Proteomic strategies for analysis of cerebrospinal fluid in neurodegenerative disorders

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Tillägnas min mormor,
Ingrid Elisabet Caspersson.
Ditt stora hjärta och din varma humor gör dig oförglömlig - trots att ditt eget minne svek dig.

”Den som ej natten gör till dag,
att leva, att leva, att leva ej förstår”

Skål mormor, i himmelen

ABSTRACT

There is a great need for biomarkers to diagnose neurodegenerative disorders, such as the cognitive disorders Alzheimer's disease (AD) and frontotemporal dementia (FTD). Cerebrospinal fluid (CSF) is in contact with the extracellular fluid of the brain and is consequently a valuable medium for identifying biomarkers for neurological disorders. Biomarkers can be used for early identification of disease, to facilitate homogenous classification and to extend our basic knowledge of disease pathogenesis. Proteomics, an approach for biomarker discovery, generally combines various separation techniques with mass spectrometry (MS) and bioinformatics to identify and characterize proteins, reflecting a defined state at a specific time point. The aim of this thesis was to develop and evaluate proteomic strategies for analysis of CSF proteins to reveal disease mechanisms and identify potential biomarkers to distinguish AD from FTD.

Two approaches to improve the detection of CSF proteins by two-dimensional gel electrophoresis (2-DGE) were used. First, to enrich the proteins, CSF was prefractionated using liquid phase isoelectric focusing followed by 2-DGE profiling. Secondly, zoom 2D gels increased protein separation directly in the gels. These studies showed that in the CSF proteome of AD and FTD patients several proteins were differentially expressed, suggesting that different mechanisms are involved in the pathogenesis of these disorders.

To validate some of the findings from the 2-DGE studies, β-trace, transthyretin (TTR), α-1-antitrypsin and cystatin C (CysC) were quantified in CSF. The concentrations of all these proteins, previously shown to bind amyloid-beta (Aβ) peptides, were reduced in AD CSF, while only CysC and β-trace were reduced in FTD. Furthermore, we found a strong positive correlation between β-trace, TTR and CysC, and levels of Aβ peptides specifically in the AD group, suggesting that a lack of proteins binding to Aβ peptides in AD CSF might cause increased extracellular Aβ aggregation, a major pathological hallmark in the AD brain.

Additionally, we showed that incorrect storage conditions can influence the isoform levels of some CSF proteins. Thus, standardization of CSF sample handling is important in avoiding ambiguous results. Furthermore, very low-abundant neuron specific tau protein isoforms, were for the first time characterized in CSF using a targeted immunoprecipitation-MS approach, opening up new possibilities for further differentiation of tauopathies, including AD and FTD.

Key words: Alzheimer's disease, cerebrospinal fluid, frontotemporal dementia, neurodegeneration, proteomics, mass spectrometry, prefractionation, protein identification, quantification.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Orsakerna till att människor drabbas av demenssjukdomar som bryter ner nervcellerna i hjärnan, till exempel Alzheimers sjukdom (AD) och frontallobsdemens (FTD) är fortfarande i de flesta fall okända. För att studera orsaken till sjukdomarna kan man undersöka ryggvätskan, som flödar runt hjärnan och ryggmärgen. Ryggvätskan kan spegla de processer som sker i hjärnan. Syftet med denna avhandling var att undersöka om det finns några proteiner som är förändrade i ryggvätskan hos sjuka patienter jämfört med jämngamla ej dementa personer och om dessa proteinförändringar även kan användas för att skilja AD och FTD åt.

Proteomik är ett forskningsfält där identifiering, kvantifiering och karaktärisering av proteiner i biologiska material är i fokus och ofta kombineras olika analytiska tekniker. En svårighet med att studera proteiner i ryggvätska är att de proteiner som finns i störst koncentrationer härstammar från blodet. Därför måste teknikerna ofta anpassas för att anrika och separera ut de låg-förekommande proteiner som kan spegla hjärnans processer. Genom två-dimensionell gelelektrofores (2-DGE), separeras proteiner i en gelmatris med avseende på laddning och sedan massa. Proteinerna kvantifieras efter infärgning av gelen och förändrade proteiner kan skäras ut. Två tillvägagångssätt användes för att öka detektionen av proteiner från ryggvätska m.h.a. 2-DGE. Dessa var antingen ett anrikningssteg före analysen eller zoom 2D geler, vilken ökade separationen av proteiner direkt i gelen. Andra metoder som har användes för att separera ut proteiner före masspektrometrisk analys var antingen olika kemiska ytors förmåga att binda upp grupper av proteiner eller specifika antikroppars förmåga att binda ett mål protein. För protein identifiering användes masspektrometri följt av databassökningar. I korta drag klyvs utseparerade proteiner först med specifika enzymer till mindre bitar, s.k. peptider. Massorna av dessa peptider bildar ett mönster som är unikt för varje protein, som ett fingeravtryck. Peptidernas massa bestäms genom masspektrometri och detta peptidmassmönster identifierar proteinet genom jämförelse med teoretiska mönster av proteiner i databaser. Peptiderna kan också, i vissa typer av masspektrometrar, sönderdelas till ännu mindre fragment som speglar aminosyrasekvensen varmed en ännu mer specifik databassökning för proteinidentifiering erhålls.

2-DGE studierna indikerade att flera proteiner förekom i olika nivåer i ryggvätska vid AD jämfört med FTD. Några av dessa förändrade proteiner, som hade förmåga att binda till amyloid-beta (Aβ) peptider (β-trace, transtyretin, α-1-antitrypsin och cystatin C) kvantifierades i ryggvätska från ett större antal AD- och FTD patienter samt ej dementa kontroll personer. Nivåerna av alla proteinerna var sänkta hos AD patienter varav transtyretin och α-1-antitrypsin var specifikt sänkta vid AD jämfört med FTD. Dessutom korrelerade nivåerna av transtyretin, cystatin C och β-trace starkt med nivåerna av Aβ peptider i ryggvätskan vilket indikerar att den Aβ-bindande förmågan kan vara sänkt i ryggvätska specifikt vid AD och vara involverad i sjukdomsmekanismen med ökad Aβ aggregation, vilket föreslagits kunna vara en initial process vid AD. Dessutom visade en studie i avhandlingen att undermålig förvaring av ryggvätskeprover kan ge upphov till proteinförändringar, och därför krävs standardisering av prov hanteringen för att undvika att resultat som inte speglar sjukdomsprocessen erhålls. Slutligen kunde vi för första gången karaktärisera låg-förekommande tau protein isoformer i CSF genom att använda en riktad immunoaffinitets-MS metod.

PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Validation of a prefractionation method followed by two-dimensional electrophoresis - Applied to cerebrospinal fluid proteins from frontotemporal dementia patients.
Hansson SF, Puchades M, Blennow K, Sjogren M, Davidsson P. *Proteome Sci. 2004 Nov 18;2(1):7.*

II. Proteomic studies of potential cerebrospinal fluid protein markers for Alzheimer's disease.
Puchades M*, **Hansson SF***, Nilsson CL, Andreasen N, Blennow K, Davidsson P. *Brain Res Mol Brain Res. 2003 Oct 21;118(1-2):140-6.*

III. Reduced levels of amyloid-β-binding proteins in cerebrospinal fluid from Alzheimer's disease patients
Hansson SF, Andreasson U, Wall M, Skoog I, Andreasen N, Wallin A, Zetterberg H, Blennow K. *Submitted.*

IV. Cystatin C in cerebrospinal fluid and multiple sclerosis.
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Related publications not included in this thesis:

Amyloid β_{1-40} quantification in CSF: comparison between chromatographic and immunochemical methods.
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Davidsson P, **Folkesson S**, Christiansson M, Lindbjer M, Dellheden B, Blennow K, Westman-Brinkmalm A. *Rapid com mass spectrom. 2002 Oct 16;2083:2088.*

Please note the change in surname from Folkesson to Hansson.

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ABBREVIATIONS

2D	Two-dimensional
2-DGE	Two-dimensional gel electrophoresis
AAT	α-1-antitrypsin
Aβ	Amyloid-β
AD	Alzheimer's disease
CID	Collision induced dissociation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CysC	Cystatin C
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FTD	Frontotemporal dementia
FT-ICR	Fourier transform ion cyclotron resonance
IEF	Isoelectric focusing
IP-MS	Immunoprecipitation-mass spectrometry
LC	Liquid chromatography
LTQ	Linear ion trap
LP-IEF	Liquid phase isoelectric focusing
MALDI	Matrix-assisted laser desorption/ionization
m/z	Mass-to-charge
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
M_W	Molecular mass
NFTs	Neurofibrillary tangles
P-tau	Phosphorylated tau
p*I*	Isoelectric point
PMF	Peptide mass fingerprint
RP	Reverse phase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELDI	Surface enhanced laser desorption/ionization
SPs	Senile plaques
T-tau	total tau
TOF	Time-of-flight
TTR	Transthyretin
QTOF	Quadrupole time-of-flight
QIT	Quadrupole ion trap

INTRODUCTION

1. The central nervous system

The human central nervous system (CNS), consisting of the brain and the spinal cord, is one of the most complex structures known. The CNS is responsible for monitoring and coordinating body functions as well as creating memories and cognition. Cognition is the mental process of knowing, including aspects of awareness, perception, reasoning and judgement.

The basic structural and functional unit of the nervous system is the neuron (figure 1). Within the CNS, neurons are interconnected by synapses, transporting the neuronal signal through an intricate network. The neuron has several dendrites and one axon, often extensively branched at the distal end. The neuronal signal enters the dendrites, passes through the cell body and along the axon as an electrical impulse, until it reaches the synapse where it causes the release of a signal substance that traverses the synaptic cleft to a receptor on the dendrite of the next neuron. This conducts the neuronal signal forward through the network.

The neurons are surrounded by other cell types, which maintain the neuronal environment and contribute to a rapid transmission of information. The basic types of non-neuronal cells in the CNS include astrocytes providing support and nourishement, oligodendrocytes for myelination of axons, microglia involved in immunological functions and ependymal cells producing cerebrospinal fluid (CSF). The complex functions of cells in the CNS demand constant and rigorously controlled synthesis, modification and degradation of proteins and peptides. Since proteins and peptides are the functional units responsible for most biological processes in an organism their expression is dynamic and will continuously be adapting to changes in the environment, state of development or disease.

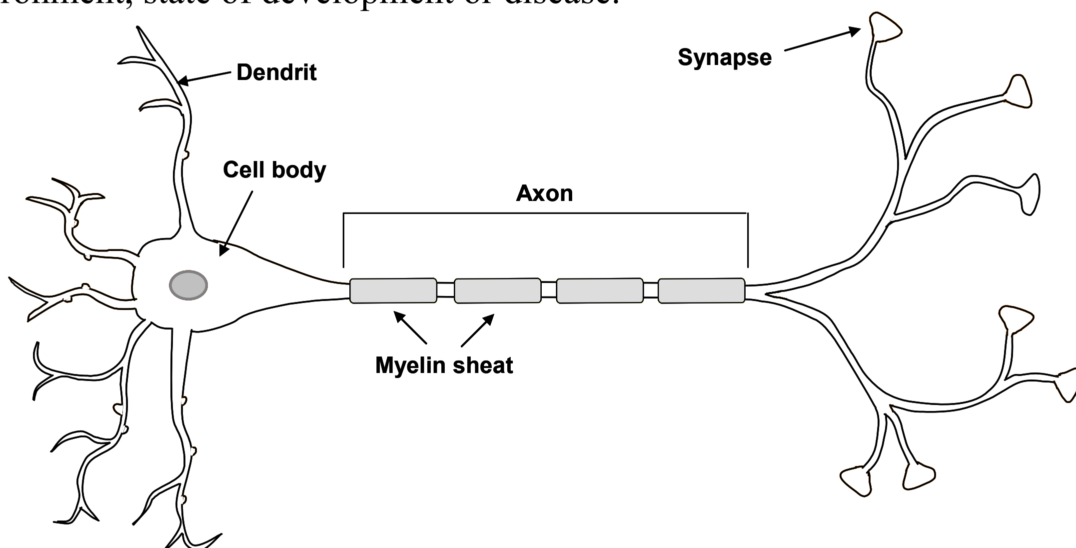


Figure 1: A schematic picture of a neuron.

2. Diseases of the central nervous system

Due to its complexity, diseases of the CNS are often devastating, affecting the very essence of the individual. In this thesis the cognitive and primary neurodegenerative disorders Alzheimer's disease (AD) and frontotemporal dementia (FTD) have been the main focus, but CSF from patients with the inflammatory, demyelinating disorder multiple sclerosis has also been studied and these disorders will be further described below.

2.1 Alzheimer's disease

AD, first described by Alois Alzheimer in 1906 [1, 2] is the most common dementia disorder, responsible for about 50-60% of all cases of dementia. The prevalence of AD increases continuously with age and is estimated to 0.5-0.8% in the age group 65 to 69 years and 20-25% at the age of 90 years and older [3-6].

2.1.1 Diagnosis and clinical manifestation

The diagnosis of AD is based on the criteria from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [7]. These criteria depend largely on exclusion of other CNS disorders or dementias. In clinical practice the diagnosis of AD is often also based on the criteria of the International Classification of Disease, 10th revision (ICD-10) [8] as well as criteria for dementia by the Diagnostic and Statistic Manual of Mental Disorders (DSM-IV) [9]. These criteria for AD have several requirements in common, including an insidious onset of the disease with a progressive loss of memory and increasing difficulties in organizing different tasks, while the absence of confusion and dementia caused by other disorders must be ruled out. At an early stage of AD motor, sensor and linguistic abilities can be relatively intact. Later stages of the disease lead to a global cognitive impairment, language disabilities, practical problems and change in personality [10].

2.1.2 Genetics and risk factors

The majority of AD cases are sporadic where no obvious genetic factors can be found. Recent twin studies however, estimated the heritability of sporadic AD to 58-79%, suggesting that mainly unidentified genetic factors are influential on the risk of developing AD [11]. Today the most confirmed risk factors for sporadic AD are increased age, first degree relative with dementia and the presence of the *ε4* allele of the apolipoprotein E gene (*APOE*). *APOE ε4* has been established as a risk factor for both sporadic AD [12, 13] and late-onset familial AD [14] with each *ε4* allele increasing the risk and reducing the age of onset.

Highly penetrant familial autosomal inherited forms of AD have been estimated to account for less than 1% of all AD cases [15, 16]. These familial forms have mutations in the amyloid-β precursor protein (APP) gene [17] located on chromosome 21, or genes involved in the processing of APP, i.e. presenilin-1 [18] or presenilin-2 [19] located on chromosome 14 [18] and chromosome 1 [20], respectively. All these mutations generally lead to increased production of amyloid-β (Aβ) [21].

2.1.3 Neuropathology

Even though the clinical diagnosis has a relatively high accuracy rate (80-90%) [22] the definite diagnosis of AD can only be made post-mortem [7]. The neurodegeneration in AD starts in structures of the medial temporal lobe (enthorinal cortex, amygdala and hippocampus), progressing into the temporal and parietal cortex and finally reaching the frontal cortex [23]. Microscopic changes characterizing AD, initially identified by Alois Alzheimer, are the presence of extracellular senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs), ultimately leading to neuronal degeneration and synaptic dysfunction [24]. The primary component of SPs is Aβ-peptides [25], whereas the NFTs consist of hyperphosphorylated and filamentous forms of the microtubule-associated protein tau [26].

