 patients with multiple sclerosis had marginally elevated levels of 0.41 and 0.47 ng/ml. Of the other neurological disease group, 18% had elevated CSF S-100b concentrations. The diagnoses of these individuals are given in Table 2. The highest value of 8.25 ng/ml was seen in a patient with neurosarcoidosis.

3.6. Serial S-100b concentrations from a patient after removal of an infected in-dwelling shunt

The renewal of ventricular fluid every 6 h has the consequence that brain proteins can be rapidly cleared from the CSF. This was demonstrated by the sharp fall in CSF S-100b concentration following the removal on day 0 of a ventricular–peritoneal shunt infected with group C Streptococci from a patient

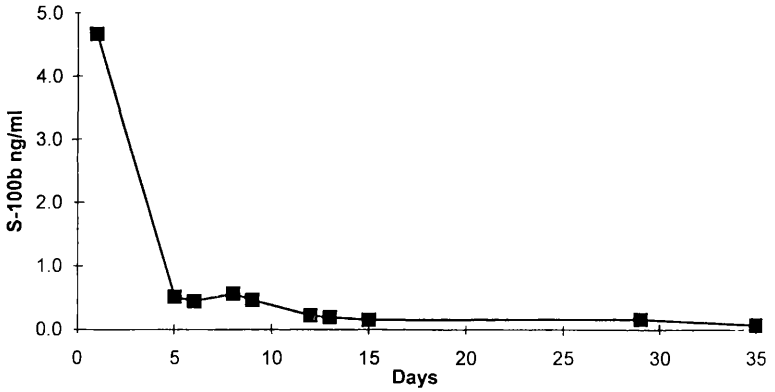

Fig. 4. Rapid decline of CSF S-100b concentration after removal of a ventricular–peritoneal shunt infected with group C Streptococci from a patient with post-traumatic epilepsy complicated by a CSF leak.

with post-traumatic epilepsy complicated by a CSF leak (Fig. 4). The CSF S-100b concentration fell from 4.67 ng/ml on day 1 to 0.56 ng/ml on day 5. The levels continue to fall over the next 30 days until a concentration of 0.07 ng/ml was seen on day 35.

4. Discussion

S-100b has been measured in CSF by a number of groups (Michetti et al., 1980; Kato et al., 1982; Sindic et al., 1982; Mokuno et al., 1983; Noppe et al., 1986; Persson et al., 1987; Aurell et al., 1989; Van Engelen et al., 1992). The reference ranges for patients with non-neurological disease quoted in these different reports differ considerably. Persson et al. (1987) quote a range of < 1 to 6.8 ng/ml, Noppe et al. (1986) a range of less than 0.8 ng/ml, Van Engelen et al. (1992) a range of 0.22–1.21 ng/ml and Mokuno et al. (1983) a range of less than 0.51 ng/ml. The reference range obtained in this study of less than 0.4 ng/ml agrees best with that of Mokuno et al. (1983). The reason for this variation probably lies with the selection of control patients and with the specificity of the assay used to measure S-100b. As S-100b is one of a family of proteins which share a large amount of sequence homology, it is possible that many of the other antisera used react not only with S-100b, but also with the closely related S-100a. The combination of antisera used in this assay results in a high degree of specificity as concentrations of the α-subunit of up to 1000 ng/ml show only 0.006% reactivity.

The lowest amount of S-100b detected in this assay was 0.04 ng/ml S-100b, which shows comparable sensitivity to the ELISA described by Griffin et al. (1993) and to the fluorescent immunoassay described by Kato et al. (1982). The ELISA described in this report demonstrates a linear relationship between absorbance and S-100b concentrations between 0.05 and 0.5 ng/ml, and is thus suitable for measuring S-100b levels in CSF. The ELISA described by Griffin et al. (1993, 1995) depends on the in-house production of polyclonal antisera and its subsequent biotinylation, which limits its use to those laboratories which have these facilities. The fluorescent immunoassay described by Kato et al. (1982) involves the immobilisation of antibody onto silicone rubber fragments. This makes the assay lengthy and cumbersome. The ELISA described in this report is not dependent on the in-house production of antisera or subsequent conjugation, is easy to perform and can be completed in less than 5 h if precoated plates are used.

This study confirms earlier reports of elevated S-100b concentrations in patients with intracranial haemorrhage (Sindic et al., 1982; Kato et al., 1982; Persson et al., 1987) and intracranial tumours (Kato et al., 1982; Sindic et al., 1982). Of the 40 patients with multiple sclerosis, only two had elevated S-100b concentrations. These results confirm other reports of marginal increases in S-100b occurring in multiple sclerosis (Michetti et al., 1979; Massaro et al., 1985; Noppe et al., 1986). Those samples from the other neurological disease category having increased S-100b concentrations were from patients with conditions associated with CNS parenchymal damage. The sharp decline in S-100b concentrations after removal of an infected ventricular–peritoneal shunt suggest that S-100b is rapidly cleared from the CSF and that elevated levels are evidence of recent brain trauma. The ELISA described in this paper is specific for the S-100b form of S-100, has a high degree of sensitivity and uses commercially available reagents. The assay is easy to perform and is suitable for use in clinical laboratories which have an interest in measuring S-100b as a sensitive indicator of brain destruction.

