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# Development of proteomic methods for studying cerebrospinal fluid proteins involved in Alzheimer´s disease.

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Maja Amedjkouh Puchades

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Avhandlingen baseras på följande delarbeten:

I. Davidsson P., Puchades M. and Blennow K. Identification of synaptic vesicle, pre and postsynaptic proteins in human cerebrospinal fluid using liquid phase isoelectric focusing. Electrophoresis 1999; 20, 431-437.

II. Puchades M, Blennow, K. and Davidsson P. Increased levels of phosphosynapsin I in cerebrospinal fluid of Alzheimer's disease patients. *Manuscript.*

III. Davidsson P., Westman A., Puchades M., Nilsson C. L. and Blennow K. Characterization of proteins from human cerebrospinal fluid by a combination of preparative two-dimensional liquid phase electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 1999; 71 (3), 642-647.

IV. Puchades M., Westman A., Blennow K. and Davidsson P. Removal of SDS from protein samples prior to matrix-assisted laser desorption/ionization mass spectrometry analysis. Rapid Commun. Mass Spectrom. 1999; 13 (5), 344-349.

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## ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia in western countries. The main neuropathological findings in the AD brain are senile plaques, neurofibrillary tangles and degeneration of neurons and synapses. Although research on AD is progressing fast, the causes and mechanisms of this disease remain to be elucidated and development of new methods is necessary to study neuron-related proteins involved in the pathophysiological mechanisms. Six low-abundance synaptic proteins in human cerebrospinal fluid (CSF), namely rab 3a, synaptotagmin, synapsin, the presynaptic protein GAP-43, the synaptosomal-associated protein 25 and the postsynaptic protein neurogranin, were detected with liquid phase isoelectric focusing and immunoblotting. An ELISA method for quantification of the phosphorylated form (Ser 9) of synapsin I was developed. Increased levels of phosphosynapsin I were demonstrated in AD patients compared to controls. These results are consistent with the hypothesis of impaired protein phosphorylation mechanisms in AD. To purify and characterise proteins in CSF, a new strategy combining two-dimensional liquid phase electrophoresis (2D-LPE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was developed. Two brain-specific proteins, cystatin C and β2 microglobulin, were isolated from CSF in sufficient quantities for analysis by MALDI-TOF MS. Special attention was needed to make 2D-LPE and mass spectrometry compatible. Chloroform/methanol/water extraction was the most efficient method for SDS removal, allowing the acquisition of good quality MALDI spectra of the tryptic digest of the proteins analysed. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry have been used for clinical screening of disease-influenced CSF proteins in AD. In order to increase the detection of CSF proteins and to improve the separation of protein isoforms, micro-narrow range immobilised pH gradient strips and prefractionation prior to 2-DE of CSF were used. Previously detected protein changes by 2-DE, between AD patients and controls, such as apoliprotein E and apoliprotein A1, were confirmed. Several new protein changes were demonstrated, including kininogen, apoliprotein J, β-trace, 1 β glycoprotein, α 2-HS glycoprotein and α-1 antitrypsin. As shown in this study, different isoforms i.e. different states of glycosylated proteins, are altered in AD. Therefore, the determination of post-translational modifications such as glycosylation and phosphorylation, is of importance for an increased understanding of the neuropathology in AD. The use of complementary strategies in proteome studies of CSF offers new perspectives on the pathology in neurodegenerative diseases and also reveals new potential biomarkers for brain disorders such as AD.

*Keywords: Alzheimer's disease, cerebrospinal fluid, proteomics, mass spectrometry, biomarkers, synaptic proteins, liquid phase IEF*

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2003

Si la theorie de l'évolution est vraie, comment se fait-il que les mères de famille n'ont toujours que deux mains ?

E. Dussault.

If the evolution theory is true, why do mothers still have only two hands ?

(translation) E. Dussault

# Contents

# Abstract

Alzheimer's disease (AD) is the most common cause of dementia in western countries. The main neuropathological findings in the AD brain are senile plaques, neurofibrillary tangles and degeneration of neurons and synapses. Although research on AD is progressing fast, the causes and mechanisms of this disease remain to be elucidated and development of new methods is necessary to study neuron-related proteins involved in the pathophysiological mechanisms. Six low-abundance synaptic proteins in human cerebrospinal fluid (CSF), namely rab 3a, synaptotagmin, synapsin, the presynaptic protein GAP-43, the synaptosomal-associated protein 25 and the postsynaptic protein neurogranin, were detected with liquid phase isoelectric focusing and immunoblotting. An ELISA method for quantification of the phosphorylated form (Ser 9) of synapsin I was developed. Increased levels of phosphosynapsin I were demonstrated in AD patients compared to controls. These results are consistent with the hypothesis of impaired protein phosphorylation mechanisms in AD.

To purify and characterise proteins in CSF, a new strategy combining two-dimensional liquid phase electrophoresis (2D-LPE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was developed. Two brain-specific proteins, cystatin C and β2 microglobulin, were isolated from CSF in sufficient quantities for analysis by MALDI-TOF MS. Special attention was needed to make 2D-LPE and mass spectrometry compatible. Chloroform/methanol/water extraction was the most efficient method for SDS removal, allowing the acquisition of good quality MALDI spectra of the tryptic digest of the proteins analysed. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry have been used for clinical screening of disease-influenced CSF proteins in AD. In order to increase the detection of CSF proteins and to improve the separation of protein isoforms, micro-narrow range immobilised pH gradient strips and prefractionation prior to 2-DE of CSF were used. Previously detected protein changes by 2-DE, between AD patients and controls, such as apoliprotein E and apoliprotein A1, were confirmed. Several new protein changes were demonstrated, including kininogen, apoliprotein J, β-trace, 1 β glycoprotein, α 2-HS glycoprotein and α-1 antitrypsin. As shown in this study, different isoforms i.e. different states of glycosylated proteins, are altered in AD. Therefore, the determination of post-translational modifications such as glycosylation and phosphorylation, is of importance for an increased understanding of the neuropathology in AD.

The use of complementary strategies in proteome studies of CSF offers new perspectives on the pathology in neurodegenerative diseases and also reveals new potential biomarkers for brain disorders such as AD.

# List of publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Davidsson P., Puchades M. and Blennow K. (1999) Identification of synaptic vesicle, pre and postsynaptic proteins in human cerebrospinal fluid using liquid phase isoelectric focusing. Electrophoresis, 20, 431-437.

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# Abbreviations

| | |
|---|---|
| Aβ | β-amyloid peptide |
| AD | Alzheimer's disease |
| ApoAI | apoliprotein AI |
| ApoE | apolipoprotein E |
| ApoJ | apolipoprotein J |
| APP | amyloid precursor protein |
| α-1 AT | α-1 antitrypsin |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| CHCA | α-cyano-4-hydroxycinnamic acid |
| C/M/W | chloroform/methanol/water |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| DTE | dithioerythritol |
| DTT | dithiothreitol |
| EAD | early onset Alzheimer's disease |
| ECF | extracellular fluid |
| ECL | enhanced chemiluminescence |
| ELISA | enzyme linked immunosorbent assay |
| ESI | electrospray ionisation |
| FTD | frontotemporal dementia |
| GAP-43 | growth associated protein 43 |
| HPLC | high performance liquid chromatography |
| IEF | isoelectric focusing |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| IPG | immobilised pH gradient |
| LAD | late onset Alzheimer's disease |
| LDS | lithium dodecyl sulphate |
| MALDI-TOF | matrix assisted laser desorption/ionisation time-of-flight |
| MES | 2-(N-morpholino) ethane sulphonic acid |
| MOPS | 3-(N-morpholino) propane sulphonic acid |
| MS | mass spectrometry |
| Mw | molecular weight |
| m/z | mass-to-charge ratio |
| NFT | neurofibrillary tangles |
| MMSE | Mini Mental State Examination |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PHF | paired helical filaments |
| pI | isoelectric point |
| PVDF | polyvinyl difluoride |
| RBP | retinol-binding protein |
| SDS | sodium dodecyl sulphate |
| SELDI | surface-enhanced laser desorption/ionization |
| SNAP-25 | synaptosomal-associated protein 25 |
| SNARE | soluble N-ethylmaleimide-sensitive factor attachment protein receptor |
| SP | senile plaque |
| TCA | trichloroacetic acid |
| TFA | trifluoroacetic acid |
| TOF | time-of-flight |
| 2-D | two-dimensional |
| 2-DE | two-dimensional gel electrophoresis |
| 2D-LPE | two-dimensional liquid phase electrophoresis |

# Background

## Alzheimer's disease

There are three major dementia disorders: Alzheimer's disease (AD), vascular dementia and frontotemporal dementia (FTD). AD is the most common cause of dementia in western countries. The disease is named after Alois Alzheimer, who, in 1907, described a 51-year old demented woman [1]. Her cognitive functions deteriorated with the progression of the disease, leading to death. It was at autopsy that Alois Alzheimer observed severe atrophy of the brain and discovered the characteristic neuropathological changes, senile plaques (SP) and neurofibrillary tangles (NFT), which are still used today to confirm the diagnosis.

### Epidemiology

AD affects 3 to 10% of the population over 65 years of age. Although ethnic differences exist, comparison of population studies from different countries shows that between 65 and 95 years of age, the prevalence and incidence rise in an exponential fashion, doubling every five years[2].

### Diagnosis

The diagnosis is today based on clinical examination and measurement of biochemical markers. However, only post-mortem identification of SP and NFT definitely confirm the diagnosis of AD. The clinical diagnosis is based on the symptomatology together with exclusion of other causes of dementia. It is made by the use of internationally accepted criteria for diagnosis of AD, described by the National Institute of Neurological and Communicative Disorders and the Stroke Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[3], the International Classification of Disease, 10$^{th}$ revision (IDC-10) [4] and the Diagnostic and Statistical Manual of Mental Disorders, 4$^{th}$ edition (DSM IV)[5]. All the systems have similar requirements: in order to be given the diagnosis of AD, the patient must have dementia with memory loss and impairment in several cognitive domains, disease onset between 40 and 90 years of age, and absence of other diseases. Results from clinical examinations are also weighted together with other examinations, such as brain imaging (magnetic resonance imaging, computerised tomography) and laboratory tests (e.g. blood and cerebrospinal fluid (CSF) analyses). The combined determination of β amyloid-42 (Aβ-42) and tau in CSF used today predicts more than 80% of AD cases [6], but in order to

further increase the diagnostic accuracy, it is important to find new, complementary biomarkers.

**Genetics**

AD can be divided into sporadic (no obvious heredity) and familial (autosomal dominant heredity) forms. For the sporadic form, several factors are considered to be risk factors for development of AD; for example, age, head trauma, female sex, low level of education and environmental factors [7]. Familial Alzheimer's disease is rare and due to mutations in specific genes. The first missense mutation was found in 1991 in the amyloid precursor protein (APP) gene, on chromosome 21[8]. Mutations in the APP gene are very rare (less than 0.1% of all AD cases), but they provide important information on the pathogenic mechanisms of AD [9]. Mutations in the presenilin 1 gene (chromosome 14) and presenilin 2 gene (chromosome 1) cause an aggressive, early-onset form of AD, usually beginning between 40 and 60 years of age [10, 11]. There is also an association between sporadic AD and the apolipoprotein E (apoE) gene located on chromosome 19. ApoE is involved in cholesterol transport and has three alleles, designated ε2, ε3 (the most common) and ε4. The frequency of the ε4 allele is increased to about 40% among AD patients [12, 13] and it has been established as a major risk factor for sporadic AD [14, 15]. The use of cholesterol-lowering drugs such as statins, was shown to be associated with a lower incidence of AD [16] and a high-cholesterol diet led to increased Aβ deposition in animal models [17].

**Neuropathology**

Although research on AD is rapidly progressing, the causes and mechanisms of this disease remain to be elucidated. The histological features of AD are complex and varied. The main neuropathological findings in the AD brain are SP, NFTs and degeneration of neurons and synapses. These changes may also be seen to a lesser extent in the brains of elderly non-demented individuals [9, 18]. SP are observed throughout the cortex [19]. They consist mostly of Aβ, a 40-42 amino acid peptide, which is a proteolytic product of APP [20, 21]. The amyloid deposits are found in different regions of the normal aging brain but are prominent in the associative areas in AD. NFT are not specific for AD, and may be found in a variety of neurodegenerative disorders [22]. In AD, NFT are initially found in the entorhinal cortex and then spread into the hippocampal formation following a specific pattern and cause progressive

cortical destruction [23-25]. NFT consist of abnormal fibrillary deposits called paired helical filaments (PHF), which are formed of abnormal aggregations of hyperphosphorylated tau-protein [26, 27]. Six major isoforms of tau are present in the human brain [28]. Tau-protein is a normal brain phosphoprotein which binds to microtubules in the neuronal axons, thereby promoting their assembly and stability [29].

