Revealing the complex nature of amyloid beta and its relation to dementia

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ἕν οἶδα, ὅτι οὐδέν οἶδα,

Σωκράτης

To my grandma

Στην γιαγιά μου

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ABSTRACT

Dementia is the clinical expression for a range of acquired progressive brain disorders that affect cognitive functions severely enough to lead to impairments in daily life. The most common type of dementia is Alzheimer's disease (AD). AD is strongly associated with the presence of amyloid plaques in the extracellular space, consisting of amyloid beta (Aβ) peptides of various lengths. Aβ is produced through enzymatic cleavage of amyloid precursor protein (APP). Increased production or reduced clearance of Aβ peptides, especially the 42 amino acid long Aβ1-42, can start an aggregation process that eventually leads to insoluble aggregates and amyloid plaques. The increased accumulation of Aβ1-42 in the brain is reflected by low Aβ1-42 levels in cerebrospinal fluid (CSF) and plasma, making it a useful AD biomarker. Amyloid pathology can also be involved in other types of dementia, either as a putative driving force, as in cerebral amyloid angiopathy (CAA), Down syndrome (DS) and familial AD (FAD), or as a coexisting pathology in for example dementia with Lewy bodies (DLB), Parkinson's disease dementia (PDD) and familial British dementia. Furthermore, there are individuals with no cognitive impairment, but with amyloid plaque pathology documented during autopsy; this is often referred to as pathological ageing (PA). The aim of this thesis was to identify possible differences/similarities in Aβ peptide composition in different types of dementia, different brain regions and different amyloid deposits. To accomplish this, human brain tissue was used and Aβ peptides were isolated by immunoprecipitation (IP) and further analysed by mass spectrometry (MS).

In an initial investigation, the diffuse 'lake-like' Aβ deposits in presubiculum were compared with cored plaques observed in the neighbouring entorhinal cortex. Aβ deposits in presubiculum consisted of Aβ1-42 and Aβ4-42, while the cored plaques in entorhinal cortex had additional AβX-42 peptides, as well

as pyroglutamate-modified Aβ peptides that according to some studies are more toxic. Next, Aβ peptides (together with synaptic loss) were investigated in AD patients and patients with Aβ deposits but no cognitive symptoms until death (PA). Aβ1-40, Aβ4-40 and pyroglutamate-modified Aβ peptides generally had higher relative abundance in AD compared with PA, while Aβ1-42 and Aβ4-42 generally had higher relative abundance in PA compared with AD. Moreover, the amount of synaptic proteins was lower in AD compared with PA brains. In AD, AβX-40 and pyroglutamated Aβ3-42 correlated negatively with the amount of synaptic proteins, indicating that the presence of these Aβ peptides is associated with synaptic loss and cognitive decline. When comparing different regions no difference was observed between frontal and occipital lobes, while cerebellum generally had less Aβ.

Since both AD and CAA are characterised by deposits of Aβ, the two diseases were compared. In AD patients with and without CAA pathology, a distinct difference in Aβ deposition was observed. For AD without CAA pathology, the relative abundance of AβX-42 peptides was higher, while for AD with CAA pathology the relative abundance of AβX-40 peptides was higher. Further, an investigation of Aβ deposition in AD and DS patients was performed. Although DS individuals have an extra copy of APP, no major difference in Aβ amounts were found between AD and DS, except for APP/Aβ(-X to 15), AβX-40 and AβX-34, for which the abundances were higher in DS compared with AD. The protofibril/oligomer Aβ composition was also investigated, showing that the main components were Aβ1-40, Aβ1-42 and Aβ4-42, with higher abundance in AD and DS compared with controls. These results suggest that monitoring DS patients from early age might contribute to our understanding of plaque formation and finally neurodegeneration in sporadic AD. They also imply the possibility that treatment with clinical benefits in sporadic AD may also be beneficial for DS individuals. Finally, Aβ deposition in DLB and PDD patients was investigated to identify possible differences in their Aβ pathology. However, no difference in regard to the Aβ peptide pattern between DLB and PDD was found, but the Aβ load was significantly higher in DLB than in PDD. From these data, it cannot be ruled out that DLB and PPD are the same disease with different progression. Further, the Aβ peptide pattern was similar to that previously measured in AD, indicating that both patient groups have AD-type Aβ deposition that likely contributes to cognitive decline.

By investigating Aβ deposition in different types of dementia and different brain regions, a great complexity of the amyloid pathology was revealed. Even though the deposits of Aβ were similar across the different dementias investigated (except for CAA), the data presented indicate that there are different toxic (and non-toxic) Aβ assemblies in the different diseases. Further

investigation of Aβ peptides in combination with other dementia-associated factors (*e.g.*, tau peptides, synaptic and microglia-associated proteins) is needed to better understand the pathophysiology of the different types of dementia.

Keywords: Amyloid beta, dementia, Alzheimer's disease, cerebral amyloid angiopathy, Down syndrome, pathological ageing, dementia with Lewy bodies, Parkinson's disease dementia, mass spectrometry

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SAMMANFATTNING PÅ SVENSKA

Demens är den kliniska beteckningen på ett antal progressiva hjärnsjukdomar som påverkar kognitionen tillräckligt mycket för att leda till problem i vardagen. Den vanligaste typen av demens är Alzheimers sjukdom (AD). AD är starkt associerad till närvaron av extracellulära amyloida plack, som består av olika långa amyloid beta (Aβ)-peptider. Aβ skapas genom enzymatisk klyvning av ”amyloid precursor protein” (APP). Ökad produktion eller minskad nedbrytning av Aβ-peptider, särskilt den 42 aminosyror långa Aβ1-42, kan starta en aggregeringsprocess som så småningom leder till förekomsten av olösliga aggregat och amyloida plack. Den ökade ackumuleringen av Aβ1-42 i hjärnan reflekteras av låga Aβ1-42-nivåer i cerebrospinalvätska (CSV) och plasma, vilket gör den till en användbar AD-biomarkör. Amyloidpatologi kan också vara involverad i andra typer av demens, antingen som en potentiell drivkraft, som vid cerebral amyloidangiopati (CAA), Downs syndrom (DS) och familjär AD (FAD), eller som en komorbiditet vid exempelvis lewykropps-demens (DLB), Parkinson-demens (PDD) och familjär brittisk demens. Dessutom finns det personer som inte har någon kognitiv försämring, men där amyloidplackpatologi upptäcks vid obduktion – detta kallas ofta patologisk åldrande (PA). Syftet med denna avhandling var att identifiera möjliga skillnader eller likheter i Aβ-peptidsammansättning för olika typer av demens, hjärnregioner och amyloidstrukturer. För detta användes mänsklig hjärnvävnad och Aβ-peptider isolerades genom immunfällning (IP) och analyserades vidare med masspektrometri (MS).

I en inledande undersökning jämfördes de diffusa "sjöliknande" Aβ-deponierna i presubiculum med kompakta plack som observerades i närliggande entorhinal cortex. Aβ-deponierna i presubiculum bestod av Aβ1-42 och Aβ4-42, medan de kompakta placken i entorhinal cortex även hade andra AβX-42-peptider, såsom pyroglutamat-modifierade Aβ-peptider, vilka är mer toxiska enligt vissa studier. Därefter undersöktes Aβ-peptider (och även synapsförlust) hos AD-patienter och patienter med Aβ-patologi men utan kognitiva symtom vid dödstillfället (PA). Aβ1-40, Aβ4-40 och pyroglutamat-modifierade Aβ-peptider hade vanligtvis högre relativ förekomst i AD jämfört med PA, medan Aβ1-42 och Aβ4-42 generellt förekom i högre relativ mängd i PA jämfört med AD. Dessutom var mängden synaptiska proteiner lägre i AD- än i PA-hjärnor. För AD korrelerade AβX-40 och pyroglutamat-modifierad Aβ3-42 negativt med mängden synaptiska proteiner, vilket indikerar att dessa Aβ-peptider är associerade med synapsförlust och kognitiv försämring. En jämförelse mellan olika hjärnregioner visade ingen skillnad mellan frontal- och occipital-loberna, medan cerebellum i allmänhet hade mindre Aβ.

