ANALYTICAL IMPROVEMENTS IN THE DETECTION OF AMYLOID-β PEPTIDES IN CEREBROSPINAL FLUID BY MASS SPECTROMETRY

by

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Abstract

Current diagnosis of Alzheimer's disease (AD) is based on physical and neurological exams, mental state exams, and brain scans. Cerebrospinal fluid (CSF) biomarkers such as amyloid-β (Aβ) peptides have been incorporated into the research diagnostic criteria for AD and clinical care. Aβ peptides are produced from the amyloid precursor protein (APP) by proteolytic cleavage. Aβ40 and Aβ42, along with the protein tau, are reliable biochemical indicators of AD. Additionally autosomal dominant AD is caused from highly penetrant autosomal dominantly inherited variants in the *APP*, presenilin 1 and 2 genes.

Biomarker detection and quantification is commonly performed by immunoassays, which provide an indirect measurement of the analyte of interest. In the case of Aβ immunoassays, high between-lab variability and lack of harmonization between platforms has been a concern. Moreover, immunoassays are known, in general, to be prone to analytical interferences – both positive and negative – from endogenous and exogenous molecules.

To overcome issues associated with antibody-based detection methods and the desire to implement AD biomarker testing in routine care, a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay was previously developed by the laboratory for detecting evidence of AD pathology by quantifying wild-type Aβ40 and Aβ42 in CSF.

The aims of this thesis with respect to the LC-MS/MS method were to (1) evaluate the surrogate matrix composition, investigate potential interferences, and identify criteria for sample acceptance/rejection in a clinical environment, and (2) to adapt the method from detecting wild-type Aβ peptides to also detecting Aβ variants. The surrogate matrix was established with protein content compatible with that observed in human CSF. The interference studies indicated all endogenous and exogenous factors tested met the pre-specified bias acceptance criteria for Aβ40 and Aβ42. Thus Aβ peptides can be accurately quantified in the presence of high but physiologically plausible total protein concentrations and anti-Aβ antibodies, enabling use in routine clinical care and clinical trials. Additionally, testing hemolysate contaminated samples indicated no interference from hemolysate in the range commonly seen by the clinical laboratory. The LC-MS/MS assay was adapted to quantify wild type-Aβ42, Aβ40 and to identify Aβ variants in CSF.

Lay Summary

Alzheimer's disease (AD) is characterized by the presence of protein clusters in the brain formed by a peptide called amyloid-β (Aβ). There is evidence that measuring the amount of Aβ peptides, more specifically Aβ42, in the fluid surrounding the brain, cerebrospinal fluid (CSF), can be indicative of cognitive decline due to AD. To measure Aβ, a technique called mass spectrometry can be used to detect and measure the amount of specific peptides present based on their mass. The objectives of this thesis were to evaluate factors such as blood, protein, and therapeutics compounds that can potentially cause interference and to adapt a previous developed method to measure both Aβ peptides and familial variants in CSF using a highly selective technique. The knowledge gained from this research aims to improve diagnostic testing for AD and related disorders.

Preface

The work presented herein was conducted as part of the research group of Dr. Mari DeMarco in the Department of Pathology and Laboratory Medicine at the University of British Columbia. In addition to the support of the research group, I would like to specifically acknowledge the contributions of Ms. Grace van der Gugten (Providence Health Care), Ms. Alice Fok, and Dr. Robin Hsiung to this work.

Chapter 2 is based on the work previously conducted by Ms. Grace van der Gugten and Dr. Mari DeMarco, which lays the groundwork for the Aims pursued in this work. A subset of the experiments in chapter 3 were also supported by the efforts of Ms. van der Gugten.

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List of Abbreviations

Alzheimer's disease (AD)

amyloid-β (Aβ)

amyloid-β 1-40 sequence (Aβ40)

amyloid-β 1-42 sequence (Aβ42)

amyloid precursor protein (*APP*)

cerebrospinal fluid (CSF)

presenilin-1 (*PSEN1*)

presenilin-2 (*PSEN2*)

beta-site amyloid precursor protein-cleaving enzyme 1 (BACE)

soluble ectodomain known (sAPPβ)

carboxy-terminal fragment (CTFβ)

APP intracellular domain (AICD)

blood brain barrier (BBB)

Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV)

National Institute of Neurologic, Communicative Disorders and Stroke-AD and Related Disorders Association (NINCDS-ADRDA)

National Institute of Aging and the Reagan Institute (NIA-RI)

Consortium to Establish a Registry for Alzheimer Disease (CERAD)

International Working Group (IWG)

positron emission tomography (PET)

total-tau (t-tau)

phosphorylated tau (p-tau)

National Institute on Aging and Alzheimer's Association (NIA-AA)

mild cognitive impairment (MCI)

magnetic resonance imaging (MRI)

^{11}C-Pittsburgh Compound-B (PIB)

enzyme-linked immunosorbent assay (ELISA)

liquid chromatography tandem mass spectrometry (LC-MS/MS)

mass spectrometry (MS)

solid phase extraction (SPE)

multiple reaction monitoring (MRM)

mass to charge ratio (m/z)

internal standard (IS)

wild-type (wt)

intravenous immunoglobulins (IVIG)

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My committee members, Honglin Luo, Philipp Lange, Haakon Nygaard, for their outstanding knowledge, enthusiasm and guidance during my master thesis.

Chapter 1: Introduction

1.1 Neuropathology

Alzheimer's disease (AD) affects 50 million worldwide and accounts for the majority of all cases of dementia [1]. In 1906, Dr. Alois Alzheimer, who the disease was named after, characterized the abnormal deposits found in brains of individuals with cognitive impairment [2]. These abnormal deposits were identified to be extracellular plaques and intracellular neurofibrillary tangles which are the neuropathological hallmarks of AD [3]. The accumulation of plaques and neurofibrillary tangles lead to synaptic loss, plasticity changes, neuronal loss and eventually neurodegeneration [4]. In 1984, Dr. George Glenner and Dr. Cai'ne Wong identified amyloid-β (Aβ) the primary component of the extracellular plaques [5]. Soon after in 1986, researchers discovered that the neurofibrillary tangles found in AD were made of tau, a type of protein that stabilizes microtubules and gets released upon neurodegeneration [6, 7]. Abnormal hyperphosphorylation of tau results in formation of the neurofibrillary tangles.

Aβ peptides are derived via proteolytic cleavage of the trans-membrane amyloid-precursor protein (APP) by endogenous proteases, resulting in peptides of various lengths, including residues 1-40 and 1-42, referred to herein as Aβ40 and Aβ42, respectively [8-10] (Figure 1). The plaques consist of fibrillar Aβ42 formed via aggregation peptide monomers into higher order aggregates [11]. There is still debate on which forms of Aβ are the most neurotoxic; however, soluble smaller-order aggregates of Aβ have shown to be correlated with synapse loss and neuronal injury [12, 13], while Aβ fibrils have been shown to induce neuronal loss [11].

Aβ being secreted into cerebrospinal fluid (CSF) was first reported in 1992, which made CSF an important candidate biomarker for AD [14]. Measurement Aβ40 and Aβ42 peptides along with tau protein and the phosphorylated form of tau have been proven to be reliable biochemical indicators of AD with a sensitivity and specificity of 85% and 95%, respectively [15].

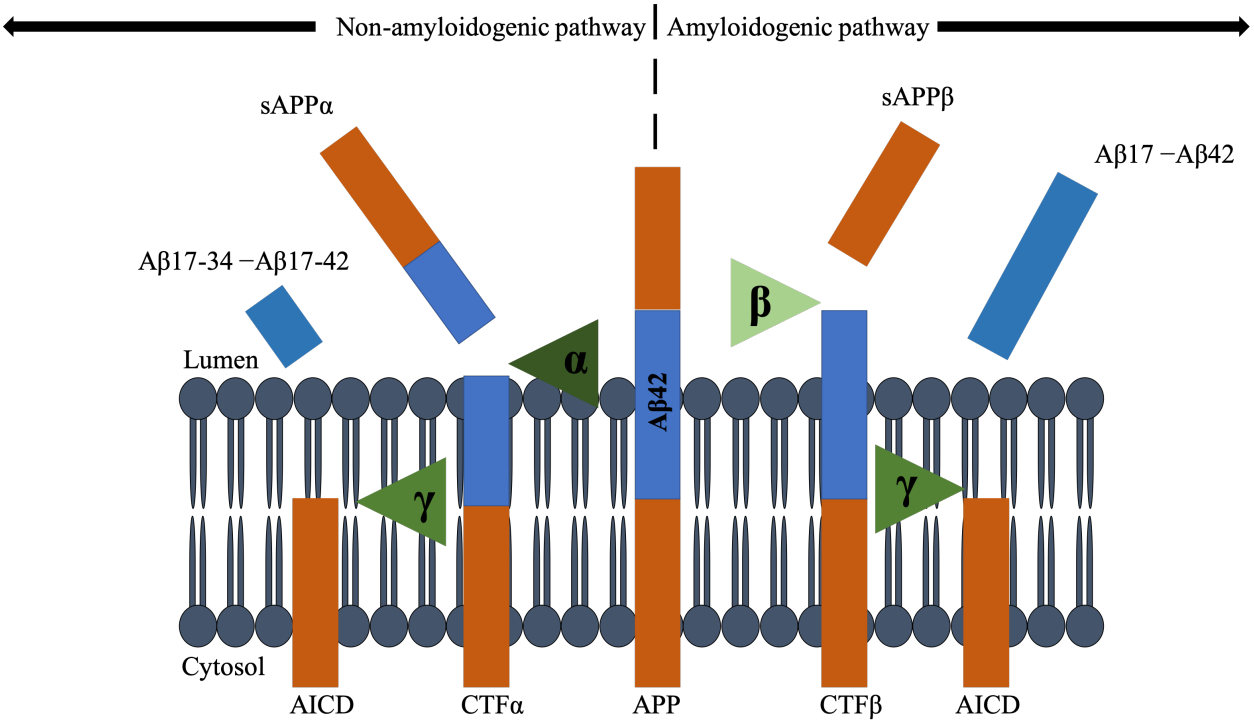


Figure 1: APP processing showing the non-amyloidogenic pathway on the left and the amyloidogenic pathway on the right.