**Synaptic pathology**

Apart from the identification of components of plaques and tangles, neuropathological studies strongly support the view that synapse loss is an essential feature of the dementia associated with AD. This was first demonstrated by ultrastructural studies [30, 31] with immunohistochemistry using the synaptic vesicle proteins synapsin I [32] and synaptophysin [33, 34] as markers. A correlation has been found between synaptic loss and severity of dementia, which suggests a close relationship between synaptic pathology and the cognitive decline in AD [35, 36]. No direct correlation was found between synaptic pathology and SP or NFT [33]. Presynaptic terminal degeneration was found to be greater than loss of large neurons in the same cortical areas, suggesting that the synapse damage occurs before any injury to the cell bodies [37, 38]. As other markers for synapse integrity (synaptotagmin, synapsin, the synaptosomal-associated protein 25 (SNAP-25), rab3a) have been used, synapse loss has been better defined and at present it is suggested that components of small synaptic vesicles are affected [35, 39]. The exact mechanism of the synaptic pathology in AD is unknown but some groups suggest that it is an early event in AD, due to abnormal APP function in the synapse [40]. Several synaptic vesicle proteins have important functions in synaptic transmission, such as vesicle trafficking, docking and fusion of the synaptic plasma membrane, and in neurotransmitter exocytosis. Some of them are illustrated in figure 1.

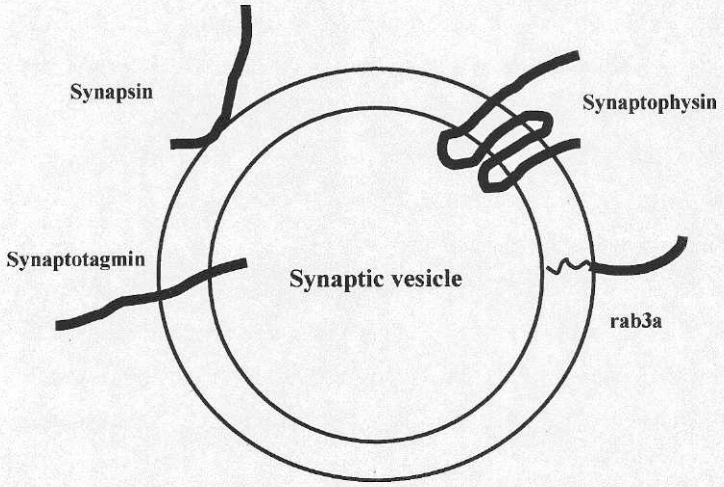


Figure 1. Schematic drawing of some synaptic vesicle proteins.

*Description of synaptic proteins studied in the study.*

*Synapsins* are a family of synaptic vesicle phosphoproteins playing an important role in regulation of neurotransmission and synaptogenesis. In mammals, three synapsin genes (I, II and III) have been identified [41-43]. By alternative splicing, these genes give rise to two protein isoforms (a and b). All synapsins share a constant N-terminal domain (A-C) containing a site for phosphorylation by cAMP-dependent protein kinase or calcium-calmodulin kinase I. This domain is important for the association of the synaptic vesicle to the plasma membrane and thereby the regulation of vesicle trafficking [44, 45]. The C-terminal domain is variable and seems to be involved in the binding of synapsin I to the synaptic vesicle [46]. The synapsins have two main functions; regulation of transmitter release by determining the availability of synaptic vesicles for exocytosis [47, 48, 49] and the formation and maintenance of synaptic contacts as reviewed by Ferreira et al [50].

*Rab3a*, the most abundant member of the "rab3 proteins" subfamily, is only expressed in neurons and neuroendocrine cells [51]. Rab3a binds and hydrolyses guanosine triphosphate and dissociates from the synaptic vesicle membrane upon depolarisation-induced exocytosis [52]. Rab 3a is thought to play a central role in the regulation of exocytosis and recycling of small synaptic vesicles [53, 54].

*Growth-associated protein 43* (*GAP-43*) is a presynaptic membrane protein [55] considered to be involved in neuronal growth, neurite formation, regeneration and neuronal sprouting after injury [56]. GAP-43 is also thought to have a role in regulation of calmodulin availability in the cell [57].

*Neurogranin* is the dendritic analogue to *GAP-43* in the post synaptic membrane [58]. Neurogranin is also involved in the calmodulin availability in the cell and thereby is thought to have an effect on regulation of calcium signals [57].

*SNAP-25* is expressed in two isoforms (A and B) and is associated to the presynaptic membrane by palmitoylation. SNAP-25 is involved in transmitter release and exocytosis [59, 60]. This protein is a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (with synaptobrevin and syntaxin) responsible for membrane fusion [54, 61].

*Synaptotagmin,* an integral membrane protein of synaptic vesicles, is involved in the docking process of the vesicles to the presynaptic membrane and exocytosis [62]. Synaptotagmin interacts with SNAP-25 in the SNARE complex and is proposed to be a calcium sensor for regulated exocytosis [62-64].

*Synaptophysin,* an hydrophobic membran protein, contains four transmembrane regions. This protein is suggested to be involved in the regulation of the SNARE complex by binding to synaptobrevin [65] and this binding was shown to vary with development stages [66].

*Implication of synaptic proteins in AD.*

*Synapsins* have previously been implicated in AD pathology. The level of synapsin was shown to be reduced in the dentate gyrus and entorhinal cortex in AD by immunohistochemical studies [32, 67]. Reduced levels of synapsin II mRNA were also demonstrated in AD brains using cDNA microarrays [68]. In the same study, reduced levels of synapsin variants I-III of the a-type isoform were observed in the entorhinal cortex. In contrast, no differences in synapsin levels between AD and controls were found in the study by Sze et al. [36].

The synaptic vesicle proteins *rab3a*, *synaptotagmin* and *synaptophysin*, the presynaptic protein *GAP-43* and the postsynaptic protein *neurogranin* have all been found to be reduced in the frontal, temporal and parietal cortex and hippocampus in AD patients compared to controls [35, 39, 69]. Reduced levels of synaptic vesicle proteins in AD [70-73] have also been found in several other studies.

## Cerebrospinal fluid

### Description

The extracellular fluid (ECF) (estimated at 250 ml) surrounding the neurons and glia cells is continuous with the CSF filling the ventricles, the subarachnoid space and the spinal cord. There is a slow current of ECF moving into the CSF [74]. The total volume is approximately 140 ml of CSF, secreted at a rate about 0.5 ml/min by the choroid plexuses. Its function is to support the brain and prevent damage by mechanical injury. The CSF may also act as a "third circulation", providing nutrients to the brain, transporting hormones and also serving to collect waste products coming from the brain [74]. The blood-brain barrier and the blood-CSF barrier maintain the homeostasis of the brain. The ionic composition of CSF is similar to that of plasma. The concentration of glucose and amino acids is only half of the value in plasma. The protein concentration in CSF is much lower, 250 mg/L compared to 40g/L in plasma. The protein concentration in CSF shows individual variations and has a tendency to increase with age [75]. The CSF is mainly composed of albumin and IgG, approximately 80% of the total protein content [76]. The major proteins found in human CSF are listed in Table 1. Many proteins diffuse from plasma, depending on their molecular size and ability to pass through the blood-CSF barrier, but some proteins were found to be more concentrated in CSF than in plasma, probably due to intracerebral synthesis (e.g. β-trace, transthyretin, cystatin C and β–2 microglobulin)[77].

Table 1: List of major proteins found in CSF (values compiled from Thompson, 1988 and Aldred et al, 1995)[76, 77].

| Protein | mg/L |
|---|---|
| albumin | 155 |
| prostaglandin D synthase (β-trace) | 26 |
| IgG | 22 |
| transthyretin (prealbumin) | 14.7 |
| transferrin | 14 |
| cystatin C (gamma trace) | 7.3 |
| α-1 anti-trypsin | 7 |
| apolipoprotein AI | 6 |
| α-2 macroglobulin | 4.6 |
| α-1 acid glycoprotein (orosomucoid) | 3.5 |
| β-1B glycoprotein (hemopexin) | 3.0 |
| haptoglobin | 2.2 |
| α–1 anti-chymotrypsin | 2.1 |
| α–2 HS glycoprotein | 1.7 |
| complement C3 | 1.5 |
| complement C9 | 1.5 |
| IgA | 1.3 |
| β-2 microglobulin | 1.1 |
| ceruloplasmin | 1.0 |
| complement C4 | 1.0 |
| lysozyme | 1.0 |
| β-2 glycoprotein I | 1.0 |
| fibrinogen | 0.65 |

**Lumbar puncture**

CSF is usually obtained by lumbar puncture in the L3/L4 or L4/L5 interspace. However, the protein concentration in CSF varies depending on location. Lower values were found in the ventricles, intermediate values in the cisterna magna and the highest values in the lumbar sac [78]. It has also been shown that during lumbar puncture, a gradient of protein concentration occurs. A significant decrease in protein concentration was observed in the first (0 to 4$^{th}$ mL) and the last (21$^{th}$ to 24$^{th}$ mL) portions [79]. Therefore, a standardised procedure has been adopted in our laboratory, where only the first 12mL are collected, then gently mixed to avoid

gradient effects and centrifuged to eliminate cells and other insoluble material. CSF analysis provides information about possible damage to the blood-brain barrier as well as about inflammatory or infectious processes in the brain. An increased white cell count in CSF and intrathecal immunoglobulin production, evaluated using the IgG and IgM index and/or CSF-specific oligoclonal bands, are found in chronic cerebral infectious and inflammatory disorders. These disorders have to be excluded when diagnosing AD, which is most conveniently done by lumbar puncture and CSF analyses. In AD, the blood-brain barrier is considered to be intact. Perturbations in albumin levels were found only in patients having associated vascular diseases [80].

## Biochemical markers for Alzheimer's disease

The diagnosis of AD is mainly based on clinical examination and relies on exclusion criteria. Combined determination of Aβ–42 and tau in CSF has become a valuable diagnostic tool during recent years, predicting about 80 % of AD cases [6, 81]. In AD, Aβ–42 levels in CSF decrease compared to controls, while tau levels increase [6, 82-84]. Because CSF is in direct contact with the ECF of the brain, one way to detect molecular changes like plaque formation, degeneration of neurons and/or synapses is by analysing proteins in CSF. In order to increase the diagnostic accuracy, especially in the early stage of the disease, finding biochemical markers that reflect these changes is very important [85].

Other proteins involved in the neuropathology of AD, such as APP, phosphorylated tau and apoE, have previously been studied by ELISA assays. Studies on CSF-APP in AD have not given concordant results. Some studies have shown reduced levels of APP in CSF,[86-88] while others showed no significant differences between AD patients and controls [89, 90]. Phosphorylated tau levels have recently been shown to be elevated in AD compared to controls [91-94].

Measurement of CSF-apoE also showed conflicting results. Some studies demonstrated reduced levels of CSF-apoE in AD compared to controls [95, 96], while other studies showed no changes [97, 98].

Synaptic proteins in CSF might be useful as markers of the synaptic degeneration. However, most synaptic vesicle proteins are membrane proteins with low water solubility and are present in very low concentrations in CSF. Attempts to define synaptic pathology in CSF came first from other markers of synapses such as chromogranin A and the GM1 ganglioside, which were shown to be reduced in AD patients compared to controls [99, 100]. Previously, one

synaptic vesicle protein, synaptotagmin, was for the first time detected in CSF, using a procedure including affinity chromatography, micro-reversed phase chromatography (μRP-HPLC) and enhanced chemiluminescence (ECL) immunoblotting [101]. A reduction of synaptotagmin was found in both brain tissue and lumbar CSF in AD [101], implying that CSF reflects the composition of synaptic proteins in the brain under normal and pathological conditions. Other synaptic vesicle proteins, such as rab3a, synaptophysin, GAP-43 and neurogranin, were not detectable with this procedure. The synaptic proteins cannot be detected by ordinary sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting, even after CSF concentration. New methods therefore have to be developed for detection and characterisation of low-abundance neuronal proteins such as synaptic proteins in CSF.

## Proteomic studies of CSF.

A map of human CSF proteins was first presented in 1980 [102]. The identification of CSF proteins was based on the co-migration of purified proteins, immunostaining and comparison with published two-dimensional gel electrophoresis (2-DE) maps of other body fluids [103-106]. Since then, many groups have adapted this method. Today 30 to 35 proteins have been identified by 2-DE and mass spectrometry [107-111]. The CSF reference map is accessible through the World Wide Web at http://www.expasy.ch .

# Experimental theory

## Enzyme linked immunosorbent assay (ELISA)

ELISA is a technique widely used in research and clinical practice for quantitative determination of various proteins in complex biological mixtures. There are several types of ELISA methods, for example direct antigen ELISA or competitive antigen ELISA. The sandwich ELISA, which is illustrated in figure 2, has been used in the present study.

Antibodies coated on the solid phase are exposed to sample and/or standard antigens in dilutions and, after washing, the complex is further exposed to an antibody conjugate to the same antigen. With the restriction that the detected and standard antigen must have multiple epitopes for antibody binding, or a repeating single epitope, this assay is sensitive and also provides special features of specificity and low background characteristics compared to direct antigen ELISA.

