Mass spectrometric quantification of amyloid-beta in cerebrospinal fluid and plasma

Implications for Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most common neurodegenerative disease among the elderly and accounts for 60-80% of all cases of dementia. Currently, the diagnosis of AD is based on cognitive tests and mental state exams, but the peptide amyloid-beta (Aβ) in cerebrospinal fluid (CSF) is increasingly used in clinical trials and settings. As for most protein and peptide biomarkers, quantification is performed using antibody-based techniques such as enzyme-linked immunosorbent assay (ELISA). However these immunoassays suffer from high variability in measurements of Aβ concentrations, hampering its use as a diagnostic marker.

The aim of this thesis was to develop an antibody independent method for absolute quantification of Aβ in human CSF, free of the specificity and reproducibility issues associated with antibody-based quantification. The method was based on solid-phase extraction (SPE) and liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Stable isotope labeled Aβ peptides were used as internal standards, enabling absolute quantification. The method was first tested in a pilot study with CSF samples from AD patients and controls. As expected, the level of the 42 amino acid variant of Aβ ($Aβ_{1-42}$) was decreased in AD CSF as compared to controls ($p<0.01$). The results were similar to those obtained with conventional ELISA, and an even better separation between the groups was obtained when

using the $A\beta_{1-42}/A\beta_{1-40}$ ratio. To investigate whether the antibody independent method would give similar results across different research centers, an inter-laboratory study was initiated which included three other laboratories using similar LC-MS/MS methods. Results showed good agreement and highlighted the importance of a certified reference material (CRM) to further increase the agreement between laboratories and MS methods. The method was further optimized, validated and published as a candidate reference measurement procedure (RMP). An RMP is required to set the value of a CRM used as a 'gold standard' to harmonize CSF Aβ measurements. To investigate if the large number of Aβ peptides in addition to $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ found in CSF could also be found in human plasma, an immunoprecipitation-based method for enrichment of Aβ peptides was developed. Sixteen N- or C-terminally truncated Aβ peptides were reproducibly detected using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. While quantification of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ using LC-MS/MS showed no AD association, the method may be useful in clinical trials of drugs affecting amyloid precursor protein (APP) processing or Aβ homeostasis.

In summary, absolute quantification of $A\beta_{1-42}$ using the developed LC-MS-MS method overcomes many of the issues associated with antibody-based methods. The method is currently being considered for formal certification as a RMP to determine the absolute concentration of $A\beta_{1-42}$ in a CRM to harmonize CSF $A\beta_{1-42}$ measurements across techniques and analytical platforms.

Keywords: Alzheimer's Disease, Mass Spectrometry, Biological Markers, Cerebrospinal Fluid, Amyloid beta-Peptides

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SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom (AD) är den vanligaste formen av demens och globalt är över 40 miljoner människor drabbade. Karaktäristiska fynd som görs i hjärnan hos patienter med sjukdomen är så kallade plack som framför allt består av peptiden beta-amyloid och neurofibriller bestående av tau-proteinet. Beta-amyloid är en peptid, en nedbrytningsprodukt från ett större protein som heter amyloid precursor protein. Enligt den främsta hypotesen för AD, amyloidkaskadhypotesen, är det en obalans i omsättningen av beta-amyloid. Hypotesen postulerar att det antingen är en överproduktion och/eller en minskad utrensning av beta-amyloid i hjärnan, vilket leder till aggregation med påföljande skador på hjärnans nervceller och slutligen demens. Beta-amyloid förekommer i flera olika längder (antal aminosyror), där beta-amyloid 1-42 länge använts som biomarkör för sjukdomen då den återfinns i lägre koncentration i likvor hos patienter med AD. Även de relativa nivåerna sinsemellan olika långa beta-amyloid peptider har i kliniska studier visat sig vara bra markörer för diagnostik och för att visa effekter vid läkemedelsstudier. Det finns redan antikroppsbaserade metoder som kan mäta upp till tre olika former av beta-amyloid, men dessa metoder brukar ge stora variationer i resultat mellan laboratorier, vilket gör det svårt att jämföra resultat t.ex. vid större läkemedelstudier.

I denna avhandling har metoder utvecklats, användbara inom klinisk forskning och rutin, för att mäta nivåerna av de mest intressanta formerna av beta-amyloid i likvor och blod. Den antikroppsoberoende metod som utvecklats bygger på masspektrometri, ett instrument som extremt noggrant kan separera molekyler från varandra utifrån deras förhållande mellan massa och laddning. Metoden kan på sikt ersätta nuvarande metoder, men framförallt kan metoden bli en internationell referensmetod. En referensmetod kan i sin tur användas för att bestämma koncentrationen av beta-amyloid i ett referensmaterial. Referensmaterialet kan sedan distribueras till tillverkare av andra mätmetoder och laboratorier för att kalibrera dessa och därmed minska mätvariationerna mellan laboratorier över världen. Med en referensmetod kan även ett definitivt ”cutoff-värde” för beta-amyloidkoncentrationen i likvor fastställas vilket kan medföra en säkrare Alzheimersdiagnos.

LIST OF PAPERS

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

I. **Pannee J**, Portelius E, Oppermann M, Atkins A, Hornshaw M, Zegers I, Hojrup P, Minthon L, Hansson O, Zetterberg H, Blennow K, Gobom J. A selected reaction monitoring (SRM)-based method for absolute quantification of Abeta38, Abeta40, and Abeta42 in cerebrospinal fluid of Alzheimer's disease patients and healthy controls. Journal of Alzheimer's Disease 2013; 33:1021-1032.

II. Leinenbach A, **Pannee J**, Dulffer T, Huber A, Bittner T, Andreasson U, Gobom J, Zetterberg H, Kobold U, Portelius E, Blennow K, on behalf of the IFCC Scientific Division Working Group on CSF proteins. Mass spectrometry-based candidate reference measurement procedure for quantification of amyloid-beta in cerebrospinal fluid. Clinical Chemistry 2014;60:987-984.

III. **Pannee J**, Tornqvist U, Westerlund A, Ingelsson M, Lannfelt L, Brinkmalm G, Persson R, Gobom J, Svensson J, Johansson P, Zetterberg H, Blennow K, Portelius E. The amyloid-beta degradation pattern in plasma-A possible tool for clinical trials in Alzheimer's disease. Neuroscience Letters 2014;573:7-12.

IV. **Pannee J**, Gobom J, Shaw LM, Korecka M, Chambers EE, Lame M, Jenkins R, Mylott W, Carrillo MC, Zegers I, Zetterberg H, Blennow K, Portelius E. Round robin test on quantification of Aβ42 in CSF by mass spectrometry. Alzheimer's & Dementia. In Press, published online July 20th 2015.

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CONTENT

ABBREVIATIONS

AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid-beta
BACE1	Beta-site amyloid precursor protein-cleaving enzyme 1
BBB	Blood-brain barrier
CID	Collision-induced dissociation
CNS	Central nervous system
CRM	Certified reference material
CSF	Cerebrospinal fluid
CT	Computed tomography
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
EO-FAD	Early-onset familial Alzheimer's disease
ESI	Electrospray ionization
GdnHCl	Guanidine hydrochloride
IP	Immunoprecipitation
IS	Internal standard
LC	Liquid Chromatography
LOAD	Late onset Alzheimer's disease
LTP	Long term potentiation
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization

MCI	Mild cognitive impairment
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NFT	Neurofibrillary tangle
PET	Positron emission tomography
PRM	Parallel reaction monitoring
PS-1	Presenilin-1 protein
PS-2	Presenilin-2 protein
PSEN1	Presenilin-1 gene
PSEN2	Presinilin-2 gene
RMP	Reference measurement procedure
RPC	Reversed-phase chromatography
SD	Standard deviation
SIM	Single ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
TOF	Time-of-flight
TOF/TOF	Tandem time-of-flight

1 INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, affecting more than 40 million people worldwide and accounts for 60-80% of all cases of dementia [1, 2]. The diagnosis is based on patient history, cognitive tests and auxiliary investigations (*e.g.,* CT/MRI scans of the brain) to exclude other conditions [3]. Biomarkers currently used to diagnose AD are magnetic resonance imaging (MRI), amyloid positron emission tomography (PET) and cerebrospinal fluid (CSF) measurement of the 42 amino acid form of amyloid-beta ($A\beta_{1\text{-}42}$) together with total tau protein (T-tau) and a phosphorylated form (P-tau). Amyloid PET and CSF $A\beta_{1\text{-}42}$, T-tau and P-tau have recently been included in the IWG-2 criteria for AD by The International Working Group (IWG) [4] and in the National Institute on Aging–Alzheimer's Association (NIA–AA) criteria for dementia due to AD [5] and mild cognitive impairment (MCI) due to AD [6]. Using structural MRI, the rate of brain atrophy from serially acquired scans can be determined. MRI has been shown to correlate with the rate of cognitive decline in AD patients [7], predict progression from MCI to AD as well as being useful for clinical AD diagnosis [8]. Together with an amyloid tracer, *e.g.,* ^{18}F-flutemetamol [9, 10] or ^{11}C-Pittsburgh compound B (^{11}C-PIB) [11], PET can determine the Aβ deposition in cortical brain regions in AD, and brain amyloid load measured using PET correlates with lower CSF $A\beta_{1\text{-}42}$ levels [12-16].