2.1.4 Aβ peptides

Aβ is produced from APP [27] by the combined action of β- and γ-secretase (figure 2). This amyloidogenic pathway starts with the action of β-secretase producing a soluble β-sAPP fragment. The remaining membrane-bound part of APP is further cleaved inside the membrane by the γ-secretase complex, producing the Aβ peptide. Depending on the site of cleavage the Aβ peptide can be of different lengths (37-43 amino acids).

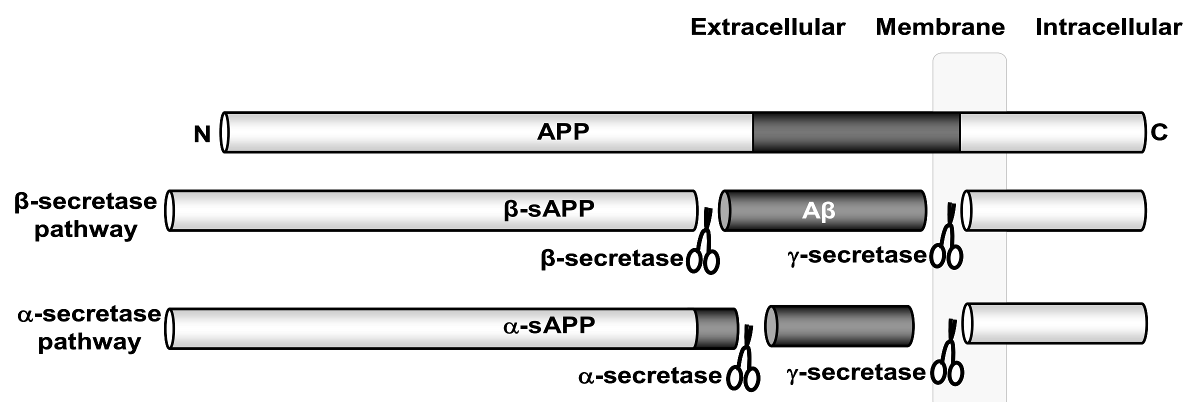


Figure 2: Proteolytic cleavage of APP. In the β-secretase pathway, APP is first cleaved by β-secretase and the following γ-secretase cleavage produces Aβ. In the α-secreatase pathway, α secreatase cleaves APP inside the Aβ region.

Among the Aβ species produced, the 42 amino acid form (Aβ1-42) has been suggested to be of major importance in the pathogenesis of AD because it has a greater tendency to form amyloid fibrils [28-30] and it is the peptide initially deposited in SPs [31, 32]. An alternative APP processing pathway is the non-amyloidogenic pathway, where α-secretase cleaves inside the Aβ region, precluding formation of the fibrinogenic Aβ1-42 and generating soluble α-sAPP, which might be neuroprotective [33, 34] (figure 2).

Aβ accumulation in the AD brain may occur by several ways, including overproduction due to increased amyloidogenic cleavage of APP in the brain, inadequate degradation, or an imbalance between import and export of Aβ or Aβ-binding proteins at the brain barriers. The amyloid cascade hypothesis suggests that abnormal Aβ accumulation is the primary event in AD pathogenesis [35, 36]. Initially, this suggestion was based on the finding that the APP gene is localized to chromosome 21 [27], coupled to the earlier recognition that trisomy 21 (Downs syndrome) leads invariably to the neuropathology of AD [37]. The hypothesis was further supported by studies showing that familial AD was caused by mutations in APP, presenilin-1 or presenilin-2, which generally increase production and accumulation of Aβ into plaques [21]. A more recent and broadly supported variation of the amyloid hypothesis identifies the cytotoxic species as an intermediate misfolded form of Aβ, neither a soluble monomer nor a mature aggregated polymer but an oligomeric specie [38]. Even if the amyloid cascade hypothesis is the prevailing idea there is still an ongoing debate and a precise mechanism between Aβ accumulation and NFTs formation has not been shown.

2.1.5 Tau protein

Tau, a protein particularly abundant in the axons of neurons, has the primary function of stabilizing and promoting the assembly of microtubules, by binding to their tubulin monomers [39]. Microtubules are key cytoskeletal elements maintaining the morphology of neurons as well as transporting nutrients, signalling molecules, vesicles and other substances. Thus, tau has an important effect on axonal transport and on the function and viability of neurons and their highly extended axons [40].

There are six major isoforms of the tau protein expressed in the adult human brain, which are derived from a single gene on chromosome 17, through alternative RNA splicing of exones 2, 3 and 10 [41]. Absence or presence of the 10th exon results in a tau protein containing three (3R) or four (4R) repeats of highly conserved microtubule binding motifs [42]. Furthermore, the N-terminal region of tau can contain both exon 2 and 3 (2N), only exon 2 (1N) or lack both of these exons (0N) [41] (figure 3).

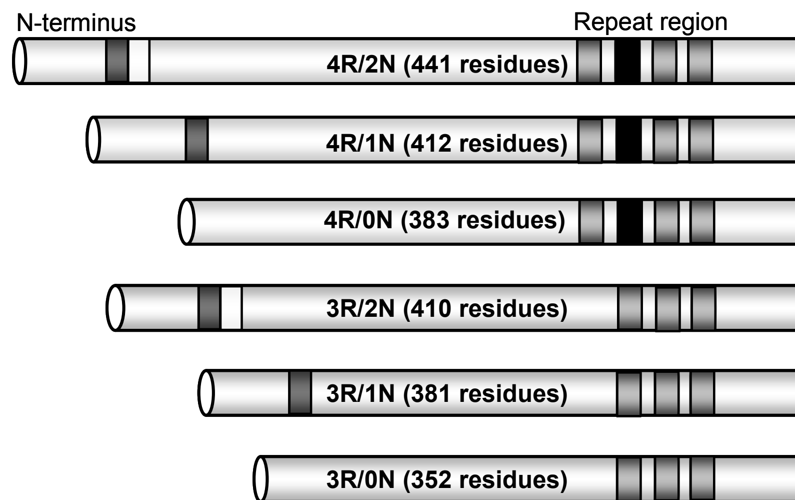


Figure 3: The six tau isoforms expressed in the adult human brain have either three or four microtubule binding repeats, referred to as 3R or 4R, respectively. Furthermore, the isoforms differ by the presence or absence of either one or two, highly acidic, N-terminal inserts, referred to as 0N, 1N or 2N, respectively.

The various isoforms are likely to have particular physiological roles since they are differentially expressed during development [43]. The 3R and 4R isoforms are generally expressed in a one-to-one ratio in most regions of the adult brain, but deviations of this ratio are characteristic for some FTD tauopathies [44]. The N-terminal inserts are highly acidic and are followed by a basic proline rich region. This N-terminal part projects from the microtubule surface and may interact with cytoskeletal elements and the plasma membrane [45, 46], while the C-terminal part with the repeat region binds to the microtubules. It has been demonstrated that isoforms with 4R are more efficient at promoting microtubule assembly than the 3R isoforms [47].

The microtubule binding ability of tau is post-translationally regulated by phosphorylation, with increased phosphorylation causing decreased affinity of tau to the microtubules. Under normal conditions, there is a constant dynamic equilibrium of tau binding to and detaching from microtubules. This equilibrium is thought to be regulated by the actions of different kinases and phosphatases. Under pathological conditions such as AD, the phosphorylation equilibrium of tau is disturbed, resulting in an abnormal level of cytosolic, hyperphosphorylated unbound tau, leading to its aggregation and fibrillization into paired helical filaments (PHFs), which further self-assemble to form NFTs. From a total of 85 possible serine (S), threonine (T) and tyrosine (Y) phosphorylation sites existing on the longer tau isoform (4R/2N), 39 have been found to be phosphorylated in PHF-tau from AD brain tissue, using mass spectrometry [48]. Apart from phosphorylation, other post-translational modifications of tau occur (see ref [49] for a recent review), including glycosylation, ubiquitylation, N-acetylation, nitration and proteolysis. Although it is conceivable that most or all of these post-translational

modifications may take place at various stages of tau pathology, their significance is yet to be fully characterized [50].

The pathological effect may arise from loss of the normal microtubule stabilizing function of tau, compromising axonal transport and thus contributing to synaptic dysfunction and neurodegeneration [40]. In addition, the relatively large size of the NFTs may cause a direct physical obstruction of cellular functions such as axonal transport in the neurons. In further support of the pathological role of tau, immunohistochemical studies of different brain regions of AD patients as well as non-demented elderly individuals, demonstrated that the number of NFTs, but not the number of SPs, correlated with the degree of cognitive impairment [51, 52]. Finally, showing that mutations in the *TAU* gene caused inherited frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [53] provided unambiguous evidence that tau malfunction is sufficient to trigger neurodegeneration and dementia.

2.2 Frontotemporal dementia

Frontotemporal dementia (FTD) is a heterogeneous group of primary neurodegenerative disorders. Picks disease (PiD), a subgroup of the FTD entity, was described already in 1892 by Arnold Pick [54]. Even though the symptoms and pathology of the disorder were described early it was not until the mid 1980s that extensive focus was directed towards the dementias of frontal and temporal type, lacking the typical hallmarks of AD [55, 56]. FTD may account for up to 20% of presenile (onset before 65 year of age) dementia cases [57] and is, after AD and dementia with Lewy bodies, the third most common form of dementia [57, 58]. In the majority of cases the onset occurs between the ages of 45 and 65 and unlike the incidence of AD, it is rare to have the onset of FTD after the age of 75 [59].

2.2.1 Diagnosis and clinical manifestation

In 1994, the Lund and Manchester groups established the first clinical and neuropathological criteria for these dementias and coined the term FTD [60], which previously had a broad and confusing terminology in medical literature due to its heterogeneity in histopathology. FTD consensus criteria included frontal lobe degeneration (FLD), PiD and FTD with motor neuron disease (MND). In 1998, the criteria were extended to include the disorders progressive non-fluent aphasia and semantic dementia [58] collected under the term frontotemporal lobar degeneration (FTLD). In 2001, McKhann et al. [59] further defined the clinical criteria to facilitate the diagnosis and reverted to the term FTD. Recently, revision of these criteria has been proposed in order to take into account current advances in molecular genetics, biochemistry and neuropathology, again using the term FTLD [44]. However,

the term FTD has prevailed in most medical literature and will be used throughout this thesis for description of these disorders.

The clinical picture in FTD is heterogeneous but is usually characterized by early changes in personality and social behaviour, signs of disinhibition and progressive language disturbances [57]. Memory deficit, which prevails in AD, may also be found in FTD but not usually to the same extent [61, 62].

2.2.2 Genetics and risk factors

FTD can occur in sporadic as well as familial forms, with 30-50% of cases having a familial history of dementia [56, 57]. Few studies of risk factors have been presented and subsequently there are no established risk factors for FTD. Inheritance of the *APOE ε4* allele shows conflicting results in FTD, with several studies showing a normal frequency [63-65], while others have shown increased allele frequency [66, 67]. For hereditary FTD, more than 40 different mutations in the *TAU* gene have been identified as causative for different familial forms of FTDP-17 [68]. Recently, mutations in the progranulin (*PGRN*) gene [69, 70] were found to cause familial FTD forms having tau-negative, ubiquitin-positive neuronal inclusions linked to chromosome 17 (FTDU-17). A rare autosomal dominant disorder belonging to the FTDU subtype of FTD is caused by mutations in the valosin-containing protein gene (*VCP*). Furthermore, a Danish familial form of FTD linked to chromosome 3 is caused by mutation in the gene *CHMP2B* (charged multivesicular body protein 2B) [71]. Finally, a genetic locus on chromosome 9 for familial FTD/MND has been described [72].

2.2.3 Neuropathology

Pathological post-mortem examination of FTD brains reveals bilateral atrophy of the frontal and anterior temporal lobes and the ventricular system is widened frontally [44, 60]. With exception of those cases where a gene deficit has been identified, examination of the brain and neuropathology are essential in order to determine the disease entity underling FTD since no clear relationship between histological changes and clinical presentation exists [44, 59].

Neuropathological findings are proposed to be of seven main types [44]. The first three types are tauopathies, having insoluble aggregates of tau protein in the brain, and the FTDP-17 disorders can belong to any of these three types depending on the nature of the causative tau mutation. The first type has insoluble 3R-tau aggregates in association with neuronal loss and gliosis, which is characteristic for PiD. Other features of PiD are inflated neurons and neuronal inclusion bodies, Pick bodies, containing the 3R-tau and ubiquitin aggregates. The second type is attributed to diagnoses such as corticobasal degeneration, progressive supranuclear palsy and argyrophilic grain disease, all having filamentous, insoluble 4R-tau aggregates. In the third

type both 3R and 4R tau is present in the aggregates and the NFT disorders belong to this group. The fourth neuropathological type, FLD, is characterized by neuron loss and gliosis in the absence of distinctive histopathology, such as NFTs or other intracellular inclusions. In the fifth type, the characteristics of FTD are generally combined with MND, adding motor difficulties to the clinical picture. This subtype has ubiquitin-positive, tau-negative inclusions and recently it was shown that these inclusions all contain major aggregates of the TAR DNA-binding protein 43 (TDP-43) [73, 74], a nuclear protein implicated in exon skipping and transcription regulation. The sixth and seventh type display neuronal loss and gliosis with ubiquitin positive, TDP-43- and tau negative inclusions [44].

2.3 Multiple sclerosis

Multiple sclerosis is the most common neurological disease among young adults, with onset at a mean age of 30 years [75]. The disease is regarded as an autoimmune-mediated inflammation of the central nervous system leading to demyelination and axonal damage [76]. Multiple sclerosis has a high heterogeneity of clinical aspects, neuroradiological appearance of the lesions, involvement of susceptibility gene loci and response to treatment [77]. Furthermore, the target of injury, myelin or oligodendrocytes [78], and the mechanism of demyelination are suggested to be distinctly different in subgroups of the disease and at different stages of multiple sclerosis development [77]. Because no single test provides a definite multiple sclerosis diagnosis, different diagnostic criteria have been used [79, 80]. These criteria also involve different paraclinical tests, such as detection of intrathecal IgG synthesis in CSF, and imaging techniques that may be used to support the diagnosis when necessary.

The rationale for treatment of multiple sclerosis is generally to reduce disease activity to protect neurons and axons from permanent damage and it has been shown that early treatment has a beneficial effect on disease progression [81, 82].

3. Cerebrospinal fluid

CSF is produced mainly by the ependymal cells at the choroid plexus, an organ protruding into the lateral and the third and fourth ventricles, while about 20-30% of the CSF comes from the extracellular fluid (ECF) of the brain. CSF fills the ventricles, enters the subarachnoidal space and flows around the brain and the spinal cord [83]. The role of the CSF includes providing buoyancy and physical protection of the CNS, bulk absorption or selective removal of various compounds and active regulation of CNS activity via circulating neuropeptides and hormones [84]. The total volume of CSF is 150-270 ml [85], with a production rate of about 0.4 ml/min. Consequently the CSF is exchanged about three to four times a day [83].

The CSF and the ECF, surrounding the neurons and glia of the brain, have a controlled composition of ions, amino acids, proteins, etc. This homeostasis mainly depends on the low permeability of hydrophilic substances due to "tight junctions" between the endothelial cells and a limited number of ion pores at the blood-brain barrier (BBB) and the blood-CSF barriers, which are located at the choroid pexus and the arachnoid membrane [86].