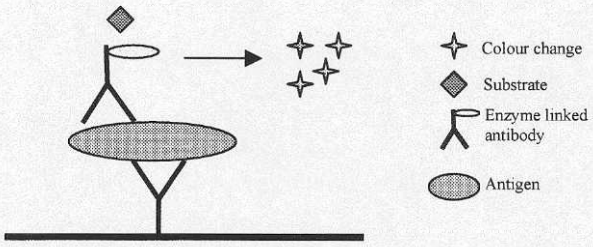


Figure 2. Schematic representation of a sandwich ELISA.

## Proteomic methods

The term "proteome", first introduced in 1995 [112], means the protein complement of a genome. In the cascade of regulatory events leading from the gene to the protein, the proteome can be seen as the end product of the genome. While the genome is static, the proteome is highly dynamic, because the protein content of a cell will vary, depending on the physiological state, stress, drug administration, health and disease.

The most widespread strategy for studying protein expression in biological systems employs 2-DE followed by enzymatic degradation of isolated protein spots, peptide mapping and bioinformatic database searches. To study low-abundance proteins such as neuron-related

proteins in CSF, some alternative proteomic methods have to be developed, i.e. combinations of analytical and preparative 2-DE methods (figure 3).

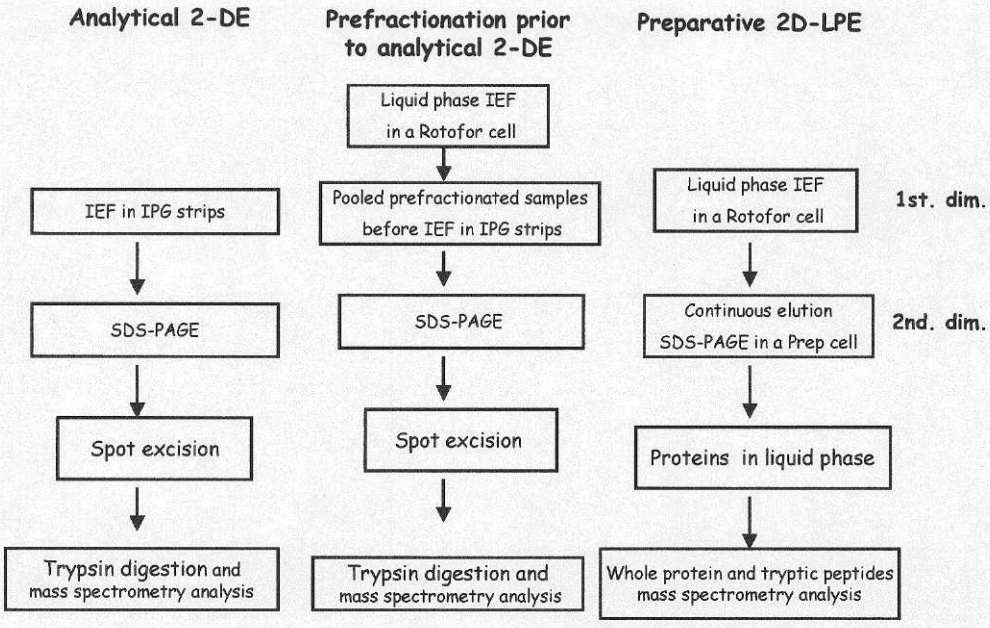


Figure 3. Combination of analytical and preparative 2-D electrophoretic methods.

**Analytical 2-D gel electrophoresis**

Two-dimensional gel electrophoresis, which combines isoelectric focusing (IEF) and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) [113, 114], is carried out under denaturing conditions. In the first dimension, IEF separates the proteins according to their isoelectric point (pI). The second dimension, SDS-PAGE, separates the proteins according to their molecular weight (Mw). The 2-D technique generates 2-D maps of the proteins in the sample (figure 4).

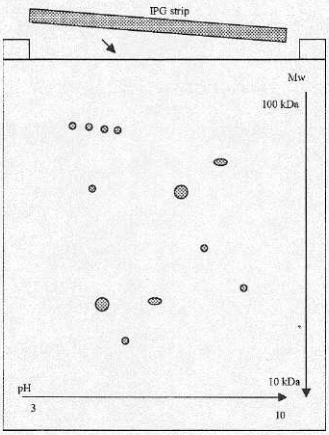


Figure 4. Schematic representation of proteins separated on a 2-D gel.

*In the first dimension: IEF*

The first dimension is performed in individual immobilised pH gradient (IPG) strips, which are 3mm wide and cast on plastic film. Before IEF, the IPG strips are rehydrated to their original thickness of 0.5 mm with an appropriate buffer. The sample is loaded onto the IPG strip containing ampholyte molecules, which can generate a pH gradient. High voltage is applied and the proteins are separated according to their pI. IPG strips are available in different lengths (7cm, 11cm, 13cm, 18cm and 24cm) and with different pH intervals. A pH 3-10 IPG strip will display the widest range of proteins on a single gel. The narrower pH ranges (e.g. 3-6 or 4-7) are used for higher resolution separation in a particular pH range. Furthermore, micro-narrow pH intervals (e.g. 3.9-5.1 or 4.7-5.9) make it possible to get a much better resolution of protein isoforms and also to distinguish faint spots that are otherwise masked.

*In the second dimension: SDS-PAGE*

The IPG strips are equilibrated in the presence of SDS, dithiothreitol (DTT), urea, glycerol and iodoacetamide, and placed onto the surface of a horizontal or on top of a vertical SDS-PAGE gel and the proteins are electrophoresed through the gel. The most commonly used buffer system for second dimension is the tris-glycine system described by Laemmli [115]. Other buffer systems can be used, particularly the Nu-PAGE system (Novex), which gives a better resolution of spots and a better reproducibility than the previous system. Upon completion of

electrophoresis, the proteins are stained with Coomassie blue, silver or fluorescent dyes. For evaluation of protein spots on the 2-D gel, different types of software can be used, performing spot detection, spot quantification, gel comparison, and statistical analysis. Protein spots on the gel can be cut out of the gel and enzymatically digested, yielding sufficient amounts of peptides to enable unambiguous protein identification through peptide mapping and/or tandem MS [116]. Post-translational modifications of proteins such as acetylation, phosphorylation and glycosylation visualised as trains of spots on the 2-D gel, can be identified and characterised using MS [117]. However, the 2-DE technique has some drawbacks that need to be considered. Proteins that are notably difficult to separate are membrane proteins, low copy number proteins, large and highly basic proteins. Therefore, some alternative separation methods including combination of analytical and preparative 2-DE have to be developed to provide complementary information to that derived from 2-DE.

**Liquid-phase IEF**

Protein purification by IEF has the advantage of being a non-denaturing technique, i.e. antibodies, antigens and enzymes usually retain their biological activity during the procedure. The Rotofor cell (figure 5) has been developed for preparative scale, free solution IEF applications. Sample preparation, prior to liquid phase IEF, is very simple. Biological fluids, like CSF or serum, are dissolved in water, and the ampholytes are added. The protein sample with ampholytes is loaded into the focusing chamber containing a cylindrical membrane core with 19 monofilament polyester membrane screens, which divide the focusing chamber into 20 compartments. Under rotation, voltage is applied, creating a pH gradient and the proteins migrate in the electrical field through the screens to their pI. At this point, the proteins will stop migrating and become focused. When the equilibrium is reached, the cell is stopped and fractions are harvested.

By liquid phase IEF, up to 55mL and 1 g of protein can be loaded using the large cell and proteins can be enriched up to 500 times, at their pI. Since the separation is performed in liquid phase, no gel elution or other steps are required.

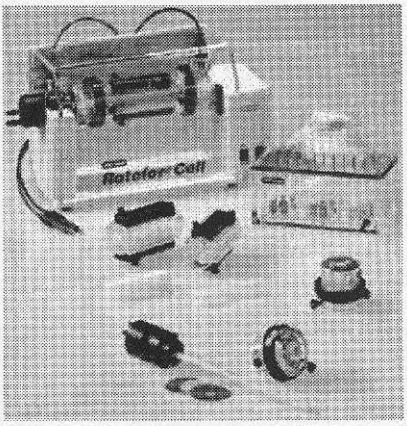


Figure 5. The Rotofor cell

**Preparative 2D liquid phase electrophoresis (2D-LPE)**

Preparative 2D-LPE is based on the same IEF and gel electrophoresis principles as the widely used analytical 2-DE, except that all separations steps occur in solution, avoiding extra steps such as extraction, electroelution or transfer of proteins to membranes before identification. 2D-LPE allows a larger volume of sample to be analysed compared to analytical 2-DE, yielding sufficient amounts of low-abundance proteins for further characterisation.

*The first dimension: liquid-phase IEF*

In the first step, the proteins are fractionated by liquid phase IEF as described above.

*The second dimension: continuous elution SDS-PAGE:*

In the second purification step, individual proteins from the Rotofor fractions are isolated on the basis of their size differences in a cylindrical SDS-PAGE gel using the Prep cell (figure 6). The best separation conditions for a specific molecular weight region (e.g. 10 kDa) are obtained by varying parameters such as the gel length, the diameter of the tube (28 or 37mm) and the acrylamide composition of the gel (e.g. 17% T/ 2.67% C). The percentage by weight of total monomer including cross-linker (N,N'-methylene bisacrylamide) is given by T, while C is the proportion of cross-linker as a percentage of total monomer. The sample is dissolved in sample buffer containing SDS, which is an anionic detergent that binds to proteins (1.4 g SDS/ 1g protein), so that all proteins in the sample will become negatively charged. Since the

shape of the protein influences the rate of migration, a reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol is usually added. The reducing agent disrupts all disulphide bonds, denaturing the protein. About 2 mg of protein can be loaded onto the Prep cell. However, the sample volume should be kept below 2 mL so that the whole sample enters the gel at the same time. When the proteins reach the bottom of the gel, they are continuously eluted with a buffer and collected automatically.

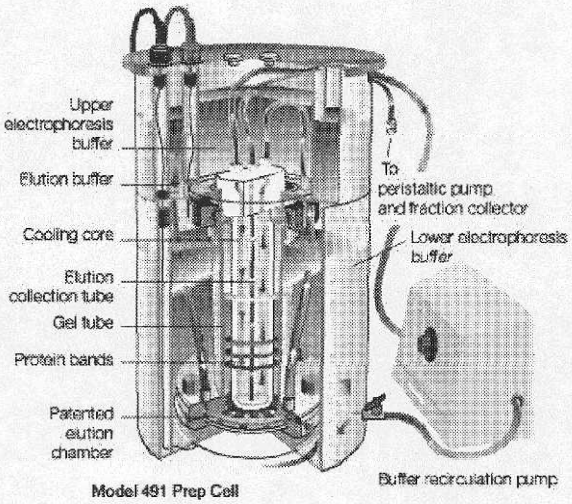


Figure 6. The Prep cell

**Mass spectrometry**

*Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)*

During the last decade, mass spectrometry has emerged as a powerful technique to identify and characterise proteins. [118] MALDI-TOF MS provides a very good mass determination of intact molecules. Furthermore, MALDI allows the detection of post-translational modifications of proteins and allows us to obtain amino acid sequence information. MALDI-MS is ideal for protein research in many ways. It is capable of generating molecular ions with minimal fragmentation and has a higher compatibility with buffers and detergents commonly used in the purifications of proteins than other mass spectrometric methods [119]. The MALDI-TOF mass spectrometer we use at our laboratory is described in figure 7.

The analytes are mixed with matrix, deposited on a stainless steel probe and introduced in the ionisation chamber, which is under vacuum. The matrix, usually low molecular mass aromatic molecules such as cinnamic acid derivatives, is important in the MALDI process. It must be possible to mix the matrix and the analyte in the same solvents; the matrix has to absorb strongly at the appropriate wavelength and be able to ionise the analyte. The sample is then bombarded with ultraviolet laser pulses, which results in the desorption and ionisation of the matrix and analyte molecules.

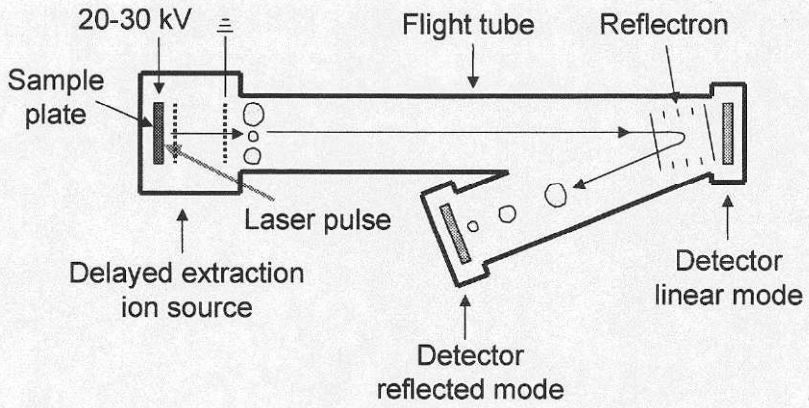


Figure 7. Schematic drawing of a MALDI-TOF mass spectrometer.

