Quantitative neuroproteomics for biomarker discovery in Alzheimer's disease

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To my family,

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia characterized by amyloid plaques and neurofibrillary tangles in the brain. Novel biomarkers for AD are needed that reflect disease progression and could identify subjects at risk of developing AD at an asymptomatic stage. The aim of this thesis was to develop methods that can be used to quantify endogenous peptides and proteins in cerebrospinal fluid (CSF) to identify potential biomarkers for AD.

A workflow was developed for preparation of peptide extracts from CSF. The endogenous peptides from CSF were identified by tandem mass spectrometry, and several novel endogenous peptides were found. To quantify the endogenous peptides in CSF, isobaric labeling for relative quantification was incorporated into the workflow. A clinical cohort with CSF samples from AD patients and controls was then analyzed with the method to identify potential biomarkers for AD. Several alterations among the endogenous peptides and proteins were found in the AD group. Altered endogenous peptides derived from proteins that affect e.g. Aβ aggregation, such as integral membrane protein 2B, and from proteins that have been reported as associated with AD, such as neurosecretory protein VGF, metallothionein-3 and secretogranin-1. Increased levels of the protein YKL-40 were found in the CSF of AD patients as well as alterations in novel potential protein biomarkers.

Human CSF samples from a γ-secretase inhibitor (GSI) trial were also analyzed with the developed workflow to identify potential biomarkers for γ-secretase activity. Endogenous peptides from amyloid precursor-like protein 1 (APLP1), apolipoprotein E, proSAAS, secretogranin-1 and metallothionein-3 were significantly lowered in subjects who received the GSI compared to those who received a placebo. Two peptides from APLP1, which is a known γ-secretase substrate, were identified as decreased and could be potential biomarkers for γ-secretase activity. The other endogenous peptides were derived from proteins that are not known γ-secretase substrates but were nevertheless decreased.

In summary, the developed method could be used to identify novel biomarkers for diseases affecting the brain, and for monitoring treatment effects of substances which have their target in the brain. Several potential CSF biomarkers were identified for AD and for γ-secretase activity.

SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom (AD) är den vanligaste orsaken till demens och karakteriseras av en nervcellsdegeneration och inlagring av så kallade senila plack och neurofibriller i hjärnan. Det finns idag ingen effektiv behandling som kan avstanna sjukdomsförloppet i AD. Nya behandlingar tros vara mest effektiva tidigt i sjukdomsförloppet, då nervcellsdegenerationen i hjärnan fortfarande är begränsad. För att tidigt kunna identifiera vilka individer som kommer att utveckla AD behövs biomarkörer som kan reflektera tidiga förändringar i hjärnan som är kopplade till sjukdomen. Ryggvätska (likvor) är i direkt kontakt med hjärnan och kan därför användas för att studera förändringar som sker i hjärnan. För att identifiera förändringar i likvor som kan användas som biomarkörer för AD, kan halterna av proteiner och peptider i likvor jämföras mellan AD patienter och kontroller.

I denna avhandling har en metod utvecklats som kan användas för att mäta de relativa halterna av proteiner och naturligt förekommande peptider i likvor. Metoden har sedan använts för att undersöka om individer med AD har förändrade halter av peptider eller proteiner i likvor. Genom detta identifierades flertalet förändringar i likvor hos AD patienter som kan vara potentiella biomarkörer och som inte tidigare har rapporterats. De förändrade peptiderna är bland annat sådana som kan påverka bildningen av de senila placken i hjärnan.

Med den utvecklade metoden analyserades även likvorprover från en klinisk läkemedelsprövning för AD. Genom att analysera likvorprover från denna studie identifierades ett flertal peptider i likvor som var förändrade som en följd av behandlingen. Två av de förändrade peptiderna har en klar koppling till läkemedlets tänkta effekt och kan därför vara biomarkörer som reflekterar hur väl läkemedlet påverkar sitt mål. Dessa peptider skulle därför kunna användas som biomarkörer vid framtida läkemedelsprövningar där liknande preparat testas.

Sammanfattningsvis har det i avhandlingsarbetet utvecklats en ny metod som kan användas för att identifiera nya biomarkörer för AD och andra sjukdomar som drabbar hjärnan. Denna metod visade sig kunna identifiera nya potentiella biomarkörer för AD och identifierade också biomarkörer som kan vara användbara i framtida läkemedelsprövningar för AD.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Hölttä M, Zetterberg H, Mirgorodskaya E, Mattsson N, Blennow K, Gobom J. **Peptidome analysis of cerebrospinal fluid by LC-MALDI MS.** PLoS One. 2012;7(8):e42555.

II. Hölttä M, Minthon M, Hansson O, Holmén-Larsson J, Pike I, Ward M, Kuhn K, Zetterberg H, Blennow K, Gobom J. **Multiplexed quantitative proteomics and peptidomics of cerebrospinal fluid identify potential biomarkers for Alzheimer's disease**. Submitted.

III. Hölttä M, Dean R, Siemers E, Mawuenyega K, Sigurdson W, May P, Paul S, Holtzman D, Portelius E, Zetterberg H, Bateman R, Blennow K, Gobom J. **Effects of γ-secretase inhibition on endogenous peptides in human cerebrospinal fluid.** Manuscript.

CONTENT

ABBREVIATIONS

CSF – cerebrospinal fluid
CNS – central nervous system
AD – Alzheimer's disease
LC – liquid chromatography
MS – mass spectrometry
MS/MS – tandem mass spectrometry
Aβ – amyloid-β
NFTs – neurofibrillary tangles
ESI – electrospray ionization
MALDI – matrix-assisted laser desorption/ionization
SELDI – surface-enhanced laser desorption/ionization
TOF – time-of-flight
CID – collision-induced dissociation
MCI – mild cognitive impairment
APP – amyloid precursor protein
APLP1 – amyloid precursor-like protein 1
PIB – Pittsburgh compound B
PET – positron-emission tomography
ApoE – apolipoprotein E
iTRAQ – isobaric tags for relative and absolute quantitation
TMT – tandem mass tags
ICAT – isotope-coded affinity tags
SILK – stable isotope-labeling kinetics
ELISA – enzyme-linked immunosorbent assay
CE- capillary electrophoresis
Da – Dalton
MWCO – molecular weight cut-off
GSI – γ-secretase inhibitor
ITM2B – integral membrane protein 2B
YKL-40 – chitinase-3-like protein 1
m/z – mass-to-charge ratio

1 INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia affecting over 24 million people worldwide [1]. AD is believed to have a latent phase of 20-30 years [2], and it has been shown that abnormal levels of biomarkers for Alzheimer's neuropathology are found up to 10 years before disease onset [3]. There is today no disease modifying treatment available. A disease modifying treatment is most likely to be effective at an early stage of the disease when the irreversible neuronal loss is limited. As AD cannot be clinically diagnosed before onset of cognitive symptoms, there is a need for biomarkers that can be used to identify subjects at risk of developing AD at an early stage.

To identify novel fluid biomarker for AD, candidate biomarkers identified in e.g. genetic studies, in cell studies, or in animal models can be analyzed in human fluid samples to evaluate their potential as biomarkers for AD. Another approach to identify novel biomarkers is the use of proteomics, which is an unprejudiced approach that tries to identify changes among all proteins in, e.g., cerebrospinal fluid (CSF). Proteomics has been used in several biomarker discovery studies for AD and several proteins have been identified as altered in the CSF of AD patients. These discovery studies have however not resulted in a clinically useful biomarker so far. Endogenous peptides in CSF have been less well studied as potential biomarkers. Changes in the endogenous peptides could reflect several disease-related processes, e.g., dysfunction of proteases, in the brain. In this thesis, quantitative methods were developed to analyze endogenous peptides in CSF to highlight their possible role as biomarkers for AD and other disorders affecting the brain.

2 THE CENTRAL NERVOUS SYSTEM

The central nervous system (CNS) consists of the brain and the spinal cord. The brain has around 100 billion neurons, and even more glial cells that provide nutrition and protection to the neurons. The CNS controls various bodily activities and mental processes such as cognition [4]. The neurons of the CNS communicate with each other through synapses, which are formed between the axon terminals and dendrites of the neurons. One neuron can have a few hundred or as many as 200,000 synaptic connections, where the connections between the neurons can take on an endless number of combinations. The synapses are involved in memory, where repeating signals through the synapse will facilitate the synapse of transmitting the same signal the next time.

Electric impulses travel through the neurons to the axon terminals and affect the release of neurotransmitter into the synaptic cleft. The neurotransmitters traverse the synaptic cleft to receptors on the dendrite of the adjacent neuron. The receptors on the dendrites are usually either cation or anion channels, which regulate the passage of ions in and out of the cells. If the dendrite has excitatory receptors that allow the passage of cations, the membrane potential rises toward the threshold level for excitation which can de-polarize the neuron and give rise to a new electrical impulse that travels towards the soma of the neurons, where it will affect whether that neuron will send the electrical impulse further on. If the dendrite has inhibitory receptors that allow the passage of anions and transmitter substances that inhibit the postsynaptic neurons are released, the membrane potential of the dendrite will be lowered which inhibits a new electrical impulse from being created.

The neurons in the brain are constantly adapting to changes in the environment and to different forms of stimuli. The proteins and peptides in the brain are responsible for many of the processes in the brain regulating the survival and function of the neurons. If the synthesis, formation, degradation, or modification of these proteins and peptides are affected it may very well cause disturbances in the functions of the neurons and their structure that eventually may result in clinically overt diseases.

2.1 Cerebrospinal fluid

Cerebrospinal fluid (CSF) fills the ventricles of the brain and surrounds the brain and the spinal cord [5]. CSF is created by passive filtration of blood in the choroid plexus and by diffusion of interstitial fluid from the brain [6].

The CSF is isolated from the blood by the blood-CSF barrier which is made up by tight junctions between the ependymal cells facing the CSF side. This prevents proteins from passing through while small hydrophobic substances can pass. There is an active transport of vital compounds such as glucose across the barrier.