1.2 Amyloid Precursor Protein Metabolism

APP can be processed and cleaved by several pathways producing Aβ peptides that vary in length. Following the amyloidogenic pathway (Figure 1), APP is cleaved by β-secretase (β-site amyloid precursor protein-cleaving enzyme 1, BACE), which results in the release of the large

soluble ectodomain known as sAPPβ. The remaining carboxy-terminal fragment (CTFβ) is then cleaved within the membrane-bound domain by γ-secretase, a complex consisting of at least four subunits: the enzymatic portion of the complex, presenilin 1 protein, presenilin 2 protein, presenilin enhancer 2; nicastrin; and anterior pharynx defective-1 [8, 10, 16-19], releasing the Aβ42 [20] and several carboxy-terminal truncated forms including Aβ40 and Aβ38 [9, 21] outside the cell and the *APP* intracellular domain into the cytoplasm. The function of APP and Aβ remains unclear; however, it is known that that the extracellular domain of the protease nexin form of APP is part of the clotting cascade [22], and it has been speculated to bind to other proteins on the surface of cells or help cells attach to one another [23]. Additionally, studies have suggested that APP helps to direct the migration of neurons during early development in the brain [23-26].

APP can also be cleaved following the non-amyloidogenic pathway, whereby cleavage is initiated by α-secretase, which cuts APP at the luminal domain releasing an extracellular sAPPα domain and leaving the carboxyl-terminal fragment, CTFα in the plasma membrane, which can be further cleaved by γ-secretase. The non-amyloidogenic processing results in the generation of N-terminally truncated Aβ peptides referred to as p3 peptides. The non-amyloidogenic processing pathway of APP, however, does not result in Aβ generation.

1.3 Amyloid Cascade Hypothesis

The central hypothesis for the development of AD is the amyloid cascade hypothesis, which states that an imbalance between the production and clearance of Aβ causes plaque accumulation in the brain and is the initiating event, ultimately leading to neuronal degeneration and dementia

[2, 27-29]. There have been several clearance mechanisms for Aβ that have been proposed such as enzymatic/proteolytic degradation, low-density lipoprotein receptor related clearance, clearance by cellular signaling pathways, and transport over the blood brain barrier (BBB) from CSF to blood. The proteolytic degradation of Aβ is a major route of clearance.

While the amyloid cascade hypothesis remains the central hypothesis in AD, there is continuing debate about the exact role of amyloid in disease as there are findings that undercut its importance in pathogenesis. For example, the amyloid plaque burden does not correlate well with the degree of cognitive impairment when compared to neurofibrillary tangle count suggesting tangles may precede amyloid plaques [30]. Along the same lines, abundant amyloid plaques have been noted in individuals with no cognitive impairment [30]. These findings and the high number of failed clinical drug trials involved in targeting Aβ emboldened critics of the amyloid cascade hypothesis; hindering its universal acceptance [31].

1.4 Types of AD

AD can be broadly divided into two types, sporadic or autosomal dominant. Sporadic AD accounts for the majority of the AD cases and typically first presents in those older than 65 years [2]. Autosomal dominant AD, accounts for less than 1% of all cases in those under 65 years of age. Autosomal dominant AD is caused by rare and highly penetrant dominantly inherited mutations referred to herein as autosomal dominant variants, in the *APP*, presenilin-1 (*PSEN1)* and presenilin-2 (*PSEN2)* genes [32-38]. Mutations in *PSEN1* account for 70% of autosomal dominant AD cases while mutations in *PSEN2* and *APP* are very rare among autosomal dominant AD cases [32, 33, 35, 39]. Mutations in *APP*, *PSEN1* and *PSEN2* genes have all been

shown to affect APP processing by altering the amounts and/or length of the Aβ peptide produced [35, 40-42]. Diagnosis of autosomal dominant AD is confirmed by the identification of an autosomal dominant variant. Variants in APP, more specifically in the Aβ42 sequence, were investigated as part of this thesis (Figure 2).

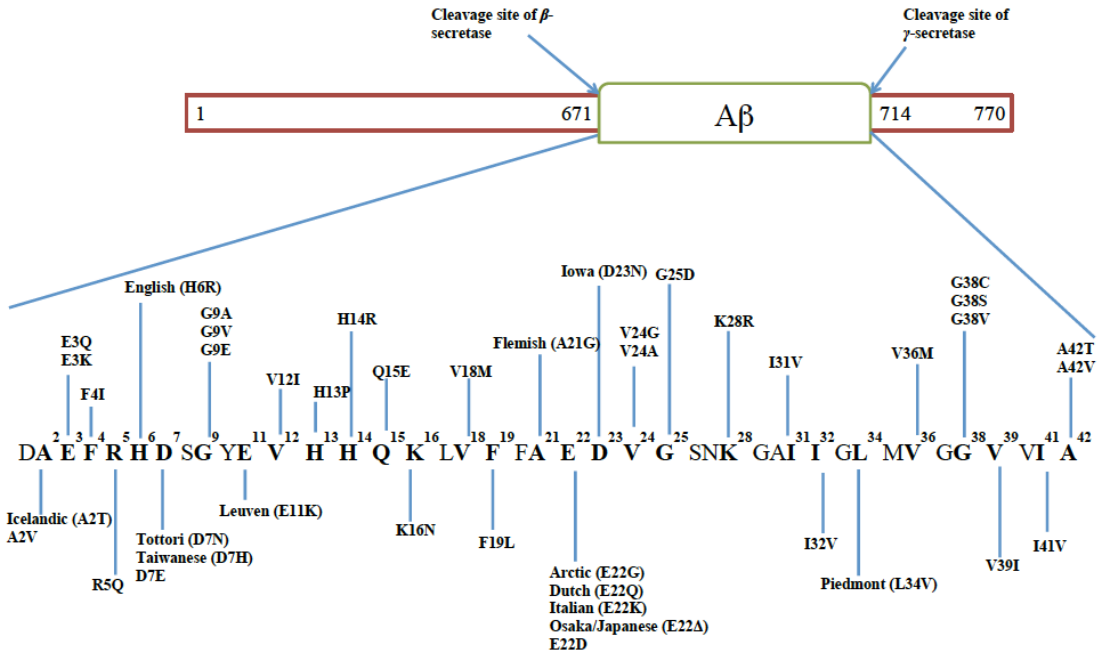


Figure 2: APP annotated with sequence variants associated with autosomal dominant AD occurring within the Aβ42 sequence.

1.5 Diagnosis

1.5.1 Post-mortem Diagnosis

To make a definitive AD diagnosis, the gold standard is identification of amyloid plaques and neurofibrillary tangles on post-mortem neuropathological examination. The recommendations of the National Institute of Aging and the Reagan Institute (NIA-RI) [43] for the neuropathological assessment for AD is to combine the Consortium to Establish a Registry for Alzheimer Disease

(CERAD) score of neuritic plaques [44] with the topographic staging of neurofibrillary tangles [45]. These criteria are divided into 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 individual had a clinical history of dementia [43].

1.5.2 Antemortem Diagnosis

Antemortem diagnosis of AD is based on medical and family history, physical exam, neurological exam, mental status tests, diagnostic tests, and brain imaging [46]. The most widely used sets of criteria for diagnosis of AD are the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V) [47] and National Institute of Neurologic, Communicative Disorders and Stroke-AD and Related Disorders Association (NINCDS-ADRDA) [48, 49]. The NINCDS-ADRDA and DSM-IV criteria both have a sensitivity of 81% and specificity of 70% for clinical diagnosis of AD [46].

In support of improving diagnostic accuracy, CSF biomarkers have been incorporated into the diagnostic criteria of AD for research in the International Working Group guidelines, National Institute of Aging, and Alzheimer's Association (NIA-AA) Research Framework and in the Canadian Consensus Conference on Diagnosis and Treatment of Dementia [43, 50, 51], which are discussed further in section 1.5.4.

1.5.3 Imaging Biomarkers

PET imaging, using tracers targeting the pathological protein aggregates, is one biomarker approach to detecting AD pathology [52]. The PET imaging of amyloid related

neuropathological changes associated with AD is performed using tracers such as ^{11}C-Pittsburgh Compound-B (PIB) or ^{18}F-labeled tracers such as flutemetamol [53-56]. These tracers enable visualization of the location of the deposits within the brain and can provide quantitative information on amyloid deposits [53, 57]. PET tracers for tau have only been recently tested in human trials, which include phenyl/pyridinyl-butadienyl benzothiazoles/benzothiazoliums [58] and 18F-T808 [59]. Limitation of the use of PET imaging in routine care is the short half-life of these tracers, which presents challenges in availability, require an on-site cyclotron, radiochemistry expertise, and is an expensive test [52, 60].

1.5.4 CSF Biomarkers

In addition to imaging biomarker, biofluid marker can also be used to assess the presence of AD neuropathology via quantitation of Aβ peptides and tau in CSF [52]. More specifically, the concentration of Aβ42 in CSF has been proven to be a reliable biomarker for AD with the concentration of Aβ42 being ~50% lower in individuals with AD compared to healthy controls [61]. Notably, changes in the concentration of Aβ42 in CSF precede changes observed via PET imaging as observed in multiple studies involving individuals with AD [55, 56, 62, 63].

The NIA-AA Research Framework defines AD by its underlying pathologic processes that can be documented by post-mortem examination or *in vivo* using biomarkers [43]. The NIA-AA Research Framework proposed the AT(N) biomarker classification system for the symptomatic or "clinical" stages of AD, that is, mild cognitive impairment (MCI) and dementia [43]. The "A" in the biomarker classification system represents CSF Aβ peptides and cortical amyloid PET ligand binding. The "T" biomarkers represent the neurofibrillary tangles and are measured via

CSF p-tau or cortical tau PET ligand binding. Biomarkers of neurodegeneration or neuronal injury (labeled "N") are measured using CSF t-tau, FDG PET hypometabolism or observation of atrophy on magnetic resonance imaging (MRI) [43]. Structural and functional MRI is used to support clinical AD diagnosis. Structural MRI visualized the cerebral atrophy characteristics for neurodegeneration [64-67]. Functional MRI measures activity within the brain by using blood-oxygen-level dependent contrast imaging [68, 69] to study functional connectivity and detect early brain dysfunction related to AD. In this classification system, the three biomarker groups are profiled as positive (+, abnormal) or negative (–, normal) and are then grouped accordingly into three possible biomarker categories (Table 1).