The ions are then accelerated by an electric field towards a grid at ground potential. The resulting velocity of the ions is inversely proportional to the square root of their mass-to-charge ratio (m/z). The ions are allowed to drift in a field-free tube and since ions with different mass have different velocity (the higher the mass, the slower the ion will be), they reach the detector at different times. However, ions of the same m/z often have different initial kinetic energies and hit the detector at slightly different times, resulting in broad peaks. Use of a time-lag-focusing ion source and/or an ion mirror can reduce this peak broadening and improve the resolving power of the spectrometer. The ion mirror [120] compensates for the difference in initial kinetic energies so that ions with the same m/z arrive at the detector at the same time. However, the sensitivity in the reflected mode (with the ion mirror on) is lower than in the linear mode (ion mirror off). The linear mode is therefore preferred for protein analysis, while the reflected mode is more often used in peptide analysis.

*Q-TOF*

The Q-TOF is a hybrid instrument consisting of a quadrupole mass filter coupled orthogonally to a time-of-flight (TOF) analyser. Electrospray ionisation (ESI) is used to create gas-phase analyte ions. Briefly, the sample is dissolved in a polar, volatile solvent and then transported through a needle placed at high potential. A spray of charged droplets is ejected from the tip of the needle by the electric field between the needle and the nozzle (entrance to the vacuum system). The droplets shrink through evaporation, finally yielding gas-phase ions. In MS mode the ions drift through the quadrupole filter (which in this case only acts as a focusing device) and are separated according to their mass-to-charge ratio (m/z) in the TOF analyser. In MS/MS mode, the quadrupole filter is set to allow only a narrow m/z range to pass for subsequent collision-induced dissociation. The fragments are then separated in the TOF analyser. Thus, amino acid sequence information can be obtained since the fragmentation occurs according to known patterns. Comparison of the obtained sequence information against a database can give protein/peptide identification. The combination of ionisation at atmospheric pressure and the continuous flow of solvent used in ESI allows direct coupling with separation techniques, such as liquid chromatography. With nano-spray, a low flow rate version of ESI, very low sample consumption as well as increased sensitivity is achieved.

**Mass spectrometric analysis of tryptic peptides (peptide mapping)**

Trypsin is preferentially used to digest proteins for MS analysis. Trypsin cleaves at the C-terminal of the two basic amino acids lysine and arginine, generating a wide distribution of peptide masses (typically 500-2500 u). Peptide mapping with a database search is often used for identification of proteins present in a biological sample. The molecular masses of the cleavage products are determined by MS and compared with theoretical masses of enzymatic peptides in protein sequence databases. Internet resources (e.g. Profound or MASCOT) used for this purpose present the results as a ranking of proteins that have the highest probability of matching the experimental data.

# Aims of the present study

## Overall aim

The overall aim of this study is to gain an increased understanding of the pathophysiological mechanisms in AD. To study these processes, neuron-specific proteins were investigated in living patients, i.e. in CSF from individual patients. New proteomic strategies for the purification, detection, quantification and characterisation of CSF proteins are needed for studying neuron-specific proteins in CSF.

**Specific aims of the study were**

- To study synaptic pathology in CSF.
- To develop complementary proteomic methods for analysing less abundant proteins in CSF.
- To reveal new potential biomarkers for AD.

**The studies were performed by:**

- Detecting several synaptic proteins (ng/L) in CSF using liquid phase IEF and immunoblotting.
- Measuring phosphorylated synapsin I in CSF of AD patients compared to controls.
- Developing preparative 2-D liquid phase electrophoresis methods for isolation, identification and increased detection of CSF proteins.
- Comparing the CSF proteome in AD patients with that in controls.

# Materials and methods

## Cerebrospinal fluid

CSF samples used were obtained from the Clinical Neurochemical Laboratory, Sahlgrenska University Hospital/Mölndal, Sweden. Lumbar puncture was performed in the L4-L5 vertebral interspace. The first 12 mL of CSF were collected, gently mixed to avoid gradient effects, and centrifuged at 2000 × g (+4°C) for 10 min to eliminate cells and other insoluble material. The samples were stored at -80°C if further analyses were carried out later on. All patients had a normal white blood cell count and blood-brain barrier function and absence of intrathecal IgG and IgM production.

CSF from AD patients and controls was analysed and more detailed information is provided below for each study. AD patients were diagnosed according to NINCDS-ADRDA criteria [3]. The severity of dementia was evaluated using the MMSE [121, 122]. The control group "healthy individuals" had no history, symptoms or signs of psychiatric or neuronal disease.

The control group "non-demented controls" consisted of patients with minor psychiatric complaints or subjective memory complaints that could not be verified by clinical examination or neuropsychological testing. All individuals had MMSE scores of 29-30.

## Ethical approval

The study was approved by the Ethics Committee of Göteborg University. All participants or their relatives gave informed consent to inclusion in the study, which was performed in accordance with the Helsinski Declaration.

## Chemicals/Antibodies

Detailed descriptions of the chemicals and antibodies used and their respective suppliers are given in the papers.

## Detection of low-abundance synaptic proteins in CSF with a combination of liquid phase IEF and immunoblotting (Papers I and II)

Pooled CSF samples from patients on whom lumbar puncture had been performed to exclude infectious disorders of the central nervous system (CNS) were used.

Liquid phase IEF was performed in the Rotofor cell (BioRad Laboratories, Hercules, CA, USA). In Paper I, The CSF sample was concentrated by precipitation using 10% trichloroacetic acid (TCA) for one hour on ice. After centrifugation (2000 × g, 10 min), the protein pellet was washed twice with ether/ethanol (1:1 v/v) and dried. The precipitate was brought to a volume of 10-12 mL with 6 M urea and 20 mM DTT and 2% Biolytes ampholytes, pH range 3-10 (Bio-Rad).

The sample preparation was slightly modified in Paper II, where CSF was dialysed for 2 hours (Mw cut-off 7.000) against distilled water. After dialysis, 20mM DTT, 1% Servalyt pH 3-10 isodalt and 0.1% n-octylglucoside were added. IEF was performed according to the manufacturer's instructions. The running conditions are described in detail in Paper I.

The proteins were electrophoresed through tris-glycine gels (Bio-Rad Laboratories, Hercules, CA, USA)(Papers I and III) or NuPAGE Bis-Tris gels (Novex, San Diego, CA, USA) (Paper II) and transferred from the gel to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using the NovaBlot System (Pharmacia, Uppsala, Sweden). Immunodetection was performed with antibodies directed against different synaptic proteins. In Paper I, synaptotagmin (clone 41.1), the rab3a MAb (clone 42.2), the GAP-43 (NM4), SNAP-25 and neurogranin were used. In Paper II, synapsin I MAb 355, synapsin I polyclonal antibody AB 1543 and synapsin I polyclonal antibody 51-5200 were used. The suppliers and the detailed immunoblotting protocols are described in the papers.

## Proteomic methods.

### Protein precipitation and desalting procedures

CSF proteins were mixed with acetone or ethanol (1:4 v/v) and precipitated at -20°C for 2 hours. The mixture was then centrifuged at 10.000g for 10 min. at +4°C.

Bio-Spin columns P-6 (cut-off 6,000 Da (BioRad)) were used to desalt CSF samples according to the manufacturer's instructions.

### Analysis of CSF proteins in AD patients by 2-DE using micro-narrow strips (Paper V)

The AD patients consisted of 7 AD patients (4 women and 3 men) and the average age was 80± 5 years. The control group consisted of 7 healthy individuals (3 women and 4 men) and the average age was 66 ± 6 years.

The 2-D gel electrophoresis method was previously described in detail [107]. Briefly, 300 µL of CSF was precipitated with acetone (1:4 v/v). The pellet was mixed with a buffer (9M urea, 35 mM tris, 42 mM DTT, 2% CHAPS, 0.66% SDS, 2% IPG buffer and bromophenol blue (BPB)). The first dimension was performed with IPG strips (pH 4.7-5.9, 7 cm (Bio-Rad)) with a Protean IEF Cell (Bio-Rad). After equilibration of the IPG strips in the buffer (50mM tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS and BPB) containing 1% DTT for 15 min and 2.5% iodoacetamide for a further 15 min, the second dimension separation was performed with the Nu-PAGE gel system (Novex, San Diego, CA, USA) combined with MOPS buffer (1.0 M MOPS, 1.0 M tris, 69 mM SDS, 20m M EDTA) for 45 min.

For detection of the protein spots, SYPRO Ruby Protein Stain (Molecular Probes) was used. Image acquisition and analysis were performed on a Fluor-S MultiImager (Bio-Rad). The protein spots were detected, quantified and matched using the PD-Quest 2-D gel analysis software, v.6. The protein spots from different gels were matched and their spot volumes determined. In the normalisation process, the integrated optical density of all spots within a gel that have been matched to the reference standard image spots are summed, and the summed values are then compared as a basis of normalisation.

The trypsin digestion method was previously described in detail [107]. Briefly, the spots were excised, placed in a siliconised tube and digested with porcine trypsin (Promega Corporation, Madison, USA). The peptides were extracted with formic acid (FA) and acetonitrile (ACN). Before mass spectrometric analysis, the peptides were purified with Zip $Tip_{C18}$ (Millipore, Bedford, USA) according to the manufacturer's instructions. The matrix used was α-cyano-4-hydroxy-cinnamic acid (CHCA) saturated (15g/L) in ACN:0.1% FA (1:1).

**Analysis of CSF proteins in AD patients using liquid phase IEF in combination with 2-DE (Paper V)**

CSF from individual patients, 5 AD patients (2 women and 3 men), average age 81± 5 years, and 5 non-demented controls (3 women and 2 men), average age 62± 12 years, was analysed. The CSF sample (3 mL) was prefractionated using a Rotofor cell as mentioned above. Briefly, the CSF sample was brought up to a volume of 12 mL with ultrapure water. Thereafter, 1% Servalyt (pH range 3-10), 20 mM DTT and 0.5 mL antiprotease solution (Complete, Roche Diagnostics, Mannheim, Germany) were added. Selected Rotofor fractions were pooled and concentrated to 300 µL in a vacuum centrifuge before 2-DE. We obtained three pools from

the prefractionation step, i.e. pool 1:fractions 2-5 (pH 1.5-4.5), pool 2: fractions 6-9 (pH 4.5-6) and pool 3: fractions 10-14 (pH 6-7.5).

The prefractionated protein fraction pools were precipitated with 900µL ice-cold ethanol (1:4 v/v) and the first dimension was carried out on IPG strips pH 3-6, pH 4-7 and pH 5-8, 7 cm for 20,000 Vh on the Protean Cell. After the first dimension, the samples were reduced and alkylated as described above. The second dimension as well as the staining procedure, image acquisition and analysis and in-gel tryptic digestion were performed as described above.

**Characterisation of CSF proteins using preparative 2D liquid phase electrophoresis (2D-LPE) (Papers III and IV)**

Pooled CSF samples from patients in whom lumbar puncture had been performed to exclude infectious disorders of the central nervous system (CNS) were used.

The whole procedure is described thoroughly in Paper III. After liquid phase IEF (described previously), the Rotofor fractions were analysed by immunoblotting using rabbit anti-serum against cystatin C and β-2 microglobulin (Dakopatts, Glostrup, Denmark). The dried Rotofor fractions containing cystatin C and β-2 microglobulin were dissolved in 1mL SDS sample buffer (0.06 M tris-HCl, pH 6.8, containing 2% SDS, 3% DTT, 10% glycerol and 0.025% bromophenol blue) and boiled for 5 min. The protein sample was applied to the 491 Preparative cell (Bio-Rad Laboratories, Hercules, CA, USA) and electrophoresed under constant power (12W) for approximately 13 hours. Fractions were collected automatically at a rate of 0.7 mL/min. The gel composition was 17% T/2.67% C, with a height of 10 cm, and with a gel tube size of 28mm (internal diameter). The stacking gel composition was 4% T/ 2.67% C with a height of 2 cm.

*Removal and quantification of residual SDS*

Three different methods were compared for SDS removal and are described in Paper IV. The chloroform/methanol/water (C/M/W) (1:4:3 v/v) extraction was the most effective. In Paper III, we used both C/M/W extraction and desalting columns for SDS removal.

A colorimetric method [123] was used to quantify the residual SDS in the samples after different SDS removal methods (Paper IV) and determine the minimal SDS amount tolerated for MALDI MS analysis of our samples.

*Protein analysis*

Quantitative determination of albumin, IgG and IgM in serum and CSF was performed using the Behring Nephelometer Analyser (Behringwerke AG, Marburg, Germany). The blood-brain barrier function was determined using the CSF/serum albumin ratio.

Silver staining of the gels was performed in order to analyse the total protein content of a fraction, using the Xpress silver staining kit (Novex, San Diego, USA). The gels were dried with the DryEase mini-gel drying system (Novex, San Diego, USA), according to the instruction manual.

*Trypsination of samples prior to mass spectrometry*

In Paper III, the protein pellets were subjected to tryptic digestion for 4 hours. The detailed procedure is described in Paper III. The samples were then dried and reconstituted in 0.1% trifluoroacetic acid (TFA) in water before deposition on the probe with the seed layer method.