The choroid plexus is located to the ventricles of the brain and contains fenestrated capillaries that are permeable to water. The water passes from the blood to the CSF as a result of active transport of ions from blood into CSF, which causes osmosis of water through the membrane into the CSF. There is around 150 mL of CSF in the CNS. As CSF is created at a rate of 400-500 mL per day, it is turned over at a rate of approximately 3 times a day.

The interstitial fluid in the brain that diffuses to become CSF brings along proteins, peptides, and metabolites from the brain, and therefore changes in the brain are reflected in the CSF. CSF has a protein concentration of ~350 µg/mL, which is low compared to serum where the protein concentration is ~70 mg/mL [7]. Approximately 80% of the CSF proteins come from serum, while only around 20 % of the proteins are derived from the brain. The two most high-abundant proteins, albumin and immunoglobulins, represent more than 65% of the total protein composition of CSF [8]. CSF is still considered the optimal body fluid to analyze to identify biochemical changes in the brain as it is in direct contact with the extracellular space of the brain. CSF can be sampled by lumbar puncture between the L4 and L5 interspace, where usually around 12 mL of CSF is collected.

3 ALZHEIMER'S DISEASE

The first case of AD was reported in 1907, when Alois Alzheimer described a demented woman with progressive memory loss and eventually the inability to perform daily activities. At post mortem analysis of her brain, Alzheimer described neuritic plaques, neurofibrillary tangles, and extensive atrophy of her brain [9]. The disease with these characteristics was later named Alzheimer's disease by Emil Kraepelin.

AD is estimated to affect over 24 million people worldwide (2001), with a predicted increase to over 42 million in 2020 [1]. It is the most common form of dementia characterized by neuronal degradation and incorporation of amyloid plaques and neurofibrillary tangles in the brain [10]. The disease commonly affects people that are 65 years or older, with a prevalence that increases exponentially with age, from under 1% in the age group 60-64 years to over 24% in people over 85 years of age [1].

3.1 Diagnosis and clinical manifestation

The diagnosis of AD is based on fulfillment of the criteria for dementia according to DSM-IV, and the outline of the AD criteria according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [11]. The diagnosis is to a large extent based on the exclusion of other forms of dementia. The criteria include symptoms such as loss of memory and difficulties in planning and executing different tasks, where exclusion criteria are signs of confusion and other causes of dementia. In general only about one third of all AD patients have pure Alzheimer pathology. The remaining part shows overlap with cerebrovascular disease, Lewy body pathology, or other types of pathology [12-15]. The diagnostic accuracy of the clinical criteria is not ideal, as the sensitivity and specificity is around 80 % and 70 %, respectively [16]. A definite diagnosis can only be made by postmortem examination, where the characteristic pathological manifestations in the brain are used for confirmation. In recent, still not established revisions of the clinical criteria, biomarker-derived information on plaque and tangle pathology has been incorporated [17, 18]. These revisions are expected to increase the specificity of the criteria and will also allow for making an AD diagnosis in pre-dementia stages of the disease [19].

3.2 Neuropathology

The main constituent of the neurofibrillary tangles (NFT) in the brain was identified in 1986 as insoluble hyperphosphorylated tau aggregates [20-23]. The normal function of tau is to stabilize microtubule in neurons and is mainly found in the distal part of axons. What causes the hyperphosphorylation of tau in AD is today not known. The formation of NFTs in the brain has been suggested to happen at a very early age, before the formation of amyloid plaques [24], and the abundance and distribution of NFTs in the AD brain correlate with the symptoms seen in AD [25, 26]. However, the NFTs are not specific for AD as they are also present in several other dementias such as frontotemporal dementia, while increased levels of phosphorylated tau are only reported in AD. In AD the NFTs are initially found in the entorhinal region of the medial temporal lobe and are then spread to the hippocampus, the limbic system and eventually to the neocortex [27].

Aβ was identified as the main component of amyloid plaques in 1984-1985 [23, 28], after which the amyloid precursor protein (APP) gene was cloned [29]. The amyloid plaques are first found in the temporal, frontal and occipital lobe and then spreads to the other parts of the neocortex, and to deeper brain areas/nuclei and the hippocampus [27]. Amyloid plaques are however also found in non-demented individuals, where around 30% have plaques and tangles that would be enough for a neuropathological diagnosis of AD [14, 15]. The levels of $A\beta_{42}$ in CSF are decreased by 50% in AD patients [30, 31], most likely due to incorporation of $A\beta_{42}$ into plaques, with less $A\beta_{42}$ available for diffusion into the CSF. It has been shown that mutations causing overproduction of Aβ increases the risk of AD and lowers the age of onset [32].

3.2.1 Processing of APP/Aβ and its homologues

Amyloid precursor protein (APP) is a transmembrane protein whose function in the brain is not fully understood. APP is processed by different proteases such as α-secretase, β-secretase, and γ-secretase which create Aβ peptides of different lengths [33-36] (Figure 1). APP is first cleaved by either α-secretase or β-secretase, followed by γ-secretase cleavage in the transmembrane region of the APP, creating the Aβ peptide. α-secretase cleavage followed by γ-secretase creates a shorter form of the Aβ peptide, which is believed to be the non-amyloidogenic pathway [37]. The 42 amino acid Aβ form, created by β- and γ-secretase cleavage, is prone to aggregation, and is the major constituent of amyloid plaques [23]. The Aβ peptides are cleared by enzymatic

degradation by insulin degrading enzyme [38, 39], neprilysin [40, 41], and other proteases or by flux across the blood brain barrier by low-density lipoprotein receptor-related protein (LRP) [42, 43].

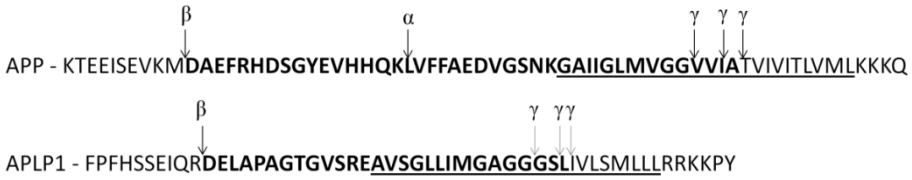

Figure 1. Cleavage sites for APP and APLP1 that creates Aβ and Aβ-like peptides. Both proteins are cleaved in their transmembrane region (underlined) by γ-secretase, while β-secretase cleaves both proteins in the extracellular region. α-secretase creates a shorter Aβ peptide and is believed to be the non-amyloidogenic pathway.

APP has two mammalian homologues, amyloid like protein 1 & 2 (APLP1 and APLP2) (reviewed in [44]). These proteins seem to have complementary functions as knocking out one of these genes in mice does not give rise to severe phenotypes, while double knock-outs for APLP1/APLP2, and APP/APLP2 are lethal, although APP/APLP1 double knockout mice are not [45]. The APLP1 protein is supposedly brain specific, while APLP2 and APP are not. In the brain APP is found in the synaptic membrane and pre-synaptic terminals, while APLP1 is found in the post-synaptic density [44]. APLP1 is processed in the same manner as APP, where β-secretase and γ-secretase creates Aβ like peptides [33].

3.2.2 γ-secretase processing of APP and its homologues

γ-secretase is a transmembranous protease consisting of four subunits, presenilin-1/presenilin-2, nicastrin, APH-1, and PEN-2 [46]. The presenilin-1/presenelin-2 is the active site of γ-secretase complex [47-50], which cleaves proteins at their transmembrane region. For APP this cleavage creates the carboxy-terminal of the Aβ peptide [47, 51, 52]. γ-secretase is rather promiscuous as it creates several truncated forms of Aβ, as $Aβ_{38}$, $Aβ_{40}$, and $Aβ_{42}$. Interestingly, it also seems to be involved in the formation of $Aβ_{17}$, where the cleavage site is well outside the transmembrane part [53, 54].

There are familiar forms of AD that are caused by mutations in the γ-secretase/presenilins which lead to increased production of $Aβ_{42}$, which increases the plaque load and lowers the age of onset of the disease [55-59]. Some of these mutations shift the $Aβ_{42}/Aβ_{40}$ ratio mainly by lowering the production of $Aβ_{40}$, which might suggest that $Aβ_{40}$ could be neuroprotective

[60]. γ-secretase has several other known substrates including notch and APLP1. APLP1 is also processed in the same manner as APP by the other enzymes creating Aβ, β-secretase and α-secretase [33, 61], which generates Aβ like peptides from APLP1 of several different lengths. Three peptides from APLP1 are known to be generated by γ-secretase cleavage in the transmembrane part, APL1β25, APL1β27, and APL1β28 [33, 62]. There are additionally several shorter APLP1 peptides generated from this region of the protein, although the proteases generating them have not been reported [63-65].

3.3 Amyloid plaque burden in aging

The amyloid plaques in the brain can be visualized in vivo by using Aβ aggregate ligands, e.g., the ^{11}C Pittsburgh Compound B (PIB), in conjunction with positron emission tomography (PET) [66]. This can be used as an aid to determine if an individual has amyloid plaques in the brain which is indicative of AD. The ^{11}C-PIB binding in AD patients has been shown to correlate with lower levels of CSF $A\beta_{42}$ [67-71]. There are however certain subtypes of amyloid plaques, e.g. diffuse plaques, that are not detected with PIB-PET [72]. Studies have shown that around 30% of cognitively normal elderly have amyloid plaques in their brains in post mortem analysis, without having developed cognitive deficiency [73, 74], which has also been reported in PIB-PET studies [75-78].

3.4 Genetic risk factors

Over 70% of all AD cases are estimated to be related to genetic components [79]. There are familial mutations that increase the risk of developing AD, which are located to the genes for PS1 [51], PS2 [58], and APP [80]. These mutations usually lead to an increased production of $A\beta_{42}$ or increase Aβs aggregation proficiency, leading to a lower age of onset. These familial mutations are however rare and account for less than 1% of all AD cases [81].

A more common genetic risk factor is the susceptibility gene coding for apolipoprotein E (ApoE). Three common polymorphisms of *APOE* are found in humans, the ε2, ε3, and ε4 alleles. The *APOE* ε4 allele is present in around 5-35% of the population, the ε3 allele in around 50-90% and the ε2 allele in 1-5% [82]. Heterozygotes for *APOE* ε4 have a decreased age of onset of about 10 years and a 3-4 folds increased risk of developing AD, while homozygotes have a decreased age of onset of about 20 years and 8-10 folds

increased risk of developing AD [83-86]. The *APOE* ε4 allele is found in about 50% of all AD cases [87].