Eftersom både AD och CAA kännetecknas av Aβ-deponier jämfördes de två sjukdomarna. Hos AD-patienter med och utan CAA-patologi kunde man se en tydlig skillnad i Aβ-mönstret. För AD utan CAA-patologi var den relativa förekomsten av AβX-42-peptider högre, medan för AD med CAA-patologi var den relativa förekomsten av AβX-40-peptider högre. Vidare utfördes en undersökning av Aβ-strukturer hos AD- och DS-patienter. Även om personer med DS har en extra kopia av APP påvisades ingen större skillnad i Aβ-halt mellan AD och DS, förutom för APP/Aβ (-X till 15), AβX-40 och AβX-34, för vilka halterna var högre för DS än för AD. Även protofibrillärt/oligomert Aβ undersöktes och det visade sig att huvudkomponenterna utgjordes av Aβ1-40, Aβ1-42 och Aβ4-42, samt att förekomst var högre för AD och DS än för kontrollindivider. Dessa resultat tyder på att man genom att undersöka personer med DS redan i tidig ålder skulle kunna erhålla ökad insikt om plackbildning och neurodegeneration vid sporadisk AD. Resultaten indikerar också att behandling med gott resultat för sporadisk AD också kan vara fördelaktig för personer med DS. Slutligen undersöktes Aβ-deponi hos DLB- och PDD-patienter för att identifiera potentiella skillnader i deras Aβ-patologi. Emellertid kunde ingen skillnad i Aβ-peptidmönstret mellan DLB och PDD påvisas, men mängden Aβ var markant högre i DLB än i PDD. Från dessa data kan det inte uteslutas att DLB och PPD är samma sjukdom men med olika progression. Vidare var Aβ-peptidmönstret liknande det som tidigare uppmätts för AD, något som indikerar att båda patientgrupperna har en Aβ-patologi av AD-typ som sannolikt bidrar till den kognitiva försämringen.

Genom att undersöka Aβ-deponier i olika demenstyper och olika hjärnregioner påvisades amyloidpatologins stora komplexitet. Även om Aβ-deponierna var likartade för de olika sjukdomarna (förutom för CAA) indikerar dessa data att det bildas olika toxiska (och icke-toxiska) Aβ-varianter i de olika sjukdomarna. Ytterligare undersökning av Aβ-peptider i kombination med andra faktorer associerade med demens (t.ex. tau-peptider, synaptiska och mikroglia-associerade proteiner) behövs för att bättre förstå patofysiologin hos de olika typerna av demens.

ΠΕΡΙΛΗΨΗ ΤΗΣ ΔΙΑΤΡΙΒΗΣ ΣΤΑ ΕΛΛΗΝΙΚΑ

«Αποκαλύπτοντας την περίπλοκη φύση των αμυλοειδών βήτα και τη σχέση τους με την άνοια»

Ο όρος άνοια αναφέρεται στην κλινική έκφραση μια σειράς από επίκτητες προοδευτικές εγκεφαλικές διαταραχές που επηρεάζουν τις γνωστικές λειτουργίες ενός ανθρώπου τόσο σοβαρά ώστε να επηρεάζουν την καθημερινή του ζωή. Ο πιο συνηθισμένος τύπος άνοιας είναι η νόσος του Αλτσχάιμερ (ΝΑ). Η ΝΑ σχετίζεται στενά με την παρουσία αμυλοειδών πλακών στον εξωκυτταρικό χώρο, που αποτελούνται από διαφόρων μηκών πεπτίδια αμυλοειδούς βήτα (Αβ). Τα πεπτίδια Αβ παράγονται μέσω ενζυματικής διάσπασης της πρόδρομης αμυλοειδούς πρωτεΐνης (ΠΑΠ). Η αυξημένη παραγωγή ή η μειωμένη εκκαθάριση των πεπτιδίων Αβ, ειδικά του Αβ1-42, μπορεί να ξεκινήσει μια διαδικασία συσσωμάτωσης που οδηγεί σε αδιάλυτα συσσωματώματα και τελικά σε αμυλοειδείς πλάκες. Η αυξημένη συσσώρευση του πεπτιδίου Αβ1-42 στον εγκέφαλο αντικατοπτρίζεται από τα χαμηλά επίπεδα του στο εγκεφαλονωτιαίο υγρό (ΕΝΥ) και στο πλάσμα του αίματος, καθιστώντας το ένα χρήσιμο βιοδείκτη για τη ΝΑ. Η αμυλοειδής παθολογία μπορεί επίσης να εμπλέκεται και σε άλλους τύπους άνοιας, είτε ως πρωταρχική παθολογία, όπως στην εγκεφαλική αμυλοειδή αγγειοπάθεια (ΕΑΑ), στο σύνδρομο Ντάουν (ΣΝτ) και στην οικογενειακή ΝΑ (ΟΝΑ), ή ως συνυπάρχουσα παθολογία, όπως για παράδειγμα στην άνοια με σωμάτια Λιούη (ΑΣΛ), στην άνοια της νόσου του Πάρκινσον (ΑΝΠ) και την Βρετανική οικογενειακή άνοια. Επιπλέον, υπάρχουν άτομα χωρίς γνωστική εξασθένηση, αλλά με παθολογία αμυλοειδών πλάκων κατά την αυτοψία. Αυτό συχνά αναφέρεται ως παθολογική γήρανση (ΠΓ). Ο σκοπός αυτής της διατριβής ήταν να εντοπίσει πιθανές διαφορές/ομοιότητες στη σύσταση των πεπτιδίων Αβ σε διαφορετικούς τύπους άνοιας, σε διαφορετικές περιοχές του εγκεφάλου και των διαφορετικών τύπων αμυλοειδών εναποθέσεων. Για την επίτευξη αυτού του σκοπού, χρησιμοποιήθηκε ανθρώπινος εγκεφαλικός ιστός, τα πεπτίδια Αβ απομονώθηκαν με ανοσοκαθίζηση (ΑΚ) και η περαιτέρω ανάλυσή τους πραγματοποιήθηκε με φασματομετρία μάζας (ΦΜ).