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References

Aurell, A., Rosengren, L.E., Wikkelso, C., Nordberg, G., Haglid, K.G., 1989. The S-100 protein in cerebrospinal fluid: a simple ELISA method. J. Neurol. Sci. 89, 157.

Fano, G., Biocca, S., Fulle, S., Mariggio, M., Belia, S., Calissano, P., 1995. The S-100: a protein family in search of a function. Prog. Neurobiol. 46, 71.

Griffin, W.S.T., Stanley, L.C., Yeralan, O., Rovaghi, C.R., Marshak, D.R., 1993. Cytokines in human neurodegenerative disease. Methods Neurosci. 17, 267.

Griffin, W.S.T., Yeralan, O., Sheng, J.G., Boop, F.A., Mrak, R.E., Rovnaghi, C.R., Burnett, B.A., Feoktistova, A., Van Eldik, L.J., 1995. Overexpression of neurotropic cytokine S-100b in human temporal lobe epilepsy. J. Neurochem. 65 (1), 228.

Kato, K., Kimura, S., 1985. S-100ao (alpha, alpha) protein in mainly located in the heart and striated muscles. Biochim. Biophys. Acta 842 (2-3), 146.

Kato, K., Nakajima, T., Ishiguro, Y., Matsutani, T., 1982. Sensitive enzyme immunoassay for S-100 protein in human cerebrospinal fluid. Biomed. Res. 3 (1), 24.

Kato, K., Haimoto, H., Ariyoshi, Y., Horisawa, M., Washida, H., Kimura, S., 1985. High levels of S-100ao (alpha, alpha) protein in tumor tissues and in sera of patients with renal cell carcinoma. Jpn. J. Cancer Res. 76 (9), 856.

Massaro, A.R., Michetti, F., Laudisio, A., Bergonzi, P., 1985. Myelin basic protein and S-100 antigen in cerebrospinal fluid of patients with multiple sclerosis in the acute phase. Ital. J. Neurol. Sci. VI, 53.

Michetti, F., Massaro, A.R., Murazio, M., 1979. The nervous system specific S-100 antigen in cerebrospinal fluid of multiple sclerosis patients. Neurosci. Lett. 11, 171.

Michetti, F., Massaro, A.R., Russo, G., Rigon, G., 1980. The S-100 antigen in cerebrospinal fluid as a possible index of cell injury in the nervous system. J. Neurol. Sci. 44, 259.

Mokuno, K., Kato, K., Kawai, K., Matsuoka, Y., Sobue, I., 1983. Neuron-specific enolase and S-100 protein levels in cerebrospinal fluid of patients with various neurological diseases. J. Neurol. Sci. 60, 443.

Noppe, M., Crols, R., Andries, D., Lowenthal, A., 1986. Determination in human cerebrospinal fluid of glial fibrillary acidic protein, S-100 and myelin basic protein as indices of non-specific or specific central nervous tissue pathology. Clin. Chim. Acta 155, 143.

Persson, L., Hardemark, H., Gustafsson, J., Rundstrom, G., Mendel-Hartvig, I., Esscher, T., Pahlman, S., 1987. S-100 protein and neuron-specific enolase in cerebrospinal fluid and serum: markers of cell damage in human central nervous system. Stroke 18, 911.

Sindic, C.J.M., Chalon, M.P., Cambiaso, C.L., Laterre, E.C., Masson, P.L., 1982. Assessment of damage to the central nervous system by determination of S-100 protein in the cerebrospinal fluid. J. Neurol. Neurosurg. Psychiatry 45, 1130.

Van Engelen, B.G.M., Lamers, K.J.B., Gabreels, F.J.M., Wevers, R.A., Van Geel, W.J.A., Borm, G.F., 1992. Age-related changes of neurone-specific enolase, S-100 Protein and myelin basic protein concentrations in cerebrospinal fluid. Clin. Chem. 38 (6), 813.

Zimmer, D.B., Cornwall, E.H., Landar, A., Song, W., 1995. The S100 protein family: history, function and expression. Brain Res. Bull. 37 (4), 417.

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Increased S100β in the cerebrospinal fluid of patients with frontotemporal dementia

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Abstract

Levels of S100β, a calcium-binding protein found in astrocytes, were measured using a sandwich ELISA in the cerebrospinal fluid (CSF) of patients with frontotemporal dementia and Alzheimer's disease and compared with controls. Mean CSF S100β concentrations were significantly raised in patients with frontotemporal dementia when compared with healthy controls (0.49 ± 0.28 vs. 0.22 ± 0.08 ng/ml, $P < 0.001$). There was no correlation between age at disease onset, disease severity or length of illness. The increased concentration of CSF S100β seen in frontotemporal dementia may reflect the marked astrocytosis seen in this condition. © 1997 Elsevier Science Ireland Ltd.