These genetic findings, together with the fact that individuals with Down's syndrome, that have an extra copy of gene 21 where APP gene sequence is located, develops amyloid plaques and AD early in life, has led to a general belief that the Aβ is the driving force in the disease [88-90].

3.5 Cerebrospinal fluid biomarkers for AD

The AD diagnosis is today based on clinical evaluation. In addition, CSF biomarkers are sometimes measured to help with the diagnostic process. The decreased levels of $A\beta_{42}$, and increased levels of phosphorylated tau and total-tau found in the CSF of AD patients reflect the incorporation of $A\beta_{42}$ into plaques [91], NFT formation [21, 22], and neuronal degeneration [92, 93], respectively. These CSF biomarkers are not included in the AD criteria today but have been proposed to be included in the new criteria discussed for AD [17], as they have shown high sensitivity and specificity.

There is a need for biomarkers that can detect individuals at risk of developing AD, as a future treatments are most likely to be effective at an early stage to prevent the irreversible neuronal degeneration from progressing [94, 95] (Figure 2). Naturally, it is not possible to diagnose presymptomatic patients by clinical evaluation. The ideal marker should reflect changes that are AD specific and can be used to evaluate disease progression and treatment efficacy.

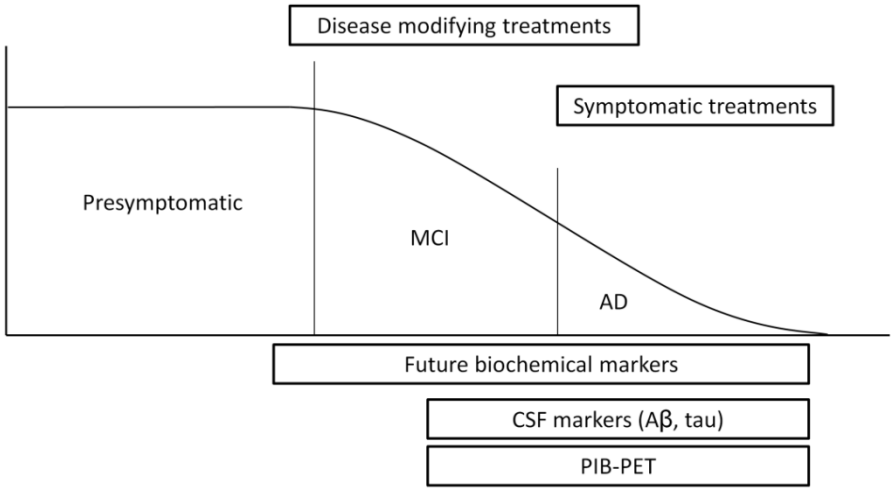

Figure 2. Diagnostic and treatment possibilities. The biomarkers used today (Aβ, tau) can distinguish individuals at risk of developing AD 10 years before disease onset. A disease modifying treatment will most likely have the best effect at an early stage of the disease, before the neuronal loss has become too extensive. Adapted from [96].

3.6 Mild cognitive impairment

Patients with mild cognitive impairment (MCI) are characterized by having objective memory impairment adjusted for age and education but do not fulfill the criteria for dementia [97]. MCI has been proposed to be a prodromal stage of dementia. Although the MCI patients progress to dementia of AD type at a rate of 6-15% annually [98-101], it has been reported that more than half of the MCI patients do not progress to dementia even after 10 years [100]. Some of the MCI patients revert to normal function as well.

3.7 Treatments

There is today no curative or disease-modifying drug available for AD. The treatments that are available are symptomatic treatments, where two different types of drugs are used. One group of drugs inhibits the activity of acetylcholine esterase which increases the amount of available acetylcholine in the synaptic cleft and thus increases the activity on the cholinergic neurons (donezepil, galantamine, and rivastigmine), while the other compound is a partial N-methyl-D-aspartate (NMDA) receptor antagonist (memantine).

These treatments are only moderately effective on symptoms, as the average effect duration is around 2 years, even if some individuals may have positive effects up to 5 years [102-104]. The acetylcholine esterase inhibitors do not differ in efficacy and give a modest effect on the patients' MMSE (mini-mental state examination) scores, as well as on behavioral and functional symptoms [105]. The NMDA receptor inhibitor also has positive effects on cognition and behavioral symptoms as well as on daily activities [106]. The use of the two different treatments in combination is more beneficial than only using acetylcholine esterase inhibitors in moderate to severe AD [107]

Several novel compounds have been developed and tested in the pursuit of preventing the disease from progressing. There have been attempts using vaccination/immunization against Aβ [108-111], using antibodies to clear out the amyloid plaques and lower Aβ levels [112-120], inhibitors against Aβ aggregation [121-125] , and attempts to inhibit or modulate proteases that create the Aβ peptides namely β-secretase (reviewed in [126]) and γ-secretase [127-137].

These compounds have shown very promising effects in animal models where they have cleared the amyloid plaques from mice brains [126, 138-148] and recovered their cognitive decline. They have however so far failed in human subjects [149].

4 BIOMARKER DISCOVERY

4.1 Biomarker discovery in CSF using proteomics

There has been an intensive search for new biomarkers for AD which can be used for early detection of the disease and that could reflect other underlying processes of the disease than plaque and tangle pathology. Several biomarker discovery studies in CSF using 2-dimensional gel electrophoresis and mass spectrometry (MS) have been performed (for review see [150]). This analytical technique has the ability to separate different and modified forms of proteins. Disadvantages are that the analyzed spots seldom only contain one protein and it is not a high-throughput technique, and therefore not applicable to larger scale clinical studies. Certain categories of proteins are also not accessible to analysis, e.g., aggregation-prone proteins and protein with extreme pI values. Another technique that has been used is Surface-Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) [151]. This technique combines affinity purification on a chip platform (based on hydrophobicity, ionic strength, antibodies affinity, DNA affinity, or protein affinity) with mass spectrometric readout of protein molecular weight profiles. While initially promising, a severe shortcoming of SELDI-TOF turned out to be the dynamic range: using only a single sample purification step based on general physicochemical properties, only high-abundant sample components can be detected. While these may in some cases differ between disease and control cohorts, they are usually not specific to one disease. Another drawback of SELDI-TOF is that it only provides protein profiles with low mass accuracy where the proteins are not identified.

The most commonly employed analytical approach used in proteomics currently is the shotgun-approach. Here, proteins in crude samples (e.g. CSF) are subjected to proteolytic digestion without preceding isolation. The resulting peptide mixture is then analyzed by LC-MS, using single or multiple chromatographic separation steps upstream to the mass spectrometer. While distinguishing among protein isoforms is often not possible with the shotgun approach, the technique is highly automatable and, arguably, is superior in terms of number of identified proteins per analysis.

Besides identification, several different strategies are being used for quantification. In the label-free approach, the precursor ion signal intensity is used to determine the relative abundance of the identified peptides in

biological samples [152]. Label-free quantification typically results in large variation because of variations in the sample preparation, electrospray ionization (ESI) signal suppression in complex samples, and ESI spray variation over time. Furthermore, label-free quantification does not permit multiplexing, leading to long analysis times for large sample cohorts.

These problems have been addressed by the implementation of isotopic labeling, such as isotope-coded affinity tags (ICAT) and dimethyl labeling [153, 154]. Here all peptides in a sample are derivatized with a peptide-reactive reagent. Different biological samples are labeled with variants of the reagent that differ in their incorporation of the heavy stable isotopes, ^{13}C, ^{15}N, and ^{2}H. After labeling, biological samples are combined and analyzed together by LC-MS. Differentially labeled forms of a peptide co-elute in the LC and are detected as multiple signals in the mass spectra, and the relative abundance of the peptide in the biological samples equals to the signal intensity ratio. While isotopic labeling enables multiplexing, this has the drawback of crowding of the MS domain, increasing the occurrence of overlapping peaks.

The most recent method has been the use of isobaric labels for multiplexed proteomics. With this approach the digested peptides are labeled with isobaric tag for relative and absolute quantitation (iTRAQ) [155], tandem mass tags (TMT) [156], or isotope coded affinity tags [153]. Using these techniques in biomarker discovery studies has identified several alterations in AD patients, however with somewhat contradictory results (reviewed in [157-162]). There seems to be some general trends visible, such as lowered levels of neurosecretory protein VGF and secretogranins, and increased levels of clusterin. Several of the changes are also found in other diseases such as Parkinson's disease and multiple sclerosis [163-167]. These proteomic biomarker discovery studies have so far not resulted in any new diagnostic marker for AD. A possible explanation for this is that it is difficult to determine which of the identified changes that are disease related and which are caused by other factors that differ between the studied groups. In the studies where peptide digests are used for quantification it is not possible to determine from which isoform, modified form, or truncated form of a proteins that the changed tryptic peptides comes from. This means that validation studies using e.g. ELISA, which identifies specific forms of a protein, might not yield similar results.

4.1.1 Biomarker discovery – endogenous peptides

CSF does not only contain intact proteins, it also has a lot of endogenous peptides which are peptides generated by specific enzymatic processes, while others are created by common degradation pathways. Some of the endogenous peptides in CSF are truncated forms of known neuropeptides that might have active functions in the CNS [63].

Endogenous peptides in CSF have however not been widely studied as potential biomarkers for neurodegenerative diseases. A few studies have been performed to identify candidate biomarkers for AD by quantifying endogenous peptides in CSF [64, 168-174]. For biomarker discovery of endogenous peptides in CSF, the label-free approach has been used ,with data produced by MALDI-MS [173], CE-ESI-TOF-MS [64], or SELDI-MS [168-172, 174]. These studies have identified alterations in the endogenous peptide between AD patients and controls (summarized in Table 1). Some of the peptides derived from the same protein do display the same direction of change in general, although there are some contradictory results. In at least two studies higher levels of peptides from complement C3a, complement C4a, integral membrane protein 2B, and lower levels of VGF have been identified.