Αρχικά, οι διάχυτες Αβ εναποθέσεις με «μορφή-λίμνης» στην περιοχή του προ-υποθέματος (μέρος του ιπποκάμπου) συγκρίθηκαν με τις αμυλοειδείς πλάκες που περιεχουν πυρήνα και παρατηρούνταιστον γειτονικό ενδορρινικό φλοιό (μέρος του ιπποκάμπου). Οι Αβ εναποθέσεις στην περιοχή του προ-υποθέματος αποτελούνταν κυρίως απο τα πεπτίδια Αβ1-42 και Αβ4-42, ενώ οι πλάκες που περιέχουν πυρήνα τον ενδορρινικό φλοιό διέθεταν επιπλέον πεπίδια ΑβΧ-42, καθώς και μετα-μεταγραφικά

τροποποιημένα με πυρογλουταμικό οξύ (πυρογλουταμινωμένα) πεπτίδια Αβ, που σύμφωνα με ορισμένες μελέτες είναι πιο τοξικά. Στη συνέχεια, τα πεπτίδια Αβ (μαζί με πρωτείνες για τον έλεγχο συναπτικής απώλειας) διερευνήθηκαν σε ασθενείς με ΝΑ και σε ασθενείς με Αβ εναποθέσεις αλλά χωρίς γνωστικά συμπτώματα μέχρι τον θάνατο (ΠΓ). Τα πεπτίδια Αβ1-40, Αβ4-40 και τα πυρογλουταμινωμένα Αβ είχαν γενικά υψηλότερη σχετική παρουσία σε ασθενείς με ΝΑ σε σύγκριση με τους ασθενείς με ΠΓ, ενώ τα πεπτίδια Αβ1-42 και Αβ4-42 είχαν γενικά υψηλότερη σχετική παρουσία σε ασθενείς με ΠΓ σε σύγκριση με ασθενείς με ΝΑ . Επιπλέον, η ποσότητα των συναπτικών πρωτεϊνών ήταν χαμηλότερη στη ΝΑ σε σύγκριση με τους εγκεφάλους στην ΠΓ. Στη ΝΑ, τα πεπτίδια ΑβΧ-40 και το πυρογλουταμινωμένο Αβ3-42 συσχετίστηκαν αρνητικά με την ποσότητα των συναπτικών πρωτεϊνών, υποδεικνύοντας ότι η παρουσία αυτών των πεπτιδίων Αβ σχετίζεται με τη συναπτική απώλεια και τη γνωστική εξασθένηση. Κατά τη σύγκριση διαφορετικών εγκεφαλικών περιοχών, δεν παρατηρήθηκε διαφορά μεταξύ του μετωπιαίου και ινιακού λοβού, ενώ η παρεγκεφαλίδα είχε γενικά μικρότερο φορτίο Αβ.

Δεδομένου ότι αμφότερες οι ΝΑ και ΕΑΑ χαρακτηρίζονται από εναποθέσεις πεπτιδίων Αβ, οι δύο ασθένειες συγκρίθηκαν μεταξύ τους. Σε ασθενείς με ΝΑ με ΕΑΑ παθολογία και χωρίς ΕΑΑ παθολογία, παρατηρήθηκε μια διάκριτη διαφορά στην εναπόθεση των πεπτιδίων Αβ. Σε ασθενείς με ΝΑ χωρίς όμως ΕΑΑ παθολογία , η σχετική αφθονία των πεπτιδίων ΑβΧ-42 ήταν υψηλότερη, ενώ σε ασθενείς με ΝΑ και ΕΑΑ παθολογία η σχετική αφθονία των πεπτιδίων ΑβΧ-40 ήταν υψηλότερη. Περαιτέρω, πραγματοποιήθηκε έρευνα στην εναπόθεση των πεπτιδίων Αβ σε ασθενείς με ΝΑ και ασθενείς με ΣΝτ. Παρόλο το γεγονός ότι τα άτομα με ΣΝτ έχουν ένα επιπλέον αντίγραφο της ΠΑΠ, δεν βρέθηκε σημαντική διαφορά στις ποσότητες των πεπτιδίων Αβ μεταξύ των ασθενών με ΝΑ και ΣΝτ, εκτός από τα πεπτίδια ΠΑΠ/Αβ (-Χ έως 15), τα ΑβΧ-40 και τα ΑβΧ-34, η παρουσία των οποίων ήταν αυξημένη στους ασθενείς με ΣΝτ, συγκριτικά με τους ασθενείς με ΝΑ. Διερευνήθηκε επίσης η σύνθεση των πρωτο-ινιδίων/ολιγομερών Αβ εναποθέσεων, δείχνοντας ότι τα κύρια συστατικά τους ήταν τα πεπτίδια Αβ1-40, Αβ1-42 και Αβ4-42, με μεγαλύτερη αφθονία σε ασθενείς με ΝΑ και ΣΝτ σε σύγκριση με υγιείς ασθενείς ελέγχου. Αυτά τα αποτελέσματα υποδηλώνουν ότι η παρακολούθηση ασθενών με ΣΝτ από νεαρή ηλικία μπορεί να συμβάλει στην κατανόηση του σχηματισμού των αμυλοειδών πλακών και τελικά στον νευροεκφυλισμό στη ΝΑ. Τα παραπάνω αποτελέσματα, υποδεικνύουν επίσης την πιθανότητα ότι μια θεραπεία με κλινικά οφέλη στη ΝΑ μπορεί να είναι επίσης ευεργετική για άτομα με ΣΝτ.

Τέλος, διερευνήθηκε η εναπόθεση των Αβ πεπτιδίων σε ασθενείς με ΑΣΛ και ΑΝΠ για τον εντοπισμό πιθανών διαφορών στην παθολογία των Αβ εναποθέσεων. Ωστόσο, δεν βρέθηκε διαφορά σε σχέση με την κατανομή των πεπτιδίων Αβ μεταξύ ΑΣΛ και ΑΝΠ, αλλά το φορτίων των πεπτιδίων Αβ ήταν σημαντικά υψηλότερο στο ΑΣΛ από ότι στο ΑΝΠ. Από αυτά τα δεδομένα, δεν αποκλείεται ότι η ΑΣΛ και η ΑΝΠ είναι η ίδια ασθένεια με διαφορετικό ρυθμό εξέλιξης. Περαιτέρω, η κατανομή των πεπτιδίων Αβ ήταν παρόμοια με εκείνη που είχε προηγουμένως παρατηρηθεί στη ΝΑ, υποδεικνύοντας ότι και οι δύο ασθένιες, ΑΣΛ και ΑΝΠ, έχουν εναπόθεση Αβ πεπτιδίων όμοια με τη ΝΑ που πιθανώς συμβάλλει στη γνωστική εξασθένηση των ασθενών.

Ερευνώντας την εναπόθεση των Αβ πεπτιδίων σε διαφορετικούς τύπους άνοιας και διαφορετικές περιοχές του εγκεφάλου, αποκαλύφθηκε μια μεγάλη πολυπλοκότητα της αμυλοειδούς παθολογίας. Παρόλο που οι εναποθέσεις των Αβ πεπτιδίων ήταν παρόμοιες στις διάφορες μορφές άνοιας που ερευνήθηκαν (εκτός από το ΕΑΑ), τα δεδομένα που παρουσιάζονται δείχνουν ότι υπάρχουν διαφορετικές τοξικές (και μη τοξικές) συγκροτήσεις των Αβ πεπτιδίων στις διάφορες ασθένειες. Απαιτείται περαιτέρω διερεύνηση των Αβ πεπτιδίων σε συνδυασμό με άλλους παράγοντες που σχετίζονται με την άνοια (π.χ. πεπτίδια ταυ, συναπτικές και μικρογλοιακές πρωτεΐνες) για την καλύτερη κατανόηση της παθοφυσιολογίας των διαφόρων τύπων άνοιας.

LIST OF PAPERS

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

I. Murray CE, Gami-Patel P, Gkanatsiou E, Brinkmalm G, Portelius E, Wirths O, Heywood W, Blennow K, Ghiso J, Holton JL, Mills K, Zetterberg H, Revesz T, Lashley T. **The presubiculum is preserved from neurodegenerative changes in Alzheimer's disease.** Acta Neuropathol Commun. 2018 Jul 20;6(1):62.

II. Gkanatsiou E, Portelius E, Toomey CE, Blennow K, Zetterberg H, Lashley T, Brinkmalm G. **A distinct brain beta amyloid signature in cerebral amyloid angiopathy compared to Alzheimer's disease**. Neurosci Lett. 2019 May 14;701:125-131.