Table 1: AD diagnostic classification system using biomarker profiles (adapted from C.R Jack Jr. *et al*. 2018).

A	**T**	**(N)**	**Interpretation**	
–	–	–	Normal Alzheimer's disease biomarkers	
+	–	–	Alzheimer's pathologic change	Alzheimer's continuum
+	+	–	Alzheimer's disease	
+	+	+	Alzheimer's disease	
+	–	+	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change	
–	+	–	Non-Alzheimer's disease pathologic change	
–	–	+	Non-Alzheimer's disease pathologic change	
–	+	+	Non-Alzheimer's disease pathologic change	

1.6 Analytical Approaches for Quantification of Aβ

1.6.1 Ligand Binding Methods

Many different approaches have been used for quantification of Aβ peptides including various ligand binding methods: enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, biochip immunoassays, electrochemiluminescence, immunoelectrophoresis, and nephelometry [15, 70-74]. Immunoassays, in general, rely on the interaction between antibodies and the analyte of interest for quantification, providing an indirect measurement of antigen quantity. Unfortunately, accuracy and precision can be affected by several factors including hemolysate, proteins, and therapeutic products [75-78]. Antibodies are also known to cross-react with substances other than the target analyte, potentially resulting in positive or negative interference [79, 80]. Additionally, immunoassays also cannot distinguish among modified and processed forms of the analyte of interest such as between the Aβ autosomal dominant variants.

1.6.2 Mass Spectrometry

Mass spectrometry (MS) is a technique that can address the many of the limitations of immunoassays and therefore has been increasingly used in clinical laboratories for absolute quantitation of peptides and proteins [81]. MS is a widely used analytical technique and is capable of providing information about the qualitative and quantitative composition of complex mixtures [82]. A central advantage of liquid chromatography tandem mass spectrometry (LC-MS/MS) is the exquisite analyte selectivity derived from the use of multiple reaction monitoring (MRM). LC is used to separate analytes in time, and MS/MS enables further selectivity by the analyte's mass-to-charge (m/z) ratio and subsequent confirmation of the analyte's primary structure (i.e. sequence) by fragmentation. The use of MRM is akin to performing a sequencing

experiment for each analyte detected where the peptide (i.e. precursor ion) is reproducibly fragmented into a series of overlapping peptide sequences, and the fragment m/z ratios are then detected to confirm the identity of the target analyte. This level of selectivity, far exceeding that of immunoassays, is the reason the laboratory medicine community has turned to MS for the development of reference methods (i.e. gold standard methods) and for quantification of clinically relevant peptide and proteins [70-74, 83], including a candidate reference method for Aβ [84]. MS being able to differentiate between Aβ isoforms can thus provide accurate quantification of total Aβ. Additionally, an MRM method allows for screening of multiple analytes in a single analysis, increasing the multiplexing capacity of the method when compared to immunoassays.

Quantification is achieved by mixing a known amount of isotope-labelled (e.g., ^{15}N or ^{13}C labelled) internal standard (IS) peptide with the sample. The ideal IS is one that is chemically equivalent to the endogenous peptide – such as an isotopically labelled version of the measured analyte – where it behaves in a similar fashion throughout the entire analytical process: sample preparation, chromatography, ionization, fragmentation and detection. The resulting LC-MS/MS peak area ratio of the endogenous peptide measured to that of its IS is then used for comparison to an external calibration curve for quantification.

1.7 Disease Modifying Therapeutics

There are several symptomatic therapies for AD; however, no disease-modifying therapies are currently available. A major focus of development of therapeutics for AD is on therapeutic monoclonal antibodies targeting Aβ and/or Aβ aggregates. In active immunotherapy, the

individual's immune system is activated to remove Aβ peptides to reduce the formation of amyloid plaques. Active immunotherapy (or Aβ vaccination) involves activation of the individual's immune system to stimulate the production of endogenous anti-Aβ antibodies [85]. These antibodies bind to the Aβ peptides and are cleared out the brain. Passive immunotherapy administrates exogenous monoclonal antibodies by infusion of humanized or human anti-Aβ antibodies produced in the laboratory to clear Aβ peptides and plaques [85]. An advantage of passive immunotherapy is being able to control adverse events by manually stopping treatments. Current passive immunotherapeutic approaches for AD require improvement with efficiency of the antibody-Aβ complex crossing the BBB (only approximately 0.1% cross the BBB) as well as the cross reactivity and the inflammatory alterations [86].

Despite the many promising therapeutic approaches, the clinical trials have failed to show positive effects on primary clinical outcome [85, 87]. This can be due to the drug treatments not being commenced early enough in the disease, before neurodegeneration is too severe and widespread [85]. In 2010, Clifford Jack proposed a hypothetical model of biomarkers of the AD pathological cascade [88]. The model relates disease stages to AD biomarkers where Aβ biomarkers become abnormal first, then neurodegeneration biomarkers and cognitive symptoms, lastly neurodegenerative biomarkers, which correlate with clinical symptom severity. This model suggests that Aβ biomarkers can be used to identify individuals in the pre-symptomatic phase of AD for clinical trial enrolment. If treatment can be initiated at an earlier stage of the disease, before neurodegeneration is too severe, it may give promising drug candidates a chance of showing a disease-modifying effect. Additionally the diagnostic procedure in enrolling individuals for clinical trials needs refinement so that only individuals designated as having AD

in the trial have objective evidence of AD pathology [85]. Thus, research on the development of more specific and accurate diagnostic tools, specifically Aβ CSF diagnostic test, are important for patient enrichment at earlier stages of the disease.

1.8 Aims and Hypotheses

The aims of this thesis were to improve the diagnostic power of CSF biomarker testing for AD by optimizing/modifying the laboratory's existing quantitative wt-Aβ LC-MS/MS method. Hypotheses tested pertaining to the LC-MS/MS Aβ method were as follows:

1. By assessing analytical performance characteristics of the LC-MS/MS method in the presence of potential interferents, criteria for sample acceptance/rejection can be developed for use in routine care.

2. If modifications are made to the quantitative wt-Aβ LC-MS/MS method, it can be adapted to also detect known Aβ variants as a diagnostic tool for autosomal dominant AD.

Chapter 2: Pre-Analytical and Analytical Considerations for Quantification of Aß40 and Aß42 by Mass Spectrometry

2.1 Introduction

An automated Aβ LC-MS/MS method to quantify and detect wt-Aβ peptides was previously developed (Figure 3) [89]. The LC-MS/MS workflow is summarized as follows: 200 µL of CSF aliquots were treated with guanidine HCl and the ^{15}N-Aβ40 and ^{15}N-Aβ42 IS added, followed by SPE by mixed-mode cation exchange, and followed by LC-MS/MS analysis on a Shimadzu high performance LC and SCIEX 5500 triple quadrupole mass spectrometer. This multiplex Aβ LC-MS/MS assay was validated in a clinically accredited laboratory following Clinical and Laboratory Standards Institute guidelines including C62, EP-5A, and EP-6A [90-92].

The first aim of this thesis was to optimizing the assay by evaluating the pre-analytical and analytical factors that could effect accurate and precise quantification of the analytes of interest [75, 93]. Endogenous and exogenous factors looked at included hemolysate (to model a traumatic lumbar puncture), total protein, and immunoglobulins. With clinical implementation in mind, the outcomes of the work would be used to develop criteria for sample acceptance/rejection.

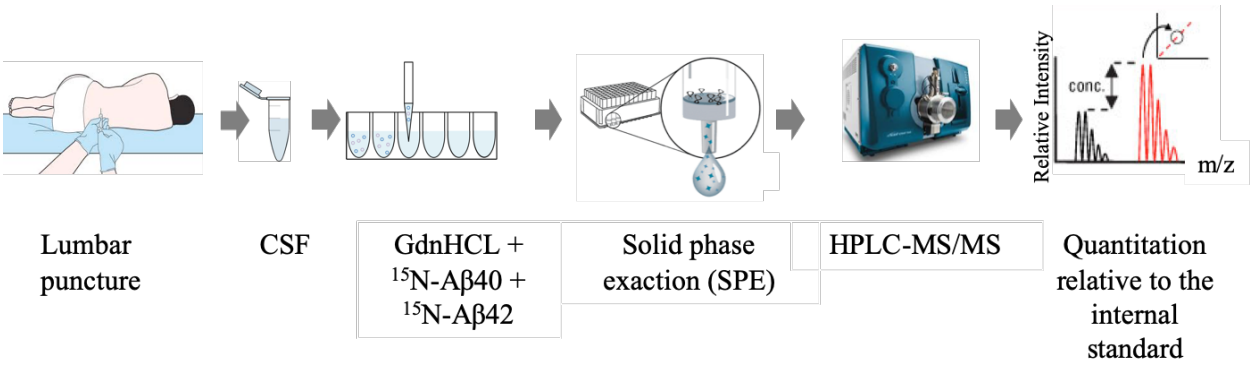


Figure 3: Automated multiplex Aβ LC-MS/MS assay workflow.

2.2 Methods

Spike and recovery experiments were performed to determine interference. Effect on Aβ quantitation was assessed as both the raw signal (peak area) and signal normalized by the internal standard (analyte concentration). Matrix interference is evaluated in the context of bias, which is the difference between the expected value and the measured value. In the interference studies performed, the expected value is the known concentration of Aβ peptides spiked into a sample. The pre-specified acceptance criteria for both Aβ40 and Aβ42 was a bias within ± 20 % of the expected concentration.

2.2.1 Samples

For the spike and recovery experiments performed, pools of human CSF collected from patients at the St. Paul's Hospital in Vancouver, Canada, following an established protocol (REB# H18-03050) were used. All samples were analyzed using the multiplex Aβ LC-MS/MS assay (Figure 3).