However, hydrophobic substances, with the ability to cross the membrane of the endothelial cells can penetrate the barriers. The BBB acts as a filter allowing only a small amount of proteins to pass from serum to the extracellular space of the CNS resulting in approximately a 200 fold lower protein concentration in CSF (about 250-300 mg/L) than serum. Both serum and CSF have a relatively high salt concentration (>150 mmol/L). However, the ionic composition is different in CSF compared with serum suggesting that CSF cannot be formed by passive ultrafiltration but is a fluid formed by active secretion [87].

CSF is in direct contact with the ECF [88]. Thus, the CSF can reflect the chemistry of the brain in living patients under various physiological and pathological states [89]. Collection of CSF samples is usually performed by lumbar puncture, interstitial at L3/L4 or L4/L5. The protein composition in CSF has been shown to vary along the draining pathway [90]. Therefore, to avoid concentration gradient effects of proteins it is generally recommended to use the first drawn 12 mL of CSF [91].

3.1 Biomarkers in cerebrospinal fluid for AD and FTD

Biomarkers are defined as cellular, biochemical or molecular alterations that are measurable in biological samples such as human tissues, cells or fluids [92]. Biomarkers indicate an alteration in physiology and can elucidate disease mechanisms, facilitate prediction, diagnosis, progression and outcome of treatment of a disease [93].

For the nervous system there is a wide range of techniques used to gain information about the brain including measurements directly on biological media, for example blood and CSF, or measurements such as brain imaging, which registers changes in the composition or function of the nervous system. Since CSF is in contact with the CNS, one excellent way of detecting molecular changes at the onset of a neurological disease is by analyzing human CSF.

Currently there are no established diagnostic tools clearly distinguishing AD from FTD [94]. The differential diagnosis is based on clinical symptoms aided by CSF biomarkers and brain imaging results. The clinical symptoms can, especially early in the disease, be similar for AD and FTD [59]. Furthermore, the molecular pathology of a neurodegenerative disease is generally present several years prior to the onset of clinical symptoms [95]. Thus, biomarkers could provide tools to better understand the disease

mechanisms and would greatly facilitate the differential diagnosis of AD versus FTD, enabling accurate diagnosis prior to the occurrence of widespread neuronal degeneration.

A set of criteria has been proposed for an ideal AD biomarker assay, namely that it should be able to detect a fundamental feature of AD pathology, be precise and reliable, non-invasive, simple to perform and inexpensive. Furthermore, it should have a sensitivity >80% for detecting AD and a specificity >80% for distinguishing AD from other dementias [96].

At present, increased levels of total tau (T-tau) and decreased levels of Aβ1-42 are the most established CSF markers in AD [97] giving a sensitivity of 89% and a specificity of 90% discriminating AD from non-dementia controls [98]. This combination of CSF markers could, with a sensitivity of 95% and a specificity of 83%, identify those patients suffering from mild cognitive impairment (MCI) that, at the 4-6 year follow-up, would have progressed to AD [99]. However due to some overlap, T-tau and Aβ1-42 fail to definitely differentiate AD from FTD, since FTD has normal to slightly increased T-tau levels in CSF [100-103] and normal to slightly decreased CSF levels of Aβ1-42 [104, 105]. In a study comparing three enzyme linked immunosorbent assays (ELISA) for quantification of CSF tau phosphorylated at different epitopes (P-tau), including P-tau_{181}, P-tau_{199} and P-tau_{231}, significantly increased levels were found for all P-tau species in AD compared with FTD [106]. The P-tau_{231} epitope was able to differentiate AD from FTD with a sensitivity of 88% and a specificity of 92% [106].

Increased levels of the cytoskeleton protein, neurofilament light, have also been found in both FTD and AD, with the highest levels in FTD [100, 101, 103, 107]. Increased tau and neurofilament levels probably reflect ongoing neuronal and axonal degeneration in the brain. The reduced levels of Aβ1-42 in AD is often hypothesised as resulting from increased deposition in SPs, with less diffusion to the CSF. The rather specific increase of P-tau epitopes in AD CSF compared with FTD may indicate a reduced phosphorylation of these sites in FTD but could also reflect that most FTD subgroups have tau deposits localised in intraneuronal inclusions, which may never reach the CSF. Thus, increased CSF P-tau levels may only be found in patients with extracellular ghost tangles as present in AD [108]. To conclude, additional markers that clearly differentiate AD from FTD giving information about the disease states are warranted. Since AD and FTD are complex disorders a panel of several changed proteins would probably be needed for complete discrimination of the disorders and such panels might also be utilized for sub-grouping of the diseases.

EXPERIMENTAL THEORY

4. Proteomic methods

The term "proteome" was used for the first time in 1994, to describe the protein complement of the genome [109]. The proteome, as opposed to the genome, is highly dynamic and varies over time, adapting to changes in the environment, state of development or disease. Consequently, proteomics is the characterization of several proteins simultaneously reflecting a state at a specific point in time in a biological fluid, tissue, cell line or organism [109]. The proteomic approach to detect protein changes in disease states compared with healthy controls is becoming an established way of identifying disease biomarkers, where the changes in the levels of expressed proteins can be used to reveal disease mechanisms and to develop new strategies for the prediction and diagnoses of diseases and their potential treatments.

Proteomics is a multidisciplinary research field generally combining various separation techniques, mass spectrometric methods and bioinformatics. Proteomic methods can be used as a toolbox of different techniques to separate, profile and identify proteins, both qualitatively and quantitatively. The choice of methods is dependent on the scientific question and the nature of the proteins to be studied. To study complex protein mixtures such as biological fluids or cell lysates a combination of separation methods is required to reduce the complexity of the sample. If the protein expressions of cells are studied, sub-cellular fractionation is frequently used, where the proteins generally are separated into cytosolic, cytoskeletal, nuclear and membrane protein fractions using different solubilization and centrifugation steps.

The wide range of protein concentrations in biological samples complicates the proteomic analysis since highly abundant proteins tend to mask less abundant proteins and thereby prevent their detection and identification. To reduce the complexity of biological samples, several prefractionation techniques such as liquid phase isoelectric focusing (IEF), different sorts of chromatography and depletion of high-abundant proteins have been developed (for a review see [110]). For protein profiling and separation of complex mixtures two-dimensional gel electrophoresis (2-DGE) is still frequently used. However, the relatively low through-put of 2-DGE and the fact that the experimental procedure is hard to automate has made techniques such as 2D-liquid chromatography [111] and surface enhanced laser/desorption ionization time of flight (SELDI-TOF) mass spectrometry (MS) [112] more frequently used in recent years.

4.1 Separation techniques

4.1.1 Liquid phase isoelectric focusing

Proteins are amphoteric molecules, containing acidic and basic groups in their amino acid sequence and consequently their net charge varies according to the surrounding pH. This property is used in isoelectric focusing (IEF), which separates proteins according to their isoelectric point (p*I*). The p*I* of a protein corresponds to the specific pH where the net charge of the protein is zero. Consequently, when an electric field is applied over a protein solution containing a pH gradient the proteins will arrest at their p*I*. IEF can be performed in the liquid phase and in this thesis liquid phase IEF (LP-IEF) was carried out using the Rotofor system. Creation of a pH gradient in the liquid phase requires the addition of ampholytes (small charged molecules/peptides) to the liquid sample, as well as filling the anode and cathode chambers respectively with acidic (0.1 M H_3PO_4) and alkaline fluid (0.1 M NaOH). LP-IEF has the capacity to enrich proteins with or without mild denaturation. Furthermore, this technique allows high protein loads (up to 1g) in 10-60 mL of solution in a procedure usually taking less than four hours. Since the separation is performed in liquid, the twenty fractions (ranging between pH 2-10) are collected without additional steps such as gel elution. Drawbacks with LP-IEF include a tendency of hydrophobic proteins to be lost, possibly due to adhesion of these proteins to the plastics in the apparatus membrane or during the sample collection procedure [113, 114].

4.1.2 Immunoprecipitation

A targeted approach to separate specific proteins or peptides from a biological sample, containing several proteins, salts and other contaminants, is to use immunoprecipitation (IP) with selected antibodies attached to beads.
Different types of beads can be used. However, in this thesis we have used magnetic beads, which respond to a magnetic field allowing bound material to be rapidly and precisely separated from the heterogenic sample. By disruption of the antibody-antigen interaction, bound proteins or peptides are eluted from the beads or the sample may be fractionated by step-wise elution. Different antigen fractions can be isolated from the same sample since beads with different antibodies attached can be used consecutively. In addition, detection of multiple antigens in a single assay is possible [115]. This means that IP can provide an adaptable preparation protocol for downstream MS analysis, liquid chromatography or 1D/2D gel electrophoresis analysis. By detecting the molecular masses of the captured molecules (or fragments thereof) with high accuracy, MS provides not only verification of the expected antigen, but also enables the identification of modified and differentially processed forms of the antigen, antibody cross-reactive species and molecules that interact with the antigen. Furthermore, separation is quite gentle and no column or

centrifugation is necessary. The disadvantage of IP is that the unbiased simultaneous identification and quantification of multiple proteins/peptides is diminished, and the method is limited by its dependence on antibodies, which must be highly specific and have high affinity for the selected antigen.

4.1.3 Two-dimensional gel electrophoresis

2-DGE, first described in 1975 [116, 117] is a powerful method for separating proteins in biological fluids or cell lysates, and a good visualization tool for protein expression where the proteome profiles of control and disease samples can be compared to differentiate physiological states [118].

2-DGE separates proteins in a two step approach according to their p*I*, and molecular mass (M_W) in a gel matrix under denaturing conditions [119, 120]. Separation in the first dimension of analytical 2-DGE is carried out using immobilized pH gradient (IPG) strips, whereas the second dimension separation is performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (figure 4).

Staining of the gels with for example organic or fluorescent dyes that interact with proteins, enables detection of the protein spots under visible or ultraviolet light. Following digitization of the gels, software-based quantification is performed, comparing staining intensities of protein spots between gels. Differentially expressed proteins between groups can be excised and subjected to enzymatic (e.g. tryptic) in-gel digestion. Extraction of the resulting peptides enables MS analysis and identification of the proteins.

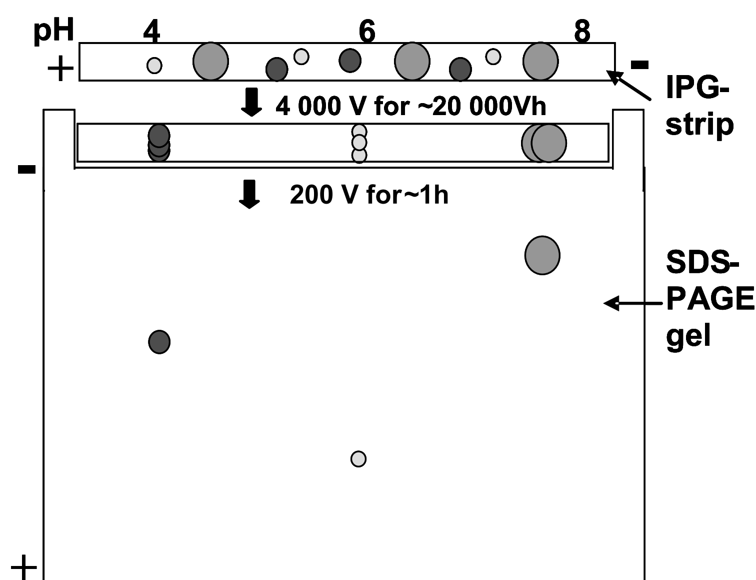


Figure 4: Proteins separated using 2-DGE. In the first dimension the proteins are separated on an IPG-strip according to their isoelectric point (p*I*). The proteins stop moving in the electric field at the pH where their net charge is zero. In the second dimension the proteins are separated on an SDS-PAGE gel according to their molecular mass. Since the anionic detergent SDS binds to the proteins in proportion to their mass and also disrupts their folding, the distance of migration through the electric field of the gel is related to the size of the protein.

Differential in-gel electrophoresis (DIGE) [121] allows co-separation of equal concentrations of two differentially labelled (Cy2 or Cy3) protein samples and an internal standard (labelled with Cy5) in the same 2-DGE experiment. Scanning the gel at Cy2, Cy3 and Cy5 excitation wavelengths, using a fluorescence imager, allows visualization of the different samples. This approach has reduced reproducibility difficulties associated with 2-DGE.

2-DGE provides information about the intact proteins, e.g. approximate mass and p*I*. Furthermore, protein isoforms with post-translational modifications changing their net charge are often well separated and can be independently quantified. Disadvantages include discrimination against certain classes of proteins such as hydrophobic, very basic and small (less than 10 kDa) proteins. Furthermore, the limited loading capacity of 2-DGE often necessitates enrichment and pre-fractionation steps in order to detect low-abundant proteins even in less complex samples. The 2-DGE procedure also has a relatively low throughput and involves several experimental steps.

4.1.4 Reversed phase liquid chromatography

Reversed phase liquid chromatography (RP-LC) is often used as the final analyte enrichment/separation step prior to MS. The peptide samples are loaded onto columns packed with solid phase adsorbents, carrying hydrophobic groups (e.g. C4, C8, C18) that bind to the peptides through hydrophobic interaction, while salts and other water soluble impurities are washed away. The peptides are eluted by applying a gradient of an organic solvent (e.g. acetonitrile). The use of aqueous organic mobile phase and the absence of salt makes RP-LC highly compatible with MS. C18 is generally used for peptide analysis. For proteins, usually being more hydrophobic, adsorbents with shorter carbon chains (e.g. C4, C18) are often used. For use as pre-separation prior to nano ESI-MS (described below) RP-LC has been scaled down, using columns with nL-bed volumes that operate at flow rates in the nL/min regime (nano RP-LC).

4.2 Biological mass spectrometry

MS is a key technique in proteomic analysis, providing accurate mass measurements, according to the mass-to-charge ratio *(m/z)*, of small quantities of proteins, peptides and peptide fragments, the latter giving information of the amino acid sequence and modifications. Three components are generally present in all mass spectrometers: an ion source, a mass analyzer and a detector. Sample molecules are introduced into the ion source where they are converted into gas phase ions. The mass analyzer separates the ionized species according to their *m/z* ratio and the detector records an ion current of

the separated analytes. Results are then plotted in the mass spectra, as the ion current against *m/z*.

The ionization methods currently most suitable for analysis of peptides and proteins are matrix assisted laser desorption/ionization (MALDI) [122] and electrospray ionization (ESI) [123] since they enable production of intact gaseous ions of large biomolecules. There are a number of different types of mass spectrometers employed in proteomic research and the ones used in this thesis are further described below.

4.2.1 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

The MALDI time-of-flight (TOF) MS instrument combines a MALDI ion source and a TOF analyzer. MALDI is a soft ionization technique, initially described in 1988, which results in most intact peptides/proteins in gas phase with little fragmentation [122, 124].