III. Gkanatsiou E, Sahlin C, Portelius E, Johannesson M, Söderberg L, Fälting J, Basun H, Möller C, Odergren T, Zetterberg H, Blennow K, Lannfelt L, Brinkmalm G. **Characterization of monomeric and soluble aggregated Aβ in Down's syndrome and Alzheimer's disease brains.** Submitted.

IV. Gkanatsiou E, Nilsson J, Toomey C, Vrillon A, Kvartsberg H, Portelius E, Zetterberg H, Blennow K, Brinkmalm A, Lashley T, Brinkmalm G**. Amyloid pathology and synaptic loss in pathological aging.** Manuscript.

V. Gkanatsiou E, Hansen D, Portelius E, Nilsson J, Zetterberg H, Blennow K, Warner T, Lashley T, Brinkmalm G. **Exploring amyloid beta peptides in Parkinson's disease dementia and dementia with Lewy bodies.** Manuscript.

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ABBREVIATIONS

AA	Amino acid
ABri	Amyloid deposition in familial British dementia
ACE	Angiotensin-converting enzyme
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease domain
ADan	Amyloid deposition in familial Danish dementia
AEP	Asparagine endopeptidase
APH-1	Anterior pharynx-defective 1
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
BACE2	Beta-site amyloid precursor protein cleaving enzyme 2
C18	Octadecyl carbon chain
CAA	Cerebral amyloid angiopathy
CD68	Cluster of differentiation 68
CSF	Cerebrospinal fluid
CTFα / C83	APP C-terminal fragment-α
CTFβ / C99	APP C-terminal fragment-β

DAB	Diaminobenzidine
DC	Direct current
DLB	Dementia with Lewy bodies
DS	Down Syndrome
ECE	Endothelin-converting enzyme
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EOAD	Early-onset AD
ESI	Electrospray ionization
FA	Formic acid
FAD	Familial AD
FBD	Familial British dementia
FDA	United States Food and Drug Administration
FDD	Familial Danish dementia
FDG	18-fluorodeoxyglucose
FTD	Frontotemporal dementia
HCHWA-D	Hereditary cerebral hemorrhage with amyloidosis-Dutch type
HPLC	igh-performance liquid chromatography
HRP	Avidin-biotinylated horseradish peroxidase
Iba1	Ionized calcium-binding adapter molecule 1
IDE	Insulin-degrading enzyme

IHC	Immunohistochemistry
IP	Immunoprecipitation
IWG	International Working Group
LB	Lewy body
LC	Liquid chromatography
LCM	Laser capture microdissection
LOAD	Late-onset AD
LP	Lumbar puncture
m/z	mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MCI	Mild cognitive impairment
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MMSE	Mini Mental State Examination
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MT5-MMP	Membrane-type 5-matrix metalloproteinase
NBB	The Netherlands Brain Bank
NEP	Neprilysin
NfL	Neurofilament light
NFTs	Neurofibrillary tangles

NIA-AA	National Institute on Aging and Alzheimer's Association
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s disease and Related Disorders Association
PA	Pathological ageing
PD	Parkinson’s disease
PDD	Parkinson’s disease dementia
PET	Positron emission tomography
pGlu	Pyroglutamation
PiB	Pittsburgh Compound B
PSEN1	Presenilin 1
PSEN2	Presenilin 2
P-tau	Phosphorylated tau
PTMs	Post-translational modification
QSBB	The Queen Square Brain Bank for Neurological Disorders
RBM3	Putative RNA-binding protein 3
RF	Radio frequency
RP	Reverse phase
SAD	Sporadic AD
sAPPα	Soluble amyloid precursor protein α
sAPPβ	Soluble amyloid precursor protein β
SNAP-25	Synaptosomal-Associated Protein, 25kDa

T21	Trisomy 21
TBS	Tris buffered saline
TOF	Time-of-flight
TREM2	Triggering receptor expressed on myeloid cells 2
Tris base	tris(hydroxymethyl)aminomethane
T-tau	Total tau
VaD	Vascular dementia

DEFINITIONS IN SHORT

APP/Aβ	APP/Aβ peptides are peptides that are N-terminally extended from the Aβ sequence
C-truncation	Truncation at the C-terminus of Aβ
N-truncation	Truncation at the N-terminus of Aβ

1 INTRODUCTION

Dementia is a general term describing a range of medical conditions characterized by cognitive symptoms (such as communication and language problems, the loss of the ability to focus, reasoning, judging and visual perception), severe enough to interfere with daily life, and with a duration of more than six months [1]. The particular symptoms depend on which brain regions that are affected. Dementia can be caused by a number of different diseases, mainly including Alzheimer's disease (AD), vascular dementia (VaD), dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), Down syndrome (DS) and Parkinson's disease dementia (PDD). All these diseases are characterized by a progressive neurodegeneration, meaning neuronal dysfunction, neuronal loss and finally brain atrophy. Through pathological studies, we now know that comorbidity (the presence of more than one disease process) is common, making it difficult to distinguish the different underlying diseases in the clinical settings.

1.1 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia-causing disease, accounting for 60-80% of all cases and currently affecting 5.8 million patients in USA and 50 million people worldwide [2]. Until the 1800s, it was believed that dementia was associated with old age. However, 1901, Dr. Alois Alzheimer identified the symptoms in a 51 year old patient named Auguste Deter. Dr. Alzheimer treated her until her death in 1906 and the same year he presented her case at a congress. He described the clinical characteristics of disturbances in memory, as well as the neuro-pathological signs that he called "military bodies" and "dense bundles of fibrils", which today are known as plaques and tangles, respectively (Figure 1) [3]. A few years later, 1910, Emil Kraepelin named the disease AD. The terminology as known today was established in 1977, where AD is described as a neurodegenerative disease with progressive pattern of cognitive and functional impairment.

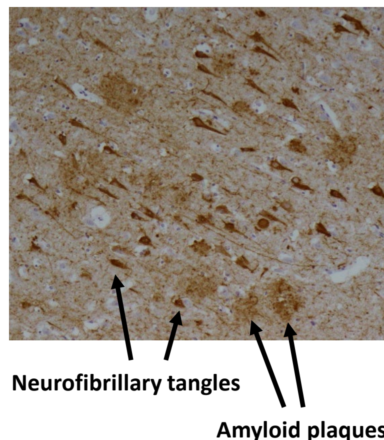


Figure 1. Plaques and tangles in AD. Staining by Tammaryn Lashley.

Despite the extensive research about AD, we are still far from fully understanding it. Today, one in three elderly die from AD and other dementias, currently with no cure [2]. In 2018, around 11 million people were affected by dementia in Europe, of which 170,000 were diagnosed with dementia in Sweden and it is estimated that this number will double by 2050 [4]. In 2005 the cost related to dementia was 50 billion SEK (≈5 billion euros) [5]. Moreover, despite the many diagnostic tools that have been developed, only 45% of people with AD or their relatives report they were aware of their condition [6].

AD is a progressive neurodegenerative disease that can be divided into two categories, sporadic AD (SAD) and familial AD (FAD). For FAD, the symptoms usually appear in people younger than 65 years. FAD has a strong genetic background (see 1.1.2) and it is estimated that less than 1% of all the AD cases has the familial form [7]. SAD is also referred to as late-onset AD (LOAD) where the symptoms appear after the age of 65. About 4-5% of all AD cases have an early-onset AD (EOAD), where symptoms appear before the age of 65 [8, 9].

AD patients can be classified into different groups depending on the disease stage [2], as summarized in Table 1.

Table 1. Stages of AD.