Keywords: Cerebrospinal fluid; S100β; Frontotemporal dementia; Alzheimer's disease; Astrocytes

S100β, a cytosolic low molecular weight calcium binding protein, belongs to a family of closely related proteins thought to play a role in cell growth and differentiation [1]. It is found primarily in the central nervous system (CNS) astrocytes and has been shown to have neurotrophic effects as well as mitogenic and morphological activity on astrocytes [15]. Alzheimer's disease is a common neurodegenerative disease which is characterised by a progressive dementia. The typical neuropathological features are amyloid plaques and intraneuronal neurofibrillary tangles [11]. Activated astrocytes have been reported to be associated with amyloid plaques and shown to contain elevated levels of S100β [12]. Increased S100β levels have been found in the brains of patients with Alzheimer's disease and the pattern of elevation across the different cerebral regions corresponds to those most affected with the disease [14]. Thus it has been proposed that S100β may be involved in the pathogenesis of the disease [12,14]. Frontotemporal dementia is associated with focal frontal and temporal lobe cerebral atrophy. Two main histological changes are seen in this disorder: first, neuronal cell loss and spongiform change (microvacuolation) together with mild to moderate astrocytosis, which has been designated frontal lobe degeneration. Secondly, intense astrocytic gliosis in the presence of intraneuronal inclusion bodies (Pick bodies) and inflated neurones, which is typical of Pick's disease [6]. This study investigated whether elevated cerebrospinal fluid (CSF) S100β is found in Alzheimer's disease and frontotemporal dementia and explored the relationship between CSF S100β concentrations and disease duration and severity.

Patients from the specialist Dementia Unit at the National Hospital for Neurology and Neurosurgery, London, UK, were selected for the study. A proportion of patients attending the clinic are admitted for investigation, including CSF examination. When CSF is collected, patients are asked to consent to the collection of an additional 3 ml for research, which is frozen at −70°C within 30 min of collection. Stored samples from 37 patients with clinically-confirmed dementia were retrospectively selected. A clinician (RH) reviewed the clinical and research notes for each case. The Mini Mental State Examination score (MMSE) [2] at the time of CSF sampling was evaluated and the final diagnosis, either from post-mortem histology or the last clinical assess-

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ment, reviewed. Diagnoses were made according to NINCDS/ADRDA criteria [7] for Alzheimer's disease and Lund and Manchester criteria [13] for frontotemporal dementia. Sixteen patients fulfilled NINCDS/ADRDA criteria for probable or definite Alzheimer's disease (mean age ± SD 58 ± 18 years; 9 males, 7 females), three of whom had autosomal dominant familial disease. Fifteen patients fulfilled criteria for frontotemporal dementia: (mean age ± SD 60 ± 20 years; 12 males, 3 females). Nineteen patients (mean age 27–76 years; 8 males, 11 females) with no known organic brain disease were used as healthy controls (eight with headache, eight with various neuralgias and three with psychological syndromes). Twenty-nine patients (mean age 26–78 years; 18 males, 11 females) with inflammatory conditions of the CNS were used as inflammatory controls (14 had multiple sclerosis, 10 had various infections and five had inflammatory neuropathy). CSF was centrifuged on receipt and the supernatant frozen at −70°C within 24 h. The S100β sandwich ELISA has been described in detail [3]. Mouse monoclonal anti S100β was purchased from Sigma (Poole, UK) and the horseradish peroxidase conjugated rabbit anti S-100 were purchased from Dako (Copenhagen, Denmark). Sodium barbitone, barbitone, o-phenylenediamine, 30% hydrogen peroxide, bovine serum albumin were of analytical grade (Sigma, Poole, UK). Calcium lactate, acetic acid, sodium acetate, hydrochloric acid (HCl), sodium phosphate, potassium phosphate, potassium chloride and sodium chloride were obtained from Merck, Germany. Nunc Maxisorb Microtitre plates were obtained from Life Technologies (Paisley, UK). A range of standards from 0.1 to 2.5 ng/ml was prepared by diluting bovine S100β (Affinity Research Products, Exeter, UK) in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin and stored in aliquots at −20°C. The microtitre plates were coated overnight with monoclonal anti S100β and washed with 0.1% bovine serum albumin in PBS containing 0.05% Tween 20 (Wash solution). Unbound sites were blocked with 1% bovine serum albumin in PBS for 30 min and subsequently rinsed with Wash solution. Fifty microlitres of standard, control or CSF sample was added in duplicate to the plate, and 50 μl of 0.06 M barbitone buffer, pH 8.6, containing 1 mM calcium lactate added to each well (Incubation buffer). The plate was incubated at 37°C for 3 h. After washing, rabbit anti S-100 antibody conjugated with horseradish peroxidase diluted 1:1000 with incubation buffer was added. The plate was incubated for 1 h at room temperature and washed with Wash solution. Enzyme substrate (1 mg/ml o-phenylenediamine in 0.05 M acetate buffer containing 0.01% hydrogen peroxide) was added. After incubation in the dark for 30 min the reaction was stopped by adding 1 M HCl. The absorbance was read at 492 nm using an Anthos 2001 Plate Reader (Denley Instruments, Sussex, UK). The within-batch and between-batch precision as measured by the coefficient of variance (CV) was <10%.