Protein/Peptide	m/z	Direction of change	Method	Ref
Phosphorylated osteopontin C-terminal fragment	7744.43	up	SELDI-TOF	[170]
Ubiquitin	8569.17	up		
C4a des-Arg	8604.57	up		
C3a des-Arg	8936.16	up		
β2-microglobulin	11734.54	up		
Cystatin C -8 AA from NT	12524.14	up	SELDI-TOF	[172]
Cystatin C	13347.95	down		
Apo C-1 (2 AA deleted from NT)	6437.28	down		
Pancreatic ribonuclease	14559.94	up		
VGF peptide	4810.349	down		
C3a des-Arg	8937.064	up		
Chromogranin A peptide (vasostatin II)	3909.032	down		
Integral membrane protein 2B CT	2430.077	up		
Integral membrane protein 2B CT	2425.06	up	SELDI-TOF	[171]
Chromogranin A, frag	3907.89	down		
Amyloid-β 1-38	4133.6	up		
Amyloid-β 1-40	4331.87	up		
Alpha-1-antichymotrypsin, CT frag	4355.54	down		
VGF, frag	4806.88	down		
ApoC1, 2 aa truncation from NT	6433.26	down		
Osteopontin, CT frag	7661.19	down		
Osteopontin, CT frag, phospho	7740.55	down		
C3a anaphylatoxin (des-Arg)	8934.82	up		
Saposin D isoform	10366.25	up		
β2-microglobulin	11726.36	down		
Cystatin C, 8 AA truncation from NT	12537.8	up		
Cystatin C	13345.43	down		
Pancreatic ribonuclease	14565.8	up		
S100A7	11.7 kDa	up	SELDI-TOF	[169]
VGF	4823.5	down	SELDI-TOF	[168]
β2-microglobulin	11786.9	up		
β2-microglobulin	11988.4	up		
Cystatin C	13416.4	up		
VGF (APPGRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEV)	3668	down	MALDI-TOF	[173]
Complement C4 factor (DDPDAPLQPVTPLQLFEGRRN)	2378.8	up		
Alpha-2-HS-glycoprotein (SVGAAAGPVVPPCPGRIRHFKV)	2272.7	up		
Alpha-2-HS-glycoprotein (TVVQPSVGAAAGPVVPPCPGRIRHFKV)	3453.1	down		
VGF (GGEERVGEEDEEAAEAEAEAEEAERARQNALLFAEEEDGEAGAED)	4807.3	down		
Chromogranin A (SGELEQEEERLSKEWEDS)	2179.03	up	CE-ESI-TOF	[64]
ProSAAS (AADHDVGSELPPEGVLGALLRV)	2214.22	up		
Phospolemman (ESPKEHDPFTYDYQSLQIGGL)	2423.19	up		
Clusterin (DQTVSDNELQEMSNQGSKYVNKEIQNA)	3068.46	up		
VGF (GRPEAQPPPLSSEHKEPVAGDAVPGPKDGSAPEV)	3401.73	down		

Table 1. A list of potential biomarkers for Alzheimer's disease from biomarker discovery studies where endogenous peptides in CSF have been analyzed.

5 METHODS

5.1 Patients and samples

The CSF samples used in the present investigation were all collected according to standardized procedures [175]. A lumbar puncture was performed in the L3/L4 or L4/L5 interspace and the CSF samples were collected in polypropylene tubes. The samples are shortly after sampling centrifuged at 2000 x g for 10 minutes to remove cell debris and more. The samples are then aliquoted in polypropylene tubes with screw-caps and stored at -80°C. The samples analyzed in this dissertation had only been thawed once.

AD patients met the criteria of dementia according to DMS-IIIR and fulfilled the criteria of AD defined by NINCDS-ADRDA [11].

5.2 Liquid chromatography

Liquid chromatography (LC) is used to separate compounds according to physicochemical properties such as their hydrophobicity, or ionic strength/net charge. In proteomic experiments, an LC is usually coupled to an MS and is the final stage of separation before MS analysis. This increases the probability of identifying low abundant proteins and peptides, which are otherwise suppressed by co-ionization of high abundant species.

Proteins and peptides are usually separated on reverse-phase columns that have non-polar carbon chains of different length to retain them (C_4, C_8, and C_{18}). The peptides and proteins interact with these adsorbents by hydrophobic interactions, where the compounds that have the strongest interactions are retained longer. A gradient with decreasing solvent polarity is used to successively elute the compounds from the column according to increasing hydrophobicity. For peptides, C_{18} columns are commonly used while proteins, which usually are more hydrophobic, are separated with C_8 or C_4 columns. Biological samples are commonly desalted prior to the LC-MS step to prevent the columns in the LC from being clogged by sample impurities, and as the ionization of peptides in ESI-MS is suppressed by the presence of salts.

The LC coupled to a mass spectrometer is often run in either µL/min or nL/min flows. Higher flow allows for faster gradients to be used which

increases sample throughput. Nano-LC is used to increase detection sensitivity as the ionization process is more efficient with small droplets in ESI, thus yielding in more charged peptides that eventually reach the mass analyzer [176, 177]. Nano-LC is thus preferred for discovery studies where the extra sensitivity is needed, as has been shown in several studies [178-184].

5.3 Mass spectrometry

Mass spectrometry is used to determine the mass to charge ratio (m/z) of a compound, and can be used to identify and quantify compounds. In this thesis MALDI-MS [185, 186] and ESI-MS [187] Orbitrap instruments were used in the experiments.

The general principle of mass spectrometry is that the sample molecules are ionized and transferred into gas phase in an ion source, and are then measured according to their mass-to-charge ratio (*m/z*) by a mass analyzer. The ion current, recorded by the detector, plotted against *m/z* constitutes the mass spectrum.

In addition to determining the molecular mass of sample constituents with high accuracy, MS can also be used to obtain structural information. This is done by isolating specific molecular ions in the mass spectrometer, and then fragmenting them by e.g., collision induced dissociation (CID) or by post source decay used in MALDI-MS. For peptides, the fragment ion spectra can be used for identification by database searching, to obtain sequence information, and to characterize posttranslational modifications.

5.3.1 MALDI-MS

MALDI is a soft ionization technique where a laser is used to desorb and ionize the analyte molecules [185, 186]. The samples to be analyzed are deposited on a MALDI target plate together with MALDI matrix solution, resulting in a solid-state mix of analyte molecules and matrix crystals. The matrix compound absorbs the energy from the laser leading to desorption of the matrix and the embedded analyte molecules. During this process, protons are transferred between matrix and analyte molecules, resulting in both positively and negatively charged analyte ions. Typically singly charged ions are produced. How ionization occurs is not fully understood. MALDI is most often used in conjunction with a time-of-flight (TOF) mass analyzer (Figure 3). In a TOF, high voltage (20-30 kV) is applied between the MALDI sample plate and a grid/extraction element in the ion source, accelerating the

generated molecular ions to a high velocity (v), which is inversely proportional the square root of the molecular mass (m) according to: v = SQRT(2qU/m), where v = velocity, q = charge, U = acceleration voltage.

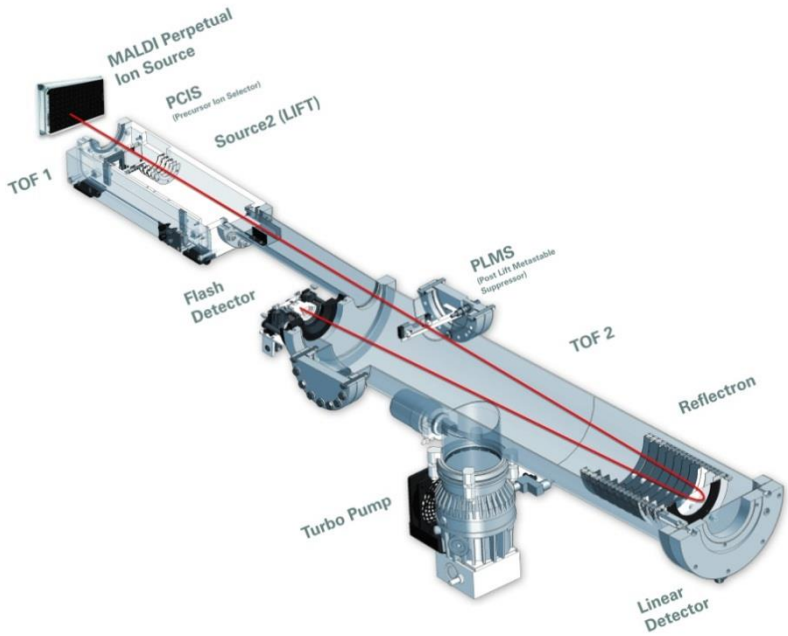

Figure 3. Schematic outline of a MALDI-TOF/TOF. The analytes are ionized and accelerated in the ion source. The ions travel through the flight tube as illustrated by the red line and reach the detector. Ions with the same ***m/z*** *reach the detector at the same time. Image courtesy of Bruker Daltonics.*

In the field-free drift-region ions separate according to their different velocities and are recorded as they reach the detector at different time points, resulting in a TOF spectrum which through calibration is converted into a mass spectrum. A dual-stage ion source for delayed ion extraction and an ion mirror (reflectron) are used to focus ions by reducing the effect of initial kinetic energy distributions, which increases the mass accuracy in MALDI MS [188].

The use of LC to separate compounds before analysis in the MALDI-MS requires offline separation onto a MALDI target plate that is then analyzed.

5.3.2 Electrospray ionization (ESI-MS)

In electrospray ionization (ESI) a liquid, e.g. the eluent from an LC, is directly introduced to a strong electric field through a thin capillary in an ion source, which creates small charged droplets with compounds in them. The protons are then transferred to the compounds as the droplets decrease in size and eventually disintegrate. This creates multiply charged ions that continuously enter the mass spectrometer. Coupling a nano-LC to ESI-MS increases sensitivity as the concentration of analyte in the droplets is higher and the ionization is believed to work better [176, 177].