The reduced CSF concentration of the AD biomarker $A\beta_{1\text{-}42}$ in AD patients compared to controls [17] is increasingly used in clinical trials and settings. Using antibody-based techniques, such as enzyme-linked immunosorbent assay (ELISA), it has been shown that CSF $A\beta_{1\text{-}42}$ combined with T-tau and P-tau are accurate biomarkers for AD with a sensitivity and specificity of 85% and 95%, respectively [18-20]. However, current ELISAs for the AD biomarkers are variable; $A\beta_{1\text{-}42}$ ELISAs show assay-dependent intra-laboratory coefficients of variation (CV) of 5-19% and inter-laboratory CVs of 20-30%. Further, due to the current lack of certified reference materials for uniform assay calibration, there is bias between the different analytical methods used to quantify the AD biomarkers (different assays for, *e.g.,* CSF $A\beta_{1\text{-}42}$ correlate but give different absolute concentrations when applied on

the same samples). All this prevents from establishing universal cutoffs for CSF tau and Aβ biomarkers [21, 22].

Mass spectrometry (MS) has been used in the clinic for quantification of small molecules for many years, and has due to advances in instrumentation now the potential to provide unbiased quantification of large peptides and proteins in clinical routine. In this thesis, an antibody-independent MS method for absolute quantification of Aβ in human CSF was developed to overcome the issues associated with antibody-based detection. An immunoprecipitation (IP)-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) method was also developed to study the patterns of additional Aβ peptides in human plasma.

1.1 Alzheimer's disease

AD was first described in 1906 at a German psychiatrists' conference, and a paper published the following year by Alois Alzheimer described extracellular "miliary bodies" (neuritic plaques) in the neuropil, "dense bundles of fibrils" (neurofibrillary tangles (NFTs)) in the interior of nerve cells and degeneration of cortical nerve cells during post mortem analysis of the brain of a patient [23]. It was however not until 1984 that the content of the plaques was discovered to be Aβ peptides [24-26]. Aβ was proposed to be the driving force of the disease (a concept later named the "amyloid cascade hypothesis" [27]). In 1986, the neurofibrillary tangles were shown to contain abnormally hyperphosphorylated tau protein [28, 29]. AD is a slowly progressing neurodegenerative disorder of the central nervous system (CNS). Characteristic clinical symptoms include impaired episodic memory, aphasia, apraxia, agnosia and other general cognitive symptoms such as impaired judgment, decision-making and orientation [30]. However, AD is believed to have a latent phase of 20-30 years prior clinical disease onset [31], a time frame which Aβ accumulates in the brain without causing any overt clinical symptoms [32].

1.1.1 Neuropathology

Neuropathologically, the disease is characterized by deposits of extracellular plaques consisting of Aβ peptides, as well as intracellular neurofibrillary tangles consisting of phosphorylated tau protein in the medial temporal lobe and cortical areas of the brain [33]. These changes are accompanied by oxidative and inflammatory components which ultimately lead to neuronal and synaptic degeneration [34]. Since the discovery of Aβ accumulation in

plaques [25], research advances widely support that it is these changes in the brain that lie at the core of the disease [35, 36].

The central hypothesis for the development of AD is that the accumulation of Aβ is the primary cause of the disease due to an imbalance between the production and clearance of Aβ in the brains (see 1.1.2 below), while the neurofibrillary tangles, also found in the brain of AD patients, are believed to be a downstream effect of the amyloid pathology [27].

There are currently two established methods to determine the degree of Aβ accumulation in the brain *in vivo*: by PET imaging of the brain using different Aβ-binding tracers and by measuring the concentration of $A\beta_{1-42}$ in the CSF. For PET imaging, the amount of Aβ deposition in the cortex is determined by using tracers such as ^{11}C-PIB or ^{18}F-flutemetamol. Both tracers have been validated against histopathologic findings as well as to each other with good agreement [10, 11, 37-39].

The levels of $A\beta_{1-42}$ in CSF are inversely correlated with the plaque load and the concentration of $A\beta_{1-42}$ in CSF is around 50% lower in AD patients compared to age-matched controls [40, 41]. This data together with the studies showing that brain amyloid measures using PET correlates with lower CSF $A\beta_{1-42}$ levels indicate that it is the deposition of the peptide into plaques that leads to a reduced $A\beta_{1-42}$ concentration in CSF [12-15].

1.1.2 The amyloid cascade hypothesis

According to the amyloid cascade hypothesis, the main hypothesis on AD pathogenesis, an imbalance between the production and clearance of Aβ causes Aβ accumulation in the brain [27, 42, 43]. A conformational change of Aβ into high β-sheet content is believed to increase its propensity to self-aggregate from soluble monomers into dimers and higher order of aggregates and ultimately into insoluble fibrils and plaques, leading to synaptic dysfunction, neurodegeneration and, in the extension, dementia [44]. Which of these forms of Aβ that are neurotoxic is still uncertain, but levels of soluble Aβ dimers and oligomers has been shown to correlate with clinical symptoms and synaptic loss [45] as well as inhibiting long term potentiation (LTP) and disrupting synaptic plasticity [46], while Aβ fibrils have been shown to induce neuronal loss [47].

1.1.3 Amyloid precursor protein metabolism

The Aβ peptides are natural metabolic products of the transmembrane glycoprotein amyloid precursor protein (APP). The Aβ peptide is generated through the amyloidogenic pathway by consecutive actions of β-secretase (beta-site amyloid precursor protein-cleaving enzyme 1, BACE1) and γ-secretase, the latter of which is a complex consisting of at least four essential components: the homologous presenilin-1 (PS-1) and presenilin-2 (PS-2), nicastrin, Aph-1 and Pen-2, with PS-1 or -2 at its active site [48-52]. In the amyloidogenic pathway (Figure 1A, right), β-secretase cleaves off a large part of the extracellular domain of APP (sAPPβ). The remaining carboxy-terminal fragment (CTFβ) is cleaved within the membrane-bound domain by γ-secretase, releasing $A\beta_{1-42}$ [53] and several carboxy-terminal truncated forms including $A\beta_{1-40}$ and $A\beta_{1-38}$ [54, 55].

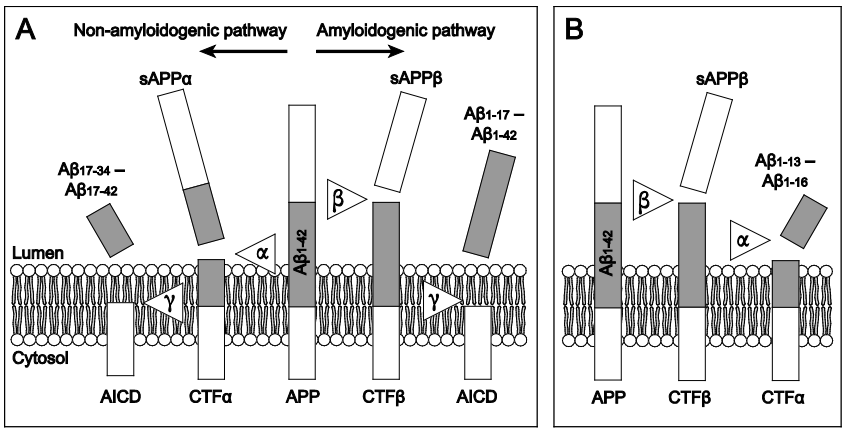

Figure 1. APP-processing showing the non-amyloidogenic pathway involving α- and γ-secretase cleavages (A, left) and the amyloidogenic pathway where β- and γ-secretase cleavages release Aβ (A, right). In a newly discovered γ-secretase independent pathway, β- and α-secretase cleavages release $A\beta_{1-13}$ to $A\beta_{1-16}$ (B).

The non-amoyloidogenic pathway, however, prevents Aβ generation since the cleavage of APP occurs in the middle of the Aβ sequence by α-secretase (Figure 1A, left) [56, 57]. An extracellular sAPPα domain is released leaving the carboxy-terminal fragment CTFα in the plasma membrane which can be further cleaved by γ-secretase, releasing $A\beta_{17-42}$ [56, 58]. In another α-secretase-dependent degradation pathway for APP, in which α- and β-secretase act on the same APP molecule, several shorter Aβ peptides including $A\beta_{1-13}$ up to $A\beta_{1-16}$ are generated (Figure 1B) [54, 55, 59]. Phase II clinical trials studying CSF biomarkers have shown that this APP processing

pathway is favored in AD patients receiving treatment with γ-secretase inhibitors, suggesting that the shorter Aβ peptides may be used as indicators of target engagement [60, 61]. In addition, it has been shown that APP processing is more complex than just these three major pathways. Several Aβ peptides ending at amino acid Q15 in the Aβ sequence and starting N-terminally of the β-secretase cleavage site, so called APP/Aβ(-x to 15) fragments, have been identified. These fragments have also been shown to increase in response to inhibition of BACE1 in cell models [62]. Several of these N-terminally extended peptides are also present in CSF [63].