## MALDI-TOF mass spectrometry (Papers III, IV and V)

### Sample preparation.

The MALDI matrix used was α-cyano-4-hydroxycinnamic acid (CHCA). Detailed preparation procedures are described in the papers. In Papers III and IV, the protein pellets were dissolved in 50 µL of 0.1% TFA in water or 20 mM *n*-octylglucoside and deposited on the stainless steel probe according to the matrix seed layer method [124]. In Paper V, the peptides were purified with Zip $Tip_{C18}$ (Millipore, Bedford, USA) according to the manufacturer's instructions and eluted with matrix directly on the probe.

### Apparatus

All MALDI analyses were performed with a Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with a two stage electrostatic reflectron and a delayed extraction (time-lag-focusing) ion source. In Paper IV, all spectra were recorded in linear mode at an acceleration voltage of 20 kV, in order to see whole proteins. In Paper III, all spectra were acquired in reflectron mode at an acceleration voltage of 20 kV. In Paper V, the mass spectrometer had been upgraded with a high resolution reflector detector and a 2 GHz digitizer. The mass spectra, acquired in reflectron mode, were analysed using Bruker software and were initially calibrated by external calibration using a

mixture of known peptides and later recalibrated using two autodigestion products of porcine trypsin as internal calibrants. Before the list of m/z values was subjected to the database search, tryptic peptides from keratin and porcine trypsin were removed using software developed in-house.

**Data analysis**

The monoisotopic masses of the peptides obtained experimentally were compared with the predicted monoisotopic *m/z* values of tryptic peptides of all known proteins assembled in a specific database. We used the Internet resource "MS-Digest" (http://prospector.ucsf.edu/) to compare the theoretical tryptic peptide masses of cystatin C and β-2 microglobulin with the experimental values (Paper III). In Paper V, the protein database search tool "ProFound" (http://129.85.19.192/profound_bin/WebProFound.exe) was used to compare the monoisotopic m/z values of the tryptic fragments with those of known proteins in the NCBI database. A mass deviation of 50 ppm was tolerated and Homo sapiens was specified.

## Q-TOF mass spectrometry (Paper V)

Samples for which MALDI-TOF did not provide unambiguous protein identity were further investigated throughout acquisition of fragment ion data in an electrospray quadrupole time-of-flight (ESI-QTOF) instrument (Q-tof, Micromass, Manchester, UK). Zip-Tip-enriched samples in ACN: 0.1% FA (1:1 v/v) were sprayed from gold-coated glass capillaries using a nanoflow electrospray source. Argon was used as the collision gas. Instrument calibration was performed using fragment ions from Glufibrinopeptide B and fourth-order polynomial fit. MS/MS spectra were post-processed using MaxEnt3 (Micromass) and used without further interpretation for database searches using MASCOT (http://www.matrixscience.com) against all entries in the NCBInr database.

## Quantification of phosphorylated synapsin in CSF with an ELISA method (Paper II)

We studied if there is a difference in CSF phosphorylated synapsin levels between AD patients and controls. The AD group consisted of 20 patients, 13 women and 7 men, average age 75± 8 years, and the control group consisted of 14 individuals, 12 women and 2 men,

average age 66± 12 years, without history, symptoms or signs of psychiatric or neurological disease.

The sandwich-ELISA was based on a rabbit anti-phosphophorylated synapsin (Ser 9) antibody (Chemicon) and a synapsin I specific MAb (MAb 355) (Chemicon). Then, a goat anti-mouse IgG (Fab-specific) biotin conjugate (Sigma-Aldrich Chemie, Gmbh) was used. Thereafter, avidin-HRP (Sigma-Aldrich Chemie, Gmbh) was incubated for 1 hour at +37 °C. Development occurred by adding 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (TMB Peroxidase EIA Substrate Kit, BioRad). The reaction was quenched with $H_2SO_4$ (0.5 M) after 30 min. The absorbance was measured at 450 nm.

The detailed procedure is described in Paper II. RIPA-buffer (150 mM NaCl, 50mM tris-HCl (pH 8.0) 1% Triton X100, 0.5% cholic acid, 0.1% SDS, 5 mM EDTA) extracted brain homogenate in a serial dilution was used as standard.

# Results and discussions

Studies of neuron-specific proteins in CSF, markers for neuropathological processes, require developments in proteomic methodology. Therefore, complementary separation methods to those derived from 2-D gels, including combinations of analytical or preparative methods, have been developed, as illustrated in figure 8. Direct 2-DE, liquid phase IEF in the Rotofor cell or SDS-PAGE in the Prep cell, followed by immunoblotting, mass spectrometry and database searches have been employed for studies on CSF proteins in AD.

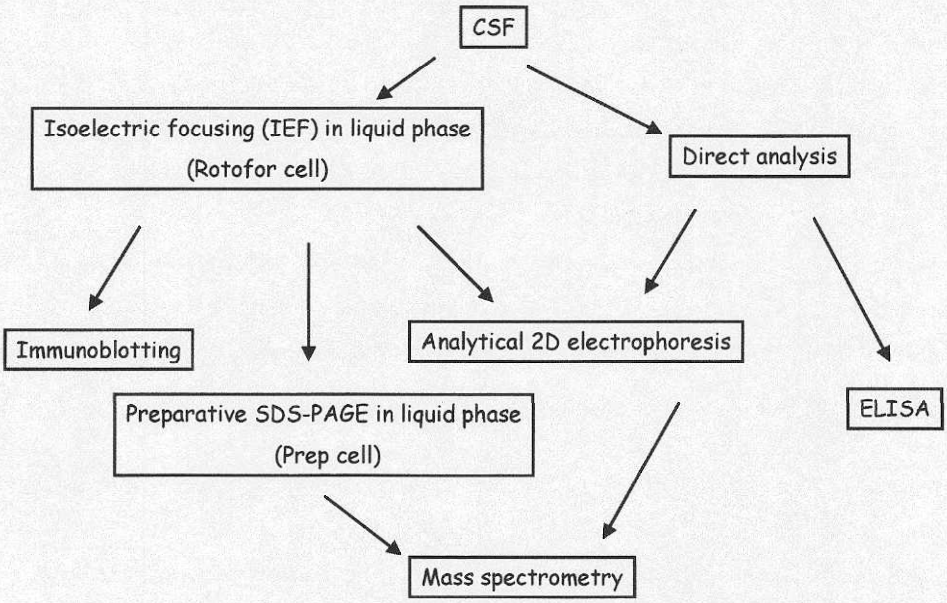


Figure 8. Combination of methods used for studying CSF proteins.

## Detection of synaptic proteins in CSF with a combination of liquid phase IEF and immunoblotting (Papers I and II)

Liquid phase IEF in combination with immunoblotting has been used to enrich trace amounts (ng/L) of neuron-specific proteins that are involved in AD. Six synaptic proteins, namely rab3a, synapsin, synaptotagmin, SNAP-25, GAP-43 and neurogranin, were detected in nanogram per litre quantities in human CSF (figure 2, Paper I; figure 1a, 1b and 1c in Paper II). Rab 3a was found in Rotofor fractions (4-6) as a single band at approximately 24 kDa. The anti-synaptotagmin antibody recognised two bands, one of 65 kDa (in fractions 9-11) and another of aproximately 110 kDa (Rotofor fractions 5-6). The higher molecular band of 110 kDa is probably a dimer of synaptotagmin. SNAP-25 was detected as a single band (in Rotofor fractions 2-3) with a Mw of 28 kDa. GAP-43 was found in Rotofor fractions 2-4, as one band (50 kDa), and neurogranin was detected as a single band at 18 kDa (in Rotofor fractions 4-5). Synapsin was detected as a doublet band with a Mw of 80 kDa approximately (Rotofor fractions 17-19).

Synaptophysin was not detectable with our method and this may be explained by its hydrophobic nature, leading to very poor solubility in CSF. In another study, tau protein, present at μg/L, has previously been detected in CSF with our technique as four bands with Mw of 25-80 kDa, using different types of antibodies that recognise both the phosphorylated and unphosphorylated forms [90].

Because albumin and IgG represent 80% of the total amount of CSF proteins, it is difficult to detect and study low-abundance proteins such as synaptic proteins in CSF. Several approaches have previously been adopted to circumvent this problem, such as elimination of plasma proteins by affinity chromatography [125, 126] or high performance electrophoresis chromatography [127]. The synaptic proteins could not be detected with these methods, or by common SDS-PAGE and immunoblotting. However, using affinity chromatography followed by HPLC and chemiluminescence immunoblotting [101], synaptotagmin was detected for the first time in human CSF. No other synaptic proteins were observed using this method.

Albumin can be removed from CSF using affinity columns [126, 128]. However affinity columns are not ideal because of non-specific binding to the column and loss of proteins. Preparative IEF has a unique ability to enrich low-abundance proteins from complex biological mixtures in sufficient quantities for detection by immunoblotting (the present study) or MALDI-MS [129],

without further purification. Other apparatus like the Gradiflow [130] or the μsol-IEF device [131] also showed the necessity of prefractionation when analysing complex samples like CSF or serum.

Synaptic proteins have previously been shown to be reduced in AD brain, and are correlated with the severity of dementia [31]. Previously, a marked reduction of synaptotagmin was found in the hippocampus and frontal cortex of EAD patients compared to controls [101]. In the same study, reduced levels of synaptotagmin were found in pooled CSF of EAD patients compared to controls by SDS-PAGE and ECL immunoblotting, supporting the idea that alterations in the protein composition of the brain are reflected, and can be detected, by analysis of human CSF. Our study showed that several of the synaptic proteins can be identified in CSF to study the synaptic function and pathology in different brain disorders.

## Detection of phosphorylated synapsin in CSF with an ELISA method (Paper II)

A sandwich-ELISA, based on a rabbit anti-phosphorylated synapsin antibody and a synapsin I specific Mab, has been developed for determination of phosphosynapsin I in individual CSF samples. Using this assay, we have measured phosphosynapsin I in CSF of 20 AD patients and 14 controls. The level of phosphosynapsin I was increased in the AD group (0.164±0.15) compared to controls (0.033±0.05) (Figure 9). Phosphosynapsin CSF levels in almost all controls were so low that the protein could not be detected with our ELISA. The synapsin ELISA was standardised using RIPA-buffer-extracted brain homogenate in a serial dilution. Earlier attempts to measure synapsin in individual CSF samples also showed undetectable levels in healthy individuals [132]. In brain tissue, either reduced or unchanged levels of synapsin have been demonstrated in AD [32, 67, 133]. In vitro studies showed reduced phosphorylation of synapsin I in AD postmortem tissues [134] and immunohistochemistry experiments on rats also showed a decreased phosphorylation of synapsin I after transient moderate ischemia [135], suggesting that perturbations of synapsin phosphorylation occur early in pathological states such as AD.

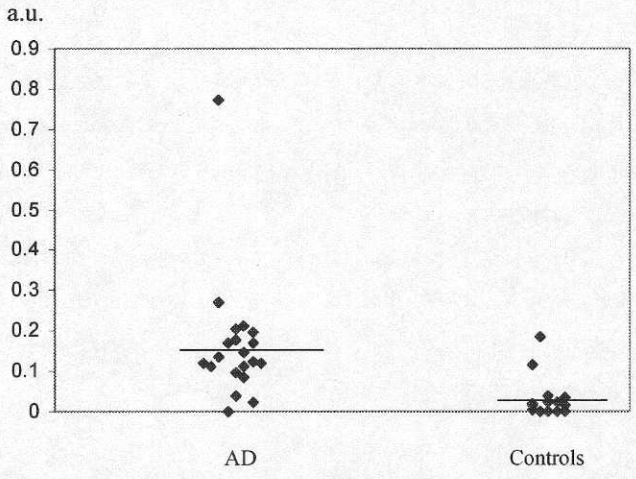


Figure 9. Individual values of phosphorylated synapsin I in AD patients compared to controls measured by ELISA. Values are given as arbitrary units (a.u.) per 70 μg/mL of total protein.

Other proteins involved in AD also showed perturbations in their phosphorylation status, such as the microtubule-associated protein tau [26] and β-tubulin [136], which are abnormally hyperphosphorylated in AD. An imbalance in the protein kinase-protein phosphatase system has been implicated in AD [137], suggesting that other phosphorylated proteins might have phosphorylation perturbations during pathological conditions, such as synapsin.

## Characterisation of CSF proteins by 2D-LPE and MALDI-TOF MS (Papers III and IV)

### 2D-LPE

A strategy employing 2D-LPE and MALDI-TOF MS was used to characterise tryptic digest of proteins in human CSF (Paper III).

In the first dimension, cystatin C was localised in Rotofor fractions 5 to 20, as a single band at approximately 15 kDa, and β–2 microglobulin was localised in Rotofor fractions 5 to 12, as a single band at approximately 11 kDa (immunoblot analyses are shown in Paper III, figure 2).

The Rotofor fractions 8-10, which contained the highest concentration of cystatin C and β–2 microglobulin, were pooled and applied to the Prep cell for separation by SDS-PAGE. Cystatin C was recovered in fractions 60-75 (figure 10a), and β–2 microglobulin in fractions

45-60 (figure 10b). No contaminants were present in those fractions as assessed by inspection of silver-stained SDS-PAGE gels, on which cystatin C and β–2 microglobulin stained as distinct bands (figure 10c).