In this thesis work, a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Scientific) was used for ESI-MS (Figure 4). The Orbitrap is a high resolution electrostatic ion trap. Ion populations injected into the trap are trapped in rings orbiting the Orbitrap core, and oscillate along the core axis with frequencies $\omega = SQRT(k/(m/q))$, where k = force constant and q = charge. The instrument is equipped with a quadrupole for precursor ion selection and an HCD cell for ion fragmentation.

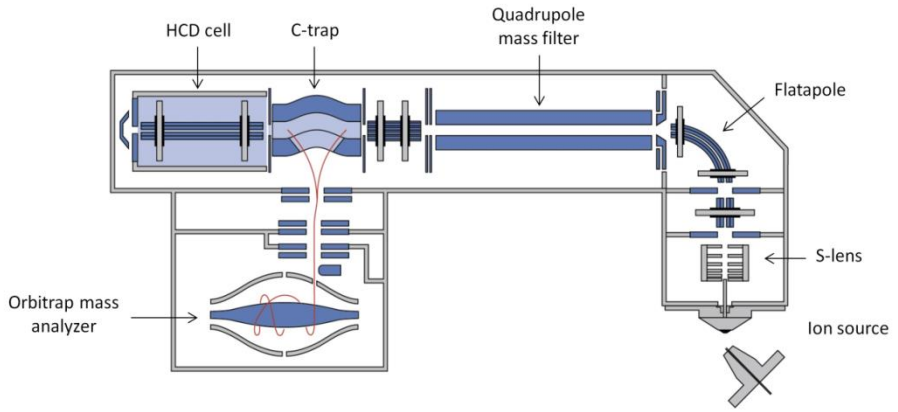

Figure 4. Schematic outline of the components of a Q-Exactive Orbitrap mass spectrometer. Image courtesy of Thermo Scientific.

5.3.3 Isolation of the low molecular weight proteome

The CSF proteome is mainly composed of a few high abundant proteins, where albumin and immunoglobulins account for more than 70% of the total protein content [8]. In biomarker studies with MS, where nano-LC is commonly used, can however only be loaded with maximum 1 µg of protein. The mass spectrometer also has a limited dynamic range, where high

abundant compounds mask the presence of low abundant compounds. To analyze low abundant species in CSF there is therefore a need to deplete the high abundant proteins from CSF. There are different strategies for doing this, for example by using multi-affinity removal columns, where immobilized ligands capture high abundant proteins, or by using molecular weight cut-off (MWCO) filters if the compounds to be analyzed have a suitable molecular weight.

5.3.4 Peptide identification

The fragment ion spectra recorded in MS/MS for selected precursor ions can be used to identify peptides. The fragment ion masses are matched against fragment ion masses created in an *in silico* generated database of all possible peptides and their theoretical fragmentation products. These searches work better for peptides that are created by e.g. trypsin compared to endogenous peptides as the former have defined amino acids at their cleavage sites (Lys and Arg) and, in ESI, give rise to more continuous fragment ion spectra.

When peptides and proteins are fragmented in the mass spectrometer they fall apart along the peptide backbone according to a certain rules (reviewed in [189]). These fragment ions are called abc/xyz ions depending on where on the peptide structure the fragmentation occurs and on where the charge is on the peptide (Figure 5) [190].

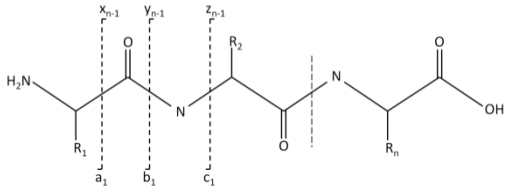

Figure 5. The peptide backbone with fragmentation sites. The peptides fragment along the peptide backbone where the fragment ions are named after the site of fragmentation. The numbers indicate the position of the amino acid in the sequence where the fragmentation occurred. The amino acid position is calculated from the N-terminus for a, b, and c ions, and from the C-terminus for x, y, and z ions.

The singly-charged peptide ions produced by MALDI fragment quite differently than the multiply-charged ions produced by ESI. Generally, fragmentation is directed to the site of protonation. When the ionizing proton is mobile along the peptide backbone fragmentation occurs at multiple sites, generating information-rich fragment ion spectra. When the ionizing proton

is fixed in place by a positive charge carrier such as Arg (and to some extent Lys), only few fragment ions are typically observed. In addition, the peptide fragmentation follows specific rules. These can be used for manually verifying the identifications of the peptides as the databases do not consider them. For peptides containing Arg, prominent charge-remote fragmentation occurs C-terminally to Asp (and to a lesser degree, Glu) [191, 192]. The peptide bond N-terminally to proline also breaks easily resulting in strong fragment ions. If there is a aspartic acid - proline sequence in the peptide, this will be the most abundant fragment ion created during the dissociation [192] (Figure 6).This type of ion spectra would give a low ion score in searches using e.g. Mascot as there are only a few fragment ions.

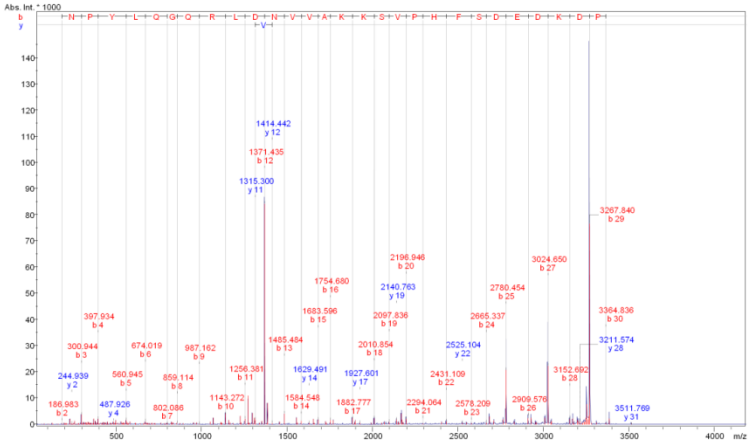

Figure 6. A fragment ion spectrum from MALDI-MS analysis with annotated peptide sequence. The fragment ion spectrum for the peptide VNPYLQGQRLDNVVAKKSVPHFSDEDKDPE. Strong fragment ions are seen c-terminally to aspartic acids (D), especially when followed by a proline (P) since there is an arginine (R) in the sequence which results is strong charge-remote fragmentation.

ESI-MS data are not as easy to manually validate as they are multiply charged and do not fragment according to specific patterns as in MALDI-MS. Quite recently a post-search algorithm called Percolator was developed that can be used to rescore peptides after they have been searched with e.g. Mascot [193, 194]. This uses a semi-supervised machine learning to automatically look for similarities among the spectra to increase the probability of a peptide to be identified correctly. This increases the number of identified endogenous peptides from ESI-MS data.

5.3.5 Isobaric labeling

The use of isobaric labels such as tandem mass tags TMT [156] or iTRAQ [155] makes it possible to use multiplexing and analyze up to 10 samples in one LC-MS run. The isobaric labeling strategies rely on the use of chemical labels that react with primary amines. The labeling reagent consists of three parts, 1) the reactive part, which reacts with primary amines found on the N-termini of proteins and peptides, and on lysines, 2) the balance group, which is used to adjust the total mass of this and the third part so that they are always equal, and 3) the reporter ion, which is released upon fragmentation and used for quantification (Figure 7).

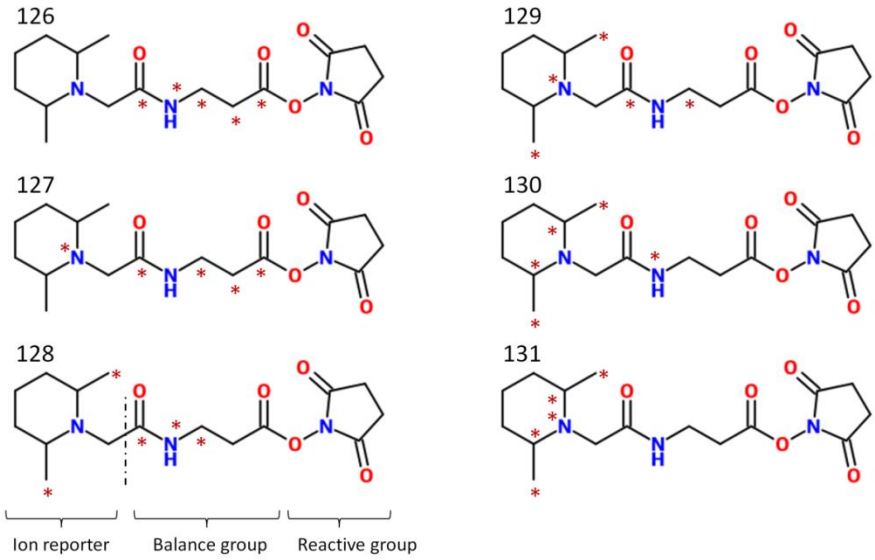

Figure 7. The structures of the TMT 6-plex reagents. The ion reporter groups are released upon fragmentation in the mass spectrometer. The amount of ^{13}C and ^{15}N is the same for all six reagents while their distribution along the ion reporter group and balance groups varies as indicated by the red stars.

Since the labels are isobaric, a given peptide will have the same nominal mass and the same retention time on the LC regardless of which of the labels in a TMT or iTRAQ it has labeled with. It is only possible to separate these by fragmenting the analyte, whereby the different reporter ions are released and show up at the low mass end of the MS/MS spectrum (Figure 8).