Several clearance mechanisms have been proposed for Aβ including enzymatic degradation as well as transport over the blood-brain barrier (BBB) from CSF to the blood. Both insulin-degrading enzyme (IDE) [64-67] and neprilysin (NEP) [66, 68-71] can degrade Aβ at multiple sites, while only NEP has been shown to degrade the potentially neurotoxic oligomeric form of the peptide [72]. Additional enzymes shown to be involved in Aβ degradation are endothelin-converting enzyme (ECE) [73, 74], plasmin [75-77], angiotensin-converting enzyme (ACE) [78], matrix metalloproteases [79, 80] and cathepsin B [81]. Clearance of Aβ via the lysosomal degradation pathway has been shown to be mediated by the low-density lipoprotein receptor-related protein (LRP) ligands α2-macroglubulin and apolipoprotein E (apoE) [82], and can also be actively transported across the BBB from CSF to the blood by LRP [83-86]. A dysfunctional BBB has been suggested to result in a reduced clearance of toxic forms of Aβ from CSF to the blood [83, 87, 88]. A pathway for fluid transport in the brain, termed the glymphatic pathway, was recently discovered in mice [89]. In this transport pathway, it is suggested that solutes (which likely include soluble Aβ) are transported from the interstitial fluid as subarachnoid CSF flows through the interstitial space in the brain, and is cleared along paravenous drainage pathways.

1.1.4 Risk factors and genes

Similar to many other age-related disorders, the greatest risk factor for AD is advanced age. Most people are diagnosed at age 65 years or older, and the prevalence of the disease increases exponentially with age [90, 91]. In the age group 60-64 fewer than 1% have AD while over 24% of people over 85 years of age are diagnosed with AD [92]. Taking into account the latent phase of the disease, when clinical symptoms are absent, the actual prevalence of AD is most likely higher [31, 93]. In addition, a very large number of other risk factors have been suggested based on association in epidemiological studies, for example low mental and physical activity, head injury, oxidative stress, arteriolosclerosis, hypertension, hyperhomocysteinemia and hypercholesterolemia

[36, 94-97], but their contributions are minor. Studies in AD transgenic mice have shown that cerebral hypoxia increases Aβ levels in the brain through induced expression of β-secretase [98]. Similarly, cerebrovascular hypoperfusion causes learning/memory impairment and a time-dependent accumulation of Aβ oligomers in rat brain [99]. In human, increased Aβ expression in hippocampus can be seen in patients who died from ischemic stroke [100], and very high levels of Aβ have been measured in human blood after resuscitation following cardiac arrest [101] as well as APP overexpression and Aβ plaques in patients who died 3-36 days after resuscitation following cardiac arrest [102]. Additionally, small brain size, low mental and physical activity and head injury could be risk factors for AD [36, 103-105].

The second strongest risk factor for AD is family history, where genetic components are estimated to play a role in over 80% of all AD cases [106]. The disease can be divided into two forms, early-onset familial AD (EO-FAD) which accounts for less than 1% of all cases under the age of 65 [36] and late-onset AD (LOAD) [107, 108] accounting for the majority of AD cases [109]. Mutations linked to EO-FAD have been located to the genes *APP*, *PSEN1* and *PSEN2,* encoding the proteins APP, PS-1 and PS-2, respectively. AD-causing mutations in *PSEN1* are the most common while mutations in *PSEN2* are very rare among EO-FAD patients [110, 111]. Whereas FAD-causative *APP* mutations either increase Aβ production in general or result in more aggregation-prone Aβ forms, *PSEN* mutations tend to alter the C-terminal truncation pattern of Aβ so that relatively more of $A\beta_{1-42}$ is produced at the expense of less aggregation-prone $A\beta_{1-37/38/39}$ forms [112-115]. The major gene polymorphism unequivocally associated with LOAD is the *APOE* gene allele ε4, which increases the risk and lowers age of onset. There are three common *APOE* gene variants in the population, the ε2, ε3 and ε4 alleles. The ε3 allele is the most common in the population (50-90%), followed by ε4 (5-35%) and the ε2 allele (1-5%) [116]. Carrying two ε4 alleles will increase the risk of developing AD by greater than 10-fold, while heterozygote allele carriers will have a 4-fold increased risk. The ε2 allele on the other hand seems to be protective [117-119]. Additional, weak but significant gene polymorphisms association with LOAD have been reported and can be found in the AlzGene database (http://www.alzgene.org), a continuously updated database that catalogs all genetic association studies in the field of AD [120].

1.1.5 Diagnosis

The diagnosis of AD is currently based on medical history, cognitive tests and mental state exams [3]. Two of the most used sets of criteria for diagnosis are the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [121] and the National Institute on Neurological and Communicative Disorders and Stroke and the Alzheimer Disease and Related Disorders Association (NINCDS-ADRDA) tests [30]. Sensitivity and specificity of clinical diagnosis are 80% and 70%, respectively [122].

For a definitive diagnosis of AD however, a post mortem neuropathological examination is required. Pathological criteria for AD is a recommendation by the National Institute of Aging and the Reagan Institute (NIA-RI) to combine the Consortium to Establish a Registry for Alzheimer Disease (CERAD) score of neuritic plaques [123, 124] and the topographic staging of NFTs [125]. These criteria are divided in three categories, low, intermediate and high likelihood of AD, and a diagnosis of AD is made when the criteria for intermediate or high likelihood of AD are met and the patient had a clinical history of dementia [126].

The AD biomarker $A\beta_{1\text{-}42}$ is increasingly used in clinical trials and settings to detect the disease at an earlier stage and to evaluate the effectiveness of new drug candidates before neurodegeneration has become too severe [127]. There is an ongoing process of including biomarkers reflecting plaque and tangle pathology in the clinical criteria [5, 128], which would permit earlier diagnosis as well as increasing the specificity [6]. According to the recent IWG-2 criteria for typical AD, increased tracer retention on amyloid PET, decreased $A\beta_{1\text{-}42}$ together with increased tau in CSF, or the presence of AD autosomal dominant mutation in the *PSEN1*, *PSEN2* or *APP* is *in vivo* evidence of AD pathology [4].

1.1.6 Biomarkers

In 1995, it was shown using ELISA that the concentration of $A\beta_{1\text{-}42}$ in CSF of AD patients was reduced compared to controls [17] and this finding has since then been replicated in numerous independent studies, also using different assay platforms [129-132]. A widely accepted explanation for the reduced concentration of $A\beta_{1\text{-}42}$ in CSF is that the peptide accumulates in plaques in the brain [133-136]. The concentration of $A\beta_{1\text{-}42}$ in CSF is approximately 50% lower in AD patients compared to healthy controls [137] and has also been shown in numerous studies to be a good biomarker for prodromal AD [133, 138]. Combined with the microtubule-stabilizing tau protein, including

P-tau and T-tau that reflect tangle pathology and cortical axonal degeneration, respectively, the biomarkers have shown a high diagnostic accuracy of AD and they are since 2007 included in the diagnostic research criteria for AD [18-20].

There are several immunoassays, such as ELISA, available which routinely are used in many clinical laboratories to measure the concentration of $A\beta_{1-42}$ [133]. These assays, however, suffer from high variability, especially between laboratories, and also between platforms and different lots within the same assay platform, which hamper the use of $A\beta_{1-42}$ as a diagnostic marker [21, 22]. Both CSF and plasma contains a variety of Aβ peptides, where $A\beta_{1-40}$ is around ten times more abundant than $A\beta_{1-42}$ [139], and there is a variation between individuals regarding the amount of all (total) Aβ peptides produced [140]. Consequently, when using only $A\beta_{1-42}$, low producers might be false positive for AD while the opposite might be true for high producers. Since the levels of $A\beta_{1-40}$ in CSF are unchanged in AD, the ratio of $A\beta_{1-42}/A\beta_{1-40}$ has been shown to improve the diagnostic accuracy of AD [140-143].

Measurement of Aβ peptides in plasma, with the possible exception of a recently described Aβ peptide spanning the β-secretase cleaving site [144], has not yet shown to be useful as biomarkers of AD [145, 146]. Plasma Aβ is mainly derived from different extra-cerebral cell types, *e.g.,* blood platelets [147, 148] and thus does not reflect plaque pathology in the CNS as CSF $A\beta_{1-42}$ does [133]. However, plasma Aβ might be useful in therapeutic clinical trials to monitor pharmacodynamics for dose setting in early clinical trials as well as to monitor the potential clearance of Aβ from the brain into the blood [149].