The CSF S100β concentration in control subjects was 0.22 ± 0.08 ng/ml (mean ± SD) (Fig. 1 and Table 1). The reference range for patients greater than 50 years old was 0.24 ± 0.07 ng/ml. The CSF S100β concentration gradually increased with age ($y = 0.0021x + 0.107$, $R^2 = 0.14$) but was not considered significant. The CSF S100β concentrations in patients with inflammatory diseases of the CNS were not increased at 0.19 ± 0.13 ng/ml. There was no difference between mean CSF S100β in patients greater than

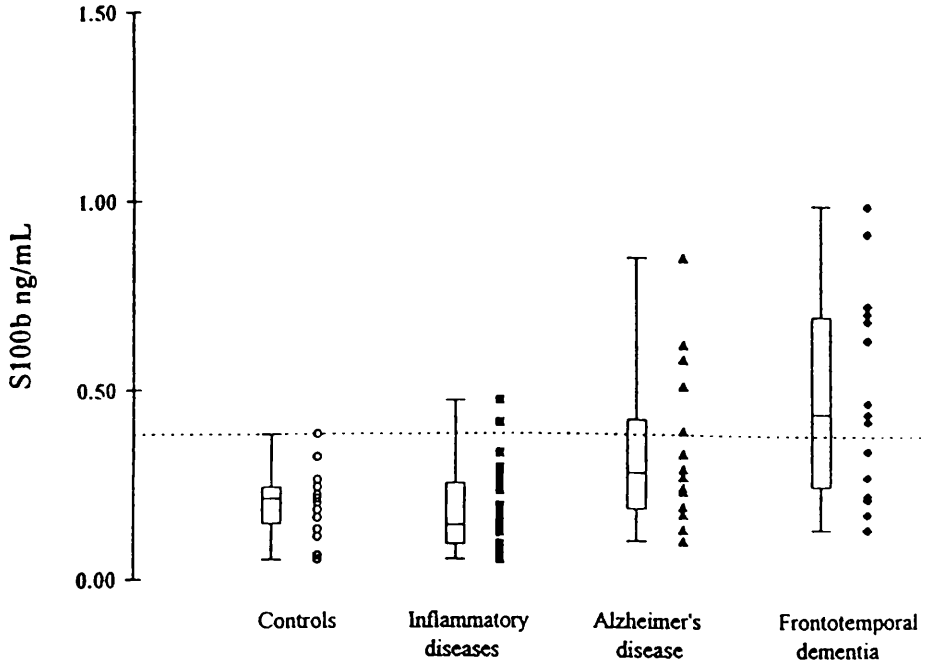

Fig. 1. Box and whisker plot of individual CSF S100β concentrations in different types of dementia. Plot shows 0, 25th, 50th, 75th and 100th percentiles. Dotted line represents the upper reference limit in healthy controls.

Table 1
CSF S100β concentrations in control subjects and patients with dementia

Patient group (years)	CSF S100β ng/ml (mean ± SD)	% samples with increased S100β (*n*)	Range
Healthy controls (27–76)	0.22 ± 0.08		0.05–0.38
Healthy controls (52–76)	0.24 ± 0.07		0.10–0.39
Inflammatory controls (26–78)	0.19 ± 0.13	7 (2)	0.05–0.47
Inflammatory controls (50–78)	0.19 ± 0.13	9 (1)	0.05–0.47
All dementias	0.41 ± 0.25*	42 (13)	0.10–0.99
Alzheimer's disease	0.33 ± 0.21**	25 (4)	0.10–0.85
Frontotemporal dementia	0.49 ± 0.28***	60 (9)	0.13–0.99

*$P < 0.001$ (one-way ANOVA); **$P < 0.05$ (Mann–Whitney); ***$P < 0.001$ (Mann–Whitney).

50 years old with inflammatory conditions when compared to all controls. The mean concentration of CSF S100β was significantly raised in patients with frontotemporal dementia (0.49 ± 0.28 ng/ml, $P < 0.001$; Mann–Whitney). The mean concentration of CSF S100β was also significantly raised in patients with Alzheimer's disease (0.33 ± 0.21 ng/ml, $P < 0.05$; Mann–Whitney). The proportion of patients having raised CSF S100β was greater in frontotemporal dementia than in Alzheimer's disease (60% vs. 25%, respectively). When considered together, there was a significant increase in CSF S100β in all dementia patients when compared to controls ($P < 0.001$; one-way ANOVA). There was no correlation between CSF S100β and age at disease onset ($R^{2=}0.006$), MMSE ($R^{2=}0.12$) and duration of illness ($R^2 = 0.0004$).