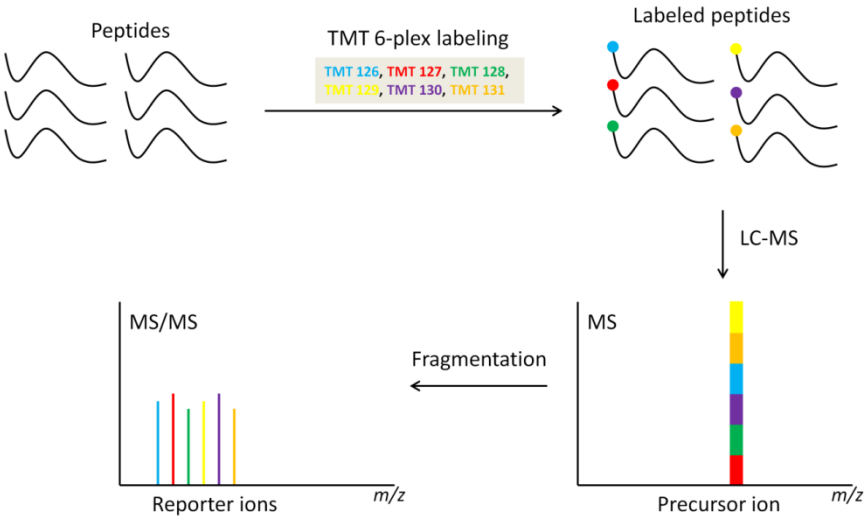

Figure 8. The TMT 6-plex workflow. Six different samples can be used where each individual sample is labeled with one of the six TMT reagents, after which the samples are mixed. Since the labels are isobaric a given peptide will have the same retention time regardless of which of the six TMT labels it has, and show up as one peak in MS analysis. Once the peptides are fragmented the reporter ions are released and their intensities can be compared to each other for relative quantification of the peptide. Adapted from [195].

The relative intensities between the reporter ions can then be compared to relatively quantify the peptides between the different samples. If more than one TMT or iTRAQ set is needed to fit all samples, a common reference sample can be labeled with one of the tags of each TMT or iTRAQ set, against which the samples then are compared. Relative quantification with isobaric tags is however affected by interferences from co-isolated peptides which may introduces ratio distortion [195]. The ratio distortion can be avoided by using MS3 fragmentation for quantification, but will results in around 20% less quantified peptides [195, 196].

5.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) is an antibody based assay used to quantify e.g. peptides and proteins in fluid samples. It is usually designed in a sandwich manner, where specific antibodies are used both to capture the target analyte and to detect it. An ELISA plate is coated with an

antibody that is specific for a region of the analyte to capture it. Thereafter the samples are added onto the plates where the target analyte is captured by the antibody immobilized on the plate. The rest of the sample constituents are washed away. A second antibody, specific for another region of the analyte, is then added for detection of the analyte. The detection antibody is usually biotinylated. After that avidin, conjugated with e.g., horse radish peroxidase (HRP), is added where avidin binds to the biotin on the detection antibody. Thereafter a chromogen solution is added which HRP converts, whereby the solution takes on a blue color. The intensity of the color correlates to the amount of analyte in the samples. This reaction is stopped by adding 2 M H_2SO_4 which turns the solution into a yellow color. The optical density of the solution is then compared against the values from a standard curve, created of titrated amounts of e.g. a synthetic or recombinant protein, to determine the quantity of the analyte in the samples. The quantification of three APLP1 peptides, APL1β25, APL1β27, and APL1β28, were done in this thesis using commercial ELISAs (IBL international, Hamburg) according to this principle. These ELISAs use C- and N-terminal specific antibodies for the peptides.

5.5 Statistics

The distribution of the values obtained for the endogenous peptides from the isobaric labeling combined with LC-MS did not show a Gaussian distribution why non-parametric test were used to evaluate the results. For calculating p-values between groups the Mann Whitney U-test was used. Correlations were evaluated with the Spearman rank test. For evaluating the changes in the time study from the γ-secretase inhibitor experiment the Friedmann test was used.

6 AIM

The aim of the thesis was to study endogenous peptides in CSF as potential biomarkers for AD.

The specific aims for the studies were as follows:

Paper I – Establish a workflow for isolating endogenous peptides from larger high abundance proteins in CSF, and to develop a platform with nano-LC combined with MALDI MS for separation and identification of the endogenous peptides.

Paper II – Develop a method to quantify endogenous peptides in CSF that can be used for biomarker discovery for disease affecting the brain such as AD.

Paper III – Study how a γ-secretase inhibitor affects the composition of endogenous peptides in human CSF, and to identify potential biomarkers that can be used to evaluate the drug's target engagement.

7 RESULTS

7.1 Paper I

The aim of Study I was to establish a workflow to isolate the endogenous peptides from high abundant proteins in CSF, and to identify the endogenous peptides.

Different MWCO filters were tested to evaluate their ability to eliminate high-abundant proteins, such as albumin, in CSF while letting through endogenous peptides. The 30 kDa filters were found to be the best choice as they did not let through albumin, which the 50 kDa filters did, while more endogenous peptides passed through this filter compared to a 10 kDa filter. The recovery of endogenous peptides was greatly increased if the CSF samples were mixed with acetonitrile or formic acid before centrifugation. The largest increase was obtained by the addition of 20% acetonitrile to CSF.

The endogenous peptides in CSF were identified using LC-MALDI MS. The endogenous peptides were separated on a nano-LC which was coupled to a droplet dispenser that dispensed the LC effluent onto AnchorChip MALDI target plates with 1536 spots. One droplet was dispensed every ten seconds during the 160 minute gradient used for separation. Matrix solution was then dispensed on the spots and the plate was analyzed with a MALDI-TOF/TOF MS.

Endogenous peptides commonly receive low identity scores when the MS/MS data for the endogenous peptides are searched, as the lack of enzyme specificity in the searches increases the number of peptide sequences to be considered by a factor 100-1,000. The results from these searches were however manually validated according to known fragmentation rules that apply for MALDI MS fragmentation. This increased the number of endogenous peptides that could be considered as correctly identified. In this paper, more endogenous peptides were identified than in any previous study where the composition of endogenous peptides in CSF has been analyzed [65, 197-199]. Several endogenous peptides of interest were identified, e.g. truncated forms of neuropeptides, and endogenous peptides that were created by specific proteases and might thus directly reflect the activity of these enzymes.

A drawback with this approach is that it is labor intensive, and analyzing MALDI plates with several hundred spots on each plate requires more

instrument time than would be needed with an online system were the LC effluent is directly analyzed with a mass spectrometer, e.g. LC-ESI-MS. The workflow in this paper was not suitable for label-free quantification of the endogenous peptides as the amount of recovered endogenous peptides differed between the MWCO filters.

7.2 Paper II

As the identified endogenous peptides in Paper I contained truncated form of neuropeptides and peptides that were created by specific proteases, they might reflect disease relevant processes and were thus worth quantifying. The workflow developed in the Paper I was not suitable for label-free quantification as the recovery of endogenous peptides from the MWCO filters were not reproducible. To overcome this issue, isobaric labeling of the CSF samples was implemented into the workflow prior to the ultrafiltration step (Figure 9). As the CSF samples were labeled and mixed at the earliest possible stage, the subsequent sample work-up steps affected all samples in the same manner. This made it possible to avoid the introduction of errors due to varying recovery of the MWCO filters.

The amount of acetonitrile used to increase recovery of the endogenous peptides from the MWCO filters in Paper I, was close to the amount of acetonitrile as is recommended for the labeling reaction with TMT 6-plex. This made it possible to combine TMT 6-plex labeling with the workflow established in Paper I. In addition, since the proteins retained on the MWCO filters had incorporated TMT 6-plex labels they could be digested and the protein digest could be used to quantify the proteins. This required that the peptides created by digestion contained a lysine as they had incorporated TMT 6-plex tags. Around a third of the identified peptides created by digestion contained a lysine and could thus be used for quantification.

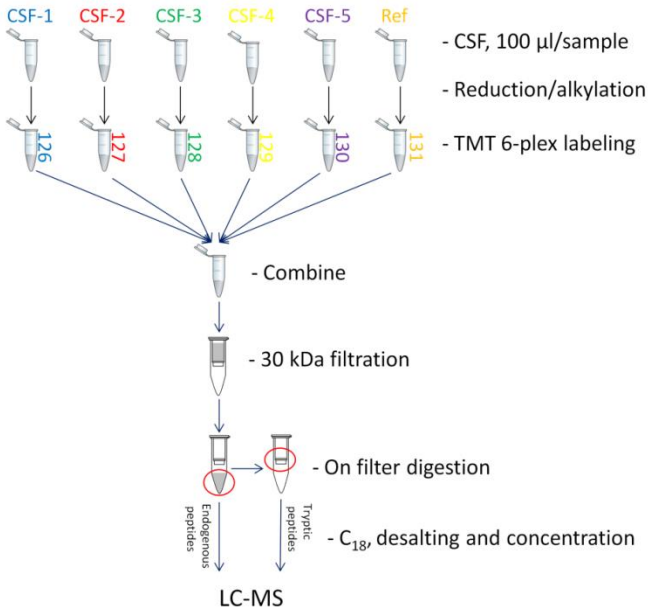

Figure 9. The developed workflow for quantification of endogenous peptides and proteins in CSF using isobaric labeling. The CSF samples are first reduced to disrupt disulfide bonds after which the sulfur groups are alkylated to prevent the formation of new disulfide bonds. The CSF samples are then labeled with the TMT 6-plex reagents, mixed, and centrifuged through a MWCO filter. The endogenous peptides are recovered, desalted and analyzed by LC-MS. The retained proteins on the MWCO filters are digested, and the protein digests are centrifuged through the filter and recovered, after which the protein digests are desalted and analyzed by LC-MS.

The use of isobaric labeling with TMT 6-plex proved to work in a reproducible manner (Table 2), where the standard deviations between samples were similar or better than have been reported for other methods used to quantify peptides and proteins [200-203].

	Endogenous peptides	Tryptic peptides
126/131	1.002 (0.094)	0.998 (0.113)
127/131	1.066 (0.096)	1.059 (0.125)
128/131	1.095 (0.098)	1.089 (0.134)
129/131	1.085 (0.097)	1.066 (0.112)
130/131	1.075 (0.104)	1.078 (0.132)

Table 2. Mean value and standard deviation for quantified endogenous and tryptic peptides. Aliquots of the CSF sample was used in all six TMT 6-plex channels.

To investigate if relative quantification of the endogenous peptides could identify any differences in biomarker discovery experiments, a clinical cohort with 8 AD patients and 8 controls was analyzed using the developed workflow. The CSF samples were distributed into four TMT 6-plex sets and the endogenous peptides were analyzed with both LC-MALDI MS and LC-ESI MS, while the protein digest were only analyzed with LC-ESI MS. By this, several alterations in the endogenous peptides were identified between the AD and control group (Table 3).