Computed tomography (CT) was first used to exclude other causes of dementia by studying structural alterations of the brain, and was later replaced by MRI. Today, structural and functional MRI is used to support a clinical AD diagnosis. Structural MRI visualizes the cerebral atrophy characteristic for neurodegeneration. In AD, a progressive atrophy is first seen in the medial temporal lobe [150] typically originating in the entorhinal cortex, followed by the hippocampus, amygdala, and parahippocampus [151-154]. Functional MRI (fMRI) measures neuronal activity indirectly by using blood-oxygen-level dependent contrast imaging [155-157], either acquired during cognitive tasks compared to a control condition, or during resting conditions (resting state functional MRI (rsfMRI)) [158] to study functional connectivity and detect early brain dysfunction related to AD.

Since glucose is the brain's primary source of energy, uptake of the glucose analog fluoro-deoxy-D-glucose (FDG) can be used in combination with PET to study brain metabolism. In AD patients, a decreased glucose metabolism can be seen in the brain, which worsens as the disease progress and correlate with AD pathologic diagnosis after autopsy [159-162].

Table 1. Summary of biomarkers used for AD

Type	Biomarker	Change in AD
CSF	$A\beta_{1-42}$	↓ concentration
CSF	$A\beta_{1-42}/A\beta_{1-40}$ ratio	↓ ratio
CSF	T-tau	↑ concentration
CSF	P-tau	↑ concentration
Imaging	Structural MRI	↓ volume
Imaging	Functional MRI	↓ functional connectivity
Imaging	FDG-PET	↓ glucose metabolism
Imaging	Amyloid PET	↑ Aβ retention
Imaging	Tau PET	↑ intracellular tau

Amyloid imaging with PET was first achieved with ^{11}C-PiB, a tracer which has a high specificity for fibrillar Aβ [163-166], and has shown significantly higher cortical retention in AD patients compared to controls [11, 167]. More recently, the three ^{18}F-labeled tracers florbetaben [168-170], florbetapir [171, 172], and flutemetamol [10] have been introduced which show similar cortical retention properties. However, ^{18}F tracers have a half-life of around 110 minutes compared to 20 minutes for ^{11}C-PiB, which allows centralized production of ^{18}F tracers for distribution to remote PET-scanning facilities while ^{11}C require an on-site cyclotron.

There are two PET tracers for tau which recently have been trialed in human subjects. Phenyl/pyridinyl-butadienyl-benzothiazoles/benzothiazoliums may be able to distinguish Aβ from tau *in vivo* [173], while ^{18}F-T808 has been shown to bind tau in brain tissue sections [174] and *in vivo* [175].

1.1.7 Treatments

There are currently four drugs available that temporarily may improve the symptoms of AD patients: three acetylcholinesterase inhibitors and one NMDA-receptor antagonist, but to date there is no therapy that slows or stops the progression of AD. The two main treatment strategies currently evaluated in clinical trials are active and passive Aβ immunotherapy. In active Aβ immunotherapy, the patient is immunized with Aβ peptides or fragments to stimulate the generation of endogenous anti-Aβ antibodies, while in passive

immunotherapy, the patient is instead treated with intravenous infusions of monoclonal anti-Aβ antibodies [176]. Almost all clinical trials with potential anti-Aβ disease-modifying drugs have failed to show positive effects on primary clinical outcome in recent years, despite the fact that these drugs have shown to prevent and in some cases clear amyloid plaques in AD mouse models [177, 178]. One possible explanation could be that these drug treatments were not commenced early enough. AD is believed to have a presymptomatic phase of 20-30 years [31], thus neurodegeneration might have been too severe and widespread in these clinical trials [176]. Another explanation might be that not all patients were diagnosed correctly, but might also have included patients with other forms of dementia. More specific diagnostic tools are needed for patient enrichment, where AD biomarkers will be vital to include subjects at an earlier stage.

2 AIMS AND OBJECTIVES

The aim of this thesis was to develop an antibody-independent MS-based method for absolute quantification of Aβ peptides in human CSF and plasma, and ultimately certify the developed method as a reference measurement procedure.

The specific aims of each paper were:

Paper I – To develop an antibody independent LC-MS/MS method for absolute quantification of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ in human CSF and test its performance in a small clinical cohort.

Paper II – To perform a full validation of the $A\beta_{1-42}$ method as a reference measurement procedure.

Paper III – Optimize the LC-MS/MS method to enable absolute quantification of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ in human plasma, as well as explore the pattern of additional N- and C-terminally truncated Aβ species peptides using MALDI-TOF.

Paper IV – Perform an inter-laboratory study involving other laboratories using similar LC-MS/MS methods, to determine the inter-laboratory variation using these methods and to examine if these methods are suitable to set the level of a certified reference material.

3 METHODS

In clinical research, proteins and peptides are still mostly measured using immunoassays owing to their high sensitivity and sample throughput. However, antibodies can cross-react with substances other than the target analyte, the target epitope can be blocked by endogenous antibodies (autoantibodies) and non-specific antibodies present in the sample can bind to the antibodies in the assay, creating a complex with the capture and detection antibodies [179, 180]. There is also the hook effect, according to which analyte concentrations above a certain point give a falsely low result due to saturation of the assay antibodies [181]. Immunoassays for $A\beta_{1-42}$ might also be influenced by matrix effects, and only measure the free fraction of the analyte revealed by the non-linearity upon dilution of samples [21].

MS based methods has been increasingly used in clinical laboratories [182]. Compared to immunoassays, they have higher specificity and multiplexing capacity with respect to different analytes. With immunoassays, quantification relies on the interaction between antibodies and the target molecule, and calibration is performed by separately measuring a series of standard samples of known concentration. The interaction between antibody and antigen provides an indirect measurement of antigen quantity that may be affected by a multitude of factors [181]. One is the specificity of the antibodies: if they cross-react with other sample components, it will affect the measurement. Antibodies often also do not distinguish among modified and processed forms of the target molecule; if present they are likely contribute to the measurement in an undefined manner. Furthermore, the interactions of antibodies and their target molecules can be highly sensitive to changes in the reaction conditions: even slight variations, such as using a different type of sample tube, or reagents and solvents of a slightly different quality may affect the result. As a result, while a laboratory can achieve high precision by establishing rigorous protocols, results will often differ from those obtained in other laboratories. The sample composition also affects antibody based quantification. Because antibody binding takes place under non-denaturing conditions, sample molecules that affect the solubility, conformation, or aggregation state of the analyte may significantly affect the measurement. These effects, which are often not well-understood, are collectively termed

matrix effects. To avoid errors due to matrix effects, it is imperative that the standards are prepared in a matrix highly similar to that of the sample.

With MS, quantification, with the use of an internal standard (IS), is achieved by directly counting the analyte molecules or fragments thereof, at their mass-to-charge (*m/z*) ratio, giving a high specificity of detection. Because mass spectrometric quantification is based on directly counting targeted molecular ions, quantification is absolute and robust. A heavy, stable isotope-labeled version (*e.g.,* ^{15}N or ^{13}C) of the analyte is often used as an IS, which is added to the samples prior to sample preparation. The IS is detected simultaneously with the endogenous peptide, at a different *m/z* and quantification is performed by taking the ratio of the endogenous peptides LC-MS peak area to that of the IS. The heavy peptide, being chemically equivalent to the endogenous peptide, has identical yield through all sample purification steps, the same ionization efficiency and fragmentation behavior in the MS. Since the IS and endogenous peptide are prepared and analyzed together, variations in sample preparation have little or no effect on quantification. Sample preparation can be performed under denaturing conditions, reducing the risk of matrix effects. In the CSF Aβ method developed in this thesis work, guanidine hydrochloride (GdnHCl) was added to the sample to break up possible protein interactions and minimize enzymatic activities during sample preparation [183], and ammonium hydroxide (NH_4OH) was used during SPE elution and LC separation to avoid aggregation of Aβ peptides [184]. When sample preparation is performed under these conditions, non-linear dilution effects as seen in immunoassays are absent, indicating that a larger proportion of the analyte may be available for quantification (the total fraction).

3.1 Patients and samples

CSF used for method development were de-identified samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden.

For AD and control studies, all patients had undergone a thorough clinical investigation, including a medical history, physical, neurological and psychiatric examination, screening laboratory tests, and CT of the brain. AD patients fulfilled the DSM-III-R criteria of dementia [185] and the criteria of probable AD defined by NINCDS-ADRDA [30]. The control individuals were cognitively normal research volunteers.

3.2 Solid phase extraction

In SPE, affinity-based separation is performed with a stepwise elution of the analyte (compared to gradient or isocratic elution in LC), often by vacuum or centrifugal force. The solid phase is typically packed in disposable syringe-barrel cartridges or in the microtiter plate format. SPE is mostly used early in the sample preparation and can be used to concentrate the analyte by eluting it in a volume smaller than the original sample volume [186].