The sample subjected to MALDI analysis is placed on a sample plate. The sample plate is then inserted into the ion source of the instrument through a vacuum interlock, as the MALDI ion source operates under conditions of high vacuum. The technique involves co-crystallization of the sample on the sample plate with a large molar excess of a matrix compound, which strongly absorbs energy at the wavelength of the ultraviolet (UV) laser. Alpha-cyano-4-hydroxycinnamic acid (CHCA) is frequently the matrix of choice for peptides. CHCA is particularly good for generating ions above 700 Daltons (Da) since lower masses can be masked by the relatively high matrix background. The 2,5-dihydroxybenzoic acid (DHB) matrix produces less interference than other matrices in the low *m/z* range and induces less fragmentation than CHCA. Therefore, DHB is often used for analyses of glycopeptides and phosphopeptides, while 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) is suitable for intact protein analysis.

The exact desorption/ionization mechanism for MALDI is not known. However, it is generally thought that the absorbed energy after laser irradiation of the analyte-matrix mixture results in vaporization and ionization of the matrix, carrying the analyte into the gas phase. The resulting ionization of the analyte probably occurs through proton transfer during the desorption process [125]. Predominately, MALDI results in formation of peptide/protein ions carrying a single positive charge, although ions having two or three charges can be formed.

The TOF mass analyzer is well suited for pulsed ion sources, such as the MALDI technique. The analyte ions are accelerated by an electrical field between the sample plate and an extraction element prior to entering the field-free drift region. Thus, the analyte velocities become a function of their *m/z* ratio. As a result the analyte ions arrive at the detector at different times. For ions with the same charge, the ones with lower mass acquire higher velocity

and reach the detector faster. However, during the desorption/ionization process ions of the same *m/z* often acquire different initial kinetic energy, and thus hit the detector at slightly different times, causing peak broadening and a lowering of the resolution. The introduction of an electrostatic ion reflector often referred to as an ion mirror, consisting of a series of grids or ring electrodes, not only lengthens the flight path but also compensates for the difference in initial kinetic energy so that ions with the same *m/z* hit the detector at almost the same time [126]. Ions with a higher initial kinetic energy will penetrate deeper into the ion mirror before reversing. Consequently, the more energetic, faster ions will have a longer flight path to the detector than the less energetic, slower ions (figure 5).

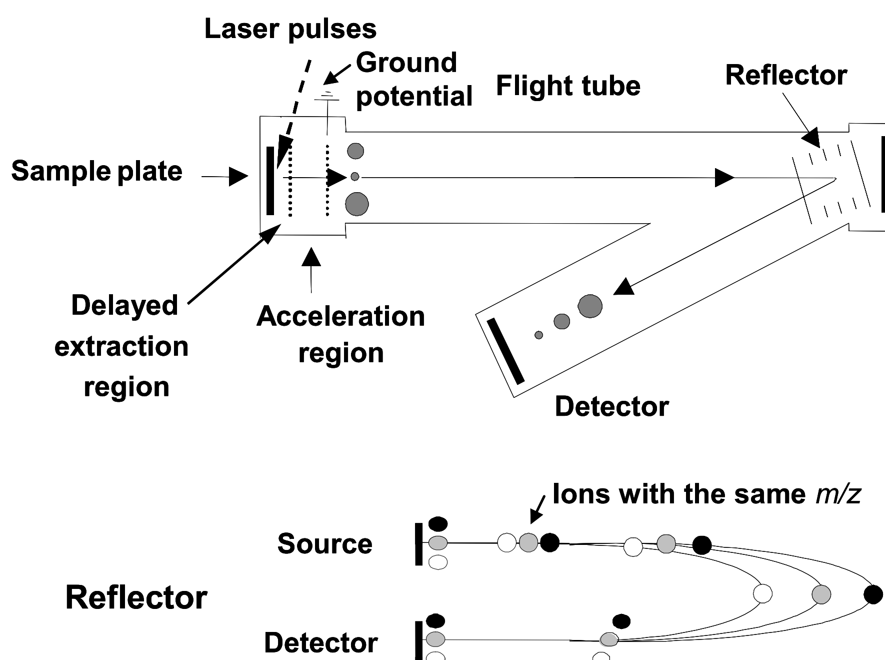


Figure 5: Schematic illustration of a reflector MALDI-TOF mass spectrometer. Ionised analytes with different *m/z* are separated in the flight tube and arrive at the detector at different times. The reflector compensates for different initial velocities of ions with the same *m/z* and improves the resolution. Ions with higher initial kinetic energy will penetrate deeper into the reflector and travel a greater distance than the less energetic ones. Thus, the ions will reach the detector at the same time.

Furthermore, the introduction of delayed extraction or time-lag focusing has provided remarkable improvement in both resolution and mass accuracy [127] and has become a standard feature of MALDI-TOF mass spectrometers. In delayed extraction, ionization occurs with no electrical field applied between the sample plate and the first extraction element. After a short time delay the electric field is switched on and the ions are accelerated towards ground potential. Ions with lower initial velocity will have travelled a shorter distance

from the sample plate and will suddenly be at higher potential than the initially faster ions. Consequently, the originally slower ions will instead be the faster ones when they exit the acceleration region, and with properly set delay time and source voltages they will reach the detector simultaneously with the initially more energetic ions.

The average of several hundreds of laser shots produces the final mass spectrum, increasing the signal. Performance in modern reflector MALDI mass spectrometers is typically in the range of a few parts per million in mass accuracy and only about a femtomole of peptide material needs to be deposited on the MALDI target to produce a signal.

More recently, MALDI ion sources have also been coupled to QTOF [128] and to two TOF analysers (TOF/TOF instruments) [129] allowing fragmentation of MALDI-generated precursor ions and subsequently providing information about the amino acid sequence for more reliable protein identification.

4.2.2 Surface enhanced laser desorption/ionization time of flight mass spectrometry

SELDI-TOF is an affinity-based MS method, initially described by Hutchens and Yip in 1993 [130], and further developed by Ciphergen Biosystems into a protein Chip MS technology platform [112]. Intact native proteins are selectively adsorbed to chemically modified array surfaces followed by the addition of an energy-absorbing matrix solution. The MS part of the SELDI technique is based on the principles of MALDI-TOF MS, but modified so that the chromatographic capture step takes place on the same sample support that is subsequently used for laser desorption MS, thereby simplifying the experimental procedure, increasing reproducibility and facilitating automated analysis. The ability of the selective array surfaces to retain subsets of the proteome allows the analysis of complex biological specimens, such as serum, CSF and cell lysates. By combining different chromatographic arrays (e.g. anion exchange (Q10), cation exchange (CM10), metal affinity (IMAC) or reverse phase (H50)) and matrix molecules, a broad range of the proteome can be analyzed. The system is favorable for proteins and peptides with a M_W lower than 20 kDa and is therefore a good complement to 1D/2D electrophoresis. Furthermore, small total protein quantities (~1.5 μg), and quick laboratory procedure favor SELDI-TOF compared with 2-DGE followed by MS. However, protein identities are not revealed in the process since the analytes cannot be subjected to any digestion or tandem mass spectrometry in the process. Thus, SELDI-TOF MS can be regarded as a profiling technique complementary to the 2-DGE procedure, requiring downstream isolation, digestion and identification of analytes after the quantification step.

4.2.3 Electrospray ionization quadruple time-of-flight mass spectrometry

ESI, first described in 1984 [131, 132], produces gaseous ionised molecules directly from a liquid solution of the analytes at atmospheric pressure. The sample solution is sprayed from the tip of a thin capillary and a strong electric field is applied between the capillary and a counter electrode. The ionization process is not yet fully understood. However, a fine spray of charged droplets is produced and dry gas or heat facilitates evaporation of solvent, reducing the size of the droplet. This results in increasing charge-density at the surface of the droplet and when the electrostatic repulsion between like charges exceeds the surface tension in the droplet it disintegrates. Repeated disintegrations will occur and ultimately solvent-free gas-phase ions are produced. A characteristic feature of ESI is the multiple charging of analytes, which increases proportionally with molecular mass. This multiple charging allows for mass determination of proteins within the limited *m/z* range of quadrupole analysers.

Low-flow electrospray, nano ESI, initially described by Wilm and Mann [133], is generally used, where the spray needle is extremely thin and positioned close to the entrance of the mass analyser. These adjustments give very small droplets and hence a reduction in the amount of sample needed, enabling longer measurement times and more accurate and sensitive mass measurements. In addition, the electrospray process itself creates the sample flow through the capillary and thus no external pump is needed [133]. The combination of ionization at atmospheric pressure and the continuous flow of solvent used in ESI allows for direct coupling with separation techniques, such as nano LC and capillary electrophoresis.

In contrast to MALDI, ESI produces a continuous beam of ions and is most compatible with mass spectrometers that operate in a similar continuous fashion, such as quadrupole mass filters, while the TOF analyser requires a pulsed operation. Thus, orthogonal voltage pulsing of ion-packages into the TOF analyser was invented [134]. The quadrupole (Q)TOF instrument combines the ability to obtain efficient precursor ion selection by the use of the quadrupole mass filter and dissociation in a hexapole collision cell with the high sensitivity of the TOF analyser (figure 6). The quadrupole mass filter in the QTOF separates ions according to their *m/z* ratio by utilising the stability of their trajectories in an oscillating electrical field. Ions that do not have a stable trajectory through the quadrupole will collide with the rods, not reaching the detector.

Compared with earlier ESI MS instruments, the advantages of the QTOF hybrid include better sensitivity, improved resolving power and mass measurement accuracy, attributed mainly to the narrow beam packet, pushed down into the TOF analyser which is equipped with a reflectron orthogonally to the transfer ion optics. Another advantage of the QTOF is the easy switching between MS and MS/MS modes and that fragmentation of a

specific *m/z* can be carefully controlled. In MS mode, the ions drift through the quadropole mass filter, which acts as a focusing device transmitting all ions to the TOF analyser, where they are separated according to their *m/z* ratio. In the MS/MS mode the quadrupole mass filter is set to allow only ions within a very narrow *m/z* range to pass through to the collision cell for subsequent fragmentation. The precursor ion dissociates into product ions, whose ion trajectories are stabilised in the hexapole, and the *m/z* of the fragment ions are then measured in the TOF analyser.

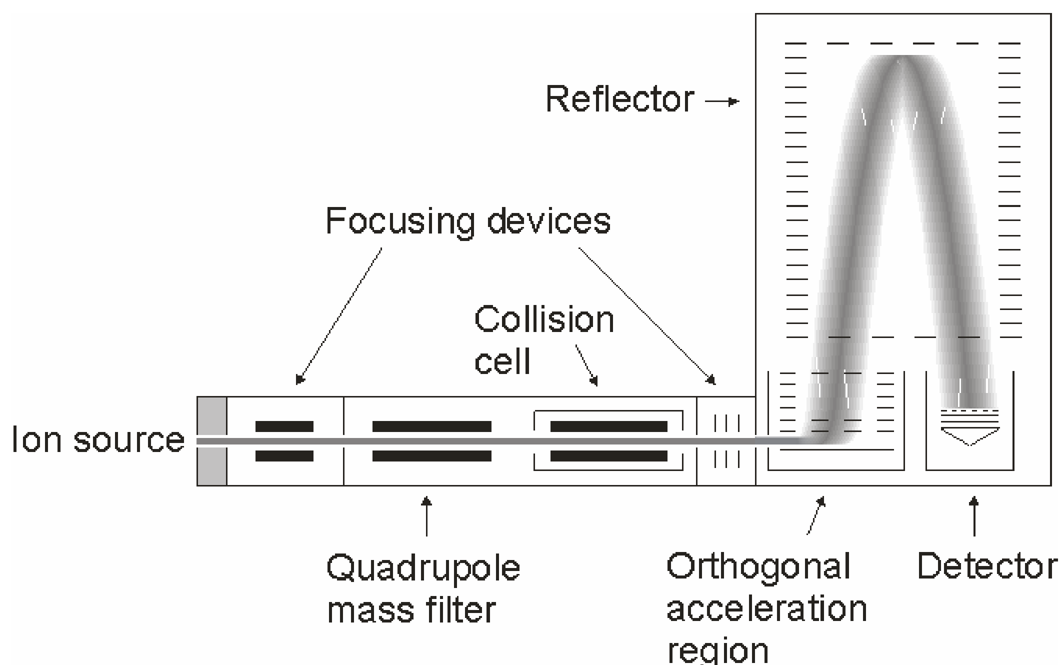


Figure 6. Schematic illustration of the principal components of an ESI-QTOF mass spectrometer.

4.2.4 Linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectromety

The linear quadrupole ion trap (QIT) Fourier transform ion cyclotron resonance (FTICR) MS instrument (figure 7) generally also employs nano ESI for ionization of analytes. It is a hybrid instrument, consisting of a linear ion trap capable of fast, sensitive peptide sequencing combined with an ion cyclotron resonance (ICR) cell, generating extraordinary resolving power and mass accuracy [135]. Ionised analytes are transmitted into the quadrupole iontrap through focusing optics e.g. quadrupoles, hexapoles or octopoles. The ion trap uses the same principles as the quadrupole filter, where different combinations of direct current and radio frequency (RF) potentials are used to select analytes of a particular *m/z* range, however in this case the ions can be trapped, forced to move back and forth in the quadrupole by applying

appropriate potentials at the entrance and exit of the trap. The ability to trap ions makes it possible to select which ions should either be ejected to the detector directly, passed forward into the ICR cell or fragmented prior to detection or ICR analysis. Analytes or their fragments selected for ICR analysis are again transferred through focusing optics into the ICR cell, where ions are trapped by a strong magnetic field (in our case seven tesla). The magnetic field will cause the ions to be trapped in a circular motion. For detection, coherent ion motion must be generated by applying a RF voltage to the exitation plates of the ICR cell, making the ions move closer to the detection plates. Then a small current will be induced in the plate each time an ion passes by. Since the ions with different *m/z* have different ion cyclotron frequencies, each generated current frequency will correspond to a certain *m/z* value. The number of ions that enters the cell can be selected by the QIT. This is important because too many ions will dramatically reduce the resolution and mass accuracy of the measurements in the cell. If an appropriate number of ions are transferred to the ICR cell the high accuracy of the precursor ion, usually less than 2 ppm, enables an accurate database search for protein identification. The QIT can also be used on its own, resulting in better sensitivity but significantly reduced mass precision and resolution. Consequently, the increased sensitivity is at the expense of losses in resolution and mass accuracy.

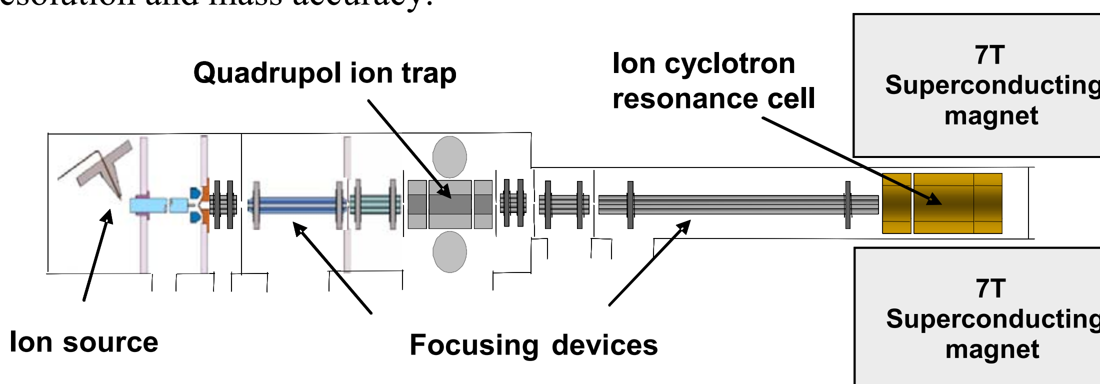


Figure 7: Schematic illustration of a QIT-FTICR MS instrument equipped with a seven tesla (T) magnet.