2.2.2 Surrogate Matrix

A spike and recovery experiment with bovine serum albumin (BSA) was used to determine the optimal range of total protein for the surrogate matrix used to make up the calibrators. Samples with concentrations of ranging from 0 to 20 g/L of BSA were prepared in phosphate buffered saline (PBS) and each sample was spiked with a final concentration of the following synthetic peptides: 15 ng/mL for Aβ40 and 1000 pg/mL for Aβ42. Samples were analyzed in triplicate using the LC-MS/MS method to identify the optimal range for Aβ recovery.

2.2.2.1 Statistical Analysis

The Mann-Whitney test was used to compare peak area signal between different BSA concentrations. Statistical differences were noted if the calculated $P < 0.05$.

2.2.3 Total Protein Matrix

A 'high' total protein CSF pool was created by pooling individual samples with high total protein concentration and spiking in plasma to create a pool with a concentration of ~10 g/L total protein. A 'low' total protein CSF pool was made by pooling individual samples with low total protein concentration. Using the 'high' and 'low' CSF pools, various mixed solutions were prepared with concentrations ranging from undetectable (i.e., less than the lower limit of the analytical measuring range of 0.01 g/L) to 10.0 g/L of total protein. Each sample was then spiked with synthetic Aβ peptides to a final concentration of 20 ng/mL Aβ40 and 3 ng/mL Aβ42. Three technical replicates were run for each sample and analyzed by LC-MS/MS.

2.2.4 Hemolysis Interference

To test the visual cut off, a spike and recovery experiment was then performed using four human CSF samples with various concentrations of hemolysate contamination determined visually from its color and appearance. Each sample was then spiked with synthetic Aβ peptides to a final concentration of 20 ng/mL Aβ40 and 3 ng/mL Aβ42. Three technical replicates were run for each sample and analyzed by LC-MS/MS. Percent bias was calculated by setting the sample with no hemolysate contamination by visual inspection as 100% recovery.

2.2.5 Anti-Amyloid Therapeutics

To mimic an anti-Aβ monoclonal antibody therapeutic, an anti-Aβ antibody (i.e., clone 4g8 an anti-Aβ (17-24) mouse IgG monoclonal antibody) that binds to the same epitope as a known candidate therapeutic antibody (i.e., crenuzumab) was spiked into a CSF pool at concentrations ranging from 0.05 – 0.6 ng/mL [94]. As a control, intravenous immunoglobulin (IVIG) was also spiked into a CSF pool ranging from at 0.05 ng/mL to 0.6 ng/mL. Aβ42 and Aβ40 were spiked into the samples at 3 ng/mL and 20 ng/mL respectively. Samples were analyzed in triplicate by LC-MS/MS. Percent bias was calculated by setting the control sample, which was CSF pool with no immunoglobulins spiked in as 100% recovery.

2.3 Results

2.3.1 Surrogate Matrix

Testing various concentrations of BSA indicated the optimal Aβ recovery is between 0.5 – 2 g/L BSA. There was also no significant difference in Aβ recovery between 0.5 – 2 g/L BSA.

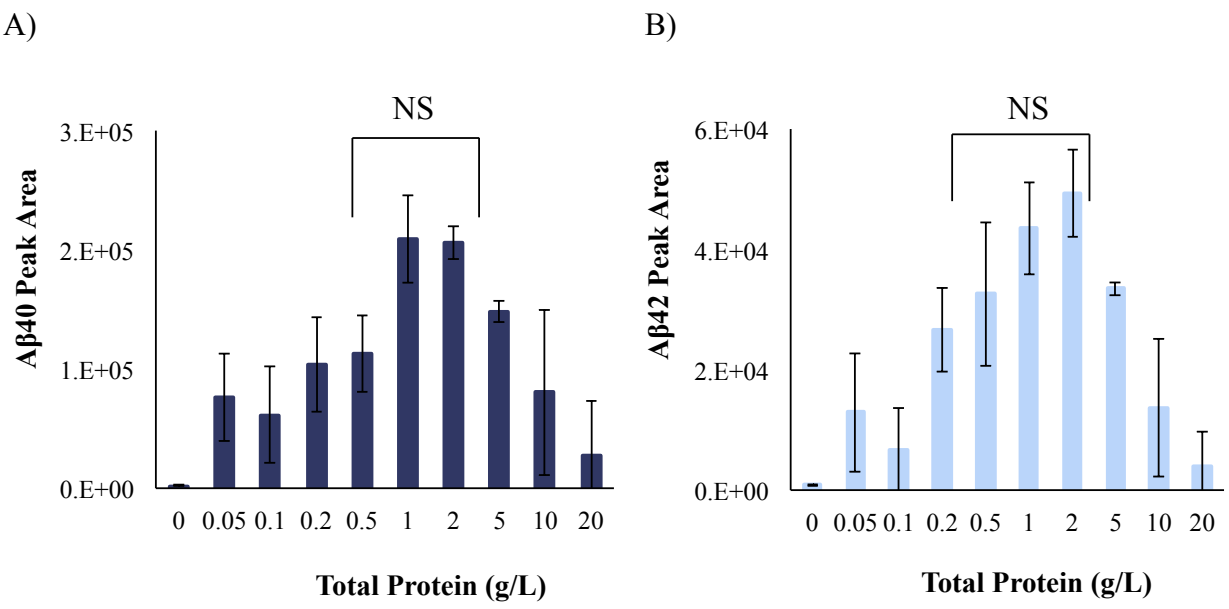


Figure 4: The optimal concentration for the surrogate matrix is between 0.5 – 2 g/L BSA. Average peak area for A) Aβ40 and B) Aβ42 over total protein concentration (g/L), represented as the mean of three technical replicates and the standard deviation. NS: Not significant.

2.3.2 Total Protein Matrix

Peak area was inversely proportional to the total protein concentration over the range of 0.2 – 12 g/L; however, there was no effect on quantitation of Aβ (Figure 5) as recovery of Aβ for all samples was within ± 20% of the expected concentration (Table 2).

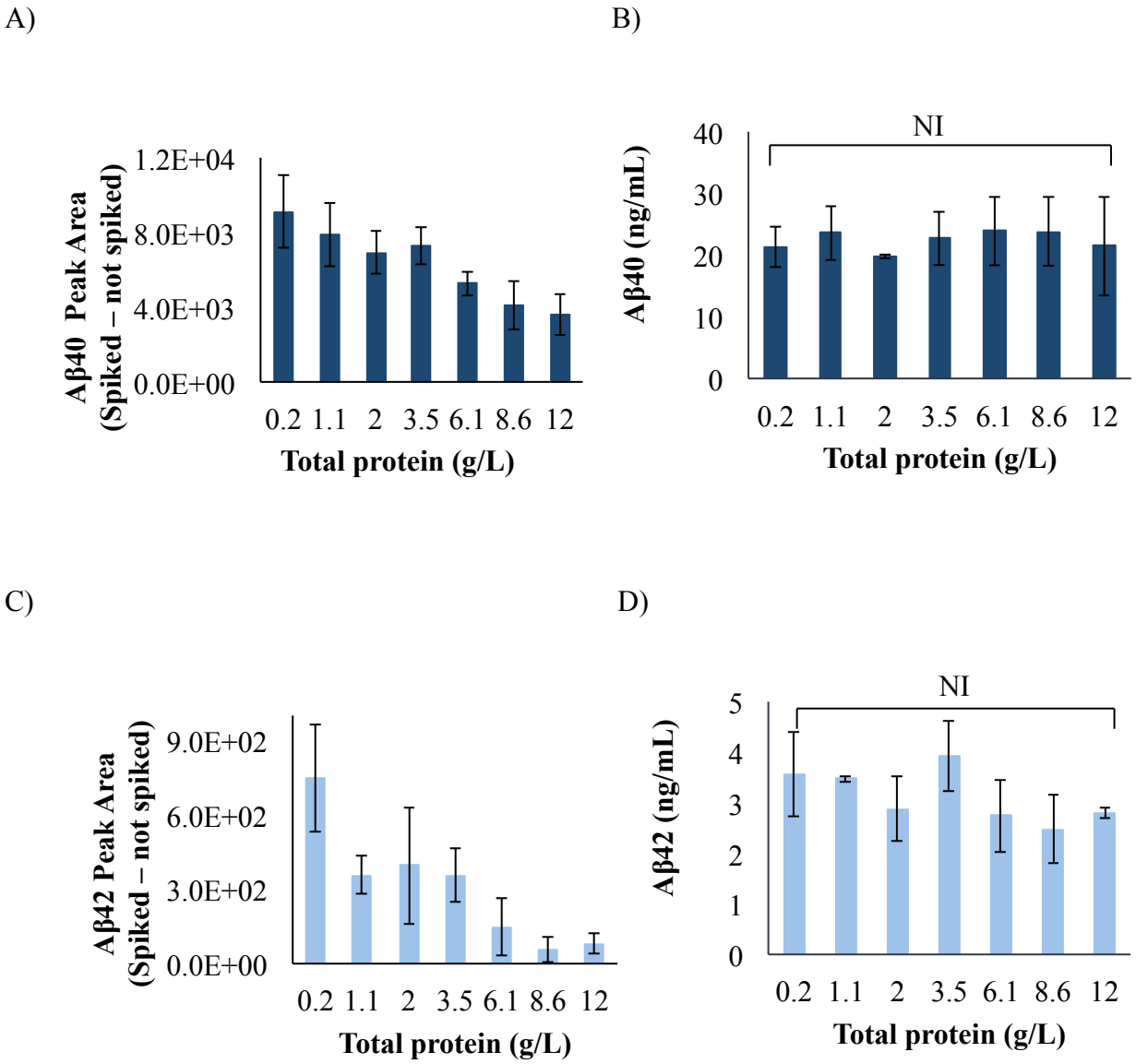


Figure 5: Total protein concentration affects raw peak area signal but not quantification of Aβ peptides. Average peak area and average Aβ concentration versus total protein concentration (g/L), represented as the mean of three technical replicates and the standard deviation. A and B) Aβ40 and C and D) Aβ42. NI: No interference.

Table 2: Calculated % bias for total protein interference study.