As with LC, SPE can be performed in different modes. For reversed-phase chromatography (RPC), a non-polar hydrophobic stationary phase such as silica bonded carbon chains is used to separate compounds according to their hydrophobicity. Ion exchange chromatography use electrostatic interaction between the analyte and the stationary phase to isolate ionic or ionizable compounds by using ionic groups such as carboxylic or sulfonic acids or amine groups on the surface of the stationary phase. Mixed mode (or multimodal) SPE utilizes different combinations of functional groups in the same stationary phase, *e.g.,* reversed-phase and cation-exchange properties.

Due to its physicochemical properties, Aβ easily self-aggregate and nonspecifically bind to other proteins and surfaces [187, 188]. To overcome these issues, the samples were pretreated with GdnHCl to minimize nonspecific protein interactions and enzymatic activities while elution was performed with an alkaline solution to avoid aggregation [184, 189, 190].

3.3 Immunoprecipitation

IP (also known as immunoaffinity capture) is a method used to isolate a particular analyte from a complex sample matrix such as CSF or plasma. Antibodies against a specific analyte are immobilized onto a support material such as magnetic beads, and incubated with the sample on a mixer. The analyte bound to the antibody can then be collected from the sample matrix using the magnetic properties of the beads. After extensive washing, the analyte is eluted and analyzed using MS. IP has previously been successfully used in combination with MS to analyze Aβ [139, 191].

3.4 Liquid chromatography

LC separate compounds according to their chemical properties where a fluid (mobile phase), carrying the sample, is pumped through a column containing a porous bed (stationary phase). The sample components are separated

according to their degree of exchange (mass transfer) between the mobile phase and the stationary phase.

In proteomics, RPC is the most commonly used mode of LC which relies on hydrophobic interaction, where a non-polar hydrophobic stationary phase, usually silica with carbon chains of varying lengths ranging from C_4 to C_{18}, is used to separate compounds according to their hydrophobicity. By pumping a solvent with a decreasing degree of polarity, retained compounds are successively eluted from the stationary phase, separated from each other by their different degrees of interaction with the sorbent, *i.e.* hydrophobic compounds are retained longer due to stronger interaction with the stationary phase. The most widely used stationary phase in RPC columns are particle-based, where the carbon chains are bonded to silica particles, but monolithic columns also exist, made of a single cylindrical polymer rod with an uninterrupted, interconnected network of flow-through channels.

For particle-based columns, higher column efficiency is achieved by using smaller particles. However, the backpressure increases proportionally as the particle size decreases. Advantages with monolithic columns over particle based columns include that they are extremely permeable, offering higher separation capabilities at higher flow without a high backpressure and tolerate a wider pH range [192, 193].

For untargeted discovery proteomics experiments, nanoflow LC (< 1 µL/min) is generally used, where sensitivity is prioritized at the cost of throughput [194, 195], while it is more common to use higher flow rates (> 1 µL/min) for high-throughput targeted assays in clinical routine. The decrease in signal intensity moving from nano to higher flow rates is partially cancelled out by the higher loading capacity of the larger columns used in comparison to a capillary column used for nano-flow LC [196].

3.5 Mass spectrometry

MS is a technique used to determine the molecular mass of a compound. It can be used to analyze molecules ranging from atoms to metabolites, and further to peptides, intact proteins, protein complexes, and even virus particles. MS instruments consist of three parts: an ion source, one or more mass analyzers and a detector. The ion source transfers the analyte molecules into gas-phase ions, which are then separated according to their *m/z* using a mass analyzer, fragmented to product ions in a fragmentation cell and finally detected either by an image current detector or an impact detector.

The introduction of the soft ionization techniques such as electrospray ionization (ESI) and MALDI enabled MS analysis of peptides and proteins. Using MS/MS, structural information of proteins and peptides can be obtained by their predictable fragmentation behavior [197, 198], and is a crucial first step in the development of a targeted MS assay. The fragmentation of peptides and proteins along the peptide backbone follows specific rules. These fragment ions are called abc/xyz ions depending where on the peptide structure the fragmentation occurs and on where on the peptide the charge is located [197, 199].

ESI and MALDI can be coupled to different types of mass analyzers [200]. Recently, hybrid instruments have been introduced, in which different types of mass analyzers are combined (*e.g.,* the Q Exactive which has a quadrupole and a high-energy collision cell in front of an Orbitrap mass analyzer). MS is under strong development, with continuous improvements in sensitivity, mass accuracy, resolution and speed, and today LC-MS/MS is being used as an alternative to some immunoassays for peptide and protein quantification [201].

3.5.1 Ionization techniques

There is currently a wide range of methods to ionize the compounds of a sample. Earlier desorption ionization techniques include fast atom bombardment (FAB) [202] and plasma desorption ionization (PDMS) [203]. For the analysis of proteins and peptides however, soft ionization methods are required to transfer the analytes to the gas phase without degradation. At present there are mainly two ionization techniques used for proteins and peptides: MALDI [204, 205] and ESI [206-208].

3.5.1.1 ESI

In ESI, ions are produced from a solution by an electric field (several kV) applied between the emitter, to which the sample is transported in a volatile, polar solvent, and the inlet of the mass spectrometer. The electric potential produces an aerosol spray consisting of small charged droplets from the liquid, carrying an excess positive or negative charge depending on the polarity of the spray needle. Ions are formed at atmospheric pressure as the droplets shrink through evaporation, which can be aided by heating the emitter and applying a flow of heated nitrogen gas between the emitter and the inlet to the mass spectrometer. The ions enter an intermediate vacuum region through a narrow inlet where several lenses focus the ion beam, and are further transported into the high vacuum region of the mass analyzer. ESI

can be operated in the positive or negative ionization mode, where positively or negatively charged molecules, respectively, enter the mass spectrometer. To aid protonation or deprotonation of the analyte during ESI, an acid or base is usually added to the mobile phases.

3.5.1.2 MALDI

In MALDI, ions are created when short pulses from a laser irradiate a sample mixed with a light-absorbing, low molecular mass matrix deposited on a MALDI target in high vacuum [209-211]. To desorb and ionize the sample molecules while avoiding decomposition, they are homogenously mixed with a matrix, most often in crystalline form, which is instead excited by the laser. When the matrix molecules absorb the laser energy, a plume of sample and matrix molecules are ejected from the sample. Photochemical reactions in the plume involve proton transfer between analyte and matrix molecules, leading to the formation of positively and negatively charged ions. Peptide gas-phase ions predominately carry a single charge, while proteins often acquire multiple charges [212]. MALDI is most commonly combined with a time-of-flight (TOF) mass analyzer, although it can be combined with other mass analyzers such as trapping analyzers [200].

3.5.2 Mass analyzers

The mass analyzer, a central part of the mass spectrometer, separates any charged particle according to its *m/z*. Since all types of mass analyzers use electric and/or magnetic fields for separation, the analytes have to be ionized, which is the role of the ion source. Different types of mass analyzers have different properties with respect to mass accuracy, resolution, speed and the ability to perform fragment ion analysis. In MALDI, discrete gas-phase ion packages are produced, which makes the technique highly compatible with pulsed TOF mass analyzers, in contrast to quadrupole mass analyzers which are typically used with a continuous ion source such as ESI. Many new MS instruments are equipped with several types of mass analyzers, so called hybrid instruments.

3.5.2.1 Quadrupole

The quadrupole mass analyzer, first described in 1953 [213], isolates ions within a very narrow *m/z* range. It consists of four parallel rods with oscillating direct-current (DC) and radio frequency (RF) potentials, where the pair opposite to each other will have the same potential at any given time.

Ions with a specific *m/z* can be isolated using the ratio between DC and RF potential. These ions will have stable trajectories through the quadrupole (Figure 2) while ions with higher or lower *m/z* will bend off and not be able to reach the detector. A mass spectrum (intensity vs. *m/z* plot), or full scan, with a range of *m/z* can be acquired by scanning the ratio of the DC and RF potentials while recording the abundance of detected ions. The strength of the quadrupole mass analyzer lays in its low cycle time and high detection sensitivity when monitoring a fixed *m/z* (single ion monitoring (SIM)) or when scanning over a narrow *m/z* range. However, scanning over a broad *m/z* range leads to a decrease in sensitivity, since only ions with the selected *m/z* are isolated at any given time while most of the ions produced are not detected.

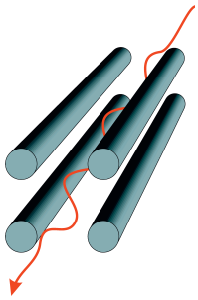

Figure 2. Schematic of a quadrupole mass analyzer. Ions selected according to their m/z will have a stable trajectory through the quadrupole.