4.3 Protein identification

A key advance in biological mass spectrometry was the development of algorithms for the identification of proteins by matching mass spectrometric data to databases containing known protein sequences.

4.3.1 Peptide mass fingerprinting

Enzymatic cleavage of proteins prior to MS analysis is usually performed by trypsin, producing a specific peptide pattern for each protein, generally referred to as a peptide mass fingerprint. Trypsin cleaves proteins C-terminally of the positively charged amino acids arginine (R) and lysine (K),

giving a relatively large number of peptides available for MS analysis. Alternative endoproteases for protein digestion include Lys-C (cleaves after K), Arg-C (cleaves after R) and chymotrypsin (cleaves after F, Y, W, L and M), producing other peptide patterns. The MS-detected *m/z* values of the peptide pattern can then be matched to theoretically expected enzyme digests for each protein sequence present in a database. A ranked list is then produced according to the number of peptides from the sample that match a specific protein (figure 8) [136]. The limit of peptide mass fingerprinting is normally reached when the sample contains a complex protein mixture, or if the protein yields too few digestion products. Therefore, this method is best suited for proteins separated by 2-DGE or other multi-dimensional protein separation strategies, usually yielding less complex tryptic digests [137].

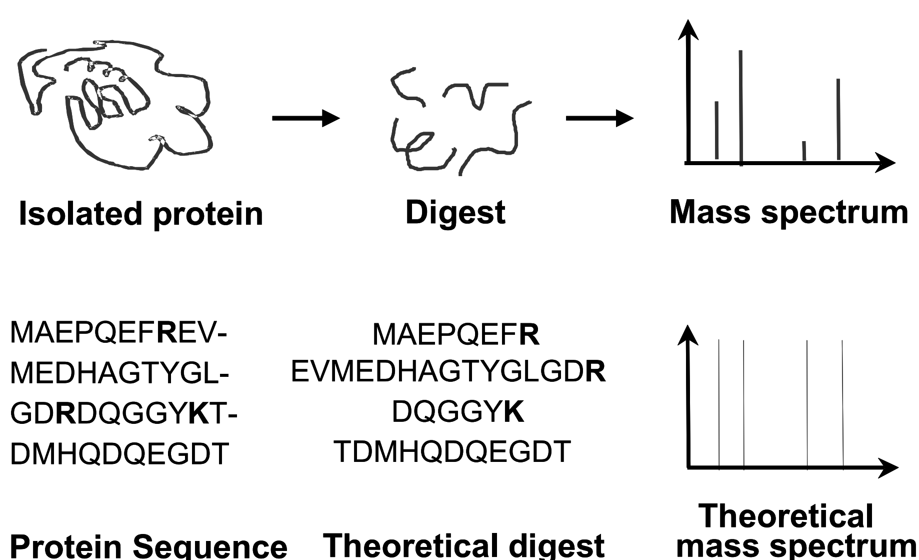


Figure 8. Protein identification by peptide mass fingerprinting. The isolated protein sample is enzymatically digested by trypsin. The resulting peptides are mass determined by MS. The peak list of the mass spectrum is compared with calculated masses of theoretically digested protein sequences present in databases.

4.3.2 Amino acid sequence analysis by tandem mass spectrometry

For protein identification using MS/MS, a single peptide precursor ion is isolated and fragmentations, mainly along the peptide bonds, are induced, resulting in a spectrum containing a pattern more or less unique for the individual peptide [137]. The fragment ion masses are then compared with theoretical fragmentation patterns of proteins in a database search. Protein identification can be achieved by MS/MS analysis of even a single peptide [136]. Consequently, and in contrast to peptide mass fingerprinting, protein identification can be obtained from complex mixtures of proteins.

The most common method of fragmentation is collision induced dissociation (CID). Other examples of fragmentation methods are laser-

induced fragmentation (LIF), electron capture dissociation (ECD) [138] and infra-red multi-photon dissociation (IRMPD) [139]. The CID process includes multiple low-energy collisions of the peptide precursor ion with an inert gas, usually argon, which finally leads to dissociation of the precursor ion. The fragments mainly produced are the result of N-terminal and C-terminal fragmentations across the peptide bond to give sequence ions with charge retention on either the acylium ('b') or ammonium ('y') ion fragments [140] (figure 9). Subfragments associated with loss of water or ammonia from these ions are commonly observed as well. The b-ions can lose carbon monoxide and become a-ions. Immonium ions ($H_2N{=}CHR$, where R is the amino acid side chain) are also seen in the spectra.

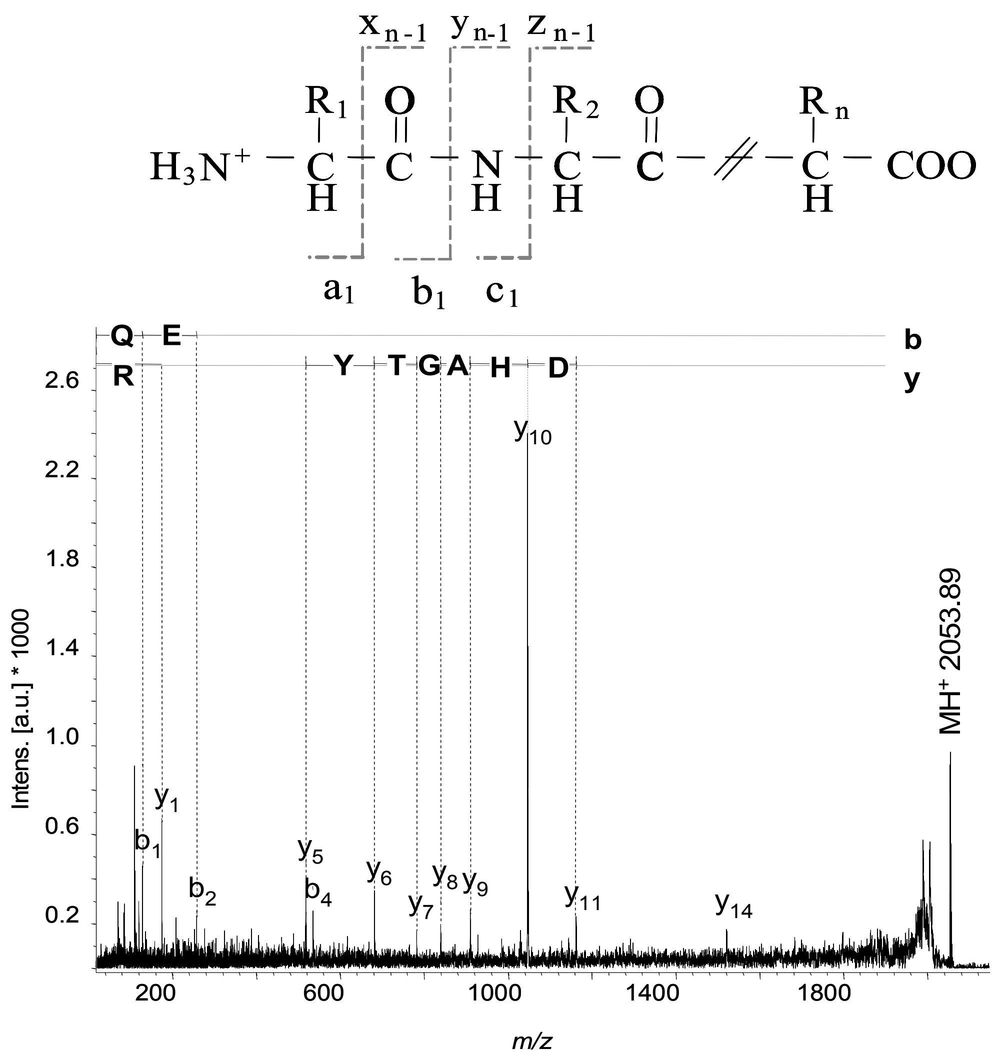


Figure 9: Nomenclature of peptide fragmentation. For protonated peptides cleavage of the peptide backbone will generate different series of fragment ions. In the formation of a-, b-, c-ions the charge is retained at the N-terminus, and the major N-terminus containing ion series is the b-ion series. For x-, y- and z-ions the charge is retained on the C-terminus, and the major C-terminus containing ion series is the y-ion series. MS/MS spectrum of the selected peptide at MH^+ 2053.89 of the tau protein analysed by MALDI-TOF/TOF MS. The y-ion series (C-terminal fragments), and the b-ion series (N-terminal fragments), are shown. The amino acids matching to this MS/MS data are given in the spectra.

5 Immunobased quantification

5.1 Nephelometry

The immunonephelometric assay employs affinity-purified polyclonal antibodies for quantification of proteins in biological fluids, for example CSF. In the immunochemical reaction selective antibodies form immunocomplexes with the protein of interest. The formed complexes scatter a beam of light passed through the sample. The intensity of the scattered light is measured and corresponds to the concentration of the protein, e.i. antigen, in the sample. No isoforms are quantified since polyclonal antibodies are used. Polyclonal antibodies are derived from different B-cell lines and thus they are a mixture of immunoglobulin molecules directed against a specific antigen, each recognising a different epitope of the protein. The advantage of the method is high reproducibility and fast and automated experimental procedure, however, it has a relatively low sensitivity (in the mg/L range).

5.2 Enzyme linked immunosorbent assay

ELISA is a valuable diagnostic tool in medicine since it allows for rapid quantification of an analyte or antibody in a large number of samples. The method is based on the specific recognition of an antigen by an antibody. The sensitivity of an ELISA may be very high. The main factor determining the sensitivity of the method is the strength and specificity of the antibody-antigen interaction, whereas modifications of buffer conditions, washing solutions and enzymatic substrates may increase the sensitivity to some extent. In an ELISA either polyclonal or monoclonal antibodies can be used. Monoclonal antibodies are identical, directed against the same epitope, since they are produced by immune cells of one type, all of which are clones of a single parent. Thus monoclonal antibodies can be produced to recognise specific isoforms of a protein and are often used in highly sensitive ELISA applications.

The method is quite straightforward, briefly the liquid sample with an unknown amount of antigen is immobilized on a solid support, usually a microtiter plate, either non-specifically, via adsorption to the surface, or specifically, via a capture antibody specific to the antigen, in a sandwich ELISA. After immobilisation the detection antibody is added, forming a complex with the antigen. In a sandwich ELISA the detecting antibody must be directed against a different epitope than the capture antibody. The detecting antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme. The plate is developed by adding an enzymatic substrate producing a signal, indicating the quantity of antigen in the sample. The limitation of the ELISA technique is

that it only provides information on the presence of an analyte but gives no structural information.

5.2.1 Electrochemiluminescence

One recent development of the ELISA method is electrochemiluminescence detection (by Meso Scale Discovery, Gaithersburg, Maryland, USA). Chemical labels on the detecting antibodies emit light when electrochemically stimulated and background signals are minimal because the electric stimulation mechanism is decoupled from the generated light signal.

The sensitivity is improved due to multiple excitation cycles of each label that amplifies the signal by increasing the light levels. The electrical stimulation can be achieved since the microtiter plates have electrodes, made from carbon, integrated into the bottom of the plate. This method can be multiplexed, i.e. has the ability to measure several analytes simultaneously in a single well of the microtiter plate. Several electrodes can be incorporated into each well and one specific antibody species is coupled to the region of each electrode, enabling specific stimulation of the antibody-antigen complex attached to each electrode.

5.3 Western blot

The Western blot method can detect, in a mixture of proteins or fragments of proteins, those that react with a specific antibody. The proteins are separated using SDS-PAGE and are transferred to a membrane, generally polyvinylidene fluoride (PVDF). The immobilised proteins are irreversibly bound to the membrane and incubated with specific antibodies for immunological detection. Visualisation of the protein-antibody complex involves the binding of a secondary antibody linked to a marker enzyme that converts a substrate into a colorimetric or photometric signal. The advantage of Western blotting is that protein identification simultaneously gives information about the M_W as well as possible identification of isoforms of the protein. Western blot may also give a semi-quantitative measurement of the protein concentration.

AIM

The overall aim of this thesis was to develop and evaluate proteomic strategies applied to CSF proteins in order to reveal disease mechanisms and potentially identify new biomarkers to distinguish AD from FTD.

Specific aims:

- To determine the reproducibility and improve LP-IEF prefractionation combined with 2-DGE and MS for individual CSF samples and to apply this strategy to investigate differences between the CSF proteome of FTD patients and non-demented controls (Study I).
- To explore disease-influenced proteins in CSF of AD patients compared with non-demented controls using the prefractionated 2-DGE procedure as well as direct 2-DGE with micro-narrow range gels (Study II).
- To validate some changed proteins, with Aβ-binding capacity, from the 2-DGE studies and explore their correlation with Aβ peptides in CSF from AD patients, FTD patients and non-demented control subjects (Study III).
- To explore the general effect of preanalytic storage conditions on CSF proteins and more specifically on cystatin C by SELDI-TOF MS (Study IV).
- To develop an IP method for characterization of the tau protein in CSF by MS (study V).

RESULTS AND DISCUSSION

The workflow for analysis of CSF proteins in this thesis included 2-DGE, SELDI-TOF-MS, MALDI-TOF(TOF) MS, ESI-QTOF MS, ESI-QIT-FTICR MS and immunological methods (figure 10).

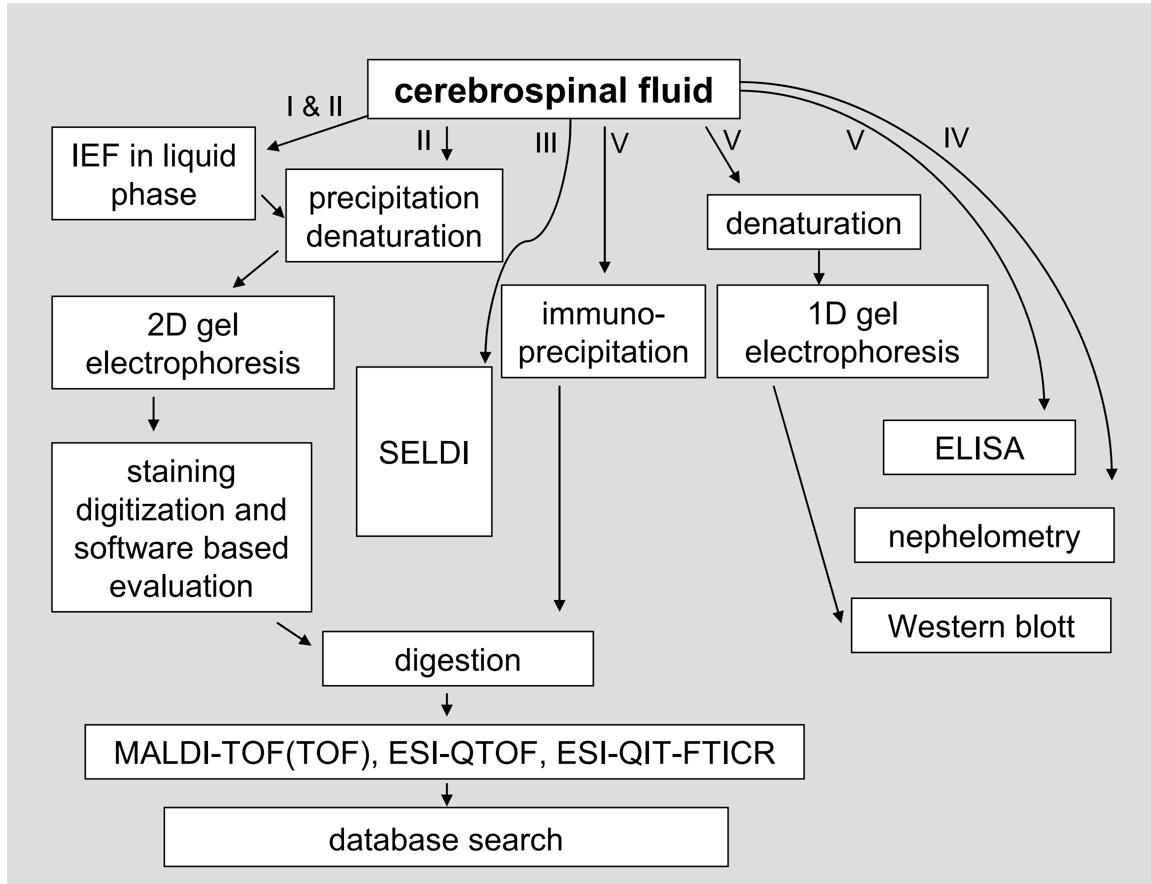


Figure 10: Schematic presentation of the workflow for analysis of CSF in the different studies (I-V) included in this thesis.