Total protein (g/L)	Aβ42 % bias	Aβ40 % bias
12.0	-6.8	7.5
8.6	-17.4	19.2
6.1	-8.5	19.5
3.5	13.0	13.5
2.0	-3.9	-1.6
1.1	15.7	18.1
0.2	18.6	6.4

2.3.3 Hemolysis Interference

There was variability in raw peak area signal between the hemolysate contaminated samples; however, there was no effect on quantitation of Aβ (Figure 6) as the recovery of Aβ for all hemolysate samples were within ± 20% of the expected concentration (Table 3).

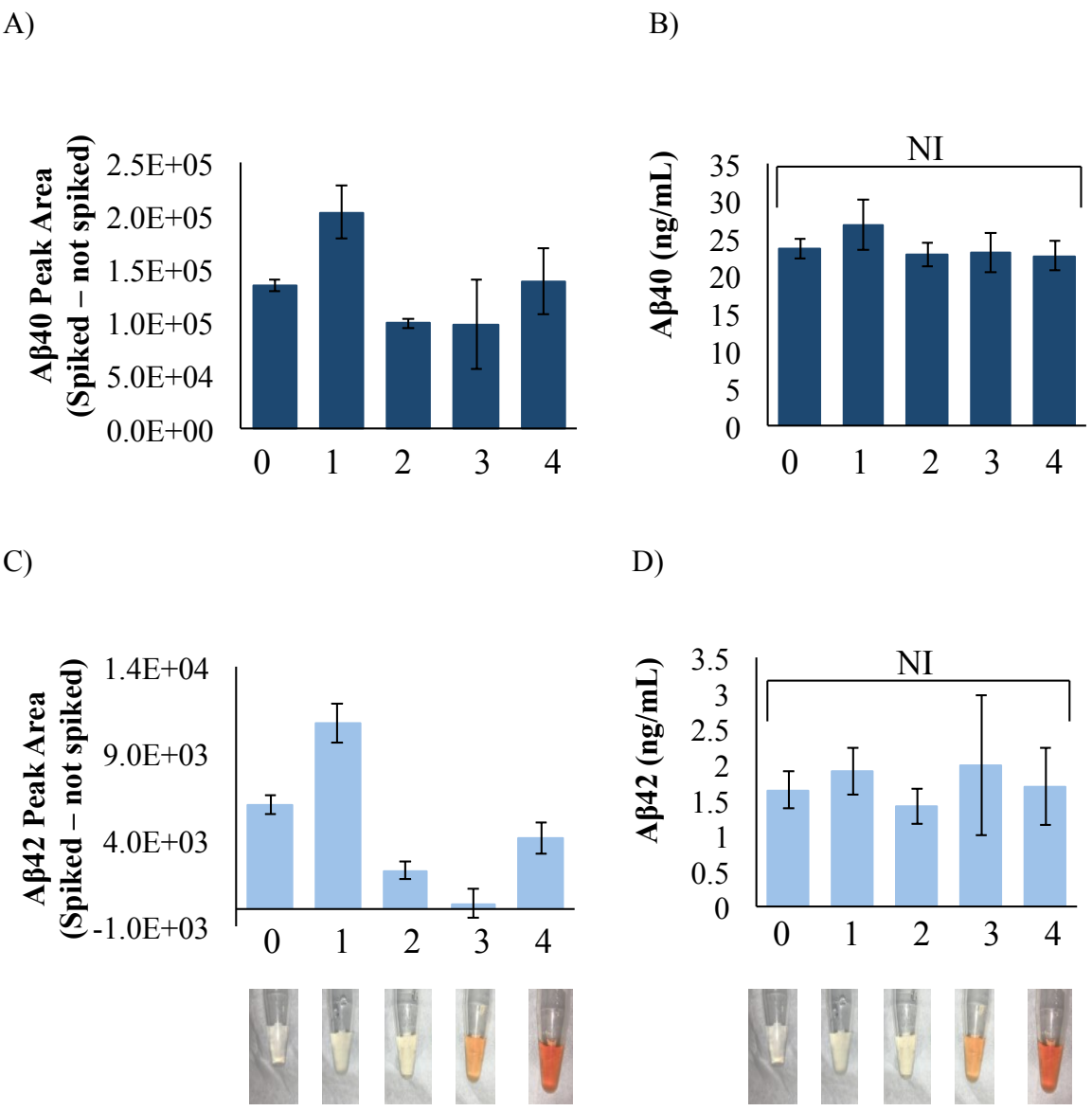


Figure 6: Hemolysate contamination affects raw peak area signal, but not quantification of Aβ peptides. Peak area adjusted for endogenous Aβ and Aβ concentration is shown for each individual sample tested, represented as the mean of three technical replicates and the standard deviation. A) and B) Aβ40. C) and D) Aβ42. Hemoglobin contamination, but not necessarily total hemolysate contamination, increased from patient sample 0 to 4 as observed visually. The control sample contained no hemolysate by visual inspection. NI: No interference.

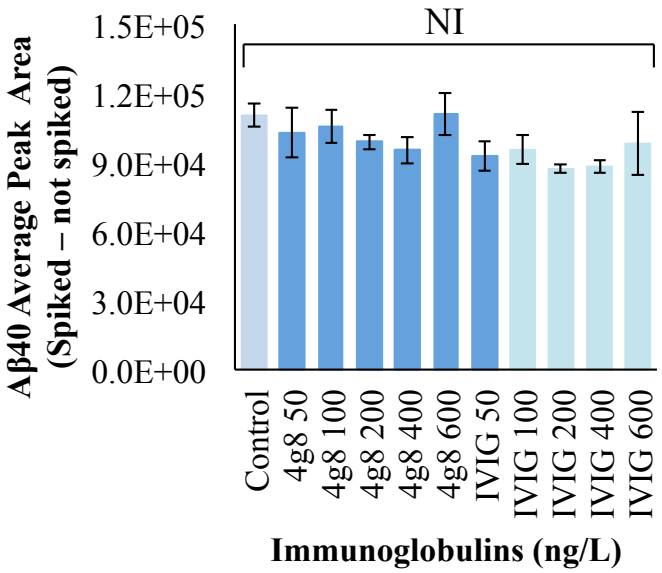
Table 3: Effect of hemolysate on accuracy of Aβ quantitation, as compared to a sample with no hemolysate contamination by visual inspection.

Hemolysate Sample	Aβ42 % bias	Aβ40 % bias
1	15.1	13.9
2	-13.2	-3.5
3	19.9	-2.2
4	3.0	-3.8

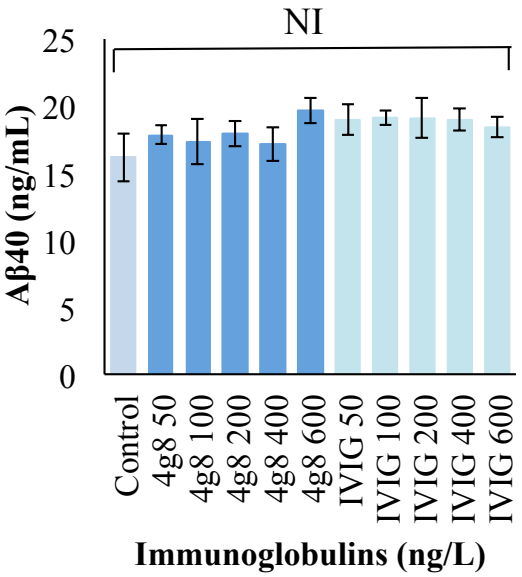
2.3.4 Anti-Amyloid Therapeutics

There was no interference observed as all immunoglobulin samples tested were within the pre-specified acceptance criteria of ± 20 % bias for both Aβ40 and Aβ42 (Table 4 and 5). Figure 7 demonstrates the minimal differences in raw peak area signal and Aβ concentration between the samples and the control (CSF pool).

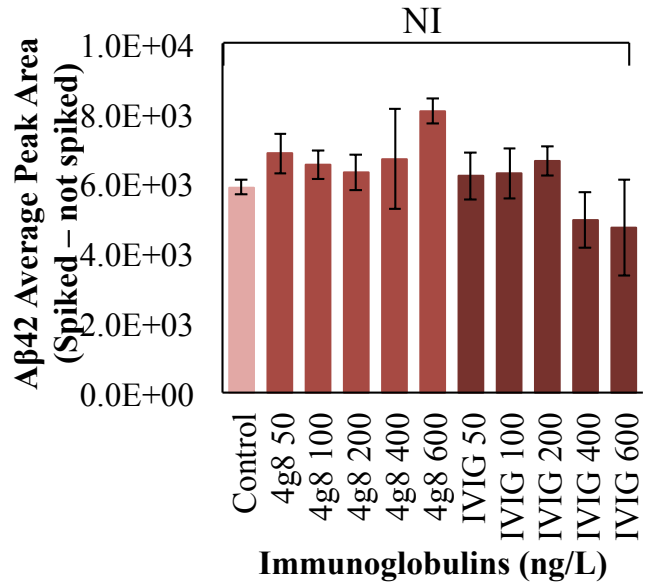
A)


NI
1.5E+05
1.2E+05
9.0E+04
6.0E+04
3.0E+04
0.0E+00
Aβ40 Average Peak Area (Spiked – not spiked)
Control
4g8 50
4g8 100
4g8 200
4g8 400
4g8 600
IVIG 50
IVIG 100
IVIG 200
IVIG 400
IVIG 600
Immunoglobulins (ng/L)

B)


NI
25
20
15
10
5
0
Aβ40 (ng/mL)
Control
4g8 50
4g8 100
4g8 200
4g8 400
4g8 600
IVIG 50
IVIG 100
IVIG 200
IVIG 400
IVIG 600
Immunoglobulins (ng/L)

C)


NI
1.0E+04
8.0E+03
6.0E+03
4.0E+03
2.0E+03
0.0E+00
Aβ42 Average Peak Area (Spiked – not spiked)
Control
4g8 50
4g8 100
4g8 200
4g8 400
4g8 600
IVIG 50
IVIG 100
IVIG 200
IVIG 400
IVIG 600
Immunoglobulins (ng/L)

D)