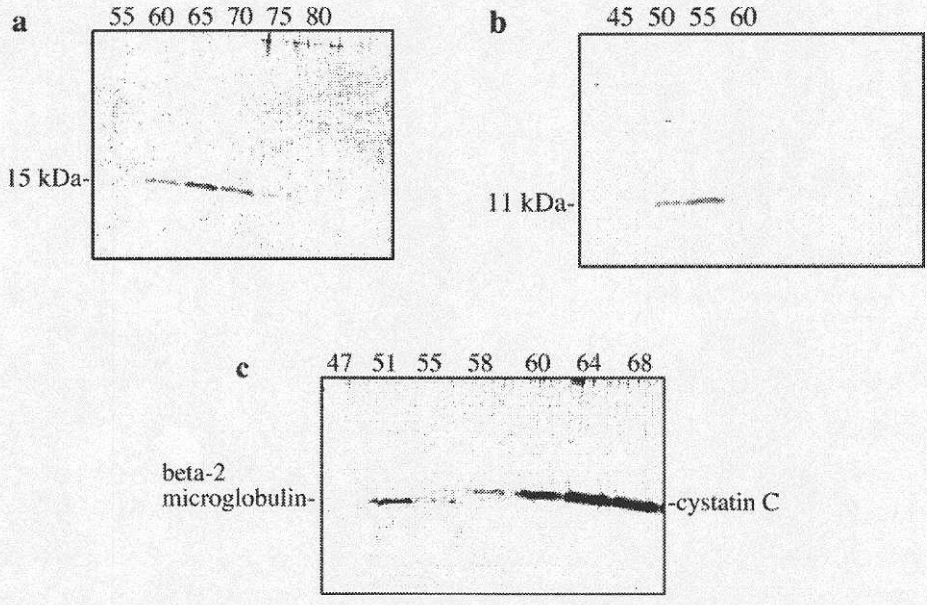


Figure 10. Immunoblot analysis of Prep cell fractions containing (a) cystatin C and (b) β–2 microglobulin. (c) Silver-stained gel analysis of Prep cell fractions

Two CSF proteins, cystatin C and β–2 microglobulin, with concentrations of 3-20 mg/L have been isolated and identified. As shown in this study, proteins with small differences in Mw (13,347 Da and 11,731 Da) can easily be separated by the Prep cell. A limitation of this method was that very low-abundant proteins were diluted in the final elution volume (a few mL), thereby making their detection very difficult.

**Removal of SDS to allow the interface between electrophoresis and mass spectrometry (Paper IV)**

*SDS removal methods*

The presence of ionic detergents, such as SDS, usually severely compromises the MALDI analysis technique [138, 139]. Therefore, SDS has to be removed from the samples before MS

analysis. We tested three different SDS removal methods; solvent extraction (C/M/W), cold acetone precipitation and desalting columns. The C/M/W extraction was the most efficient method of removing SDS, achieving a 1000-fold reduction, regardless of the initial SDS concentration. However, a protein recovery of 50% was obtained, as estimated by examination of silver-stained gels, where both the pellets and the supernatant were analysed (figure 2 in Paper IV). The cold acetone precipitation procedure was less efficient for removal of SDS, but higher amounts of proteins were recovered (80%). The use of desalting columns did not improve either SDS removal or protein recovery. Other methods, such as ion-pair extraction [140] or dialysis, failed to remove SDS efficiently and caused large protein losses.

*Influence of SDS on the sensitivity of protein detection by MALDI MS*

In order to investigate how the sensitivity of MALDI-TOF MS was affected by the presence of SDS in the sample, we analysed cytochrome C in different concentrations of SDS in water. We found that the signal-to-noise ratio of the cytochrome C peak decreased with increasing SDS concentration up to 0.1% SDS (figure 6 in Paper IV) and then increased again when 1% or 5% SDS was present in the sample. However, the high concentrations of SDS resulted in broad peaks due to adduct formation, decreasing the ability of the MALDI MS analysis to distinguish between different protein isoforms. These results are in agreement with results reported by Amado et al.[141]. Using the C/M/W procedure for SDS removal, we obtained a MALDI signal only with the lowest concentration of SDS (approximately 2 $x10^{-4}$%). When we then added the neutral detergent *n*-octylglucoside to the sample, MALDI spectra could be obtained in all protein samples (Paper IV, figure 7), even those containing 0.1% SDS (figure 11).

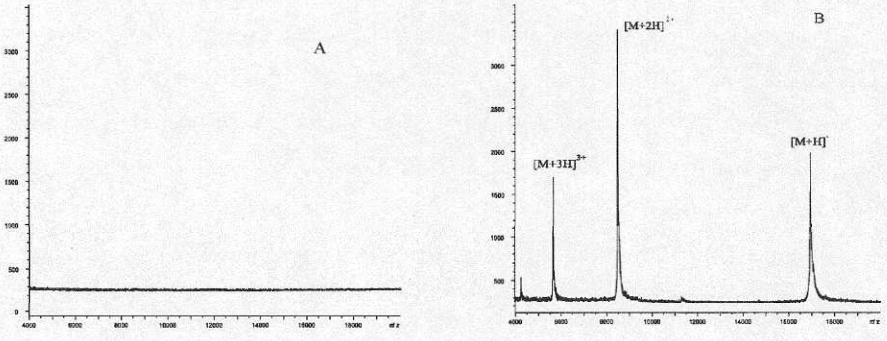


Figure 11. MALDI MS analysis of myoglobin after SDS removal by the C/M/W procedure in the absence (A) and presence (B) of *n*-octylglucoside.

This study showed that even after different types of SDS removal procedures, trace amounts of SDS are still left in the sample, which impairs the MALDI signal. We circumvented this problem by adding *n*-octylglucoside, which attenuated the negative effect of SDS. *N*-octylglucoside is a neutral detergent often used in MS analysis for its solubilisation properties [142, 143]. Comparing the methods, the C/M/W procedure was the most efficient way to remove SDS but the cold acetone precipitation gave a higher protein recovery (80%) and should be used in cases where the initial protein concentration in the sample is low.

**Analysis of Prep cell fractions after SDS removal (Paper III)**

The C/M/W method was chosen to remove SDS from cystatin C and β–2 microglobulin Prep cell fractions. However, one problem observed with the precipitation techniques is that some proteins, like β–2 microglobulin, co-precipitated with SDS, leaving only trace amounts of the protein for MALDI analysis. We therefore used desalting columns to reduce the amount of SDS in the β–2 microglobulin samples and collected only the first 3 mL eluted from the column. MALDI analysis of the tryptic digest from Prep cell fractions confirmed the presence of cystatin C and β–2 microglobulin. The MALDI spectra of tryptic peptides from cystatin C are shown in figure 12. The sequence coverage was 58/120 amino acids or 48 %, which was sufficient to identify the protein by high-accuracy peptide mass mapping and database searching.

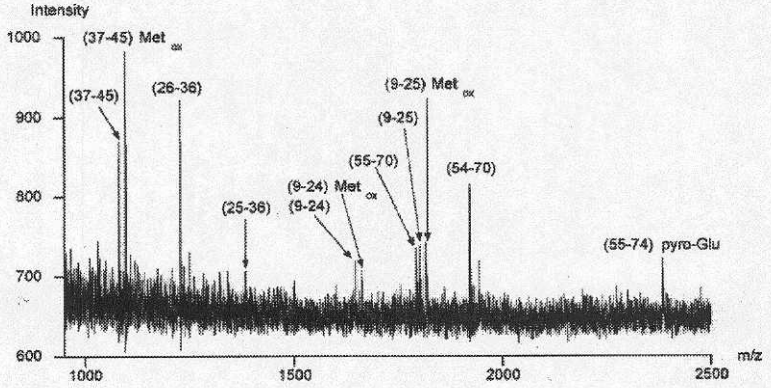


Figure 12. MALDI MS analysis of tryptic digest from a Prep cell fraction containing cystatin C.

In this study, we showed that the combination of 2D-LPE and MALDI-TOF MS is a valuable tool for purification and characterisation of small amounts of proteins. Indeed, proteins can be purified in sufficient amounts to allow both identification and characterisation by MS. 2D-LPE and MALDI-TOF MS have also been used for the characterisation of low-abundance proteins in pleural exudates [129]. Different strategies have been used by other groups, e.g. 2-D preparative electrophoresis for isolation of recombinant virus proteins [144] and in combination with electroblotting of the proteins to a PVDF membrane for purification of hydrophobic proteins from *Candida albicans* [145]. A combination of preparative IEF and reverse-phase HPLC has recently been described for the separation of proteins from a HEL cell line lysate [146].

## Proteome studies of CSF in AD patients and controls

### Protein precipitation procedures before 2-D electrophoresis

CSF contains a high salt but low protein concentration. A high concentration of salt affects the IEF of proteins in the first dimension of 2-DE. For high-quality 2-D gels, the salt has to be removed or decreased and the protein content has to be enriched. Several protein precipitation methods have been compared, including acetone precipitation, ethanol precipitation and Bio-Spin desalting columns.

When analysing 300 µL CSF directly on 2-D gels, no major differences were observed in the 2-D protein pattern between the three different procedures, (figure 13).

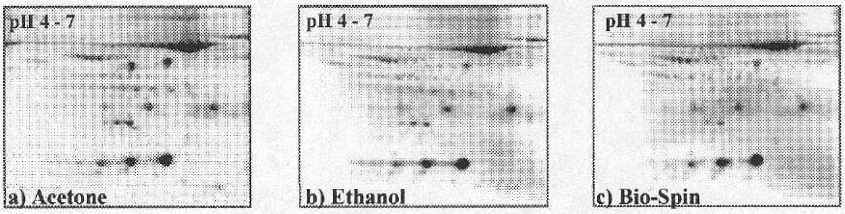


Figure 13. SYPRO Ruby-stained 2-D gels separated on IPG-strips, pH 4-7 in the first dimension with three different sample preparation methods: a) acetone precipitation (1:4 v/v), b) ethanol precipitation (1:4 v/v) c) salt removal with a Bio-Spin column (BioRad). An aliquot of 300 µL CSF was used for each experiment.

Our results were not consistent with results published by Yuan et al.[111], who observed a greater number of spots and a higher protein recovery using Bio-Spin columns compared to acetone precipitation. Many factors might have affected the results, like different gels types, staining procedures or laboratory practice. We chose to use acetone precipitation in direct 2-DE experiments in order to maintain the consistency in our methods and previous studies.In contrast, when analysing prefractionated CSF proteins, the use of ethanol instead of acetone for precipitation improved protein spot focusing, as illustrated in figure 14. It seems that ethanol precipitation removes the ampholytes present in the sample after the liquid phase IEF more successfully. May be are the ampholytes causing some perturbations in the focusing process in the first dimension? When using Bio-Spin columns on prefractionated CSF proteins, a low protein recovery was obtained (data not shown).

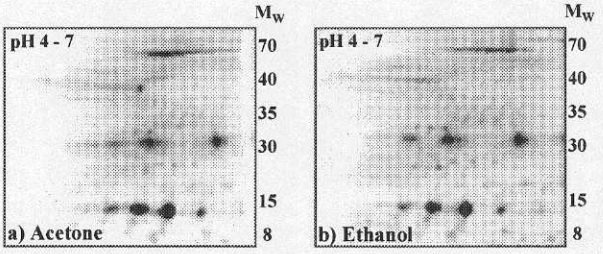


Figure 14. a) acetone precipitation (1:4 v/v) and b) ethanol precipitation (1:4 v/v) of prefractionated CSF proteins (pH 4.5-6.0), separated on IPG-strips pH 4-7.

**2-D gel electrophoresis using micro-narrow range IPG strips (Paper V)**

The partial CSF proteomes of 7 AD patients and 7 controls were compared by loading 300 μL unfractionated CSF on micro-range narrow IPG strips (pH 4.7-5.9). Comparing the intensity of spots between AD and normal controls, 18 spots were found to be significantly up- or down-regulated (Student's t-test, $p<0.05$). The altered proteins are illustrated in figure 15 and those which were identified are summarised in Table 2. Eight proteins were significantly reduced in CSF of AD patients. They were identified as kininogen, one isoform of α-1β glycoprotein, two isoforms of apolipoprotein J (apoJ), three isoforms of apoE, two isoforms of apolipoprotein A1 (apoA1), β-trace (protaglandin D2 synthase), cell cycle progression 8

protein and retinol-binding protein (RBP). Only one protein, one isoform of α-1 antitrypsin (α-AT), was increased in AD patients compared to controls.

Previously detected protein changes including apoE and apoA1 between AD patients and controls [107] were confirmed. Some new proteins altered in AD have also been identified, like kininogen, α-1β glycoprotein, apoJ, β-trace, cell cycle progression 8 protein and α-AT.