6. Analysing CSF proteins in AD and FTD by 2-DGE

Study I and II

6.1 Combining LP-IEF prefractionation with 2-DGE

Analysis of brain-specific proteins in CSF by 2-DGE is difficult because 80% of CSF proteins, mostly albumins and immunoglobulines, are derived from plasma [91, 141]. Since 2-DGE has relatively low sample loadability, low abundant, brain-specific CSF proteins can be difficult to visualize on the 2D gels and the spots contain insufficient quantities for successful identification by MS. Therefore, we used LP-IEF as a prefractionation step of intact CSF proteins prior to 2-DGE [142], which increased the intensity of the protein spots and improved MS identification. The prefractionation step enabled

enrichment of CSF proteins from individual samples, according to their p*I*, using a tenfold larger volume than in direct 2-DGE. Furthermore, proteins outside the selected pH interval of the IPG-strip could be excluded, avoiding extensive protein precipitation at the electrodes in the first dimension separation.

When CSF proteins are enriched using LP-IEF the ampholytes as well as the intrinsic high salt concentration of native CSF must be removed prior to 2-DGE since these charged molecules interfere with protein focusing in the first dimension. Therefore, different clean-up procedures were tested, including trichloroacetic acid (TCA)-acetone precipitation, ethanol precipitation, chloroform/methanol/water precipitation, acetone precipitation and a micro Bio-Spin desalting column. After comparison of these procedures, we chose precipitation using ice-cold ethanol (> 70 %) for further experiments since it generated well focused protein spots (Study I).

The reproducibility of prefractionated and directly analyzed CSF on 2D gels was compared. Coefficients of variation (CVs) were calculated for quantities in 20 protein spots present in the four gel replicates from each approach. The prefractionated 2-DGE approach gave CVs ranging from 1-33% (mean 14.6%) comparable to that of direct 2-DGE with CVs ranging from 1-35% (mean 15.4%). Generally, faint spots had CVs in the higher range. Consequently, the extra prefractionation step did not introduce additional variation and this method was subsequently used for enrichment of low-abundant proteins in individual CSF samples.

6.2 Application of the prefractionated 2-DGE method

The prefractionated 2-DGE method was used to screen for changes in the CSF proteome of five AD patients and five FTD patients. Both groups were compared with the CSF proteome from five non-dementia controls on gels with pH intervals 3-6, 4-7, and 5-8.

In the AD group, the expression of five protein spots was up-regulated whereas ten was down-regulated, at least twofold, compared with non-dementia controls. Nine of the protein spots, corresponding to seven different proteins were identified by MS. Two isoforms of α-1-antitrypsin (AAT) precursor and one isoform of β-2-microglobulin (β-2-m) were increased in AD while reduced levels of two isoforms of α-1β-glycoprotein, one isoform of α-2-HS-glycoprotein, transferrin precursor, albumin precursor and transthyretin (TTR) were found (figure 11, table 1).

Analyzing the expression of CSF proteins in the FTD group, ten protein spots were up-regulated and sixteen protein spots down-regulated compared with non-dementia controls. Thirteen of the protein spots, corresponding to seven different proteins, were identified by MS. In the FTD group the following protein spots were increased: one isoform of Zn-α-2-glycoprotein, apolipoprotein (apo) A1, β-2-m and two isoforms of TTR, while a reduction

was seen in four isoforms of serum albumin, two isoforms of alloalbumin and retinol binding protein, compared with controls (figure 11, table 1).

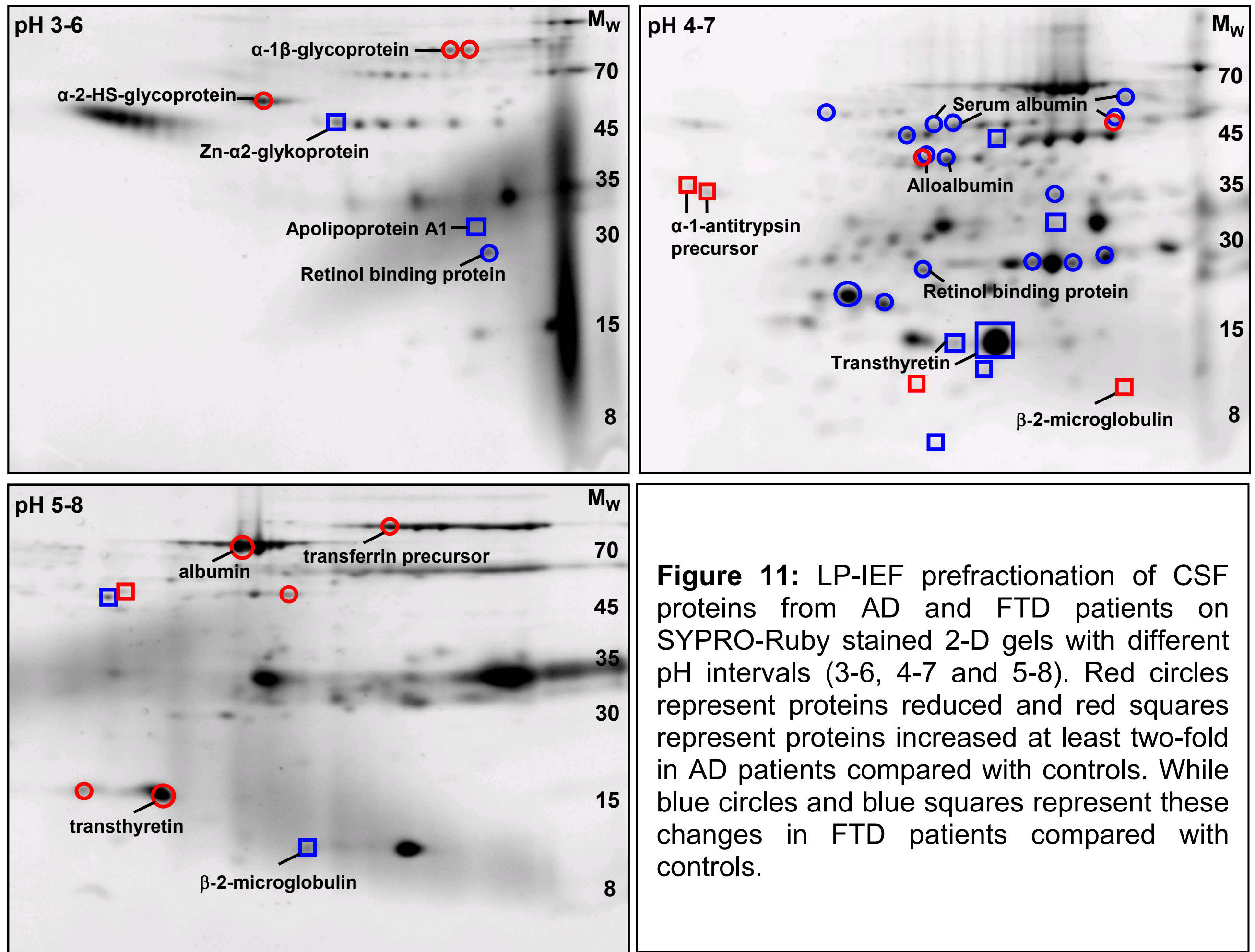


Figure 11: LP-IEF prefractionation of CSF proteins from AD and FTD patients on SYPRO-Ruby stained 2-D gels with different pH intervals (3-6, 4-7 and 5-8). Red circles represent proteins reduced and red squares represent proteins increased at least two-fold in AD patients compared with controls. While blue circles and blue squares represent these changes in FTD patients compared with controls.

6.3 Micro-narrow range 2-DGE

Another approach to increase the 2-DGE detection of proteins was applied to CSF from AD patients. In this study the separation of proteins was increased by micro-narrow range IPG strips in the first dimension (zoom gels). The CSF proteome from seven AD patients and seven controls were compared and 17 protein spots were significantly increased or decreased (Student's t-test, $p<0.05$). Of these, eight reduced proteins were identified by MS, including two isoforms of apoA1, apoE and apoJ as well as one isoform of kininogen, α-1β-glycoprotein, β-trace, retinol binding protein and cell cycle progression 8 protein. One identified protein, AAT precursor, was significantly increased in AD (table 1).

Comparing the CSF proteomes of AD and FTD showed that several proteins were differently expressed in the two disorders. This has also been shown in previous studies by our group using direct 2-DGE of AD and FTD CSF [143, 144], suggesting that different disease mechanisms can be involved in the pathology of AD compared with FTD. However, the reports from different proteomic studies have many times been conflicting (table 1). The contradictory results may have several explanations including the fact that AD and FTD are relatively heterogeneous disorders and the results can depend on selection of patients included in the studies. Furthermore, different isoforms may have been quantified in separate studies, but the nature of the isoform is not always reported and thus comparison with different studies may be difficult.

Table 1: Protein changes identified with the 2-DGE methods in AD and FTD CSF.

CSF protein	Levels in AD vs. control		Levels in FTD vs. control	Expression in other proteomic studies
	"micro-narrow" 2-DGE	IEF+ 2-DGE	IEF+ 2-DGE	
Albumin		↓	↓	↑[145, 146]AD,↑[143]FTD
α-1-antitrypsin precursor	↑	↑		↑[145, 146]AD,–[147]AD
α-1β-glycoprotein	↓	↓		↓[148]AD,–[146]AD
α2-HS glycoprotein		↓		↑[146]AD
Apolipoprotein A-I	↓		↑	↓[144]AD –[147]AD
Apolipoprotein E	↓			↓[144], ↓[143]FTD
Apolipoprotein J	↓			
β-trace protein	↓			↓[147]AD,↑[148]AD,–[146, 147, 149]AD
Cell cycle progression 8 protein	↓			
Kininogen	↓			
Retinol binding protein	↓		↓	↑[144]AD ↓ [145, 146]AD,↓[143]FTD
Transferrin		↓		↑[146]AD
Transthyretin		↓	↑	↑[144, 146]AD,↓ [96]AD,–[145, 147]AD,↑[150]FTD
β-2-microglobulin		↑	↑	↑[144-146, 148]AD, –[148, 151]AD
Zn-α2-glycoprotein			↑	↑[144]AD

Using prefractionated 2-DGE many glycosylated proteins, including α-1-antitrypsin, α-1β-glycoprotein, α-2-HS glycoprotein and Zn-α-2-glycoprotein, and circulating carrier proteins, including retinol binding protein, TTR, transferrin and albumin were found to be changed in AD and FTD. Proteins favored by prefractionation by LP-IEF are thus hydrophilic proteins, while the more hydrophobic proteins such as apolipoproteins are better visualized using direct 2-DGE. It is important to note that different

kinds of prefractionation or other attempts to increase the sensitivity in many proteomics methods can lead to loss of specific proteins or groups of proteins. Furthermore, direct and prefractionated 2-DGE have different analytical windows demonstrating that it is important to combine several proteomic methods to study as much of the proteome as possible.

Proteins with levels in the low mg/L range could be identified using the 2-DGE approaches. However, using 2-DGE to search for low-abundant neuron-specific disease markers in CSF, has not yet been successful. Disease influenced proteins that can be detected by immunological methods in CSF, for example tau, neurofilament, APP and Aβ could not be visualized using 2-DGE. Thus, in subsequent CSF analyses other proteomic and immunological methods were explored, with the aim of increasing the sensitivity in the detection of low-abundant CNS-specific proteins.

7. Validation of selected protein changes found in the 2-DGE study

Study III

Proteomic results need to be validated using complementary methods, especially when the proteome of relatively few patients has been examined as in studies I, and II. Furthermore, many proteomic approaches do not have sufficiently high through-put to be suitable for larger-scale analyses of biological fluids from patients. Therefore, other methods generally must be applied for quantifying newly discovered disease-influenced protein changes. Immunological methods are often sensitive and relatively robust for quantifying proteins, but information about the analyte's molecular mass or isoform, as can be gained by 2-DGE and/or MS, is generally not obtained.

TTR, β-trace and AAT were chosen for validation from the 2-DGE studies since these proteins were differentially expressed in AD compared with FTD. Furthermore, these proteins have been shown to bind Aβ peptides and to reduce Aβ aggregation and polymerization *in vitro* (β-trace, [152]; TTR, [153, 154]; AAT, [155]). Thus, changes in these Aβ-binding proteins might be directly linked to the disease mechanism of AD since the formation of insoluble aggregates of Aβ has been suggested as an early or even an initial event in AD [35] but not in FTD [156]. Furthermore, TTR [157] and AAT [158] have been localized to SPs in AD. TTR, β-trace and AAT are also present in CSF at relatively high concentrations (5-25 mg/L, [141, 159]) and might thus be present in sufficient amounts to play an important role in Aβ-sequestering in the brain under normal conditions.

Cystatin C (CysC) was added to the study since the protein fulfilled the relevant criteria, including the potential to inhibit Aβ aggregation and polymerisation [160, 161], its presence in SPs [162] and at relatively high concentrations in CSF (7 mg/L, [163]).