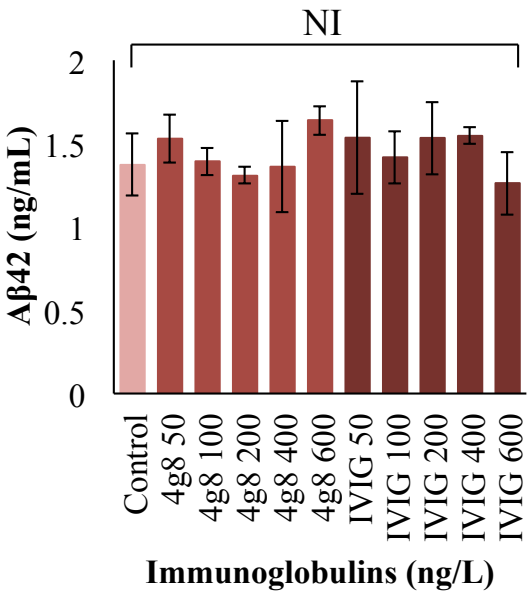

NI
2
1.5
1
0.5
0
Aβ42 (ng/mL)
Control
4g8 50
4g8 100
4g8 200
4g8 400
4g8 600
IVIG 50
IVIG 100
IVIG 200
IVIG 400
IVIG 600
Immunoglobulins (ng/L)

Figure 7: Presence of immunoglobulins do not interfere with the LC-MS/MS method. Average peak area (adjusted for endogenous Aβ) and Aβ concentration for the control, 4g8 and IVIG samples, represented as the mean of three technical replicates and the standard deviation. A and B) Aβ40. C and D) Aβ42. The control sample was CSF pool with no 4g8 antibody and IVIG. NI: No interference.

Table 4: Calculated % bias for samples containing the 4g8 antibody.

4g8 (ng/L)	**Aβ42 % bias**	**Aβ40 % bias**
50	11.5	10.2
100	1.6	7.4
200	-4.8	10.9
400	-0.8	5.8
600	19.6	20.9

Table 5: Calculated % bias for samples containing IVIG.

IVIG (ng/L)	**Aβ42 % bias**	**Aβ40 % bias**
50	11.9	17.1
100	3.3	18.1
200	11.8	17.8
400	12.7	17.1
600	-8.0	13.6

2.4 Discussion

In a quantitative LC-MS/MS method, calibrators are made by spiking in a known amount of the analyte into the appropriate matrix. In the case of Aβ, the matrix is CSF and as this already contains endogenous Aβ, it is not ideal to then spike in synthetic Aβ; therefore, an alternate matrix must be sought. Commercially available synthetic CSF, termed 'artificial CSF' is an option; however, these solutions contain only buffering small molecules and ions, and no proteins. Aβ peptides, in particular Aβ42, are hydrophobic and prone to aggregation, thus using artificial CSF with no proteins may lead to low recovery of the analyte [95]. Therefore, to better mimic CSF, an in-house surrogate matrix was designed for the LC-MS/MS calibrators. Testing various concentrations of BSA spiked into a buffered solution showed Aβ recovery was optimal in the range of 0.5 – 2 g/L of total protein. The concentration of the surrogate matrix was set to 1 g/L total protein. This concentration was similar to the normal physiological range of total protein CSF, where the reference cut-point most commonly used is < 0.45 g/L and samples received in clinically laboratories typically fall in the range of 0.2 to 0.8 g/L of total protein for a population that excludes individuals with disorders that are known or suspected to increase CSF total protein [96].

Samples with endogenous total protein concentrations within and above the reference cut-point, were also tested to assess potential impact of total protein content on routine clinical samples which vary in total protein concentration. These analyses indicated that raw peak area signal decreased with increasing total protein concentration for both Aβ40 and Aβ42; however, quantification of both Aβ40 and Aβ42 was not affected by total protein concentration, due to

normalization by the IS. Thus, the LC-MS/MS method was not affected by physiologically plausible extremes in total protein concentration.

CSF samples containing hemolyzed red blood cells were also investigated, which can occur due to a traumatic lumbar puncture or leakage of the BBB. In routine clinical analysis, hemolysate contamination is judged based on color and appearance of the CSF sample. Real patient specimens with hemolysate contamination based on visual inspection were also evaluated to test for potential interference in in the LC-MS/MS method. The results from this interference study indicated that raw signal was affected by the hemolysate contamination; however, Aβ quantification was not affected as a result of the IS normalization process (i.e., use of ion peak area ratios of analyte/IS). From previous experiments performed in the laboratory, a visual cut off for hemolysate contamination was determined, which visually corresponds to the color and appearance of patient sample 2 (Figure 6). The previous experiment performed used whole blood spiked into a CSF pool at various proportions; the solutions were then centrifuged and the supernatant analyzed. The color and appearance at this pre-determined visual cutoff is considered a physiologically plausible extreme for hemolysate contamination. In a clinical setting, in particular, out-patients being investigated for signs and symptoms of cognitive impairment, samples exceeding this amount of hemolysate contamination are unlikely.

In addition to potential endogenous interferents, plausible exogenous interferents, such as relevant therapeutic molecules, must be investigated as part of method validation [77]. In the context of AD, therapeutic molecules targeting Aβ are of particular interest. While there are many anti-Aβ antibody therapeutics under evaluation, they are not commercially available for purchase. As a surrogate, anti-Aβ monoclonal antibody 4g8 was purchased for testing. 4g8 binds

residues 18-22 of Aβ, sharing the same epitope with crenezumab, a passive immunotherapy that uses monoclonal antibodies to target multiple forms of aggregated Aβ [94]. IVIG was used as a non-Aβ-specific polyclonal antibody source. In the analyses, neither 4g8 or IVIG affected raw signal nor quantitation of Aβ by LC-MS/MS; suggesting that the assay was not susceptible to anti-Aβ therapies and the method can be used for not only in routine care, but also in clinical trials for anti-Aβ therapeutics. It was anticipated that anti-Aβ therapeutics would not interfere in the LC-MS/MS method due to the assay workflow, which includes, for instance, incubation with a strong salt denaturant (i.e., 5 M GdnHCl). For confirmation, the interference studied performed herein, can be directly applied to the investigation of other anti-Aβ therapeutics.

2.5 Conclusion

Pre-analytical and analytical steps of the LC-MS/MS method were optimized by identifying the optimal surrogate matrix concentration as well as by investigating potential interference. The surrogate matrix was established with protein content comparable to that observed in human CSF. Quantification of Aβ40 and Aβ42 was not affected by differences in total protein concentration. No interference from hemolysate was observed within the range of physiologically plausible contamination tested. Additionally, the presence of either non-specific or anti-Aβ immunoglobulins did not affect raw signal or quantification of Aβ peptides by LC-MS/MS. Thus Aβ peptides can be accurately quantified in the presence of high total protein concentration and anti-Aβ therapeutic antibodies, enabling use in routine clinical care and clinical trials. From the studies performed, pre-analytical requirements and sample acceptance/rejection criteria for the LC-MS/MS method were established.

Chapter 3: Identification Autosomal Dominant Variants and Wild-type Amyloid-β Peptides in Cerebrospinal Fluid as a Novel Diagnostic Approach for Autosomal Dominant AD

3.1 Introduction

Currently published mass spectrometric methods do not include MRMs of known autosomal dominant Aβ variants [70-74] and due to the high analytical selectivity of the MRM approach, Aβ variants are "invisible" (i.e. not detected). Immunoassays for their part cannot differentiate between Aβ variants that occur outside of the assay epitope regions, or if the mutation does occur within the epitope region (e.g. H6R variant) it may abrogate binding/detection. The Aβ variants will thus not be detected and will result in falsely low Aβ. Therefore, a method that identifies Aβ variants within the Aβ42 sequence addresses accurate quantification of total Aβ and also enables confirmatory diagnosis of AD.

The second aim of this thesis was to adapt the automated multiplex LC-MS/MS assay previously developed in the laboratory to identify known autosomal dominant variants within the Aβ42 sequence in CSF (Figure 2) [89]. As mentioned previously, *APP* autosomal dominant variants are 100% penetrant and result in autosomal dominant AD. Thus, identification of these variants in CSF can identify autosomal dominant AD. As a proof of concept study, nine pathogenic variants found in the Aβ42 sequence were tested to determine whether they could be identified and distinguished from wt-Aβ.

3.2 Methods

3.2.1 Variants Within the Aβ42 Sequence

To develop a comprehensive Aβ LC-MS/MS method that included both wt-Aβ and variant sequences, a list of known variants within the Aβ42 sequence was developed by searching existing databases including: Alzforum, Single Nucleotide Polymorphism Database (dbSNP, NCBI), Exome Aggregation Consortium (ExAC). An APP variant was included in the MRM method if it satisfied the following criteria: (1) contained a sequence variation within the Aβ42 sequence, and (2) was either identified as non-pathogenic or of unknown significance with an average frequency in the global population of ≥ 4.0E-05, or was identified as pathogenic (average frequency not considered in this case).

3.2.2 Heterozygous Aβ Samples

To test the multiplex Aβ LC-MS/MS method, CSF representative of this heterozygous Aβ expression state was used for testing – consistent with genetic make-up of individuals with autosomal dominant AD due to mutations in *APP*, who carry a single variant *APP* allele. As proof-of-concept, nine of these variants were synthesized as Aβ40 peptides and used to create the representative autosomal dominant AD CSF samples. No known pathogenic variants resulted in a mutation to residues 41 or 42, therefore all variants were captured within the Aβ40 sequence. To create a series of CSF samples characteristic of this heterozygote state, synthetic Aβ40 variants were spiked into a human CSF pool (containing only wt-Aβ) at equimolar concentration to the endogenous wt-Aβ40 (0.157 nM). An MRM method was developed for

these nine variants using MS. A predictive software, Skyline [97] was then used to build an MRM method with the remaining variants meeting the inclusion criteria be included in the adapted multiplex LC-MS/MS assay. Samples were then analyzed using the LC-MS/MS method shown in Figure 3. MRM peaks were considered within a retention time window of ± 0.2 minutes of the expected peak.