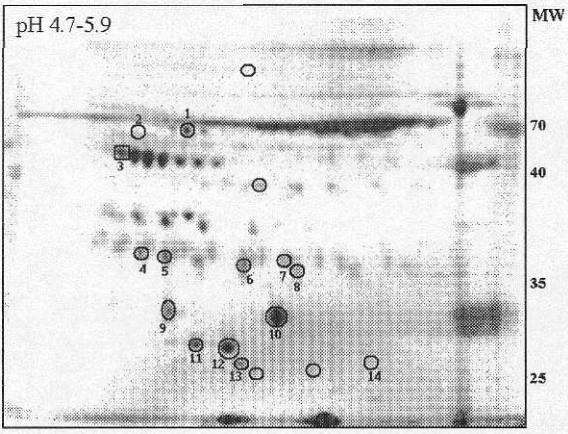


Figure 15. Direct 2-DE analysis of 300 μL CSF on pH 4.7-5.9 IPG strips (7cm) followed by 10% NuPAGE gels with MOPS buffer. Circles represent proteins with reduced levels and squares represent increased levels in AD patients compared to controls.

Table 2. List of identified proteins that are statistically ((t-test) $p<0.05$) altered in CSF from AD patients compared to controls using micro- narrow range strips pH 4.7-5.9 in the 2-DE procedure, and identified by MS as described in Materials and methods. Each protein is listed with the p-value from the Student's t-test. (* MS analysis from an earlier study [147]).

| *Spot* | *Acc nb NCBI* | *Protein identity* | *Theor. Mw* | *Theor. pI* | *Matched peptides* | *Sequence coverage* | *Mass spec.* | *P value* |
|---|---|---|---|---|---|---|---|---|
| 1 | 23503038 | α-1β glycoprotein | 51.87 | 5.51 | 8 | 21 | MALDI | 0.04 |
| 2 | 125507 | kininogen precursor | 69.88 | 6.23 | - | - | MS/MS | 0.01 |
| 3 | 177836 | α-1 antitrypsin precursor | 44.32 | 5.37 | 15 | 47 | MALDI* | 0.01 |
| 4 | 178855 | apoJ | 50.06 | 5.89 | 5 | 20 | MALDI | 0.04 |
| 5 | 178855 | apoJ | 50.06 | 5.89 | - | - | MS/MS | 0.01 |
| 6 | 4557325 | apoE | 34.23 | 5.52 | 10 | 34 | MALDI* | 0.005 |
| 7 | 4557325 | apoE | 34.23 | 5.52 | 10 | 34 | MALDI | 0.02 |
| 8 | 4557325 | apoE | 34.23 | 5.52 | 9 | 34 | MALDI | 0.002 |
| 9 | 730305 | β-trace (protaglandin D2 synthase) | 18.69 | 8.37 | 7 | 40 | MALDI* | 0.04 |
| 10 | 730305 | β-trace | 18.69 | 8.37 | 7 | 40 | MALDI* | 0.006 |
| 11 | 178775 | apoA1 | 28.07 | 5.27 | 8 | 33 | MALDI | 0.02 |
| 12 | 178775 | apoA1 | 28.07 | 5.27 | 6 | 24 | MALDI | 0.006 |
| 13 | 20141667 | retinol binding protein | 21.07 | 5.27 | 6 | 63 | MALDI* | 0.03 |
| 14 | 4758048 | cell cycle progression 8 protein | 44.33 | 9.43 | 7 | 18 | MALDI | 0.02 |

The most clearly affected proteins in AD were several isoforms of apoE and apoA1, as previously shown [107]. They are the major apolipoproteins identified in CSF. In the brain, they're thought to be involved in cholesterol transport and several studies have suggested that the cholesterol metabolism is disturbed in AD [17, 148]. ApoJ, also called clusterin, has been shown to be present in the senile plaques and increased levels have been reported in AD brain [149]. It has also been shown that apoJ is associated with soluble Aβ in CSF [150]. The conflicting data between our study and the previous study on the unchanged CSF apoJ levels in AD [151] might be due to the more sensitive detection of different isoforms of apoJ by 2-DE. Immunological determination of apoJ levels only gives information about the total apoJ immunoreactivity and not the specific differences between the apoJ isoforms. One isoform of RBP was found to be decreased in this study but another isoform was increased in CSF of AD patients in our previous study [107], showing also the importance of studying isoforms of proteins. Increased levels of RBP has been found in AD brains [152].

Several new protein changes associated with AD were demonstrated in CSF using the micro-narrow range IPG strips.

Kininogen, involved in the kallikrein/kinin system, has earlier been linked to AD [153]. Increased cleavage of kininogen was found in CSF of AD patients [154, 155] and is thought to interact with β-amyloid in AD [156].

The function of α-1β glycoprotein, which has sequence similarities with immunoglobulins, is unknown and this protein has never been linked to AD.

Only one protein was significantly increased in CSF from AD patients, i.e. α-AT, a serine protease inhibitor which has previously been localised in NFTs and SPs [157].

**Prefractionation procedure of CSF proteins before 2-DE**

We used 3 mL CSF, which is a clinically available CSF volume. Since the Rotofor cell requires up to 12-15 mL sample volume, the CSF was diluted to 12 mL with water.

When comparing direct and prefractionated 2-D gels (figure 16), it is clear that the prefractionated gels have an increased number of protein spots. Furthermore, the increased intensity of these spots indicates a higher protein concentration. Prefractionation before 2-DE also increases the ability of 2-D gels to separate protein spots that contain different proteins and thereby reduces the risk of having a mixture of enzymatically derived peptides from different proteins in the sample. Furthermore, isoforms of post-translational modifications of different proteins in CSF can be studied with high sensitivity [147].

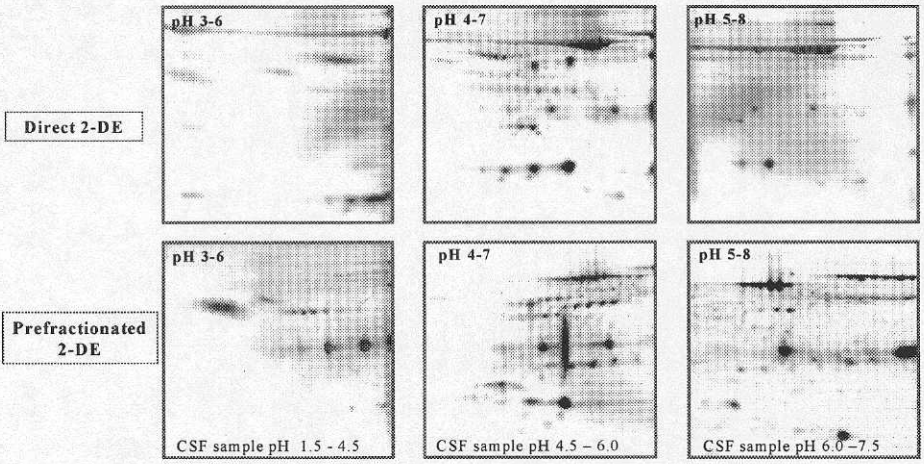


Figure 16. Comparison of direct and prefractionated CSF on SYPRO Ruby-stained 2-D gels. The pH interval of the IPG strips is denoted in the upper left corner of the gels and the pH range of the prefractionated CSF samples at the bottom of the gel images.

The advantages of prefractionation methods are that they reduce the sample complexity and facilitate detection of less abundant proteins. Liquid phase IEF also minimises non-ideal behaviour of proteins such as precipitation or aggregation.

**Analysis of prefractionated CSF proteins before 2-DE in AD patients compared to controls (Paper V)**

CSF samples (3 mL) from 5 AD patients and 5 controls were prefractionated by liquid phase IEF using the Rotofor. For each patient, twenty Rotofor fractions were obtained. They were then pooled and analysed as follows: fractions 2 to 5 on pH 3-6 strips; fractions 6 to 9 on pH 4-7 strips and fractions 10 to 14 on pH 5-8 strips. We found 37 protein spots which were up- or down-regulated at least two times in AD patients compared to controls. 23 spots were down-regulated and 14 spots were up-regulated. The identified altered proteins are presented in Table 3 and illustrated in figure 17 a, b and c.

Table 3. Proteins up or down-regulated at least two times in CSF in AD patients compared to controls using prefractionation of CSF before 2-DE, and identified by MS as described in Materials and methods. (* MS analysis from an earlier study [147]).

| *Spot* | *Acc nb.NCBI* | *Protein identity* | *Theor. Mw* | *Theor. pI* | *Matched peptides* | *Sequence coverage* | *Mass spec. analysis* |
|---|---|---|---|---|---|---|---|
| 1,2,3 | 23503038 | α-1β glycoprotein | 51.87 | 5.51 | 4 | 11 | MALDI |
| 4,5 | 2521983 | α-2-HS glycoprotein | 40.20 | 5.4 | 4 | 9 | MALDI* |
| 6 | 339685 | transthyretin | 13.76 | 5.3 | 6 | 60 | MALDI |
| 7,8 | 177836 | α-1 antitrypsin precursor | 44.32 | 5.37 | 7 | 27 | MALDI |
| 9 | 229995 | β-2 microglobulin | 11.58 | 6.5 | 4 | 46 | MALDI |
| 10,11 | 4557871 | transferrin precursor | 77.03 | 6.9 | 21 | 31 | MALDI |
| 12 | 6013427 | albumin precursor | 69.21 | 5.9 | 13 | 19 | MALDI |
| 13 | 339685 | transthyretin | 13.76 | 5.3 | 8 | 81 | MALDI* |

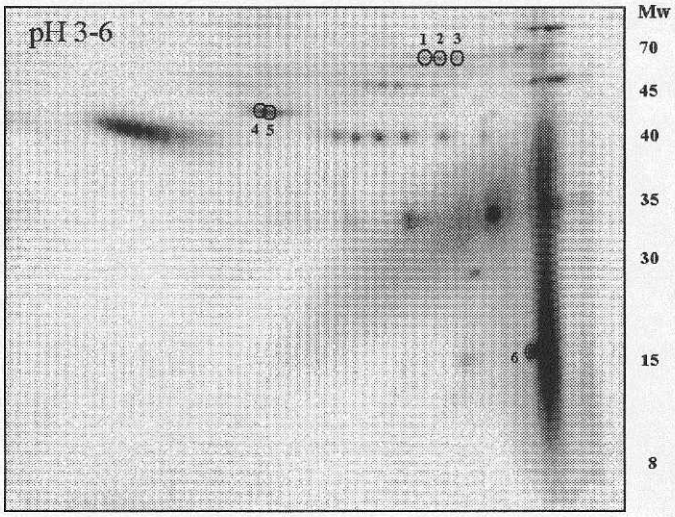


Figure 17a

Figure 17: 2-DE gel of prefractionated CSF proteins. Fraction 2-5 (a) fraction 6-9 (b) fraction 10-14 (c) were analysed on pH 3-6(a), pH 4-7 (b) and pH 5-8 (c) IPG strips respectively, followed by 10% Nu-PAGE gels. Circles denote the protein spots decreased two times in intensity and squares the protein spots increased two times in intensity in CSF of AD patients compared to controls.

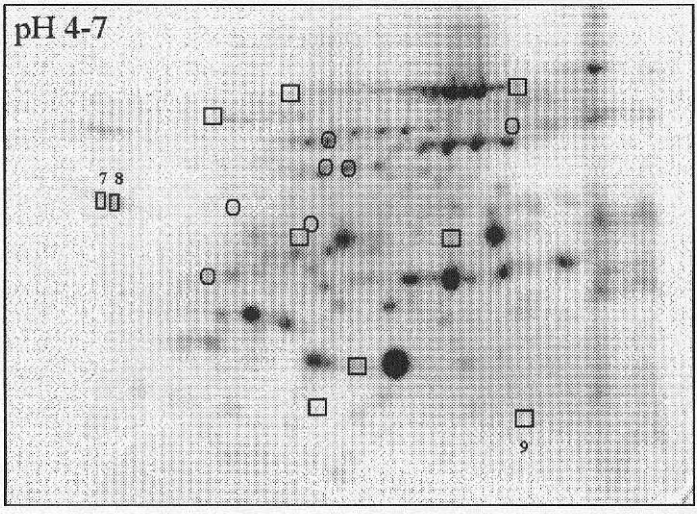


Figure 17b

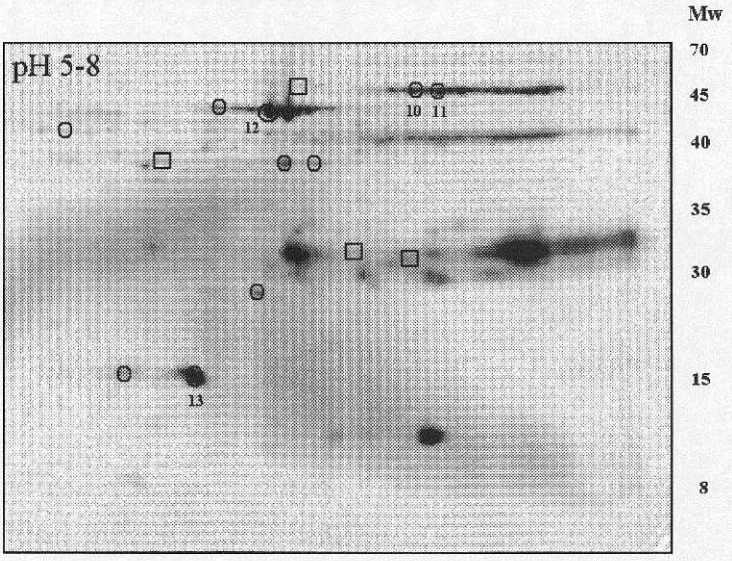


Figure 17c:

Since only 5 AD patients and 5 controls respectively were used in the prefractionation study, we did not perform statistical analyses. Instead, two-fold increase or decrease of the proteins intensity was taken in account in order to identify all possible protein candidates. Our results are consistent with our previous results using direct 2-DE, which further indicates the implication of those proteins in AD pathology. One protein, α-2-HS glycoprotein (or fetuin), implicated in bone growth and remodelling [158], has never previously been associated to AD.