3.5.2.2 Orbitrap

The orbitrap was introduced in 1999 [214] and is a high resolution electrostatic ion trap mass analyzer, where incoming ions are trapped and measured around a central electrode with an electrostatic field (Figure 3). Ions oscillate along the core axis with frequencies $\omega = \sqrt{(k/(m/q))}$, where k = force constant and q = charge. Compared to the quadrupole mass analyzer, the orbitrap has a much higher resolving power (resolution) and mass range as well as better mass accuracy, while having a lower dynamic range [215, 216].

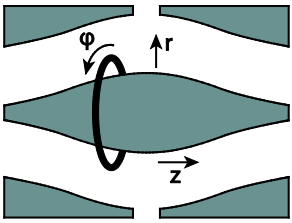

Figure 3. Schematic of an orbitrap mass analyzer. Ions move in stable trajectories both around (r, with the angular φ-motion) and along (z) the central electrode.

3.5.2.3 TOF

The TOF mass analyzer was first described in 1946 [217]. It uses a high voltage (20-30 kV), applied between the sample plate and a grid/extraction element, in the ion source. The generated ions are accelerated to a high velocity, which is inversely proportional the square root of the molecular mass according to the equation $v = \sqrt{(2qU/m)}$, where v = velocity, q = charge, U = acceleration voltage. The ions are separated according to the elapsed time between a start signal and the pulse generated when an ion impacts the detector [218]. TOF analyzers are fast and have a very high *m/z* range. They are also sensitive in full scan mode, since almost all ions generated in the ion source are detected in contrast to the quadrupole mass analyzer operated in scanning mode. Structural information (MS/MS data) of compounds can be obtained from post-source decay of the analyte, or by coupling two TOF analyzers to a tandem time-of-flight (TOF/TOF) MS.

3.5.3 Operation modes for quantification

In MS, the recorded ion current of the analyte molecule is used for quantification. While quantification can be performed in the SIM mode, there is a high risk of interfering molecules in a complex biological sample being co-isolated, since two completely different molecules can have identical *m/z*. In MS/MS, the precursor ion is isolated, fragmented and quantification is performed by monitoring specific fragments to increase selectivity. An IS can be added to a sample to correct for variations in sample preparation and fluctuation in MS signal associated with analyte ionization and matrix effects. When coupled to LC, quantification is performed by integrating the area under the chromatographic peak acquired by plotting the total ion current against time during LC separation.

3.5.3.1 Selected reaction monitoring

Selected reaction monitoring (SRM), sometimes referred to as multiple reaction monitoring (MRM), is a targeted MS/MS method that has been used for over three decades in small molecule analysis, and has also emerged as an alternative to immunoassays for protein and peptide quantification [219].

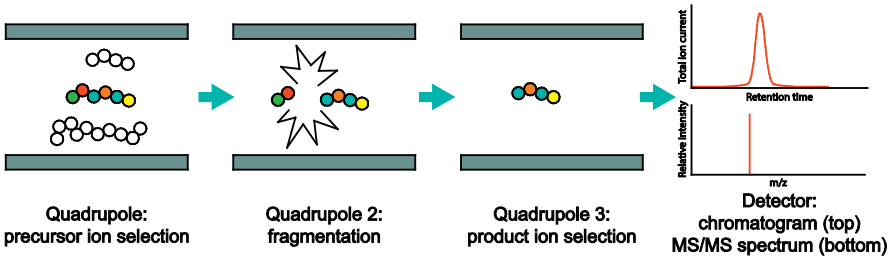

Figure 4. Selected reaction monitoring mode on a triple quadrupole mass spectrometer.

This type of targeted MS analysis is performed using a triple quadrupole mass spectrometer which consists of three consecutive quadrupole mass analyzers [220]. The first quadrupole is set to transmit an ion (precursor ion) according to its *m/z*. The ion is then fragmented in the second quadrupole, filled with an inert gas (*e.g.,* argon or nitrogen), by collision induced dissociation (CID) as it collides with the gas molecules. The third quadrupole is set to transmit one or more specific product ions generated in the second quadrupole to the detector (Figure 4). The combination of a precursor ion and a specific product ion is referred to as a transition. SRM greatly increases selectivity compared to SIM, since interfering ions with identical *m/z* co-isolated in the first quadrupole will most likely not give rise to product ions with identical *m/z* to that of the analyte of interest.

3.5.3.2 Parallel reaction monitoring

The new generation of high resolution hybrid instruments enables new approaches for targeted quantitative proteomics [216, 221, 222]. Using the parallel reaction monitoring (PRM) mode on a quadrupole-orbitrap hybrid instrument addresses some of the limitations associated with SRM [223, 224]. PRM is performed by isolating the precursor ion in the quadrupole mass analyzer. Precursor ions are accumulated in the collision cell and fragmented before the product ions are transmitted to the orbitrap mass analyzer where the MS/MS mass spectrum are recorded (Figure 5). In contrast to SRM, a PRM method does not require any prior information on

the fragment ions which is especially useful when establishing new assays. Because a full fragment ion spectrum is recorded and permits parallel detection of all target product ions, it can be used to confirm a compounds identity, and selection of fragment ions used for quantification can be done post acquisition.

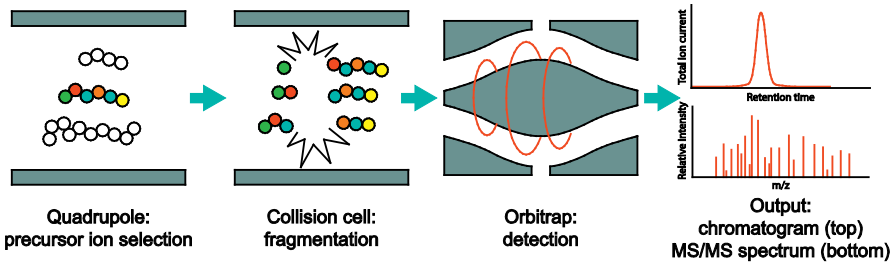

Figure 5. Parallel reaction monitoring mode on a quadrupole-orbitrap hybrid instrument.

3.6 Internal standards

An IS, corresponding to the analyte to be measured, is used to control for variability in sample preparation and analytical procedure, depending on at which stage the standard is introduced. For absolute quantification of peptides, recombinant or synthesized peptides with incorporated stable isotopes such as ^{15}N or ^{13}C are most commonly used. These standards, often called "heavy" standards due to their mass increment, are easily differentiated from the endogenous peptide in the mass spectrometer [225]. Being chemically equivalent to the endogenous peptides, as their amino acid sequence is identical to the target peptide, they will have identical yield through all sample preparation steps, co-elute with the target peptide during LC as well as having an identical ionization efficiency and fragmentation behavior in the mass spectrometer, and therefore accounts for any variations in the analytical procedure [226-228]. Quantification is performed by extrapolating the concentration using the MS signal ratio of the endogenous peptide to the IS from a calibration curve constructed with known concentrations of the analyte [229].

3.7 Calibration procedures

Calibrators used to construct a calibration curve should, according to international guidelines for bioanalysis, be prepared in an identical analyte free matrix, or as close as possible to that of the samples ("matrix matching")

to eliminate matrix-related effects. Matched analyte-free matrix for calibration has been used with LC-MS/MS, *e.g.,* for prostate-specific antigen (PSA), where calibrators were prepared in female plasma which is free of PSA, or below the level of detection [230]. Since there is no human CSF free of Aβ peptides, several surrogate matrices and calibration procedures have been used, including Aβ-depleted human CSF, rat CSF, artificial CSF and artificial CSF with 5% of rat plasma or bovine serum albumin [183, 189, 231-233].

There are several calibration approaches that can be used depending on the analyte and matrix. The most common approach for generating a calibration curve, the "normal curve", is to prepare the calibrators with variable concentrations of the endogenous (unlabeled) analyte, while a constant concentration of labeled IS is added to both the calibrators and unknown samples. When using this approach, the calibration matrix has to be free of the endogenous analyte. If there is no analyte free matrix available, preparation of calibrators in a surrogate matrix should be justified, and absence of matrix effects should be demonstrated [234, 235].

To circumvent the issue of endogenous analyte in the calibration matrix, the "reverse curve" approach can be used. With this procedure, the endogenous analyte is used as an IS when constructing the calibration curve in the same matrix as the unknown samples (from a pool of samples), and instead spiking in variable concentrations of the labeled peptide. In unknown samples, the labeled peptide is used as IS. This procedure can be valuable when the analyte is unstable in a surrogate matrix [236].

Instead of using a surrogate matrix, a surrogate analyte can be used to construct a calibration curve in the same matrix as for unknown samples (the "surrogate analyte approach") [237-239]. For this approach, two different isotopically labeled standards are needed, one (*e.g.,* ^{13}C-labeled) to construct the calibration curve, and another (*e.g.,* ^{15}N-labeled) as IS. Endogenous concentrations of unknown samples can then be determined by using the endogenous to ^{15}N-labeled ratio of the unknown samples with the ^{13}C/^{15}N-labeled ratio of the calibration curve. Using this approach, the response factor between the labeled analyte used for calibration and the native analyte has to be determined, since the isotopic distribution of a uniformly labeled peptide might be different to that of the endogenous, unlabeled peptide [231]. In summary, regardless of calibration procedure used, the response of the calibrators has to represent the response of the endogenous analyte measured [235].