There have been many reports of increased CSF S100β concentrations occurring in diseases associated with neuronal degeneration [8]. Some of these studies included patients with dementia but the numbers studied were small [4,10]. This study shows that some patients with Alzheimer's disease and frontotemporal dementia have raised CSF S100β. This is most marked in patients with frontotemporal dementia. We were unable to find a correlation between CSF S100β concentrations and age at onset of disease, severity of disease or duration of disease. This may be due to the nature of the population selected as most patients were admitted for investigation early in their illness. Patients with inflammatory diseases of the CNS do not show a significant increase in CSF S100β, suggesting that the increased concentration found in dementia is not due to an inflammatory process. It is known that reactive astrocytes express elevated levels of S100β and that astrocytosis is a common neuropathological feature in degenerative dementia [9].

The degree of astrocytosis found in patients with Alzheimer's disease is variable. At autopsy not all patients show marked astrocytosis, indeed little astrocytosis is found in association with diffuse cerebral amyloid plaques [5,9] when compared to neuritic plaques which are associated with large numbers of activated astrocytes [12]. Therefore S100β concentrations could reflect the relative proportion of these two types of plaque in patients with Alzheimer's disease.

In frontotemporal dementia marked astrocytosis is associated with Pick's disease, rather than frontal lobe degeneration where astrocytosis is a minor feature [6]. Elevated CSF S100β concentrations may be a useful predictor of Pick-type pathology and help distinguish between these two subgroups of frontotemporal dementia. Thus the raised CSF S100β concentrations found in some patients with dementia may reflect the degree of astrocytosis and may provide information about the underlying neuropathology. All these patients are subject to long-term follow-up and CSF S100β concentrations will be compared to neurohistopathological examination where possible.

[1] Fano, G., Biocca, S., Fulle, S., Mariggio, M., Belia, S. and Calissano, P., The S-100: a protein family in search of a function, Prog. Neurobiol., 4 (1995) 71–82.

[2] Folstein, M., Folstein, S. and McHughs, P., The 'Mini mental state' a practical method for grading the cognitive state of patients for the clinician, J. Psychol. Res., 12 (1975) 189–198.

[3] Green, A.J.E., Keir, G. and Thompson, E.J., A specific and sensitive ELISA for measuring S-100β in cerebrospinal fluid, J. Immunol. Methods, 205 (1997) 35–41.

[4] Lamers, K.J.B., van Engelen, B.G.M., Gabreels, F.J.M., Hommes, O.R., Borm, G.F. and Wevers, R.A., Cerebrospinal neuron specific enolase, S-100 and myelin basic protein in neurological disorders, Acta Neurol. Scand., 92 (1995) 247–251.

[5] Mandybur, T.I. and Chuirazze, C.C., Astrocytes and the plaques of Alzheimer's disease, Neurology, 40 (1990) 635–639.

[6] Mann, D.M.A., South, P.W., Snowden, J.S. and Neary, D., Dementia of frontal lobe type: neuropathology and immunohistochemistry, J. Neurol. Neurosurg. Psychiatry, 56 (1993) 605–614.

[7] McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M., Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease, Neurology, 34 (1984) 939–944.

[8] Mokuno, K., Kato, K., Kawai, K., Matsuoka, Y. and Sobue, I., Neuron-specific enolase and S-100 protein levels in cerebrospinal fluid of patients with various neurological diseases, J. Neurol. Sci., 60 (1983) 443–451.

[9] Mrak, R.E., Sheng, J.E. and Griffin, W.S.T., Glial cytokines in Alzheimer's disease: review and pathological implications, Hum. Pathol., 26 (1995) 816–823.

[10] Noppe, M., Crols, R., Andries, D. and Lowenthal, A., Determination in human cerebrospinal fluid of glial fibrillary acidic protein, S-100 and myelin basic protein as indices of non-specific or specific central nervous tissue pathology, Clin. Chim. Acta, 155 (1986) 143–150.
[11] Rossor, M.N., Molecular pathology of Alzheimer's disease, J. Neurol. Neurosurg. Psychiatry, 56 (1993) 583–586.
[12] Sheng, J.G., Mrak, R.E. and Griffin, W.S.T., S-100β protein expression in Alzheimer's disease: potential role in the pathogenesis of neuritic plaques, Neurosci. Res., 39 (1994) 398–404.
[13] The Lund and Manchester Groups., Clinical and neuropathological criteria for frontotemporal dementia, J. Neurol. Neurosurg. Psychiatry, 57 (1994) 416–418.
[14] Van Eldik, L.J. and Griffin, W.S.T., S-100β expression in Alzheimer's disease: relation to neuropathology in brain regions, Biochim. Biophys. Acta, 1223 (1994) 398–403.
[15] Zimmer, D.B., Cornwall, E.H., Landar, A. and Song, W., The S100 protein family: history, function and expression, Brain Res. Bull., 37 (4) (1995) 417–429.