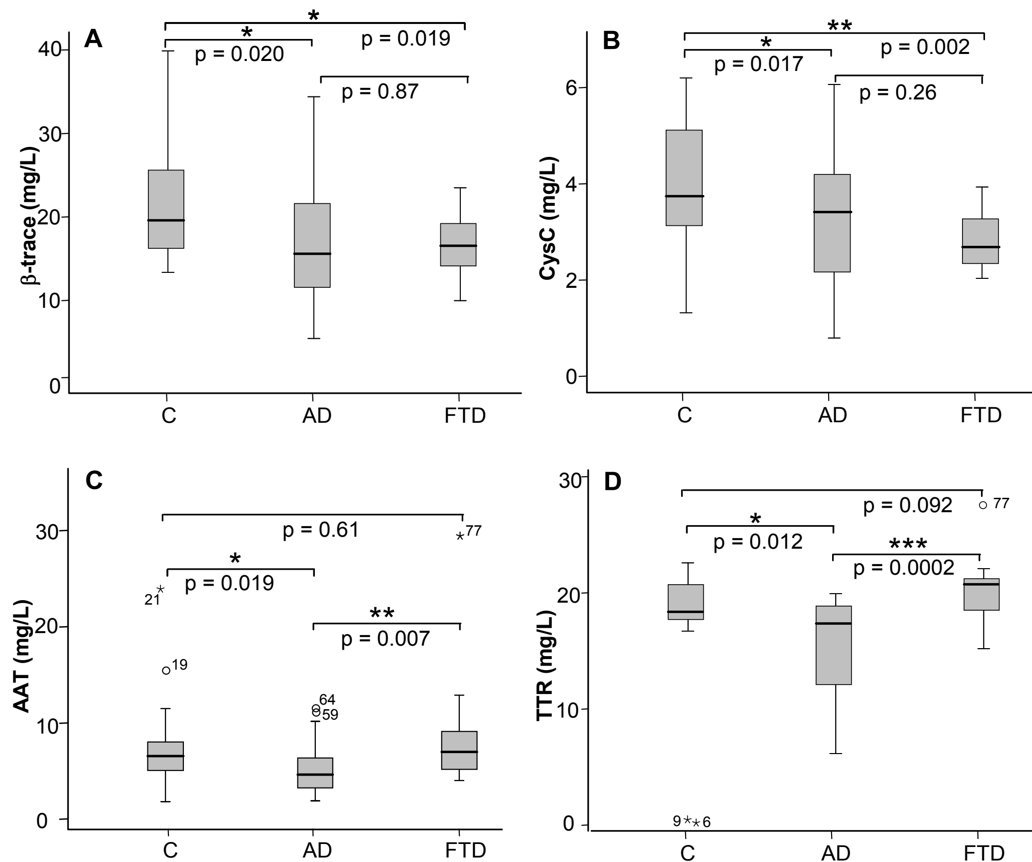


Figure 12: Concentrations of indicated CSF proteins in non-dementia controls (C), Alzheimer's disease (AD) and frontotemporal dementia (FTD) patients. Nephelometric quantification in mg/L of A) beta-trace (β-trace), B) cystatin C (CysC), C) α1-antitrypsin (AAT) and D) transthyretin (TTR). Changes between the groups were analysed using the Mann-Whitney test and results are given as horizontal brackets with *** indicating significance at the 0.001 level, ** indicating significance at the 0.01 level and * indicating significance at the 0.05 level, while p values are given below each bracket. Outliers are indicated as discrete points.

Nephelometric quantification of TTR, β-trace, AAT and CysC was performed on CSF samples from 35 subjects with AD, 18 subjects with FTD and 29 non-demented controls. Reduced CSF levels of β-trace, CysC, AAT and TTR were found in AD compared with controls. In the FTD group, CSF levels of β-trace and CysC were also reduced compared with controls, while TTR and AAT had levels similar to controls (figure 12). Subsequently, significantly reduced levels of TTR and AAT were specifically found in the AD CSF compared with FTD (figure 12).

In this study, the 2-DGE finding of reduced β-trace and TTR levels in CSF of AD patients was established. In contrast, the levels of β-trace were also reduced in FTD, not previously noted. The TTR levels in FTD were not increased, nevertheless, TTR was still differently expressed in the CSF of AD and FTD patients as suggested in the 2-DGE study. Reduced levels of AAT

were found in the AD group, while increased levels of AAT precursor were found in the 2-DGE study. Some of the discrepancies between this study and the 2-DGE study may be because no isoforms were separately quantified in this study. However, since both AD and especially FTD are heterogeneous disorders some of the changes may also be attributable to the selection of patients and when few subjects are included in a study the risks of spurious findings increase.

Next, the CSF levels of Aβ1-38, Aβ1-40 and Aβ1-42 were quantified to investigate the relationship between the potential Aβ-binding proteins and the Aβ peptide levels in CSF. As expected, CSF Aβ1-42 was highly reduced in the AD patient group compared with controls ($p=0.00005$) and FTD ($p=0.015$). This reduction may reflect increased Aβ1-42 aggregation into plaques in the AD brain. In the AD CSF there was a strong positive correlation, between all quantified Aβ peptides and β-trace, CysC and TTR levels, respectively. In contrast, no positive correlation between any Aβ species and Aβ-binding proteins was seen in CSF of FTD patients. These results suggest that reduced Aβ-binding capacity in CSF may be a specific feature of AD, which might result in an increased formation of SPs and reduced Aβ1-42 levels in CSF.

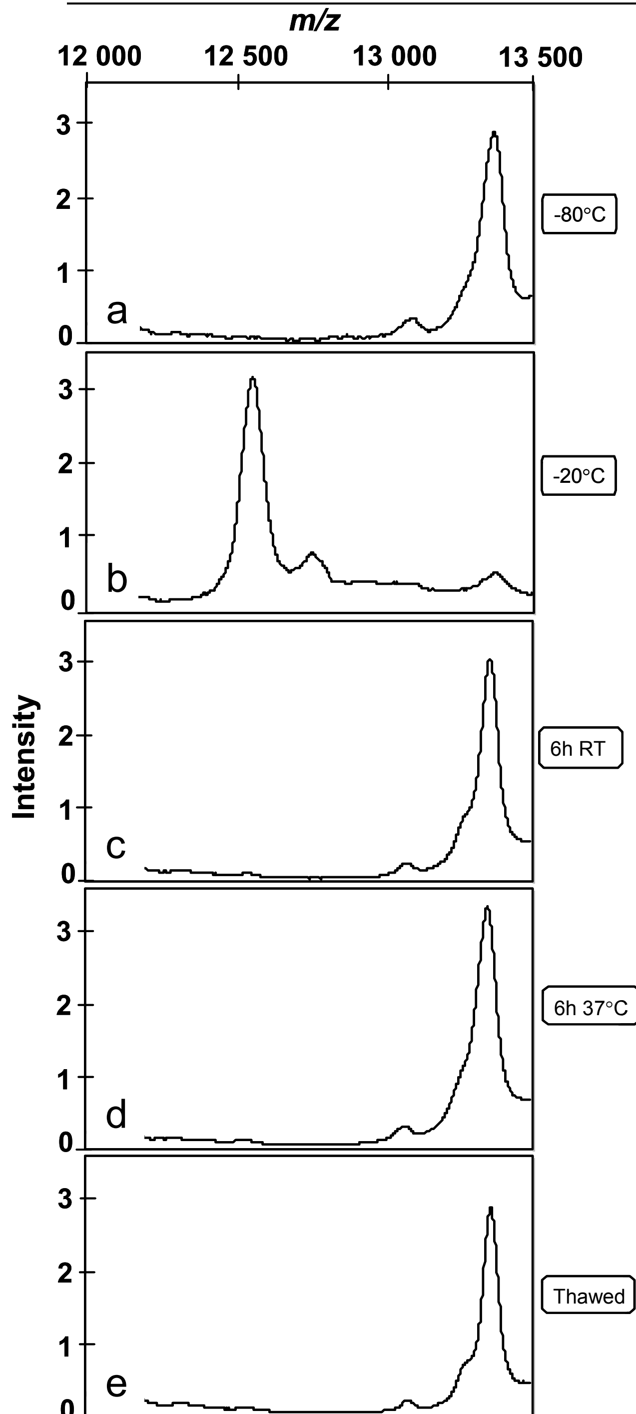
Even though the slight reduction of AAT was AD specific, these levels did not correlate with any Aβ species, suggesting a minor Aβ related role of this protein *in vivo.* TTR was the only protein specifically reduced in the AD group compared with FTD that also positively correlated with the Aβ species, thus clarifying a possible mechanistic pathway. However, neither of the protein changes could without overlap separate the groups from each other. Therefore, further larger studies are needed in order to assess any of these proteins as potential differential biomarkers for the disorders.

8. Effect of sample storage conditions on CSF proteins

Study IV

MS protein profiling and pattern analysis offer opportunities in disease diagnostics because the functional end products (i.e. the proteins), their isoforms and post-translational modifications can be studied. Concerns regarding this approach, however, include the possibility that it is limited to analysis of only the most abundant proteins. It may also reflect changes occurring as secondary systemic effects or due to the pre-analytical handling of the samples rather than disease-related processes.

Study IV was undertaken to investigate how CSF proteins were affected by different storage conditions. Carrette and co-workers [164] had recently shown that a truncated 12.5 kDa form of CysC could be produced from the full-length form (13.4 kDa) by storage-related factors not reflecting a biological process. Furthermore, preanalytical variables had been shown to



Figur 12: Cystatin C protein profiles generated by SELDI-TOF MS. The 12.5 kDa peak corresponds to truncated cystatin C and the 13.4 kDa peak corresponds to full length cystatin C. a) Spectrum from a CSF sample stored at -80°C and b) at -20°C for 3 months. c) represents a CSF sample stored at room temperature (RT) and d) at 37°C, both for 6 h, and d) represents CSF freeze-thawed four times.

markedly influence the results of SELDI-TOF-MS analysis of serum samples [165]. We therefore, raised the question of to what extent preanalytical factors, focusing on storage conditions, influence the stability of the CSF proteome in general and CysC in particular. Our results show a highly significant increase of truncated CysC in CSF after three months storage at -20°C compared with storage at -80°C, confirming that CysC is unstable at sub-optimal storage temperatures. Surprisingly, we found that CysC was not degraded in CSF stored at room temperature, incubated at 37°C or exposed to repeated freeze/thawing cycles (figure 12). Furthermore, no truncated CysC protein could be detected in CSF samples stored for 11 years at -80°C, indicating that proper storage of the CSF samples limits the truncation of CysC.

Shortly after completing our analysis, Irani and colleagues [166] reported that increased levels of the cleavage product of CysC, with a molecular mass of 12.5 kDa, was a 100% specific marker for multiple sclerosis. The increase in the cleavage product was accompanied by a reciprocal reduced level of full-length CysC.

Previous immunological studies of CysC levels in CSF of multiple sclerosis patients have been conflicting, with decreased levels found in some studies [167, 168] and unchanged levels in others [169, 170]. Simultaneously as these results were published our group had just completed a SELDI-TOF MS study

of CSF samples from 42 multiple sclerosis patients and 46 control individuals, where we were certain that the CSF samples had been stored continuously at -80°C and no significantly altered levels of either full-length CysC or the cleaved 12.5 kDa CysC fragment could be found. Accordingly, the association between multiple sclerosis and processing of CysC could not be confirmed in this study and neither by two other independent studies [171, 172].

When analysing the nature of the CysC truncation we used lys-C digestion and MS identification of the peptides to show that it was an N-terminal truncation of the first eight amino acids in the sequence, as in line with Carrette and co-workers [164]. This conclusion was not drawn in the multiple sclerosis study by Irani *et al*. [166] who suggested a C-terminal removal of the last eight amino acids. In their study trypsin digestion was used to identify the truncated CysC form but trypsin digestion cannot unambiguously define the site of truncation since the eighth amino acid in the CysC sequence is an arginine (8R), a cleavage site for trypsin. However, Lys-C does not cleave after 8R. After isolation of both CysC forms and subsequent Lys-C digestion we found peptides of 3008 Da in the 12.5 kDa digest (figure 13), corresponding to cleavage between amino acids 8R and 9L, confirming that the *in vitro* truncation must be at that position.

Figure 13: Schematic presentation of the amino acid sequence and the suggested cleavage sites of cystatin C (Cys C). Tryptic digestion of the full-length CysC (13.4 kDa) would cause a cleavage at the site where the truncated (12.5 kDa) form might be cleaved. Thus unique peptides that differentiate the cystatin C isoforms from each other are not produced using trypsin. However, Lys C endoprotease (Lys-C) digestion produces unique peptides for the isoforms and showed that the cleavage was after the eighth amino acid at the amino (N) terminal. Mass spectra of Lys-C digests of full-length (left spectrum) and truncated (right spectrum) cystatin C.

With increasing sensitivity and resolution of analytical methods the demands for rigorous control of sample handling will further increase. In addition to degradation of CysC, other proteins in CSF were also affected by storage at -20°C as compared with storage at -80°C, including transthyretin, VGF N-terminal fragment, haemoglobin beta chain and phosphorylated osteopontin C-terminal fragment and other peptides remaining to be identified (table 2) (unpublished data).

Table 2: CSF proteins affected by storage at -20°C for 3 months compared with -80°C storage analysed by SELDI-TOF MS.

M_W (Da)	Array type	-20°C vs. -80°C (change)	-20°C (mean intensity ± SD)	-80°C (mean intensity ± SD)	p-value	Identification
3814	IMAC-Cu	down	1.02 ± 0.13	3.18 ± 0.25	< 0.01	N/A
3843	CM10	up	0.84 ± 0.18	0.30 ± 0.05	< 0.01	N/A
3954	CM10	up	2.04 ± 0.49	0.26 ± 0.06	< 0.01	VGF N-terminal fragment 1
7741	Q10	down	0.69 ± 0.14	2.24 ± 0.89	< 0.01	Osteopontin CT fragment phospho
7819	CM10	down	0.65 ± 0.09	1.68 ± 0.34	< 0.01	N/A
8241	IMAC-Cu	down	1.28 ± 0.22	2.57 ± 1.07	< 0.01	N/A
8271	IMAC-Cu	down	1.15 ± 0.15	2.45 ± 0.93	< 0.01	N/A
12536	CM10	up	2.25 ± 0.77	0.07 ± 0.01	< 0.01	Cystatin C -8aa
12740	CM10	up	0.60 ± 0.19	0.01 ± 0.01	< 0.01	N/A
12913	CM10	up	1.16 ± 0.28	0.25 ± 0.05	< 0.01	N/A
13360	CM10	down	0.76 ± 0.15	2.27 ± 0.50	< 0.01	Cystatin C
13579	CM10	down	0.40 ± 0.04	0.78 ± 0.12	< 0.01	N/A
13755	CM10	down	9.00 ± 2.13	23.23 ± 7.39	< 0.01	Transthyretin
15843	CM10	down	0.10 ± 0.02	0.21 ± 0.03	< 0.01	Haemoglobin beta chain

Consequently, it is essential to ensure that control and patient samples receive the same treatment in clinical biomarker studies. It seems likely that biomarker studies employing an explorative approach, e.g. by unbiased analysis of protein profiles, are especially vulnerable to artefacts due to pre-analytical factors. Furthermore, potential CSF biomarkers must be evaluated for their stability under different pre-analytical conditions. Finally, these results highlight the importance of independent follow-up studies of potential biomarkers to eliminate spurious findings.

9. Characterization of tau from CSF

Study V

When a specific analyte is in focus and suitable antibodies exist or can be produced against the antigen, the combination of IP and MS has become a successful analytical strategy in proteome research and an important addition to established immunoassays such as ELISA and Western blot [115]. By detecting the molecular masses of the captured molecules or fragments thereof with high accuracy, MS provides not only verification of the expected antigen but also enables the identification of endogenously modified and differentially spliced forms of the antigen.

Up until now, neither 2-DGE studies nor SELDI assays or other MS methods have been successful in characterizing tau protein and its potential multi-modified forms in CSF. Tau is a particularly interesting target protein since it is involved in the neurodegenerative process both in AD and in several sub groups of FTD as described above. Furthermore, it is plausible that tau isoforms and posttranslational modifications are differently expressed in neurodegenerative disorders.