3.8 Applications in clinical chemistry

Mass spectrometry is since a long time a mainstay technique for quantification in the clinical laboratory, particularly for small molecule analysis. Owing to their robustness and relatively low cost, triple quadrupoles have been the mass spectrometric instruments of choice, achieving high specificity and detection sensitivity. The use of MS/MS is now common in many clinical laboratories as a valuable diagnostic tool where its use has grown dramatically over the last 15 years, *e.g.,* in therapeutic drug monitoring, endocrinology, toxicology, pediatrics and microbiology [240], while protein and peptide quantitation is a more recent advance [225, 241, 242]. For example, a recently developed method demonstrated how two forms of soluble recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL), used in cancer therapy, differing by only a few amino acids can be quantified simultaneously in serum with LC-MS/MS [243]. Improvements in sample preparation has also been possible with MS, such as quantifying the therapeutic peptide romplanin in human dried blood spot rather than liquid plasma and serum, with similar limits of quantification [244]. Another example is a multiplexed MS method for absolute quantification of apolipoproteins, important biomarkers for predicting cardiovascular risk, with inter- and intra-assay CVs of <12% and <6%, respectively, and showing good agreement with established immunoassay [201].

4 RESULTS AND DISCUSSION

4.1 Paper I

The aim of this paper was to develop an antibody free LC-MS/MS method for quantification of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ in human CSF. GdnHCl was used during sample preparation to dissociate Aβ peptides from other sample components prior to SPE extraction, and a triple quadrupole MS operated in the positive ion mode was used for quantification.

Human CSF was chosen as the calibration matrix since Aβ peptides, especially $A\beta_{1-42}$, are very hydrophobic and difficult to keep in solution, and using a surrogate matrix such as artificial CSF might lead to low recoveries of the analyte. To enable absolute quantification, a recombinant ^{15}N uniformly labeled Aβ peptide was used as IS and was spiked in to samples prior to sample preparation. Calibrators were prepared in human CSF using the reverse curve method [236], where the labeled peptide was used to construct the calibration curve and the endogenous Aβ was used as IS.

One of the parameter that had the highest impact on sensitivity and stability during method development was the composition of the mobile phases. Mobile phases using additives that were either acidic, *e.g.,* formic acid or trifluoroacetic acid (TFA), or basic (NH_4OH) were evaluated. Using mobile phases with high pH increased the signal-to-noise ratio, but most importantly dramatically increased the stability of $A\beta_{1-42}$ in solution [184, 245, 246].

Using mixed-mode SPE with both reversed-phase and strong cation-exchange properties in the 96-well format increased the sample preparation throughput without compromising sensitivity or analyte stability, since elution could be performed in high pH.

Another key aspect during method optimization was the selection of LC separation column. Several types of reversed-phase columns stable at high pH were evaluated, including particle-based and monolith separation columns. While both silica-based C_{18} columns and monolithic column (based on a polystyrene divinylbenzene copolymer bed) performed similarly regarding peak shape and sensitivity, a gradual increase in back pressure was

observed after only 20 injections when using a particle based column. This was most likely caused by irreversible binding of various sample impurities. The monolithic column was chosen for this method since the gradual increase in back pressure was significantly lower, most likely due to its weaker hydrophobic retention characteristics, while peak shape and sensitivity were equally good for both types of column.

A dramatic increase in sensitivity was achieved when operating the instrument in the negative ion mode, but the y and b product ions generated in the positive ion mode are much more specific compared to the water loss product ions generated in the negative mode [233].

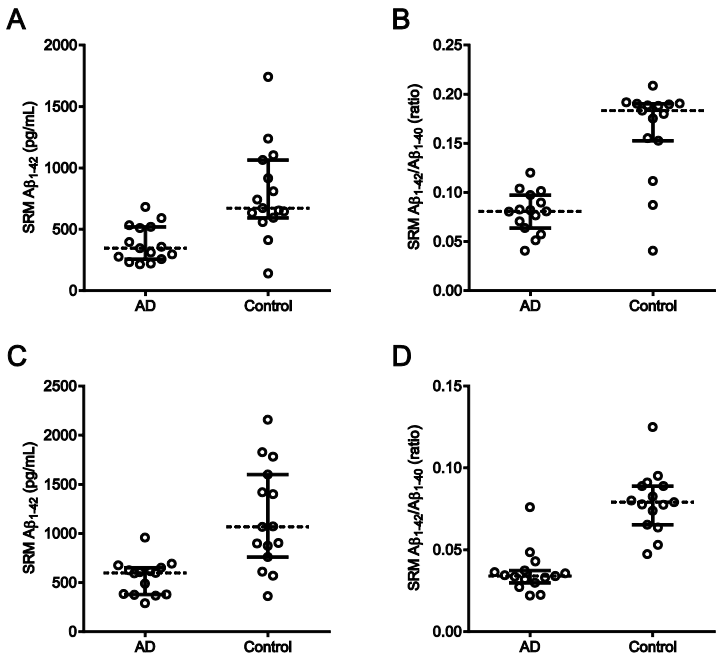

Figure 6. Separation of AD and control groups in two sample sets using $A\beta_{1-42}$ (A & C) and the $A\beta_{1-42}/A\beta_{1-40}$ ratio (B & D). Error bars are interquartile ranges, dashed line are medians.

CSF samples from one set with 15 AD patients (mean age ± SD: 75.6 ± 6.6 years) and 15 healthy controls (mean age ± SD: 66.3 ± 9.8 years), and a second set with 15 AD patients (mean age ± SD: 81 ± 5.0 years) and 15 healthy controls (mean age ± SD: 63.9 ± 9.4 years) were analyzed in this study. The MS method showed similar sensitivity and specificity compared

to ELISA measurements when separating AD patients from healthy controls (Figure 6), which indicates that SRM-based MS can be used in the clinic for $A\beta_{1-42}$ measurement in human CSF. Utilizing the multiplexed abilities of MS, the two shorter Aβ peptides $A\beta_{1-38}$ and $A\beta_{1-40}$ were also quantified. By using the $A\beta_{1-42}/A\beta_{1-40}$ ratio, the differentiation of AD from controls was better than by using $A\beta_{1-42}$ alone.

4.2 Paper II

The aim of this paper was to publish a candidate RMP by improving the method published in Paper I and performing a full method validation according to international guidelines.

The reverse curve method used in Paper I relies on endogenous $A\beta_{1-42}$ as IS in the calibrators, which has to be determined using single-point calibration. For this paper, the surrogate analyte approach was implemented [237-239], where one stable isotope-labeled (^{15}N) $A\beta_{1-42}$ was used as a surrogate for the native $A\beta_{1-42}$ to construct the calibration curve, and using another isotope-labeled (^{13}C) $A\beta_{1-42}$ as IS in both calibrators and unknown samples. By using the area ratio of endogenous to IS, the concentrations of $A\beta_{1-42}$ in unknown samples were determined from the calibration curve constructed using the area ratio of ^{15}N-$A\beta_{1-42}$ to IS.

Table 2. Imprecision data of endogenous $A\beta_{1-42}$ of 6 pools (on 6 days) with n = 12 for each pool.

	Endogenous $A\beta_{1-42}$ concentration in pool used for calibrators, pg/mL					
Replicate	1	2	3	4	5	6
1	390	410	653	780	705	702
2	410	400	708	666	708	708
3	399	413	686	824	704	737
4	378	411	653	793	703	744
5	382	408	681	748	731	673
6	407	399	692	750	700	747
7	387	421	660	771	670	738
8	371	403	691	786	648	728
9	405	366	664	749	686	728
10	336	412	737	765	673	656
11	388	379	724	777	708	710
12	360	380	707	760	677	744
Mean	384	400	688	764	693	718
CV%	5.5	4.1	4.0	4.9	3.3	4.1

The method was transferred to a quadrupole-orbitrap hybrid instrument, which has significantly higher resolution and mass accuracy compare to the triple quadrupole instrument. Another benefit with the quadrupole-orbitrap hybrid instrument is that it analyzes all fragments generated in the collision cell, thus fragments used for quantification can be selected post acquisition. For this method, 15 fragments were used for quantification. Runtime for each sample was reduced from 20 to 15 minutes by optimizing the chromatographic method, and the quantification range was expanded to 150-4000 pg/mL. Validation results showed good analyte recoveries, sample stability, method linearity and precision (Table 2), as well as absence of ion suppression and interferences.