	Prodromal AD	**Dementia due to AD**		
Preclinical AD	MCI due to AD	Mild	Moderate	Severe
No symptoms	Very mild symptoms that do not interfere with everyday life	Symptoms interfere with some everyday activities	Symptoms interfere with many everyday activities	Symptoms interfere with most everyday activities

AD is a continuum, with preclinical AD being the first stage. In this stage, individuals can function normally despite the presence of measurable brain changes, such as Aβ and tau depositions [measured by abnormal CSF biomarkers and/or positive positron emission tomography (PET) scans)] and decreased glucose metabolism (measured by PET scan). However, it is important to note that not all individuals with AD-related brain changes will develop symptoms of mild cognitive impairment (MCI) or dementia due to AD [10, 11]. These individuals will be referred to as pathological ageing (PA) in this thesis.

The majority of the preclinical AD individuals will progress to MCI due to AD. Apart from the brain changes characteristic of AD, MCI individuals also exhibit mild memory and thinking problems. The cognitive problems in MCI are mostly noticeable to close relatives, as individuals can perform everyday activities normally. The memory complaints start occurring when the brain can no longer compensate for the neuronal damage due to AD [12]. Longitudinal studies have shown that 15% of MCI individuals older than 65 years will develop dementia due to AD within two years of the first symptoms [13]. This number increased to 32-38% after a 5 year follow up [14, 15]. However, some MCI individuals will remain cognitively stable or even revert to normal cognition.

In addition to the AD-related brain changes, individuals with dementia due to AD show noticeable dysfunction in memory, thinking or behaviour that interfere with their daily functionality. The symptoms that an individual experiences can change over time and reflect the degree of neuronal damage in the different brain regions. Depending on the severity of the symptoms, AD dementia can be divided into mild, moderate and severe [16, 17]. In the mild stage, people can function independently in many daily activities, but will need assistance with some activities. Individuals that have moved to the moderate stage start having personality and behavioural changes and also difficulty to communicate and perform routine tasks. This stage is often the longest one. The final stage is the severe AD dementia, where people are incapable to perform everyday activities and need help on a daily basis. The general deterioration in physical health of the patient becomes especially apparent and neurodegeneration can result in movement difficulties. At this stage, patients become bed-bound, a situation that can cause further health problems for the patient. Neurodegeneration will expand to more brain areas and the loss of the functions they control may finally cause the death of the patient.

1.1.1 **Pathophysiology**

Neuropathological hallmarks of AD are the presence of amyloid plaques and neurofibrillary tangles in the parenchymal tissue in the brain. Amyloid plaques mainly consist of aggregated beta amyloid (Aβ) peptides (see 1.5.1), while the neurofibrillary tangles (NFTs) consist of phosphorylated tau protein. A spatiotemporal distribution of amyloid plaques and NFTs is observed in AD brain. NFTs are spreading 'outwards' in 6 stages, as described by Braak & Braak [18]. NFT spreading correlates with cognitive decline in AD patients [18, 19]. Contrary to this, amyloid pathology spreads in an opposite 'inwards' direction (see 1.5.6), as described by Thal [20]. While amyloid plaques are

mainly localised between the cells, neurofibrillary tangles are formed within the neurons. Figure 2A shows the location of tangles and plaques in/at the neuron. At the same time, degeneration of neurons and synaptic loss occurs, which correlates with cognitive decline [21]. On a macroscopic level, cortical atrophy (reduced cortical brain volume) is observed, together with the enlargement of the ventricles [22], as shown in Figure 2B.

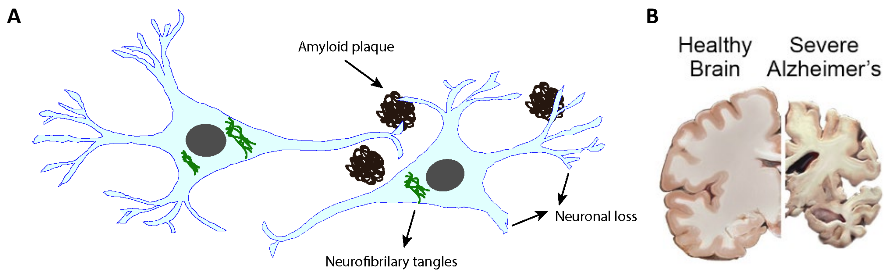


Figure 2. (A) Neuronal spatial localization of neurofibrillary tangles, amyloid plaques and neuronal loss in AD patients. (B) Brain atrophy in AD. In part adapted from the National Institute on Aging.

1.1.2 **Genetics**

The first gene identified to be associated with AD was the amyloid precursor protein gene, *APP*, which is located on chromosome 21. Today, 58 mutations in *APP* have been reported, most of which lead to FAD [23]. Missense mutations in *APP* may lead to an increased Aβ42/Aβ40 ratio while others increase total Aβ production, which may directly promote oligomer and plaque formation [24]. Moreover, mutations in the presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) genes also lead to FAD. Presenilin 1 (PSEN1) and presenilin 2 (PSEN2) proteins function as catalytic subunits of the γ-secretase complex (see 1.5.2).

The strongest genetic risk factor in SAD is the apolipoprotein E gene, *APOE* [25]. *APOE* is a polymorphic gene with three major alleles, ε2, ε3, and ε4; where ε3 is the most common allele worldwide while the *APOE* ε4-allele is strongly associated with SAD. The risk of developing AD is approximately 15 times higher in homozygotes of the ε4 allele compared with non-carriers [26]. Another gene that may modify the risk of developing AD is the *TREM2* gene that is coding for Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), which has a role in microglia activity modulation and survival [27].

1.1.3 **Diagnosis**

The majority of demented patients have mixed pathologies, making diagnosis difficult [28, 29]. When diagnosing AD, a physician initially investigates the medical history, performs physical and neurological examination, as well as several cognitive tests to evaluate the cognitive state of the patient. The most common diagnostic criteria used are the NINCDS-ADRDA Alzheimer's Criteria. They were first introduced in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [30]. These criteria were updated in 2007 and include the following eight cognitive domains: memory, problem solving, attention, language, constructive abilities, functional abilities, perceptual skills and orientation (see Table 2). The criteria were updated in 2011 and biomarkers [CSF Aβ42, total-tau (T-tau), phosphorylated tau (P-tau), PET, 18-fluorodeoxyglucose (FDG) uptake on PET, and magnetic resonance imaging (MRI) measures of atrophy of the brain] were introduced as evidence of the AD pathophysiological processes. However, the recommendation was to not use biomarkers in clinical routine.

Table 2. The NINCDS-ADRDA Alzheimer's disease criteria, 2011.

Unlikely Alzheimer's disease	The patient presents a dementia syndrome with a sudden onset, focal neurologic signs, or seizures or gait disturbance early in the course of the illness.
Possible Alzheimer's disease	There is a dementia syndrome with an atypical onset, presentation or progression; and without a known etiology; but no co-morbid diseases capable of producing dementia are believed to be the origin of it.
Probable Alzheimer's disease	Dementia has been established by clinical and neuropsychological examination. Cognitive impairments also have to be progressive and be present in two or more areas of cognition. The onset of the deficits has been between the ages of 40 and 90 years and finally there must be an absence of other diseases capable of producing a dementia syndrome.
Definite Alzheimer's disease	The patient meets the criteria for probable Alzheimer's disease and has histopathologic evidence of AD via autopsy or biopsy.