Our group has previously described a method for IP-MS of Aβ, generating an Aβ peptide signature pattern [173-175].

The development of an IP-MS procedure for tau in CSF was in several aspects complex, not least because of the low concentrations of total-tau in CSF (~300 ng/L in healthy elderly individuals and ~900 ng/L in AD patients). The largest CSF volume usually available from one individual is about 3 mL, which would contain approximately 1-3 ng tau or 20-65 fmol. Moreover, this amount is probably distributed between several post-translational modified forms and splice isoforms, reaching the detection limit of MS methods. The sensitivity of MS methods is higher for peptides than intact proteins, thus these low tau concentrations necessitate tryptic digestion of the eluted tau protein before MS analysis. However, the enzyme would also cleave antibodies co-eluted in the IP process and other contaminants unspecifically bound to antibodies or the bead material, or keratins from the laboratory environment. To reduce the co-elution problem, the first step was to cross-link the anti-tau antibody to the beads. However, neither Western blots of the intact tau eluate (figure 14a, laine 5) nor MALDI MS of digested eluates were able to identify tau in the IP-ed CSF sample (3 mL, ~3 ng T-tau). IP of high concentrations (2 μg) of three different recombinant tau isoforms yielded tau (figure 14a, lanes 6-8), showing that the IP procedure had the potential to work but was not sufficiently sensitive. One concern was that the cross-linking procedure might have reduced the binding capacity of the antibody. However, this was not the case since Western blott (figure 14b, lanes 7-9), MALDI and T-tau ELISA results showed that the yield was reduced when cross-linking was omitted.

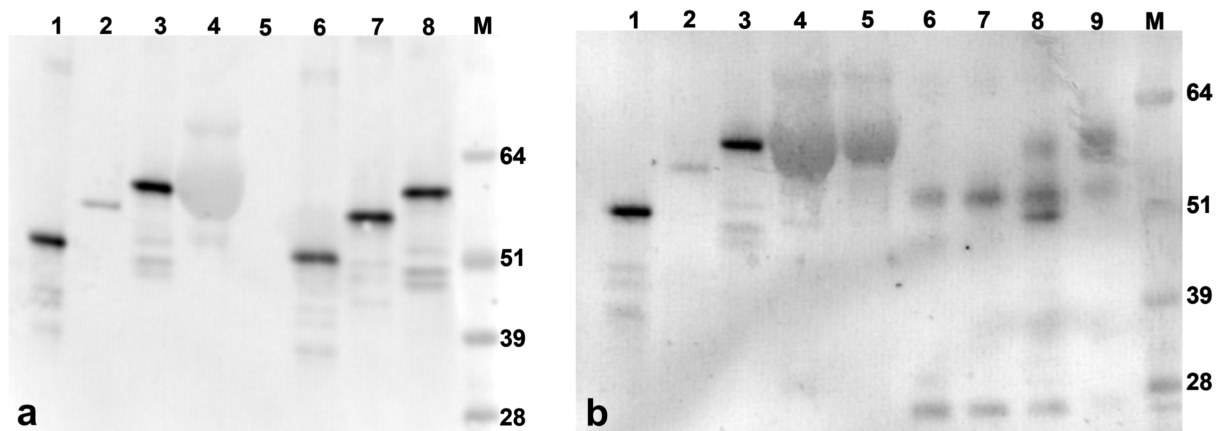


Figure 14: Western blot with the BT2 anti-tau antibody. a) Lanes 1-4 represent non-IP samples of recombinant tau proteins 3R0N, 4R0N, 4R2N and 25µl CSF, respectively and lanes 6-8 represent cross-linked BT2-IP precipitates of the corresponding recombinant proteins. Lane 5 represents the cross-linked BT2-IP CSF sample (3 mL). b) Lanes 1-5 represent non-IP samples of recombinant tau proteins 3R0N, 4R0N, 4R2N and two 25 µl CSF respectively. Lanes 7-9 represent un-linked BT2-IP precipitates of the recombinant proteins. Lane 6 represents an un-linked BT2-IP precipitated CSF sample (3 mL).

We then hypothesized that the binding capacity of the antibody to tau might be reduced due to competing high-abundance proteins in the CSF (e.g. albumin and IgG) or that these proteins bound unspecifically to the bead material, masking the tau signal. To reduce high-abundance proteins, 2.5% perchloric acid precipitation was used, previously used when purifying tau from brain tissue [176], followed by neutralization of the supernatant with potassium hydroxide prior to IP. Tau was soluble in 2.5% perchloric acid while most other CSF proteins were precipitated, since the T-tau levels, as determined by ELISA, were the same in the precipitated sample compared with the initial CSF sample. Furthermore, a reduced number of peaks from contaminating proteins were detected in the MALDI mass spectra of tau obtained from precipitated CSF by the crosslinked IP method and three tau-derived peptides (*m/z* 1393.70, 1411.73 and 1420.81) were clearly visible. The total yield of the developed tau-IP procedure applied to the supernatant of perchloric acid treated CSF was determined to 78% by the T-tau ELISA.

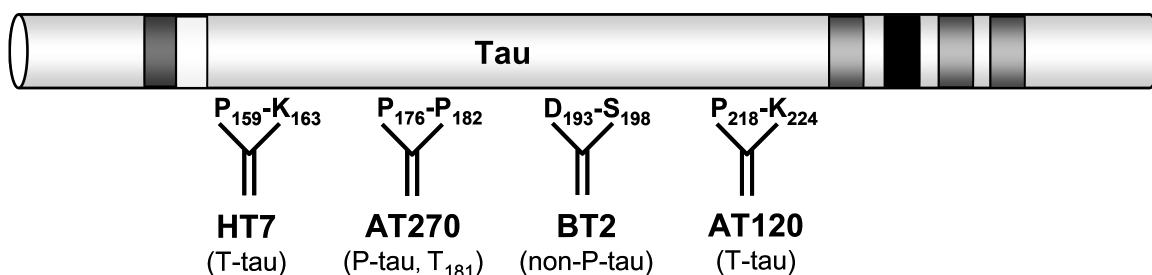


Figure 15: Epitopes of the anti-tau antibodies, numbered according to the sequence of the 441 amino acid tau isoform (4R/2N). HT7 and AT120 detect total tau (T-tau), BT2 non-phosphorylated tau forms (non-P-tau) and AT270 tau phosphorylated (P-tau) at threonin 181 (T_{181}).

In all initial experiments the BT2 anti-tau antibody was used. BT2 has been reported to have a very high affinity for tau, but was also suggested to only detect non-phosphorylated tau forms [177, 178]. Subsequently, additional tau-antibodies, including HT7, AT120 and AT270 [179] (figure 15) were compared with BT2 using the optimized IP-MS procedure. BT2 was still the most efficient antibody for detection of tau-related peptides in the MALDI mass spectra compared with the other anti-tau antibodies (figure 16).

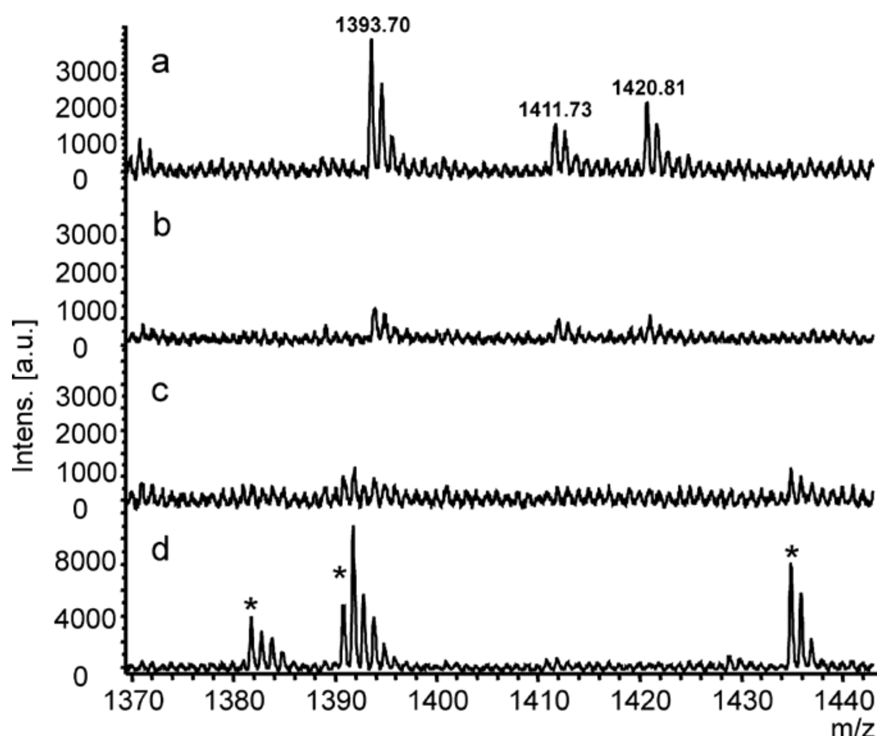


Figure 16: CSF was precipitated by perchloric acid (2.5%). Then the supernatant was subjected to immunoprecipitation using cross-linked antibodies. Four different anti-tau antibodies were tested: a) BT2, b) HT7, c) AT120 and d) AT270. Using BT2 and HT7, 3 peaks corresponding to tau were detected and their protonated monoisotopic molecular masses (MH^+) are indicated in a). The peaks labelled with * in d) were not identified.

The three tau derived peptides identified using direct MALDI MS were not sufficient for in-depth characterization of the tau protein. To increase the detection sensitivity, nano RP-LC separation of the tryptic tau peptides was performed prior to MS resulting in decreased occurrence of overlapping signals in the mass spectra, which facilitated peak detection and selection of precursor ions for MS/MS analysis.

The LC-MALDI-TOF/TOF MS analysis resulted in detection of 13 peptides with *m/z* values that matched tryptic peptides of tau with a mass accuracy of less than 30 ppm and of these, six identities were confirmed by MS/MS data. Three of the identified peptides were isoform specific with monoisotopic mass (Mr) 2423.12, 3009.38 and 3954.85, corresponding to 0N, 1N and 2N respectively. To confirm and extend our search LC-ESI-QIT-

FTICR MS was also performed resulting in the identification of six additional tau peptides and confirmation of eight peptides (table 3).

Table 3: LC-MALDI-TOF/TOF MS and ESI-QIT-FTICR MS analysis of CSF-tau immunoprecipitate digested with trypsin*.

Mr	aa sequence	MALDI ion score	ESI ion score	3R/0N	3R/1N	3R/2N	4R/0N	4R/1N	4R/2N
995.49	TPPSSGEPPK	ND*	31	123-132	152-161	181-190	123-132	152-161	181-190
998.54	TPPKSPSSAK	ND	D**	173-182	183-192	231-240	173-182	202-211	231-240
1065.58	TPSLPTPPTR	D	40	154-163	183-192	212-221	154-163	183-192	212-221
1308.71	LQTAPVPMPDLK	ND	56	185-196	214-225	243-254	185-196	214-225	243-254
1330.69	AKTDHGAEIVYK	ND	D	295-306	324-335	353-364	326-337	355-366	384-395
1392.63	SGYSSPGSPGTPGSR	54	83	137-151	166-180	195-209	137-151	166-180	195-209
1410.67	TPPSSGEPPKSGDR	27	38	123-136	152-165	181-194	123-136	152-165	181-194
1419.77	TPSLPTPPTREPK	26	36	154-166	183-195	212-224	154-166	183-195	212-224
1422.73	GAAPPGQKGQANATR	ND	D	98-112	127-141	156-170	98-112	127-141	156-170
1662.91	SRTPSLPTPPTREPK	D	19	152-166	181-195	210-224	152-166	181-195	210-224
1780.90	MVSKSKDGTGSDDKKAK	D	ND	69-85	98-114	127-143	69-85	98-114	127-143
1993.01	SKDGTGSDDKKAKGADGKTK	D	ND	73-92	102-121	131-150	73-92	102-121	131-150
2052.88	QEFEVMEDHAGTYGLGDR	62	35	6-23	6-23	6-23	6-23	6-23	6-23
2180.98	QEFEVMEDHAGTYGLGDRK	33	ND	6-24	6-24	6-24	6-24	6-24	6-24
2209.10	MVSKSKDGTGSDDKKAKGADGK	D	ND	69-90	98-119	127-148	69-90	98-119	127-148
2291.20	DRVQSKIGSLDNITHVPGGGNK	ND	D	259-280	288-309	317-338	290-311	319-340	348-369
2423.12	AEEAGIGDTPSLEDEAAGHVTQAR	D	D	45-68	-	-	45-68	-	-
3009.38	STPTAEAEEAGIGDTPSLEDEAAGHVTQAR	85	134	-	68-97	-	-	68-97	-
3954.85	QAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQAR	D	ND	-	-	88-126	-	-	88-126
Sequence coverage:				52%	49%	48%	48%	46%	45%

*The reported molecular mass values are the calculated monoisotopic masses (Mr) of the matched tryptic peptides. For peptide identities confirmed by MS/MS, the corresponding Mascot ion score is given. The position of the matched peptides in the amino acid sequence is given for each of the six human tau isoforms.
D = Peptide only assigned by molecular mass. ND = Not detected.

In conclusion, if sensitive, high affinity antibodies exist or can be produced, IP-MS is a promising approach allowing identification and characterization of isoforms and posttranslational modified forms of very low-abundant proteins in combination with state of the art MS methods. In this study we were able to characterize tau using MS for the first time in CSF.

CONCLUSIONS

Proteomics is a rapidly developing methodological field, meeting the demands for detection and quantification of low-abundance proteins. The sensitivity and mass accuracy of modern mass spectrometers are increasing and currently low femto- to attomoles can be detected in pure samples. Therefore, the limitation of protein detection/identification depends largely on sample preparation, keeping analyte losses to a minimum, while still producing pure samples with reduced complexity prior to the MS analysis. Thus, prefractionation is generally necessary but current approaches are unable to encompass the complete proteome. A combination of complementary methods will probably be needed, each displaying a different analytical window of the proteome.

The profiling methods, 2-DGE and SELDI-TOF-MS are currently limited to detection of proteins in the low mg/L range in CSF. For detection of less-abundant proteins a more focused perspective is needed and by developing an IP-MS method for characterization of tau in CSF we were able to detect tau isoforms in the ng/L range in CSF samples.

Explorative proteomic studies also demand careful handling of the CSF samples since increased sensitivity of the methods may also increase the risk for biased findings. Proteins found to be differentially expressed in the CSF of diagnostic groups should be meticulously investigated, preferably with complementary MS and/or immunological methods. A panel of several altered proteins will probably be needed to fully distinguish different neurodegenerative disorders.

Recent advances in proteomic techniques offer new opportunities for discovering biomarkers of complex neurodegenerative disorders such as AD and FTD and for unravelling the disease pathogenesis by studying their CSF proteome. Even if AD and FTD are heterogeneous and multifactor disorders, the fact that several proteins are differentially expressed indicates that there are different pathological mechanisms behind the two neurodegenerative disorders. Reduced levels of TTR separated AD from FTD and the strong correlation of TTR with Aβ peptides in CSF supported the suggestion that reduced Aβ-binding capacity in the AD brain may be involved in the AD disease mechanism.

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