4.3 Paper III

While $A\beta_{1-42}$ levels in human plasma has shown contradictory results as a diagnostic marker of AD [146], plasma is an attractive alternative for pharmacodynamic studies [149, 247-249] which prompted us to modify the method published in Paper I to be able to quantify Aβ peptides in human plasma. Compared to CSF, the total protein concentration in plasma is orders of magnitude higher, while the concentrations of Aβ peptides are approximately 10-20 times lower [145, 250-254]. To overcome these analytically challenging circumstances, IP was used instead of SPE. Absolute quantification of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ was performed using an optimized version of the LC-MS/MS method described in Paper I. To study the patterns of additional Aβ peptides, MALDI-TOF was used as described elsewhere [255] and high resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI–FTICR) MS/MS was used to verify the identities of the Aβ peptides detected with MALDI-TOF [256].

The clinical study included 9 AD patients (5 female, mean age ± SD: 81 ± 6 years) and 10 healthy controls (5 female, mean age ± SD: 77 ± 6 years). The absolute concentrations of $A\beta_{1-38}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ showed no significant differences between AD and control subjects. Similarly, the Aβ peptide patterns acquired using MALDI-TOF showed no difference in relative quantities (Figure 7). All Aβ peptides could be detected in AD and control plasma, signifying that the Aβ peptide pattern is a consistent finding.

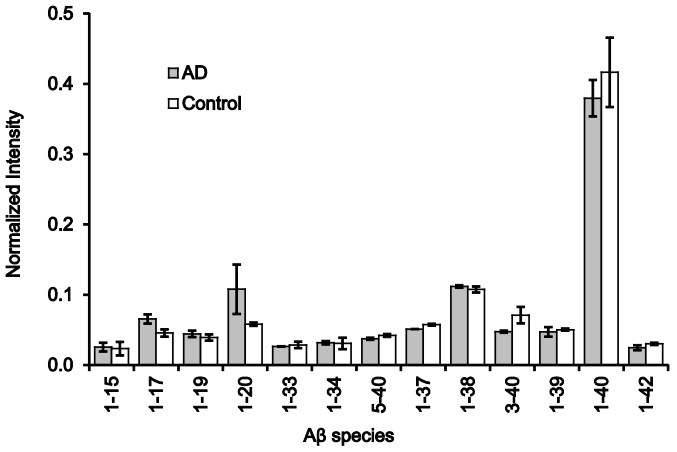

Figure 7. Relative abundance of Aβ peptides detected with MALDI-TOF in AD and control samples (n=2 in each group). Data are means, error bars are SD.

By using this approach with different MS techniques, we show that more than 15 N- and C-terminally truncated Aβ species can be reproducibly detected within a single analysis. Clinical trials have shown that $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ concentrations in plasma can increase several orders of magnitude in response to treatment [149], making the described method ideal for studying the whole spectrum of Aβ species present in plasma in response to treatment.

4.4 Paper IV

For this paper, an inter-laboratory study was performed with laboratories involved in the Global Biomarker Standardization Consortium (GBSC) of the Alzheimer's Association [257]. While all four involved laboratories used similar sample preparation procedures, different calibration procedures and LC-MS/MS methods were used. We prepared calibrators in human CSF, the other three labs used different compositions of artificial CSF. Twelve pools of human CSF were distributed to the laboratories to test the inter-laboratory measurement variation for the different mass spectrometric methods.

The main findings in this study were that despite the different methods and instrumentations used, a good agreement was seen between the laboratories, with an average inter-laboratory CV of 12.2% (Figure 8). The main difference was observed in the slopes of the linear regressions between the concentrations determined at each laboratory compared to the average concentration calculated from all laboratories. This could be explained by the

use of different batches of isotope labeled standards, and how the concentrations were set for these standards at each site.

To evaluate the potential benefit of a CRM for CSF $A\beta_{1-42,}$ one of the samples (sample 11, having an $A\beta_{1-42}$ concentration above the average of the sample set [258]) was designated as a reference and used to calculate a response factor specific for each laboratory. This factor was then used to adjust the results of the other samples, which decreased the average inter-laboratory CV to 8.3% (Figure 9).

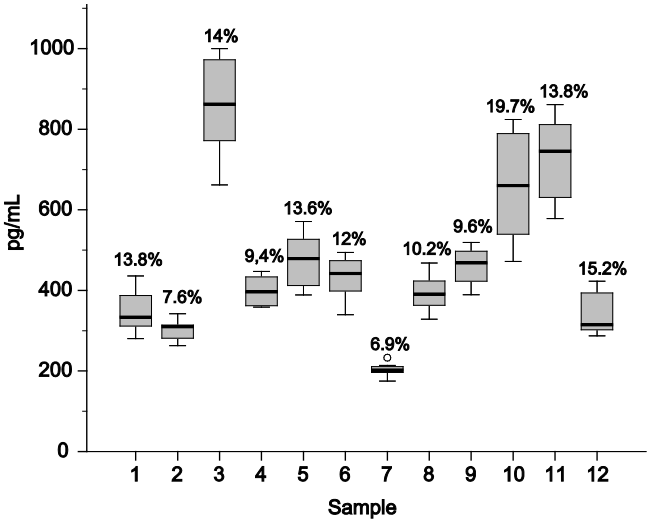

Figure 8. Inter-laboratory CV (%) for twelve CSF samples.

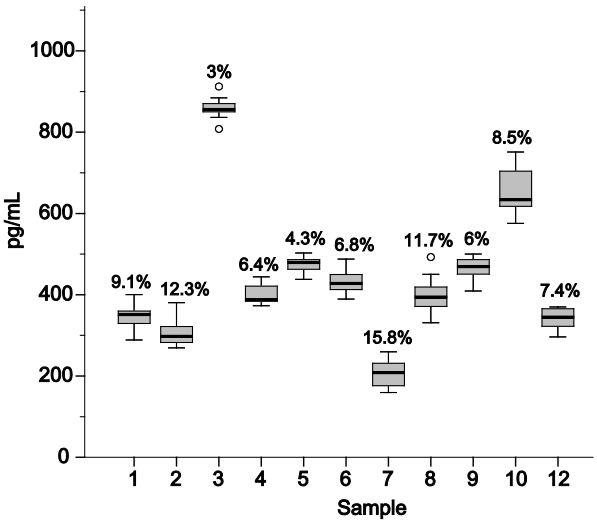

Figure 9. Inter-laboratory CV (%) for eleven CSF samples after adjusting the results using the calculated response factor obtained from the reference sample (sample 11).

5 CONCLUSION

Quantification using immunoassays is dependent on the interaction between the antibody and the antigen. This interaction could be affected by the presence of sample components that may interfere or compete with the interaction. In addition, the interaction may also be affected by the conformation of the antigen. These effects are difficult to control and are probably the main reason why it has been difficult to harmonize results between laboratories and between immunoassay platforms. Because MS quantification is based on directly counting the target molecules, usually relative to a stable-isotope labeled standard, quantification is absolute and generally unaffected by such matrix effects. In addition, diagnostic protein measurements by immunoassays should be supported by an unbroken chain of higher-order of measurement procedures and material, from validated LC-MS/MS and stable isotope-labeled internal standards to RMPs and a CRM, thus improving results comparability and reliability [259-262]. The results presented in this work indicate that LC-MS/MS is suitable for absolute quantification of $A\beta_{1-42}$ in CSF. The method will help set the value of CSF $A\beta_{1-42}$ in a CRM, which could be used to harmonize assays and facilitate the introduction of general cutoff levels in clinical trials and practice. Additionally, this method can be used to quantify different Aβ species in human plasma, and could be useful to detect pharmacodynamics effects in clinical trials of drugs affecting APP processing or Aβ homeostasis.

6 FUTURE PERSPECTIVES

While recent developments in instrumentation have enabled the use of MS for absolute quantification of proteins and peptides in clinical samples, sample throughput of immunoassays is still unmatched, and thus it will be the platform of choice for large sample numbers in clinical laboratories for the near future. Instead, it is the multiplexing ability with respect to analytes and the high specificity, without the need for antibodies, which will make MS the method of choice in clinical research, and may gradually establish MS in many routine laboratories for protein quantification. As new candidate biomarkers for AD are discovered, these can be added to a panel, which ultimately will cover many aspects of the AD pathology including Aβ, tau and markers for inflammation and synaptic loss without the need to develop specific detection antibodies.

The standardization efforts by the Alzheimer's Association Quality Control program for CSF biomarkers and recent developments of automated immunoassays together with the production of a CRM with exact levels of $A\beta_{1-42}$ determined using a LC-MS/MS RMP, will enable the introduction of global cutoff levels, and a more general use of $A\beta_{1-42}$ in CSF as a diagnostic tool. In addition, in order to monitor the biochemical effects in clinical trials for new disease-modifying drugs, an exact cutoff level for CSF $A\beta_{1-42}$, or the $A\beta_{1-42}/A\beta_{1-40}$ ratio, is needed for inclusion of "true" AD dementia cases and also AD patients in the predementia stage of the disease. In conclusion, these biomarkers will be important to provide correct treatment while neurodegeneration is still limited once these new drugs are available.

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