3.3 Results

3.3.1 Variants Within the Aβ42 Sequence

Table 6 lists all variants in the Aβ42 region, phenotype (if reported), relevant disease (i.e., AD, cerebral amyloid angiopathy [CAA] and/or Parkinson's Disease Dementia [PDD]) and the global population frequency from Database searches (Genome Aggregation Database (GnomAD-Exomes), Trans-Omics for Precision Medicine (TOPMed), Exome Aggregation Consortium (ExAC), and the NHLBI GO Exome Sequencing Project (GO-ESP) Exome Variant Server.

Table 6. Published variants within the Aβ42 sequence.

Variant	Phenotype	Disease Relevance	Frequency				Average Frequency
			GnomAD-Exomes	TOPMED	ExAC	GO-ESP	
Icelandic (A2T)	Protective	AD	4.6E-04	1.1E-04	4.7E-04	1.0E-04	4.6E-04
A2V	Pathogenic	AD					
E3Q	Unknown		4.0E-05	2.0E-05	1.0E-05		2.3E-05
E3K	Unknown		4.0E-05	2.0E-05	1.0E-05		2.3E-05
F4I	Unknown				1.0E-05		1.0E-05
R5Q	Unknown		2.0E-05	3.0E-05	4.0E-05		3.0E-05
English (H6R)	Pathogenic	AD	1.0E-05				1.0E-05
Taiwanese (D7H)	Pathogenic (AD)	AD					
Tottori (D7N)	Pathogenic (AD)	AD					
D7E	Unknown			1.0E-05			1.0E-05
G9A	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05
G9V	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05

Variant	Phenotype	Disease Relevance		Frequency			Average Frequency
G9E	Unknown			1.0E-05	1.0E-05	1.0E-04	4.0E-05
Leuven (E11K)	Pathogenic (AD)	AD					
V12I	Unknown		1.0E-05	6.0E-05	2.0E-05		3.0E-05
H13P	Unknown						
H14R	Unknown			1.0E-05	1.0E-05		1.0E-05
Q15E	Unknown			1.0E-05			1.0E-05
K16N	Pathogenic (AD)	AD					
V18M	Unknown		1.0E-05	1.0E-05	2.0E-05		1.3E-05
F19L	Unknown						
Flemish (A21G)	Pathogenic (AD)	AD & CAA					
E22D	Unknown				1.0E-05		1.0E-05
Osaka (E22Δ)	Pathogenic (AD)	AD					
Arctic (E22G)	Pathogenic (AD)	AD & CAA					
Dutch (E22Q)	Pathogenic (CAA)	CAA					

Variant	Phenotype	Disease Relevance	Frequency			Average Frequency
Italian (E22K)	Pathogenic (AD)	CAA				
Iowa (D23N)	Pathogenic	AD & CAA				
V24G	Unknown		2.0E-05			2.0E-05
V24A	Unknown		2.0E-05			2.0E-05
G25D	Unknown					
K28R	Unknown	1.0E-05				1.0E-05
I31V	Unknown		2.0E-05	1.0E-05		1.5E-05
I32V	Unknown	1.0E-05		1.0E-05		1.0E-05
Piedmont (L34V)	Pathogenic (CAA)	CAA				
V36M	Unknown			1.0E-05		1.0E-05
G38C	Unknown			8.2E-06		8.2E-06
G38S	Not Pathogenic (AD)	PDD		4.1E-05		4.1E-05
G38V	Unknown			1.0E-05		1.0E-05
V39I	Unknown	2.0E-05		3.3E-05	1.0E-04	5.1E-05

Variant	Phenotype	Disease Relevance	Frequency			Average Frequency
I41V	Unknown					
A42T	Unknown	AD	1.0E-04	3.0E-05	5.8E-05	6.3E-05
A42V	Not Pathogenic (AD)		4.0E-05	2.0E-05	6.6E-06	4.2E-05

From the database search, a total of 20 Aβ variants were found to meet the inclusion criteria. This included 13 pathogenic variants and 7 variants of not pathogenic or unknown significance. The developed comprehensive MRM method for variants within the Aβ42 sequence that met the inclusion criteria was listed in Table 7.

Table 7. MRM transitions of wt-Aβ40, wt-Aβ42, the ISs and the Aβ40 variants meeting the selection criteria for MRM development.

Variant	**Precursor ion (m/z)**	**Product ions (m/z)**
wt-Aβ40, E22K/D7N/E22Q/D23N/E11K*	1083	1054.1, 1029.1, 1000.8
wt-Aβ42	1129.4	1107.1, 1078.6, 1053.8
^{15}N-IS-Aβ40	1096	1066.9
^{15}N-IS-Aβ42	1143	1092.1
H6R	1088.2	1058.9, 1033.9, 1005.4
L34V/A21G/ K16N	1079.96	1050.5, 1011.5, 997.4
E22G	1065.45	1036.2, 1011.5, 997.1
E22Δ	1051.19	943.9, 914.8, 882.7
A2V	1090.4	1054.1, 1029.1, 1000.8
D7H	1089	1054.1, 1029.1, 948
G9A	1087	1050.5, 1029.1, 943.9
G9V	1093.3061	1054.1, 1029.1, 943.5
G9E	1101.5	1054.1, 1029.1, 1025.8

Variant	Precursor ion (m/z)	Product ions (m/z)
G38S	1090.9	1054.1,1029.1, 1000.8
V39I	1087	1029.1, 1025.8, 1000.8
A42T	1137	1107.1, 1078.6, 1053.8
A42V	1136.5	1107.1, 1078.6, 1053.8

* Variants separated chromatographically from wt- Aβ40

3.3.2 Heterozygous Aβ Samples

By LC-MS/MS analysis, all nine Aβ variants tested were resolved chromatographically from wt-Aβ. For example, Figure 8 illustrates the heterozygote sample for the E22G variant. E22G resolved chromatographically from wt-Aβ40 and can be distinguished based on mass and by retention time. Additionally the E22G, D23N and E22Q variants co-eluted, with E22G readily identifiable by its unique MRMs. However, E22Q and D23N share precursor masses and transitions (as currently selected) within the prescribed m/z tolerances, and therefore if this MRM were present, would be reported as "E22Q or D23N variant" (Figure 10).

The example of variant E22Q is shown in Figure 9. E22Q has a similar molecular weight as wt-Aβ40, at 4,328.88 Da and 4,329.90 Da respectively. In a heterozygote sample containing E22Q and wt-Aβ40, the chromatogram of the E22Q variant MRM would demonstrate both the wt-Aβ40 and variant peaks. Fortunately, E22Q is resolved chromatographically from wt-Aβ40, that is, not located within each other's respective MRM retention time windows. Thus, E22Q can be readily identified and distinguished from wt-Aβ40 in the LC-MS/MS assay.

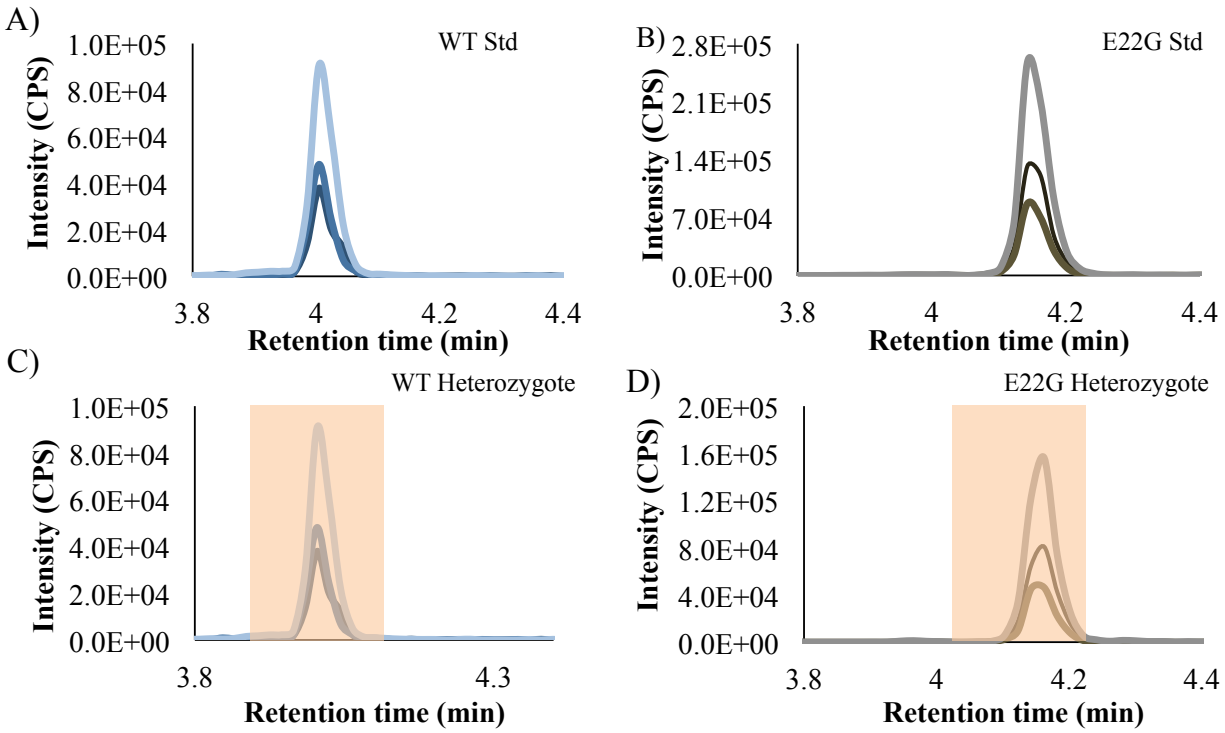


Figure 8: Wt-Aβ peak and E22G variant can be distinguished based on mass and retention time. Chromatograms of pure solutions of A) wt-Aβ40 and B) E22G variant. MRMs of the heterozygote CSF sample showing the (C) wt-Aβ40 MRM and the (D) E22G variant MRM, where the semi-transparent boxes indicate the retention time window considered for peak detection.