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Increased tau in the cerebrospinal fluid of patients with frontotemporal dementia and Alzheimer's disease

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Abstract

Cerebrospinal fluid (CSF) concentrations of tau protein were measured using an enzyme-linked immunosorbent assay which measures both normal and hyperphosphorylated tau. Levels of CSF tau were measured in 17 patients with Alzheimer's disease and 23 patients with frontotemporal dementia, and were compared to age-matched healthy controls. The CSF tau concentration in control subjects was 198 ± 49 pg/ml and no relationship was found between age and CSF tau concentrations in these subjects. CSF tau concentrations were significantly raised in patients with both Alzheimer's disease and frontotemporal dementia when compared to healthy controls (802 ± 381 pg/ml, $P < 0.001$; 612 ± 382 pg/ml, $P < 0.05$, respectively). In neither disease did CSF tau correlate with disease severity as assessed by the Mini Mental State Examination score (MMSE). This study shows that CSF tau is significantly raised in patients with frontotemporal dementia.

Keywords: Cerebrospinal fluid; Tau; Frontotemporal dementia; Alzheimer's disease; Pick's disease; Dementia

Tau protein is a microtubule associated phosphoprotein which promotes microtubule assembly and stabilization [9]. A hyperphosphorylated form of tau protein, known as phosphorylated tau, self-associates to create paired helical filaments, which are the major component of neurofibrillary tangles (NFTs) [8]. These NFTs are one of the characteristic histopathological hallmarks of Alzheimer's disease and their distribution and density correlate with disease progression and severity[5]. Concentrations of tau are elevated in the CSF of patients with Alzheimer's disease and the demonstration that levels are raised early in the disease course, suggests that measurement of CSF tau maybe a diagnostic test for early disease [3,7]. Differential diagnosis of dementia is also an important issue, particularly with other primary degenerative diseases such as frontotemporal dementia which is associated with focal frontal and temporal lobe atrophy [11,16]. Two main histological patterns are seen in this disorder; firstly neuronal cell loss and spongiform changes (microvacuolation) together with mild to moderate astrocytosis, which has been designated frontal lobe degeneration. Secondly, intense astrocytic gliosis in the presence of intraneuronal inclusion bodies (Pick bodies) and ballooned, achromatic neurones, which is typical of Pick's disease [13,17]. Frontotemporal dementia (FTD) may be difficult to distinguish from Alzheimer's disease, particularly in the early stages [12], and this study investigates whether measurement of CSF tau can be used to distinguish between these two conditions.

Patients from the Specialist Dementia Clinic at the National Hospital for Neurology and Neurosurgery were recruited for the study. Patients attending the clinic were admitted for investigation, including CSF examination. When CSF was collected, patients were asked to consent to the collection of an additional 3 ml for research, which was frozen at −70°C within 24 h of collection. Stored samples from 40 consecutive patients with clinically-confirmed dementia were retrospectively selected. A clinician (R.H.)

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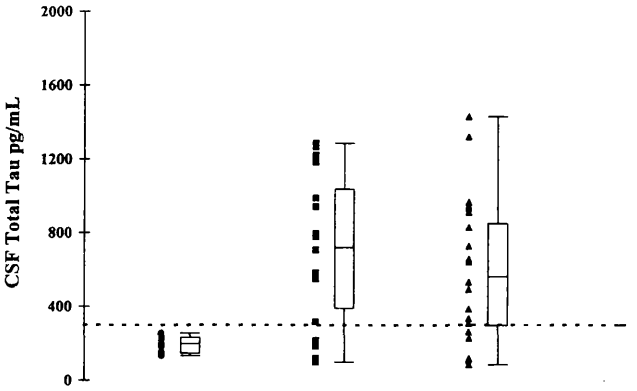

Fig. 1. Combined scatter and box and whiskers plots of cerebrospinal fluid concentrations of tau in controls subjects (●), $n = 9$), patients with Alzheimer's disease (■), $n = 17$), and patients with frontotemporal dementia (▲), $n = 23$). The box represents the 25th–75th quartile, divided horizontally by the median, the whiskers represent the range and the adjacent scatter plot represents the individual values from which the box and whiskers are derived. The horizontal dotted line represents the upper limit of normal (2 standard deviations above the mean of the control subjects).

reviewed the clinical and research notes for each case. The Mini Mental State Examination score (MMSE) [6] at the time of CSF sampling was evaluated and the final diagnosis were reviewed either from post-mortem histology in three cases or from the last clinical assessment. Diagnoses were made according to Lund and Manchester criteria for FTD [17] and NINCDS/ADRDA criteria [14] for Alzheimer's disease. Nine patients (58.8 ± 7.9 years; six male, three female) with no known organic brain disease were used as age-matched healthy controls (six with headache, one with primary hypertension, one with neuralgia and one with a psychological syndrome). Seventeen patients fulfilled NINCDS/ADRDA criteria for probable or definite Alzheimer's disease (mean age 58.5 ± 8.6 years; 10 male, 7 female), two of whom had autosomal dominant familial disease. The mean MMSE for these patients was 17/30 ± 4/30 (mean ± SD). Twenty-three patients fulfilled criteria for FTD (mean age 60.5 ± 7.8 years; 17 male, 6 female). The mean MMSE for these patients was 17/30 ± 5/30 (mean ± SD). CSF was centrifuged on receipt and the supernatant frozen at −70°C within 24 h. CSF tau levels were determined by a previously described sensitive sandwich enzyme-linked immunosorbent assay (ELISA) (Innogenetics, Belgium) according to the manufacturer's instructions [19]. This assay measures both normal and hyperphosphorylated tau.