In 2007, the International Working Group (IWG) for New Research Criteria for the diagnosis of AD introduced new guidelines. In 2014 were these criteria updated (IWG-2), consisting of two major parts; the specific clinical phenotype and the *in vivo* evidence. The clinical phenotype of the patient includes both changes in memory (for more than 6 months) and impaired performance on episodic memory test (specific for AD). The *in vivo* evidence includes a) decreased Aβ42 and increased P-tau or T-tau in CSF, b) increased

tracer retention in amyloid PET, and c) AD autosomal dominant mutation in *PSEN1*, *PSEN2*, or *APP* genes. The person must have at least one of these *in vivo* evidences. Also here, the recommendation was to use the biomarkers in research settings [31]. In 2018, the National Institute on Aging and Alzheimer's Association (NIA-AA) published a research framework in which it is recommended that the neuropathologic changes detected by biomarkers define the disease (although it is not yet intended for general clinical practice) [32]. In this framework three basic categories of AD biomarkers are examined, AT(N), each of which gets a positive or negative value. 'A' is referring to amyloid pathology and is examined either by CSF Aβ42 (or Aβ42/Aβ40) or amyloid PET. 'T' refers to tau associated pathologic changes and is examined by either CSF P-tau, or Tau PET. Finally, 'N' refers to neurodegeneration or neuronal injury and is examined by anatomic MRI (showing hypometabolism and atrophy), FDG-PET (examining glucose metabolism) or CSF T-tau.

Imaging as a biomarker can provide a clearer view of the pathological changes in individuals' brains. A very useful technique is PET, using, *e.g.*, the Pittsburgh Compound B (PiB) tracer, which has high affinity to fibrillary Aβ plaques [33, 34]. However, the use of amyloid-PET is still limited in everyday practice. Moreover, other imagining techniques can be used, such as anatomic MRI to examine brain atrophy, FDG-PET to examine glucose metabolism and finally, and more recently developed, tau-PET, to examine tau accumulation.

Even though imaging biomarkers are excellent as they combine both spatial and quantitative information, fluid biomarkers are cheaper and easier to use. Today, there are three established CSF biomarkers for AD, total tau (T-tau), phosphorylated-tau (P-Tau), and Aβ42. It is well established that the main component of amyloid plaques is Aβ42 and that the Aβ42 CSF concentration is decreased in AD patients compared with healthy controls [35]. CSF Aβ40 may be used to normalise Aβ42 concentrations to compensate for inter-individual variation in the general release of Aβ species into the CSF, making the CSF Aβ42/Aβ40 an even better marker for Aβ plaque pathology than Aβ42 alone [36]. In addition, the CSF Aβ42/Aβ40 is shown to have a high concordance with amyloid PET with a sensitivity and specificity of 96% and 91%, respectively [37]. However, lumbar puncture (the procedure of collecting CSF) can be considered to be an invasive method, and not easily assessable in many primary care facilities. For this reason, the urge for a blood biomarker is high. In 2017, Ovod *et al.* [38] showed that plasma Aβ can be used for diagnostic purposes, followed a few months later by a second study by Nakamura *et al.* [39], showing similar results. More recently several groups have worked on the development of tau plasma biomarkers, with focus on the P-tau181 and P-tau217 forms [40-43].

1.2 Cerebral amyloid angiopathy

Cerebral amyloid angiopathy (CAA) is a type of vascular disease present in more than 50% of demented elderly and in more than 80% of AD patients. CAA is characterized by deposition of Aβ peptides in the walls of cerebral leptomeningeal and cortical arteries as well as small-medium vessels. The prevalence of severe CAA is higher in demented patients [44, 45].

Aβ was first isolated from cerebral blood vessels in 1984 [46]. Aβ in the vessel walls may originate from the peripheral blood [47], from the direct production by smooth muscle cells [48], and/or from the perivascular drainage of neuronal Aβ [49]. The failure of eliminating neuronally derived Aβ by the perivascular drainage pathway results in an increase of soluble Aβ and finally to the cognitive decline in CAA [50, 51]. It has previously been shown that CAA contributes to cognitive decline and dementia, by causing vascular lesions, such as (micro)haemorrhage and cerebral ischaemia, and inflammatory changes [52]. Dysfunction in blood vessels may affect both nutrition delivery to the neurons and clearance of Aβ in the brain, thus contributing to cognitive dysfunction, a hypothesis known as the "neurovascular hypothesis" [53].

1.2.1 Pathophysiology

CAA is divided into three stages of progressive Aβ deposition in the vessels. In the first stage, leptomeningeal or parenchymal vessels of neocortex are involved, followed by allocortical areas and the cerebellum in stage two. In the final third stage, subcortical nuclei regions, white matter, and the brain stem are affected [54]. Effects on capillaries in CAA pathology can been observed at all three stages [55].

1.2.2 Genetics

Like AD, CAA exists as both hereditary and sporadic forms; the latter being most common. In hereditary CAA, missense mutations in *APP*, such as *HCHWA-D* and *BRI2* gene-related dementias, have been observed [56]. Mutations in *PSEN1* and *PSEN2* have also been reported to increase CAA [56]. A major genetic risk factor for sporadic CAA (like in AD) is *APOE*. As with AD, the *APOE* ε4 allele is associated with increased Aβ deposition, where it is thought that apoE4 contributes to the failure of efficient clearance of Aβ by causing changes in the structure and function of the capillary and arterial membranes [57-59]. Moreover, *APOE* ε2, which is protective against AD,

appears to be disease-promoting in CAA [60, 61]. *APOE* ε4 carriers are more common in CAA-type 1, while *APOE* ε2 carriers are more common in CAA-type 2 [62].

1.2.3 **Diagnosis**

In 1996, the first diagnostic criteria for CAA were developed with the latest modification in 2018, referred as the Boston criteria for cerebral amyloid angiopathy, version 1.5 [63]. Based on the Boston criteria CAA can be divided into possible CAA, probable CAA, probable CAA with supporting pathology and definite CAA, as shown in Table 3. However, a definite diagnosis can only be given after post-mortem examination. CAA can also be detectable by PiB-PET, with higher global retention compared with controls, and higher occipital-to-global retention compared to AD [64]. In CAA, the levels of both CSF Aβ40 and Aβ42 have previously been shown to be decreased compared with both AD and controls [65]. However, these data needs to be replicated to elucidate if the biomarkers can be used to assist in the diagnosis of CAA in living patients.

Table 3. Version 1.5 of the Boston criteria for cerebral amyloid angiopathy diagnosis.

Possible CAA	**Clinical data and MRI or CT demonstrating:** • **Single lobar, cortical, or cortical-subcortical ICH, CMB;** **OR** • **Presence of cSS (focal or disseminated)** • **Age ≥55 years** • **Absence of other cause of hemorrhagea or cSS**
Probable CAA	**Clinical data and MRI or CT demonstrating:** • **Multiple hemorrhages (ICH, CMB) restricted to lobar, cortical, or cortical-subcortical regions (cerebellar hemorrhage allowed)** **OR** • **Single lobar, cortical, or cortical-subcortical hemorrhage and cSS (focal or disseminated)** • **Age ≥55 years** • **Absence of other cause of hemorrhagea or cSS**
Probable CAA with supporting pathology	**Clinical data and pathologic tissue (evacuated hematoma or cortical biopsy) demonstrating:** • **Lobar, cortical, or cortical-subcortical hemorrhage (including ICH and/or CMB)** • **Some degree of CAA in specimen** • **Absence of other diagnostic lesion**
Definite CAA	**Full post-mortem examination demonstrating:** • **Lobar, cortical, or cortical-subcortical hemorrhage** • **Severe CAA with vasculopathy** • **Absence of other diagnostic lesion**

1.3 Down syndrome dementia

Down syndrome (DS) is a genetic disorder with an extra copy of chromosome 21 [trisomy 21 (T21)] and is the most prevalent genetic risk factor for EOAD [66]. Today around 6 million people have DS worldwide, with an estimated two thirds to develop dementia in their 60s [67] and with the first amyloid depositions appearing in the brain from the mid-30s [66, 68].