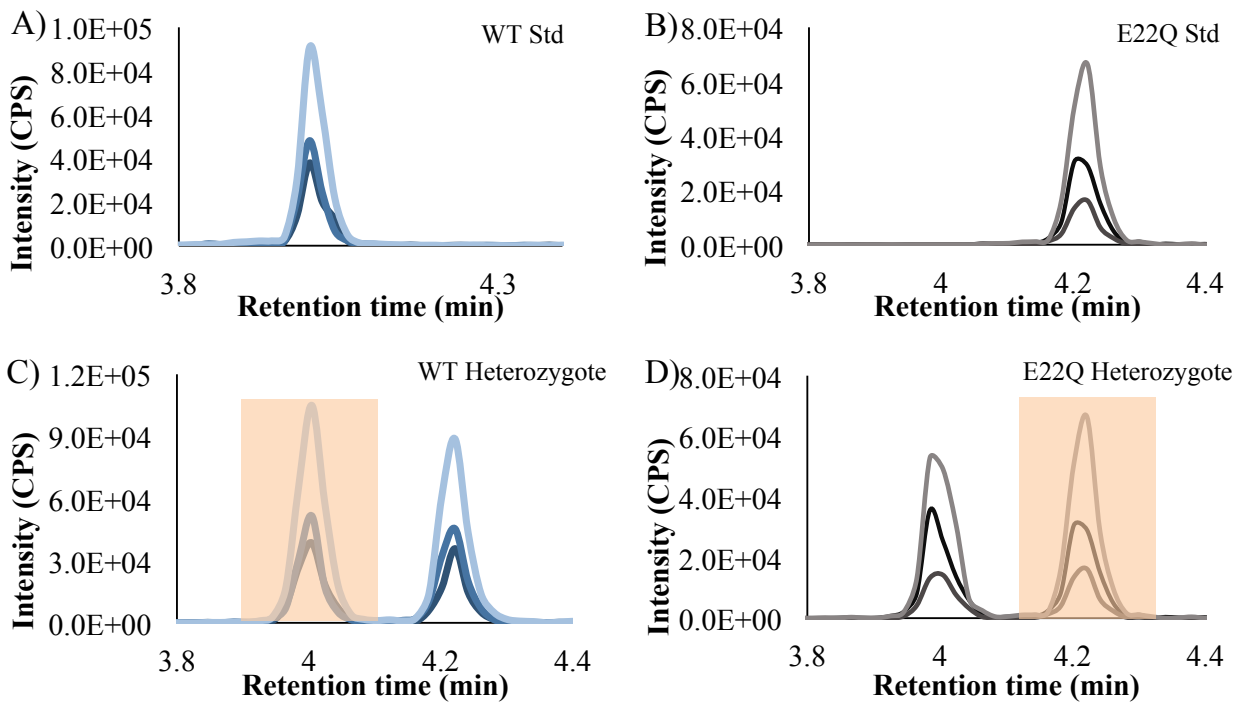


Figure 9: The variant E22Q was resolved chromatographically from wt-Aβ. Chromatograms of pure solutions of A) wt-Aβ40 and B) E22Q variant. MRMs of the heterozygote CSF sample showing the (C) wt-Aβ40 MRM and (D) E22Q variant MRM, where the semi-transparent boxes indicate the retention time window considered for peak detection.

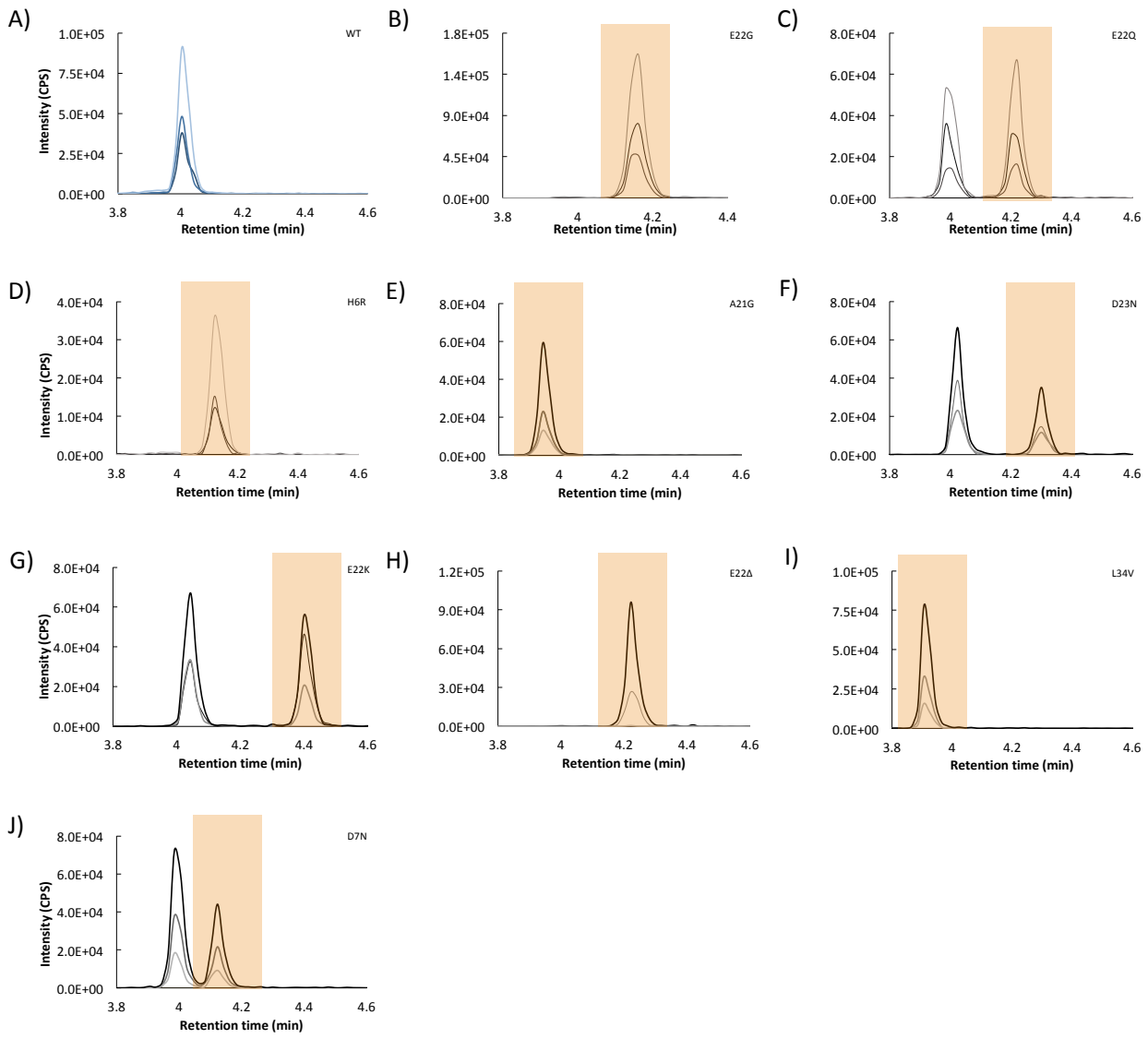


Figure 10: LC-MS/MS chromatograms of wt-Aβ40 (blue) and AD autosomal dominant variants (grey). A) Wt-Aβ40, B) Arctic (E22G), C) Dutch (E22Q), D) English (H6R), E) Flemish (A21G), F) Iowa (D23N), G) Italian (E22K), H) Osaka (E22Δ), I) Piedmont (L34V), J) Tottori (D7N). The semi-transparent boxes indicate the retention time window considered for peak detection.

3.4 Discussion

There are three types of variants: pathogenic, non-pathogenic, and unknown significance. Current MS methods for quantification of Aβ have not been designed to identify these APP variants within the Aβ42 sequence [70-74, 84]. Due to LC-MS/MS being a highly selective analytical method, only peptides included in the MRM methods are observed. If present, such variants would be missed by the methods and total Aβ content and quantification would be based on only the peptide arising from the wt allele. In the context of either a non-pathogenic variant or variant of unknown significance, a falsely low Aβ42 concentration would be reported, which could lead to a misdiagnosis of AD. Additionally screening for pathogenic variants can be used to identify autosomal dominant AD due to the variants being 100% penetrant. We thus adapted the multiplex Aβ LC-MS/MS assay to identify all known variants occurring within the Aβ42 sequence. As proof of concept, CSF samples mimicking *APP* heterozygotes (one wt and one variant allele) were tested, demonstrated that the presence of variant Aβ peptides could be readily identified. From this study, it was determined that using this adapted method, wt-Aβ and variant Aβ peptides can be distinguished by m/z ratios and/or retention time. For the remaining variants in the Aβ42 sequence that met our inclusion criteria, MRM parameters were developed for the LC-MS/MS assay.

3.5 Conclusion

The multiplex LC-MS/MS assay was adapted to quantify wt-Aβ42, Aβ40 and to identify Aβ variants in CSF. Extension of this Aβ multiplex LC-MS/MS assay to include known *APP* variants reflects an improvement in analytical accuracy, as the variants would be otherwise

missed and a falsely low Aβ concentration reported. Additionally, including known pathogenic variants enables confirmatory diagnosis of autosomal dominant (i.e. familial) AD.

Chapter 4: Summary

When developing assays in biological fluids, it is important to evaluate potential sources of analytical interference from both endogenous and exogenous sources. For the interference studies performed, the calculated % bias for the samples met the pre-specified criteria, indicating no interference for the endogenous and exogenous factors tested. Thus, the developed LC-MS/MS method was not susceptible to matrix interference from total protein content, hemolysate contamination, and immunoglobulins.

A limitation of existing LC-MS/MS methods for quantification of Aβ is that they were designed to only identify wt peptides, which can lead to quantification errors in the presence of Aβ variants and a missed opportunity to identify cases of autosomal dominant AD. Thus, the multiplex Aβ LC-MS/MS assay was adapted to create an analytical tool suitable for accurate quantification of wt-Aβ40 and Aβ42 in CSF, as well as to identify presences of any of the known Aβ variants occurring within the Aβ42 sequence. With this adaptation, the method can be used not only in its traditional role as to quantify biomarkers of AD pathology but also to identify cases of autosomal dominant AD. In addition, this method could be applied to further our understanding of Aβ metabolism – differentiating wt and variant peptides – in autosomal dominant AD.

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