The CSF tau concentration in control subjects was 198 ± 49 pg/ml (mean ± SD) (Fig. 1) (Table 1). There was no correlation between age and CSF Tau concentration ($r^2 = 0.061$). The upper limit of normal was taken to be 300 pg/ml. The CSF tau concentration was significantly raised in patients with Alzheimer's disease when compared to controls (802 ± 381 pg/ml, $P < 0.001$). There was no relationship between CSF tau concentrations and either age at onset of disease ($r^2 = 0.021$) or disease severity as assessed by MMSE ($r^2 = 0.066$) in Alzheimer's disease. CSF tau concentrations were significantly raised in patients with FTD (612 ± 382 pg/ml, $P < 0.05$). There was also no relationship between CSF tau and either age at onset of disease ($r^2 = 0.007$) or disease severity ($r^2 = 0.016$) in patients with FTD. There was no significant difference between the CSF tau concentrations seen in patients with Alzheimer's disease and FTD ($P > 0.5$).

This study demonstrates that CSF tau concentrations are significantly raised in patients with frontotemporal dementia when compared to age-matched healthy controls. No relationship exists between CSF tau concentration and disease severity as assessed by the MMSE or the age at disease onset. Although the mean concentrations of CSF tau were lower in the frontotemporal dementia patients when compared to that of the Alzheimer's group there was no significant difference between the two groups of patients. These findings expand two previous studies which reported raised CSF tau concentrations in patients with frontotemporal dementia [2] and in patients with frontal lobe degeneration [4]. These studies involved small numbers of patients and did not relate CSF tau concentrations to clinical severity. In this study 16 out of 23 patients with frontotemporal dementia had raised CSF tau concentrations, which agrees with Arai et al. (1998) who found six out of eight patients had elevated tau. A smaller proportion of patients, four out of eleven, with frontal lobe degeneration were reported to have raised CSF tau. The higher proportion of frontotemporal dementia patients with high CSF tau may be due to the inclusion of patients with Pick's disease. These patients are characterized by the presence of Pick bodies which contain hyperphosphorylated tau, and as a result may have higher tissue concentrations of tau than frontal lobe degeneration. A recent study has reported an association between

Table 1

CSF tau concentrations in control subjects and patients with dementia or other neurodegenerative diseases

Patient group	MMSE (mean ± SD)	CSF tau pg/ml (mean ± SD) (*n*)	Range
Healthy controls	nd	198 ± 49 (9)	133–255
Alzheimer's disease	17/30 ± 4/30	802 ± 381 (17)*	100–1284
Frontotemporal dementia	17/30 ± 5/30	612 ± 382 (23)**+	84–1425

*Significantly raised when compared to healthy controls ($P < 0.001$ one-way ANOVA, Bonferroni analysis). **Significantly raised when compared to healthy controls ($P < 0.05$ one-way ANOVA, Bonferroni analysis). +Not significantly raised when compared to Alzheimer's disease ($P > 0.5$ one-way ANOVA, Bonferroni analysis).

inherited frontotemporal dementia and Parkinsonism linked to Chromosome 17 and tau mutations [10]. The frontotemporal dementia patients included in this study are being investigated for these mutations, and findings will be related to CSF tau concentrations. CSF tau has been reported to be increased in other forms of dementia including vascular dementia [1], corticobasal degeneration [15] and diffuse Lewy body disease [2]. The cause of raised CSF tau in these diseases is unknown but is thought to be due to release from dying neurones [4]. Diseases associated with acute neurological destruction such as strokes [20], meningoencephalitis [3] and Creutzfeldt-Jakob disease [18] have markedly elevated concentration of CSF tau, suggesting that neuronal death may be responsible for the increase in CSF tau. The early differential diagnosis of dementia in patients who have yet to express the whole range of signs and symptoms may be difficult, and this is where a sensitive and specific diagnostic test would be most useful. This study demonstrates that elevated CSF tau concentrations are not specific for Alzheimer's disease but can occur in other dementias such as frontotemporal dementia.

[1] Andreasen, N., Vanmechelen, E., van de Voorde, A., Davidsson, P., Hesse, C., Tarvonen, S., Raha, I., Sourander, L., Winblad, B. and Blennow, K., Cerebrospinal fluid tau protein as a biochemical marker for Alzheimer's disease: a community based follow up study, J. Neurol. Neurosurg. Psychiatry, 64 (1998) 298–305.