Sequence	Protein	ESI		MALDI	
		ratio	p	ratio	P
APHGPGLIYRQPN	Alpha-2-HS-glycoprotein	0.75	0.0047	0.7	0.025
AVCKEPQEEVVPGGGRS	Tachykinin-3	0.66	0.085	0.71	0.006
AVTEDDEDEDDDKE	Testican-1	0.92	0.11	0.79	0.018
AVTEDDEDEDDDKEDEVGYIW	Testican-1	0.75	0.025	0.84	0.225
VTEDDEDEDDDKE	Testican-1	0.69	0.018	0.72	0.035
DELAPAGTGVSREAVSG	Amyloid-like protein 1	0.80	0.142	0.68	0.007
DELAPAGTGVSREAVSGLLIMGAGGGSL	Amyloid-like protein 1	0.90	0.655	0.84	0.047
DVGSELPPEGVLGALLRV	ProSAAS	0.80	0.085	0.75	0.021
APPEPVPPPR	Neurosecretory protein VGF	0.76	0.048	0.68	0.012
APPEPVPPPRAAPAPTHV	Neurosecretory protein VGF	0.76	0.003	0.78	0.007
GGEERVGEEDEEAAEAEAEAEEAERARQNA	Neurosecretory protein VGF	0.58	0.006	-	-
GGEERVGEEDEEAAEAEAEAEEAERARQNALLFAEEEDGEAGA	Neurosecretory protein VGF	0.65	0.006	-	-
GLQEAAEERESAREEEEA	Neurosecretory protein VGF	0.73	0.251	0.76	0.025
GLQEAAEERESAREEEEAEQE	Neurosecretory protein VGF	0.61	0.048	0.83	0.048
NAPPEPVPPPRAAPAPTHV	Neurosecretory protein VGF	0.70	0.018	0.75	0.003
HSGFEDELSEVLENQSSQAELKEAVEEPSSKDVME	Chromogranin-A	0.82	0.048	-	-
NCFAIRH	Integral membrane protein 2B	-	-	0.68	0.003
NCFAIRHFENK	Integral membrane protein 2B	0.77	0.035	0.71	0.012
NCFAIRHFENKF	Integral membrane protein 2B	0.79	0.18	0.68	0.006
NCFAIRHFENKFA	Integral membrane protein 2B	0.73	0.064	0.79	0.048
NCFAIRHFENKFAVET	Integral membrane protein 2B	0.8	0.121	0.82	0.006
SNCFAIRHFENKFA	Integral membrane protein 2B	0.94	0.406	0.73	0.048
SNCFAIRHFENKFAVETLICS	Integral membrane protein 2B	-	-	0.73	0.045
SSQGGSLPSEEKGHPQEESEESN	Secretogranin-1	0.65	0.035	-	-
SSQGGSLPSEEKGHPQEESEESNVSMASLGE	Secretogranin-1	0.69	0.006	-	-
TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ	Secretogranin-2	0.54	0.003	-	-
GQGSSEDDLQEEEQIEQAIKEHLNQGSSQETDKLAPVS	Secretogranin-2	0.53	0.004	-	-
ELSAERPLNEQIAEAEED	Secretogranin-3	0.86	0.1	0.8	0.028
ELSAERPLNEQIAEAEEDKI	Secretogranin-3	0.74	0.003	0.62	0.01
TCPCPSGGSCTCADSCKCEG	Metallothionein-3	0.55	0.004	0.63	0.009
VLSEEKLSEQETEAAEKSA	Neuron specific protein family member 1	0.79	0.048	-	-
ATKAVCVLKGDGPVQGIINFEQKES	Superoxide dismutase [Cu-Zn]	0.87	0.013	0.8	0.01

Table 3. Altered endogenous peptides between AD patients and controls in Paper II.

The altered endogenous peptides in the AD group were derived from proteins that have been previously reported to be altered in the CSF of AD patients, e.g. VGF and secretogranin-1 (106-111), or from proteins that have been suggested to affect Aβ aggregation as integral membrane protein 2B [204].

Several altered proteins were also identified in the CSF of the AD group (Table 4).

Protein	ratio	p-value
Mesothelin	0.62	0.01
Contactin-associated protein-like 4	0.69	0.01
Neuronal pentraxin-2	0.62	0.027
Ig alpha-1 chain C region	2.3	0.027
Neurexin-1-alpha	0.71	0.027
Chitinase-3-like protein 1	1.22	0.036
Prolow-density lipoprotein receptor-related protein 1	0.67	0.037
Galectin-1	1.06	0.043
Mimecan	1.94	0.043
Collagen alpha-2(I) chain	0.72	0.046

Table 4. Altered proteins between AD patients and controls in Paper II.

Analyzing the endogenous peptides from the CSF samples with LC-ESI MS resulted in more identified and quantified endogenous peptides than was achieved by analyzing the samples with LC-MALDI MS. The overlap of identified and quantified endogenous peptides between LC-ESI MS and LC-MALDI MS was only about 20% and highlighted that the two techniques are complimentary.

7.3 Paper III

In Paper II it was shown that the endogenous peptides in CSF could be quantified using isobaric labeling. In Paper III the method from Paper II was used to identify changes among the endogenous peptides in human CSF as a result of inhibiting γ-secretase.

γ-secretase inhibitors are one class of compounds under investigation as disease modifying treatment for AD. The goal of these compounds is to decrease the production of $A\beta_{42}$ and by that slow down the plaque formation and progression of AD. The γ-secretase inhibitor, semagacestat, went into a Phase III clinical trial which was interrupted as the treatment led to a worsening of cognitive performance in the participants [128]. The compound has not been reported to clearly decrease the concentration of $A\beta_{42}$ in CSF in previous studies [129, 135]. However, using the stable isotope-linked kinetics (SILK) technology it has been shown that semagacestat decreases the amount of newly generated $A\beta_{42}$ [127].

In Paper III, CSF samples from 15 participants from a clinical trial with semagacestat were analyzed. Five participants received a placebo, five participants received 140 mg of semagacestat, and five participants received 280 mg of semagacestat. CSF from the participants had been collected over time through an indwelling thecal sac, and CSF samples from six time points were selected to be analyzed (0h, 3h, 9h, 12h, 18h, and 36h). The relative abundances of the endogenous peptides in CSF were measured using the method described in Paper II. The CSF samples from the six time points from each individual participant were included in one TMT 6-plex set.

The analysis of the CSF samples from the γ-secretase inhibitor trial revealed decreased levels of six endogenous peptides. The changes were identified in the group who received 280 mg of semagacestat while the changes did not reach significance in the group who received 140 mg of semagacestat. A maximum decrease of the endogenous peptides in CSF was usually observed at 12-18h. Two endogenous peptides from APLP1, which is a known γ-secretase substrate, showed a clear decrease in the group that received 280 mg of semagacestat (Figure 10).

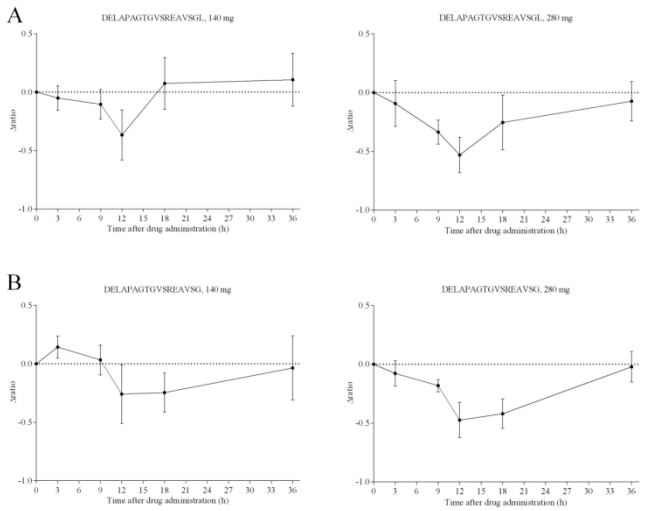

Figure 10. APLP1 peptides that were decreased as a results of γ-secretase inhibition. The effects on peptides from the Aβ-like region of APLP1, where (A) shows APL1β 1-18, and (B) shows APL1β 1-17. Data are presented as median with median absolute deviation.

The other endogenous peptides in CSF that were decreased in the group who received 280 mg of semagacestat (Figure 11), are not from proteins that are known γ-secretase substrates but they are derived from proteins that have

been related to AD pathogenesis before, namely metallothionein-3, ApoE, secretogranin-1, and proSAAS.

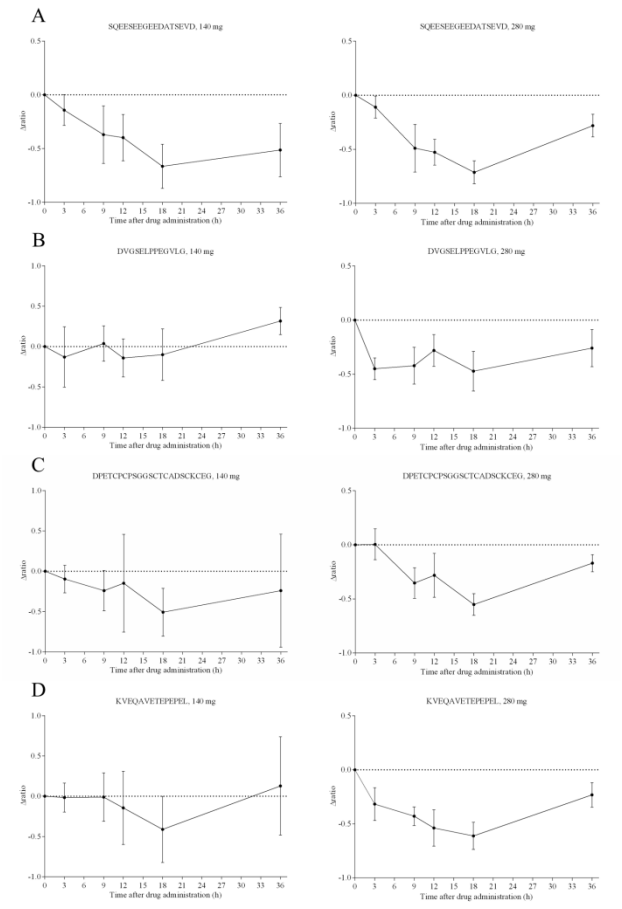

Figure 11. Endogenous peptides that were decreased as a response to treatment with the γ-secretase inhibitor. The effects of γ-secretase inhibitor treatment on the peptides from (A) position 259-275 of secretogranin-1, (B) position 225-237 of proSAAS, (C) position 2-24 of metallothionein-3, and (D) position 19-32 of ApoE, are shown. Data are presented as median with median absolute deviation.