1.3.1 Pathophysiology

DS dementia patients have similar neuropathological characteristics as AD patients. DS exhibit an early onset amyloid pathology similar to FAD, but also Aβ pathology in cerebral vasculature similar to SAD. PET studies have shown that the distribution of PiB-binding of Aβ in DS is similar to that of SAD in general, where accumulation of Aβ is observed in the cortical regions, but also with FAD, as Aβ accumulation in DS is also observed in striatum. Moreover, CAA is observed in DS, like in AD, which lowers the age of dementia onset and increases the rate of disease progression, although DS patients are protected from atherosclerosis and hypertension. Apart from the amyloid pathology, the presence of NFTs also seem to follow the same pattern as in AD, but with higher density in DS.

1.3.2 Genetics

Chromosome 21 is the genetic locus of *APP* gene, encoding APP, leading to a life-long overproduction of Aβ [66] due to the extra copy of *APP*. As APP plays an important role in amyloid depositions, the extra copy increases the risk for developing AD in DS patients. There are people that do not have trisomy 21, but instead a triplication of *APP* alone (DupAPP), developing dementia symptoms by the age of 60 with 100% penetrance. Only ~70% of DS individuals develop clinical dementia by age 60, suggesting the presence of other unknown chromosome 21-located genes that modulate the age of dementia onset [66, 69]. *APOE* ε4 also increases the risk of dementia in DS but to a lesser extent than in AD. Apart from *APP*, *BACE2* is also located on chromosome 21, with its extra copy possibly modulating the proteolytic cleavage of APP (see 1.5.2).

1.3.3 Diagnosis

The diagnosis of dementia in DS shows remarkably similar biomarker profiles to those observed in LOAD and EOAD cohorts [70, 71]. In general, low CSF Aβ42 and Aβ42/Aβ40 ratio were observed in DS individuals with AD dementia, while biomarkers of tauopathy (P-tau) and neurodegeneration (T-

tau and NfL) were higher compared with the MCI and control groups [72]. Amyloid-PET is also used to evaluate the amyloid pathology in DS patients, with rates of amyloid accumulation in DS and AD to be similar and enhanced compared to the general population [73].

1.4 Dementia with Lewy bodies and Parkinson's disease dementia

DLB and PDD are classified as α-synucleinopathies together with Parkinson's disease (PD), a group of neurodegenerative diseases characterized by the abnormal accumulation of aggregated α-synuclein in neurons. DLB and PDD have overlapping clinical and neuropathological features, accounting for 5-10% and about 2% of all dementia cases, respectively [2]. They are age related diseases, with the onset of symptoms usually occurring between 50 and 70 years of age [74]. Up to today, no cure is available for DLB/PDD; the current strategies are symptomatic treatments [75].

1.4.1 Pathophysiology

Common pathological characteristics of DLB and PDD are the presence of Lewy body (LB) inclusions, and amyloid plaque pathology. LBs are considered to be the main neuropathological hallmark of DLB and PDD, although their neuropathology is often heterogeneous [76]. LBs main component is α-synuclein and can be found in the brainstem or cortical regions. LBs are also the major pathological hallmark of PD. Apart from LBs, amyloid pathology is also commonly found in both DLB and PDD, in combination with additional AD-related neuropathologies, such as neurofibrillary tangles, amyloid plaques [76] and CAA pathology. Although DLB and PDD are considered α-synucleinopathies, the presence of amyloid pathology may be involved or drive the disease process in these diseases. The amount of cortical LBs correlate with the severity of dementia in both PDD and DLB [77, 78]. However, AD neuropathology is more severe in DLB than in PDD and leads to a worse prognosis for DLB [79].

1.4.2 Genetics

Researchers have not yet identified any specific genetic causes for DLB and PDD; most people diagnosed have no family history of the disorders [80]. However, genetic risks for both AD and PD can increase the risk for developing DLB and PDD, including *APOE* ε4 [81].

1.4.3 Diagnosis

Typical clinical features of PDD and DLB include cognitive problems with fluctuating cognition, executive dysfunction, visual hallucinations, and parkinsonism [82, 83]. Cognitive problems have been found to be more severe in DLB than in PDD [84, 85] whereas motor features are more severe in PDD than in DLB [86]. The clinical diagnosis is based on the so-called 'one year rule' [87]; in DLB the cognitive impairment is diagnosed one year before motor dysfunction symptoms appear [83], while in PDD the first cognitive symptoms appear after at least a year of a well-established PD diagnosis [82, 88]. Despite validated diagnostic criteria, only one in three cases is correctly diagnosed [89].

1.5 Amyloid beta precursor protein processing and amyloid beta peptides

Amyloid plaques are not only one of the hallmarks of AD, but is also a major neuropathological feature in many other types of dementia, as mentioned above. Although this is questionable today, early in the AD research a correlation between the amount of plaques and the severity of dementia was found [90]. In 1984 it was found that the main component of amyloid plaques is Aβ peptides, together with cellular material. Amyloid plaques were first described as sticky clumps that are formed outside and around neurons [46]. Aβ peptides are continually produced during normal cell metabolism, by enzymatic cleavage of APP [91].

1.5.1 The amyloid cascade hypothesis

There are several hypotheses proposed to explain AD pathology. In 1991 the amyloid cascade hypothesis was proposed, which up to today is the most studied one [12, 92]. The original hypothesis is based on the imbalance between production and clearance of Aβ which drives the pathological cascade of AD. The deposition of Aβ in the parenchymal space initiate and drive the rest of the AD pathology, including tau pathology, synaptic dysfunction and finally neuronal cell death, as shown in Figure 3. Several pieces of genetic evidence have supported the amyloid cascade hypothesis, such as different mutations in *APP*, an extra copy of *APP* due to DS, or in the secretases involved in the cleavage of APP (*e.g.*, *PSEN1* and *PSEN2*). The majority of the *APP* mutations increase the production of Aβ, although there are some mutations that are protective [93].