[2] Arai, H., Morikawa, Y., Higuchi, M., Matsui, T., Clark, C.M., Miura, M., Machida, N., Lee, V.M.Y., Trojanwski, J.Q. and Sasaki, H., Cerebrospinal fluid tau levels in neurodegenerative diseases with distinct tau-related pathology, Biochem. Biophys Res. Commun., 236 (1997) 262–264.

[3] Arai, H., Terajima, M., Miura, M., Higuchi, S., Muramatsu, T., Machida, N., Seki, H., Takase, S., Clark, C.M., Lee, V.M.Y., Trojanowski, J.Q. and Sasaki, H., Tau in cerebrospinal fluid: a potential diagnostic marker in Alzheimer's disease, Ann. Neurol., 38 (1995) 649–652.

[4] Blennow, K., Wallin, A., Agren, H., Spenger, C., Siegfried, J. and Vanmechelen, E., Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer's disease?, Mol. Chem. Neuropathol., 26 (1995) 231–245.

[5] Dickson, D.W., Crystal, H.A., Bevona, C., Honer, W., Vincent, I. and Davies, P., Correlations of synaptic markers with cognitive status in prospectively studied elderly humans, Neurobiol. Aging, 16 (1995) 285–298.

[6] Folstein, M., Folstein, S. and McHughs, P., The 'Mini mental state': a practical method for grading the cognitive state of patients for the clinician, J. Psychiatr. Res., 12 (1975) 189–198.

[7] Galasko, D., Clark, C., Chang, L., Miller, B., Green, R.C., Motter, R. and Seubert, P., Assessment of CSF levels of tau protein in mildly demented patients with Alzheimer's disease, Neurology, 48 (1997) 632–635.

[8] Goedert, M., Tau protein and the neurofibrillary pathology of Alzheimer's disease, Trends Neurol. Sci., 11 (1993) 460–465.

[9] Goedert, M., Crowther, R.A. and Garner, C.C., Molecular characterization of microtubule-associated proteins tau and MAP2, Trends Neurosci., 14 (1991) 193–199.

[10] Hutton, M., Lendon, C.L., Baker, M., Froelich, S., Houlden, H., Pickering Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B.J., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T. and Heutink, P., Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17, Nature, 393 (1998) 702–705.

[11] Knopman, D.S., Mastri, A.R., Frey, W.H., Sung, J.H. and Rustan, T., Dementia lacking distinctive histologic features: a common non-Alzheimer degenerative dementia, Neurology, 40 (1990) 251–266.

[12] Litvan, I., Agid, Y., Sastri, N., Jankovic, J., Wenning, G.K., Goetz, C.G., Verny, M., Brandel, J.P., Jellinger, K., Chaudhuri, K., McKee, A., Lai, E.C., Pearce, R.K.B. and Bartko, J.J., What are the obstacles for an accurate clinical diagnosis of Pick's disease, A clinicopathologic study, Neurology, 49 (1997) 62–68.

[13] Mann, D.M.A., South, P.W., Snowden, J.S. and Neary, D., Dementia of frontal lobe type: neuropathology and immunohistochemistry, J. Neurol. Neurosurg. Psychiatry, 56 (1993) 605–614.

[14] McKhan, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M., Clinical diagnosis of Alzheimer's Disease: report of the NINCDS-ADRDA work group under the auspices of The Department of Health and Human Services Task Force on Alzheimer's Disease, Neurology, 34 (1984) 939–944.

[15] Mitani, K., Furiya, Y., Uchihara, T., Ishii, K., Yamanouchi, H., Mizusawa, H. and Mori, H., Increased CSF tau protein in corticobasal degeneration, J. Neurol., 245 (1998) 44–46.

[16] Neary, D., Snowden, J.S., Northen, B. and Goulding, P., Dementia of frontal type, J. Neurol. Neurosurg. Psychiatry, 51 (1988) 353–361.

[17] The Lund and Manchester Groups, Clinical and neuropathological criteria for frontotemporal dementia, J. Neurol. Neurosurg. Psychiatry, 57 (1994) 416–418.

[18] Otto, M., Wiltfang, J., Tumani, H., Zerr, I., Lantsch, M., Kornhuber, J., Weber, T., Kretzschmar, T. and Poser, S., Elevated levels of tau-protein in cerebrospinal fluid of patients with Creutzfeldt-Jakob disease, Neurosci. Lett., 225 (1997) 210–221.

[19] Vandermeeren, M., Mercken, M., Vanmechelen, E., Six, J., van de Voorde, A., Martin, J. and Cras, P., Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay, J. Neurochem., 61 (1993) 1828–1834.

[20] Vigo-Pelfrey, C., Seubert, P., Barbour, R., Blomquist, C., Lee, M., Lee, D., Coria, F., Chang, L., Miller, B., Lieberburg, I. and Schenk, D., Elevation of microtubule-associated protein tau in the cerebrospinal fluid of patients with Alzheimer's disease, Neurology, 45 (1995) 788–793.