Surprisingly, none of the identified proteins in the prefractionation 2-DE study were apolipoproteins. This might be explained by the fact that lipoproteins tend to adhere to plastic vials [96] and could be lost in the additional sample transfer from one test-tube to another needed in the prefractionation procedure.

As shown in this study and in our previous study [107], different isoforms of the proteins are altered in AD. Several of the altered CSF proteins in AD are glycosylated, such as apoE, apoJ, α-2-HS glycoprotein, α-1β glycoprotein, α-1 antitrypsin and β-trace. Therefore, the determination of post-translational protein modifications is of importance in studies of the neuropathology in AD.

## Potential CSF biomarkers in AD

The use of micro-narrow range strips and a prefractionation step before 2-DE have increased the detection of proteins in CSF and identified several new protein changes in AD. In the present study, the levels of 14 proteins and their isoforms were altered in AD CSF compared to the controls. Some of the identified proteins have already been examined as biochemical markers for AD, including apoE [96, 159], apoJ [151] and TTR [159].

Only a few proteomic studies have been carried out to compare differences between AD patients and healthy individuals [107, 160, 161]. In the study by Johnson et al., one isoform of α-2 haptoglobin was identified and this protein was increased in AD patients compared to controls [160]. Altered CSF levels of cystatin C and two isoforms of β-2 microglobulin were recently found using the surface-enhanced laser desorption/ionization (SELDI) technique [162].

In our recent proteomic study of CSF proteins in FTD, the levels of six proteins and their isoforms were altered in FTD patients compared to controls, including granin-like neuroendocrine precursor, protein pigment epithelium-derived factor, RBP, apoE and haptoglobin [163]. All proteomic data showed that several proteins involved in FTD pathology were not influenced in CSF of AD patients, i.e one specific protein pattern was obtained for AD patients and one for the FTD patients. By using a protein pattern, like in 2-D gels, we are not limited by the sensitivity and specificity of any single biomarker. Therefore the use of a set of biomarkers derived from proteomic analysis is a good way for separating complex neurodegenerative disorders such as dementia.

As shown in this study, different isoforms of the proteins are altered in AD. Many of the altered proteins identified by 2-DE are glycosylated, e.g. apoE, apoJ, α-2-HS glycoprotein, α-1β glycoprotein, α-1 antitrypsin, and β-trace (protaglandin D2 synthase). Furthermore, our ELISA results show increased levels of phosphosynapsin I in AD compared to controls.

Therefore, the determination of post-translational protein modifications such as glycosylation or phosphorylation states is of importance for an understanding of the neuropathology in AD.

An important purpose of proteomic research is to going on understanding of the cellular function at the protein level. Table 4 correlates each protein identified in the present study with its function in an attempt to clarify the function of the CSF proteins and explore the molecular mechanisms of AD.

Table 4: Classification of altered proteins in AD by function.

| *Function* | *Proteins* |
|---|---|
| Signal protein | β-2 microglobulin, kininogen |
| Transport and binding protein | transthyretin, apoE, apoA1,apoJ, albumin, retinol-binding protein, transferrin. |
| Growth factor /regulation protein | α-2-HS glycoprotein, cell cycle 8 protein |
| Inhibitor | α-1-antitrypsin |
| Enzyme/catalysator | β-trace (protaglandin D2 synthase) |
| Unknown | α-1β glycoprotein |

The use of complementary strategies in proteome studies of CSF offers new perspectives on the pathology of neurodegenerative diseases and also reveals new potential biomarkers for AD. After further verification, proteins like α-2-HS glycoprotein, kininogen, α-1β glycoprotein or α-1 antitrypsin could perhaps be used together as biomarkers for AD.

## Analytical and /or preparative 2-DE for studying CSF proteins ?

Today, around 40 to 45 proteins have been identified in human CSF using 2-DE and MS [107-111, 161]. The identified proteins are mainly the most abundant proteins in CSF, because low-abundance proteins are masked by the highly abundant albumin and immunoglobulins. Not all CSF proteins can be detected with the 2-DE technique; indeed low-abundance, basic proteins and membrane proteins are very difficult to detect and analyse [164]. In order to detect low-abundance proteins in CSF, increased protein amounts have to be loaded on the 2-D gel, but this did not result in better detection as disturbance of the 2-D protein pattern was observed [108]. In order to facilitate the detection of low-abundance proteins in CSF, various techniques have earlier been described, e.g. affinity chromatography, gel filtration and HPLC,[103, 165] but only a few more CSF proteins could be detected [166]. Also, the risk of losing proteins in long, fastidious, multiple-step procedures is increased.

Another two-step electrophoretic procedure, involving liquid phase IEF in the Rotofor cell in combination with one-dimensional electrophoresis and electroelution, has been used for purification and identification of proteins from human CSF [167]. Several low-abundance neuron-related proteins have been identified in human CSF and human brain tissue using this

technique [168]. Recently, the use of IEF as prefractionation before 2-D electrophoresis was shown to be advantageous as less abundant proteins are enriched [147]. With preparative 2D-LPE, the conditions are optimised for separation of proteins in a specific molecular weight region and it provides a high separation capacity and higher loadability than analytical 2-DE. 2D-LPE is therefore a useful method for isolation of proteins in large quantities, allowing easier characterisation by MS or chromatography. However, 2D-LPE cannot be used for screening studies such as analytical 2-D electrophoresis.

Another challenge when analysing CSF is the high amount of salt. Several methods have been used to remove salt, such as desalting columns, dialysis or protein precipitations with acetone, ethanol or TCA/acetone [111]. In our experience, acetone was better in direct 2-DE experiments, while ethanol precipitation was more appropriate when prefractionation of CSF was used. Dialysis was used to remove salts before liquid phase IEF, as it is a smoother method, avoiding protein losses. A further limitations of 2-DE is its limited suitability for analysis of high molecular weight proteins (>100kDa) as they are often difficult to solubilise and do not enter into the IPG strip. One alternative recently proposed was the use of IPG gels containing agarose instead of polyacrylamide [169].

Basic membrane proteins are also often absent in 2-D gels and although many attempts have been made to ameliorate the resolution of commercial basic IPG strips, the results are not as good as with the acidic gels [164]. Finally, small proteins and peptides (<10kDa) are almost impossible to identify by MALDI because of the small number of peptides generated by enzymatic cleavage. In this case, other techniques could be used, like Q-TOF-MS/MS or capillary electrophoresis coupled to MS.

In a CSF proteomic approach, direct 2-DE or a combination of liquid-phase IEF and 2-D gels will be used to study the expression and comparison of disease-related proteins, while preparative 2-D electrophoresis will be used to identify and characterise, for example, low-abundance proteins or membrane proteins.

# Conclusions

To find potential biomarkers with the focus on AD, new proteomic strategies for detection, identification and characterisation of proteins in CSF have been used. With liquid phase IEF and immunoblotting, we identified six low-abundance synaptic proteins (ng/L) in human CSF, namely rab 3a, synaptotagmin, GAP-43, SNAP-25, synapsin and neurogranin. In order to purify and characterise proteins in CSF, a new strategy was developed, combining 2D-LPE and MALDI-TOF MS. Two brain-specific proteins, cystatin C and β2 microglobulin, were isolated from CSF in sufficient quantities for analysis by MALDI-TOF MS. Special attention was needed to make 2D-LPE and mass spectrometry compatible, and chloroform/methanol/water extraction was the most efficient and allowed the acquisition of good quality MALDI spectra of the tryptic digest of the proteins analysed. Comparative proteomic methods, i.e. 2-DE electrophoresis, mass spectrometry and prefractionation before 2-DE, have been used for comprehensive analysis of CSF proteins in AD patients and controls. Thirteen proteins and their isoforms were altered in AD patients compared to controls, including apoE, apoA1, apoJ, β-2 microglobulin, retinol-binding protein, transthyretin, kininogen, β-trace, transferrin and α-1 antitrypsin as well as proteins that have never been linked with AD such as, cell cycle progression 8 protein, α -1 β glycoprotein and α 2-HS glycoprotein. Many of the altered proteins in AD are glycosylated proteins. As shown in this study, determination of post-translational modifications is of importance for an understanding of the neuropathology of AD. An ELISA method for quantification of the phosphorylated form (Ser 9) of synapsin I has also been designed. Increased levels of phosphosynapsin I were demonstrated in AD patients compared to controls.

In a proteomic approach, 2-DE or a combination of liquid-phase IEF and 2-DE will be used for studying expression and post-translational modifications of disease-related proteins, while preparative 2-DE will be used to identify and characterise proteins. Complementary proteomic studies of CSF revealed new potential protein markers for AD.

# Populärvetenskaplig sammanfattning på svenska

## Bakgrund

Alzheimer's sjukdom (AD) är en den vanligaste demenssjukdom i västvärlden. Man hittar hos AD patienter en ökad förekomst av senila plack (område som består av en peptid, β-amyloid som aggregerats) och neurofibriller (ansamling av förändrade proteiner som liknar fibrer) i hjärnans celler, jämfört med vad icke-dementa äldre personer har, samt en förlust av nervceller (neuron) och minskad antal kontakter mellan neuroner (synapser). Trots omfattande forskning, är sjukdomsmekanismerna vid AD fortfarande okända. Genom att karakterisera och studera olika neuron proteiner, som på olika sätt är associerade med AD är förhoppningen att man bättre skall förstå sjukdomens patofysiologi. För att studera dessa proteiner på patienter är studier i ryggvätska (likvor) en möjlig väg. De flesta proteiner förekommer dock i mycket små mängder i likvor, och målet med denna avhandling har varit dels att utveckla analytisk och preparativ metodik för att kunna studera proteiner och dels använda dessa nya metoder för att studera eventuella förändringar i likvor hos AD patienter.

## Resultat

Med hjälp av preparativ isoelektrisk fokusering i vätskefas (där proteiner skils åt beroende på deras massa och laddning) och immunoblotting (igenkänning med hjälp av specifika antikroppar) har vi lyckats isolera och identifiera sex lågförekommande synapsproteiner i likvor, nämligen rab3a, synaptotagmin, synapsin, SNAP-25, GAP-43 och neurogranin. Vi har också satt upp en ELISA metod (baserad på antikroppar) för bestämning av den fosforylerade formen av synapsin I i likvor och funnit ökade nivåer av detta protein hos AD patienter jämfört med kontroller. Dessa resultat överensstämmer med hypotesen om att det föreligger en obalans i protein fosforylerings mekanismer hos AD patienter.

För att isolera och karakterisera proteiner i likvor, har vi utvecklat en ny strategi som kombinerar preparativ två-dimensionell gel elektrofores i vätskefas (2D-LPE) med masspektrometri. Med 2D-LPE, kan vi separera proteiner med avseende på laddning (1:a dimension), därefter med avseende på massa (2:a dimension) och till sist identifiera dem med masspektrometri. För att kunna koppla ihop elektrofores i vätskefas och masspektrometri har vi varit tvungna att ta fram en metod för eliminering av SDS (en detergent) från våra proteinprover. Två hjärn-specifika proteiner cystatin C och β-2 mikroglobulin isolerades från likvor i tillräcklig mängd för en lyckad masspektrometrisk analys.

Vi har använt analytisk två-dimensionell gelelektrofores och masspektrometri för att undersöka vilka sjukdomsspecifika proteiner som är förändrade i likvor hos AD patienter jämfört med kontroller. Tidigare kända fynd såsom att apolipoprotein E och apolipoprotein A1 var påverkade, kunde konfirmeras. Dessutom hittade vi nya proteiner såsom kininogen, apolipoprotein J, β-trace, α -1 β glycoprotein, α 2-HS glycoprotein som förekom i minskade nivåer och ett protein α-1 antitrypsin, som förekom i ökade nivåer hos AD patienter. Tre proteiner visades, för första gången ha en koppling till AD, nämligen cell cycle progression 8 protein, α -1 β glycoprotein och α 2-HS glycoprotein.

## Konklusion

Många av de proteiner som förekom i ändrad mängd hos AD patienter är fosforylerade (innehåller en fosfat grupp) eller glykosylerade (innehåller sockerkedjor) vilket visar vikten av att i framtiden, studera de post-translationella modifieringarna av proteiner.

Att använda olika "proteomik" metoder öppnar nya vägar för förståelsen av neurodegenerativa sjukdomar samt visar nya potentiella biomarkörer för demenssjukdomar som AD.

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