8 DISCUSSION

Patients with MCI who later develop AD have decreased $A\beta_{42}$, and increased total-tau and p-tau levels in CSF at an early clinical stage, which might be used to identify subjects at risk of developing AD [3, 205]. There is however a need for other biomarkers that can identify individuals at risk of developing AD at an presymptomatic stage, and biomarkers that can be used to monitor disease progression and pathologies other than those primarily related to plaques and tangles. Several biomarker discovery studies have been performed in CSF to identify novel biomarkers for AD. These have generally focused on quantifying the proteins in CSF while the naturally occurring peptides in CSF have not been widely studied in biomarker discovery experiments. CSF contains a large population of endogenous peptides that contain e.g. truncated forms of neuropeptides and peptides generated by specific proteases [63, 65, 197-199].

High-abundant proteins in CSF hamper the detection of low-abundant endogenous peptides. A fast approach to isolate the endogenous peptides from larger proteins is by using MWCO filters. In Paper I, MWCO filters with different cut-off levels were tested, where the 30 kDa filters were found to be the best compromise for excluding high abundant proteins such as albumin, while letting through smaller endogenous peptides. Additionally, increased recovery of the endogenous peptides was achieved by adding acetonitrile or formic acid to the samples to dissociate protein complexes. Similar effects have been seen when adding guanidine hydrochloride to CSF before ultrafiltration [199]. In Paper I several novel endogenous peptides in CSF were identified. The functions of these endogenous peptides are not known. It was however observed that some of them were truncated forms of neuropeptides (e.g. peptides from neuropeptide Y) and that some of the peptides are generated by the same enzymatic processing as Aβ peptides (as APLP1 peptides). The results from Paper I indicated that the endogenous peptides in CSF could be of interest as potential biomarker for neurodegenerative diseases.

The next step was to establish a method for quantification of the endogenous peptides in CSF that was suitable for clinical studies. The MWCO filters are known to have poor reproducibility [199] therefore a label-free approach was not suitable to quantify the endogenous peptides with the workflow developed in Paper I. To overcome this problem, isobaric labeling for relative quantification using TMT 6-plex was implemented at the earliest possible stage in the workflow, as reported in Paper II. The proteins and peptides in

the CSF samples were labeled and mixed the prior to ultrafiltration, which made it possible to use the MWCO filters as all samples included in one TMT 6-plex set were affected in the same manner by the MWCO filters. Additionally, it was shown that the proteins that were retained on the filters could be digested and quantified, as they had incorporated TMT 6-plex labels on the lysines. Around a third of all the peptides created by protein digestion contained a lysine and could be quantified. The TMT labeling of the endogenous peptides and proteins in CSF was shown to work reproducibly as reported in Paper II. This workflow was then used to analyze a small clinical cohort to investigate whether it could identify any interesting differences in the endogenous peptides and proteins in the CSF of AD patients. By this, increased levels of the protein YKL-40 were observed in the CSF of AD patients which is in accordance with previously published studies [206, 207]. This could indicate that the method identifies disease relevant changes. Several alterations among the endogenous peptides in the CSF of AD patients were also observed, of which several could be potential biomarkers. As an example, decreased levels of endogenous peptides from ITM2B were identified, which is a protein that has been suggested to affect Aβ aggregation [204]. The results from the clinical cohort indicate that the method has the potential to identify disease relevant alteration which merits the analysis of a larger clinical cohort to validate the results and identify other potential biomarkers. The altered endogenous peptides identified in the CSF of AD patients in Paper II are not the same as has been observed in other biomarker studies where endogenous peptides in CSF have been analyzed [64, 168-173]. The different methods used in these studies most likely identify different endogenous peptides. When quantifying proteins and peptides with TMT the ratios between samples can be distorted by interfering compounds that are co-isolated during precursor-ion selection [195]. This can compress the ratios between samples making the observed regulations between the studied groups smaller. The interferences can be reduced on ESI instruments that are capable of MS3 fragmentation, where the fragment ions in MS/MS are isolated and fragmented [195, 196]. Biomarker discovery experiments by proteomics usually identify several alterations between the studied groups. Other underlying diseases and factors can affect the CSF proteome. In AD, which generally affects people over 65 years of age, the majority of the patients have other concomitant diseases that might affect the proteome. The findings from these discovery studies therefore need to be replicated and validated to evaluate the potential of the candidate markers as clinical biomarkers for a specific disease.

There have been several attempts to find disease modifying treatment for AD that can slow down the progression of the disease. Some of these target the

enzymes that create $A\beta_{42}$ to lower the production of $A\beta_{42}$. Clinical trials have been performed with e.g. γ-secretase inhibitors to try to lower the production of $A\beta_{42}$, to modify the progression of the disease as the peptide is believed to be the driving force in AD [88, 89]. One γ-secretase inhibitor that has been tested is semagacestat which was halted because of a worsening of the cognitive decline in the participants [128]. This compound showed promising effects in animal studies [208], but did not result in a clear decrease of CSF $A\beta_{42}$ levels in human participants [129]. By using stable SILK it was however shown that semagacestat lowers the production of $A\beta_{42}$ in the brain [127]. As the levels of $A\beta_{42}$ in CSF did not show a clear decrease, $A\beta_{42}$ might not be the ideal biomarker for γ-secretase activity. In Paper III we thus tried to identify potential biomarkers for γ-secretase activity by analyzing CSF samples from individuals that had received semagacestat, using the method developed in Paper II. The participants had received a single dose of placebo, 140 mg of semagacestat, or 280 mg of semagacestat, and from the participants CSF had been sampled over time. The relative abundances of the endogenous peptides in CSF were measured from six different time points. This study showed that several of the endogenous peptides in CSF were decreased in the participants who received the 280 mg dose of semagacestat. Two peptides from APLP1, which is a known γ-secretase substrate, showed significantly decreased levels after γ-secretase inhibition and could be potential biomarkers for γ-secretase activity. Endogenous peptides from secretogranin-1, proSAAS, ApoE, and metallothionein-3 also showed decreased levels in the group who received 280 mg semagacestat. These proteins are not known γ-secretase substrates but the endogenous peptides derived from them were nevertheless decreased. The decrease in these peptides might be a secondary effect of the γ-secretase inhibition. It has been shown that inhibiting/knocking-out PS1 decreases the proteolytic activity in the lysosomes by impairing the acidification of the lysosomes, which is necessary for the acid hydrolases to be fully active [209, 210]. Lysosomes are involved in the degradation of proteins and peptides in neurons and astrocytes. It has been suggested that certain PS1 mutations causing familial AD lead to a loss of function of the lysosomes [210] and to not always lead to an increased $A\beta_{42}$ production [211]. The individuals with these mutations still develop AD and have increased formation of amyloid plaques in their brains. One could speculate whether this is caused by a decreased clearance of Aβ through the lysosomes which increases the Aβ load [212]. This might explain the observed decrease of $A\beta_{17}$ that has been reported after intake of semagacestat [213], as impaired lysosomal function might lower the production of Aβ peptides generated by degradation of longer forms of Aβ peptides in the lysosomes. Endosomal enlargement, enlarged lysosomes, and up-regulation of genes related to endocytosis have been suggested to be early

pathological events in the AD brain [214-216]. The lowering of endogenous peptides derived from the proteins that are not known γ-secretase substrates in Paper III might be explained by a loss of function of the lysosomes. This loss of function could result in a decreased degradation of proteins in the lysosomes, where some of the endogenous peptides that were formed by degradation of proteins in lysosomes would thus be decreased. Paper III showed that the method developed in Paper II could be used to quantify endogenous peptides in CSF in time studies to monitor changes in the relative abundances of the endogenous peptides in CSF. This approach can be used to identify novel biomarkers for target engagement of novel treatments, as well as to identify novel substrates of the targeted enzymes in vivo which otherwise is difficult.

9 CONCLUSION

The results presented in this thesis showed the presence of novel endogenous peptides in CSF which could contain potential biomarkers for neurodegenerative diseases. It was further shown that the endogenous peptides in CSF could be quantified using isobaric labeling, which provides a novel tool with great potential for biomarker discovery for diseases affecting the brain. It was shown this method could identify changes in endogenous peptides and proteins in the CSF of AD patients that seem to be disease relevant. The findings prompt the analysis of a larger clinical cohort to validate the results and identify other potential biomarkers. The developed methods were also showed to be useful for analyzing samples from time studies to detect differences in the abundances of endogenous peptides in CSF over time. This could be used to identify biomarkers for target engagement in clinical trials. The findings from the studies in this thesis will need to be validated in larger clinical cohorts and the potential biomarkers should further be evaluated by targeted methods to evaluate their value as clinical biomarkers for disease.

9.1 Future perspectives

There is a need for novel biomarkers in AD that can be used to study disease progression and to evaluate treatment outcomes. Quantitative proteomics has during the recent years evolved with better mass accuracy and higher throughput to make it a viable approach to discover biomarkers. The development of sample preparation and MS methodology that allows quantification of intact proteins and peptides would further improve the possibility to identify changes between different isoforms, truncated forms, and modified forms of proteins and peptides in biomarker discovery studies. This, combined with methods and instrumentation that makes it possible to identify and quantify low abundant compounds, will further push the possibility to identify biomarkers as they are most likely found among the low abundant peptides and proteins. The studies of intact peptides and proteins will most likely also increase or understanding of diseases as they directly reflect e.g. enzymatic processes in the brain, which may be affected in different diseases. Analyzing the changes in proteins and peptides during novel treatments targeting proteases will increase our understanding about the proteases function in the human body and give us a better understanding of how these affect the diseases.

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