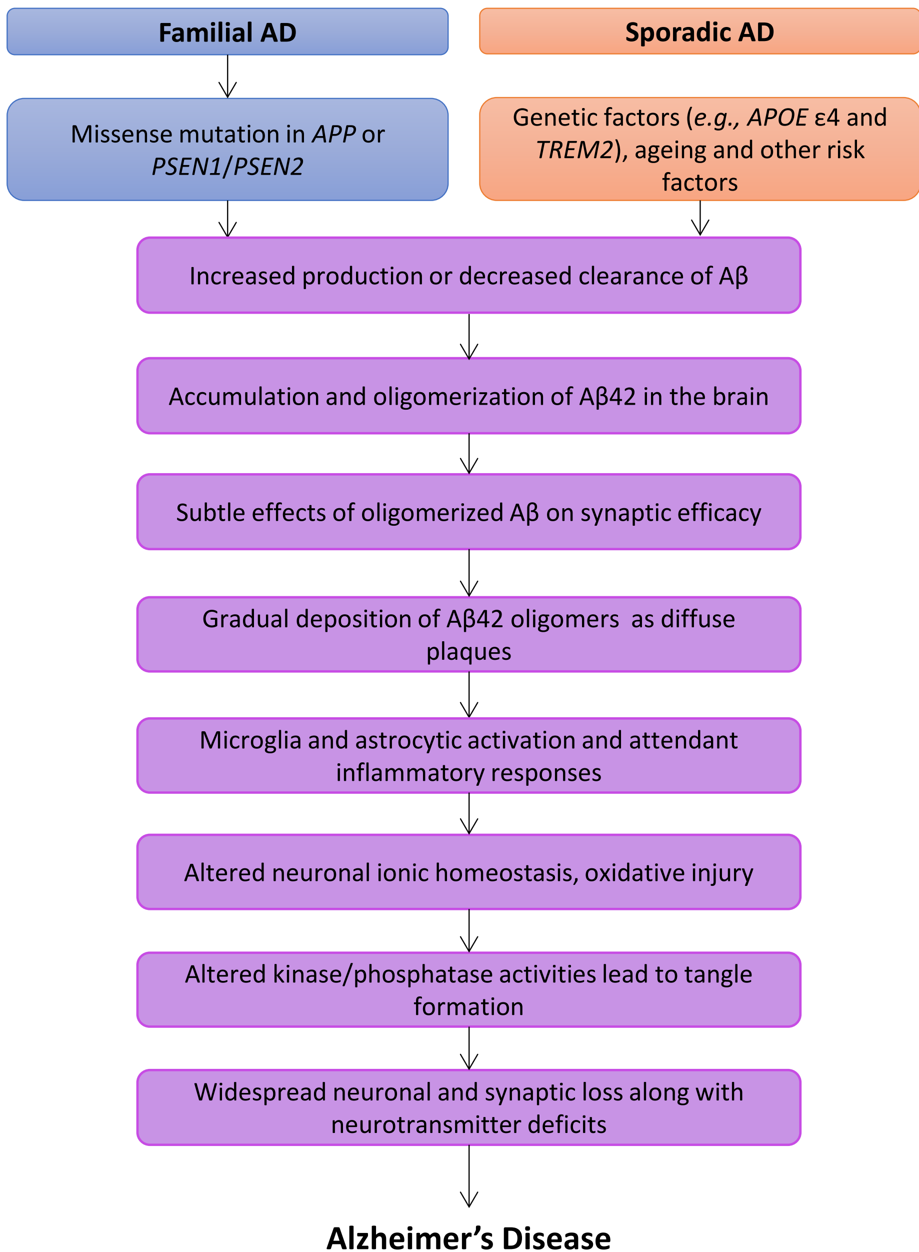


Figure 3. The amyloid cascade hypothesis.

1.5.2 APP processing and Aβ production

The *APP* gene is located on chromosome 21q21 and contains at least 18 exons. It can undergo several alternative slicing events, generating different isoforms. The major isoforms are APP695, APP751 and APP770, named after their respective number of amino acids (aa), see Figure 4. APP is a single-pass transmembrane protein with a large extracellular glycosylated N-terminus and a shorter cytoplasmic C-terminal domain and is located on the surface of the neurons [94].

The most common isoform in the brain is APP695 and this variant has been extensively investigated with regard to AD [94]. The other isoforms, APP770 and APP751, also contain the Kunitz-like serine protease inhibitory (KPI) domain and in addition APP770 has the Ox-2 antigen domain [96]. Being a transmembrane protein, APP is localized on the cell surface; it is mostly localized in the Golgi complex or internalized into endosomes in order to be processed [98-100]. APP processing is commonly categorized into the non-amylogenic pathway [101], where APP is cleaved by α-secretase (precluding the formation of full length Aβ), and the amylogenic pathway where APP is cleaved by β- and γ-secretase [102] (Figure 6).

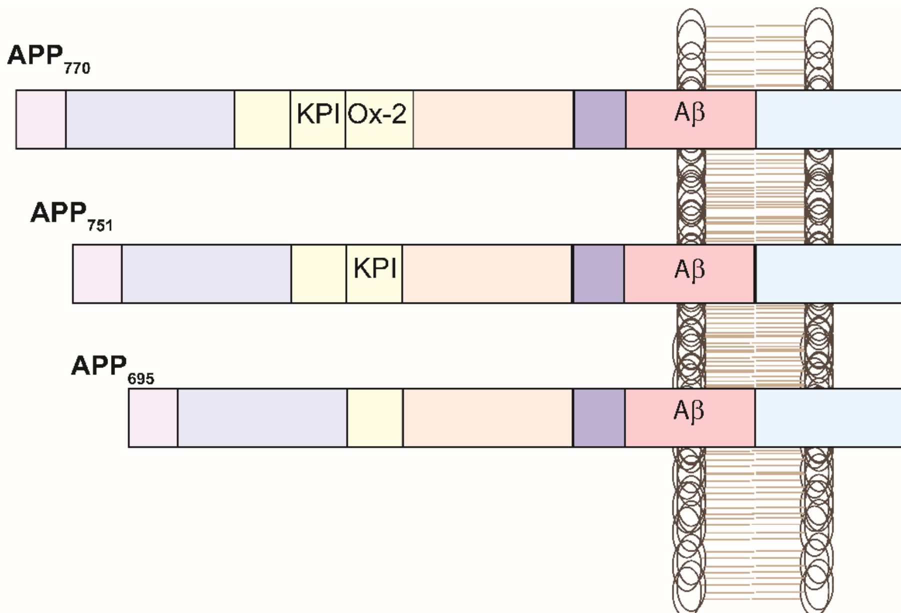


Figure 4. The major APP isoforms.

α-Secretases

The α-secretases are members of the ADAM ('a disintegrin and metalloprotease domain') family. They are expressed on the surfaces of cells and anchored in the cell membrane. The α-secretases cleave in the transmembrane region of APP, between aa 15/16 and 16/17 in the Aβ sequence [101]. Upon this cleavage, the extracellular domain, known as sAPPα is released, and a C-terminal fragment-α (CTFα or C83) is generated. α-Secretase-generated APP products can be processed further by other enzymes giving AβX-15, AβX-16, and Aβ17-X peptides. There are several ADAM proteins, where ADAM10 is the most studied of the α-secretases. ADAM10 consists of two protein domains, a disintegrin domain and a prodomain; the latter is required for APP processing [103]. Once APP reaches the cell surface, α-secretase acts rapidly to cleave it [104]. α-secretase also acts in the Golgi network (its activity depends on protein kinase C) and competes directly with β-secretase for APP during membrane protein maturation [105].

β-Secretases

There are two β-secretases mainly active in the brain, BACE1 and BACE2, which belong to the pepsin family of the aspartic proteases. BACE1 cleaves APP at the N-terminal side of aa 1 of the Aβ sequence, generating an extracellular sAPPβ fragment, and a membrane C-terminal fragment-β (CTFβ or C99). When other enzymes act on CTFβ, Aβ1-X will be generated. BACE1 can also cleave at aa 10/11 (β' site) contributing to the production of AβX-10 and Aβ11-X. BACE1 is mainly active in the Golgi network and in the endosome due to their acidic environment [106, 107], but some of the activity also occurs at the cell surface [108, 109]. BACE2 is a homologue of BACE1 and is involved in the production of AβX-19, AβX-20, and AβX-34 [110].

γ-Secretases

γ-secretase is a membrane-bound protease complex consisting of at least four essential components: the homologous presenilin-1 and -2 (PSEN1 and PSEN2), nicastrin, APH-1 (anterior pharynx-defective 1) and PEN-2 (presenilin enhancer 2) [111]. In order for γ-secretase to be active all four subunits need to be present, with PSEN1 and PSEN2 harbouring the catalytic domain [112, 113]. Although nicastrin is not directly involved in the cleavage, it acts as a gatekeeper, not allowing long ectodomain parts to reach the active side of γ-secretase. This means that APP has to be cleaved first by α- or β-secretases before γ-secretase can act on it [114]. γ-secretase acts on the CTFα or CTFβ transmembrane parts of APP, leading to the production of AβX-37, 38, 39, 40,

42, and 43 [99]. γ-Secretase is present in various subcellular compartments, and its cleavage site depend on the localization and conditions of the compartment [106, 115-118].

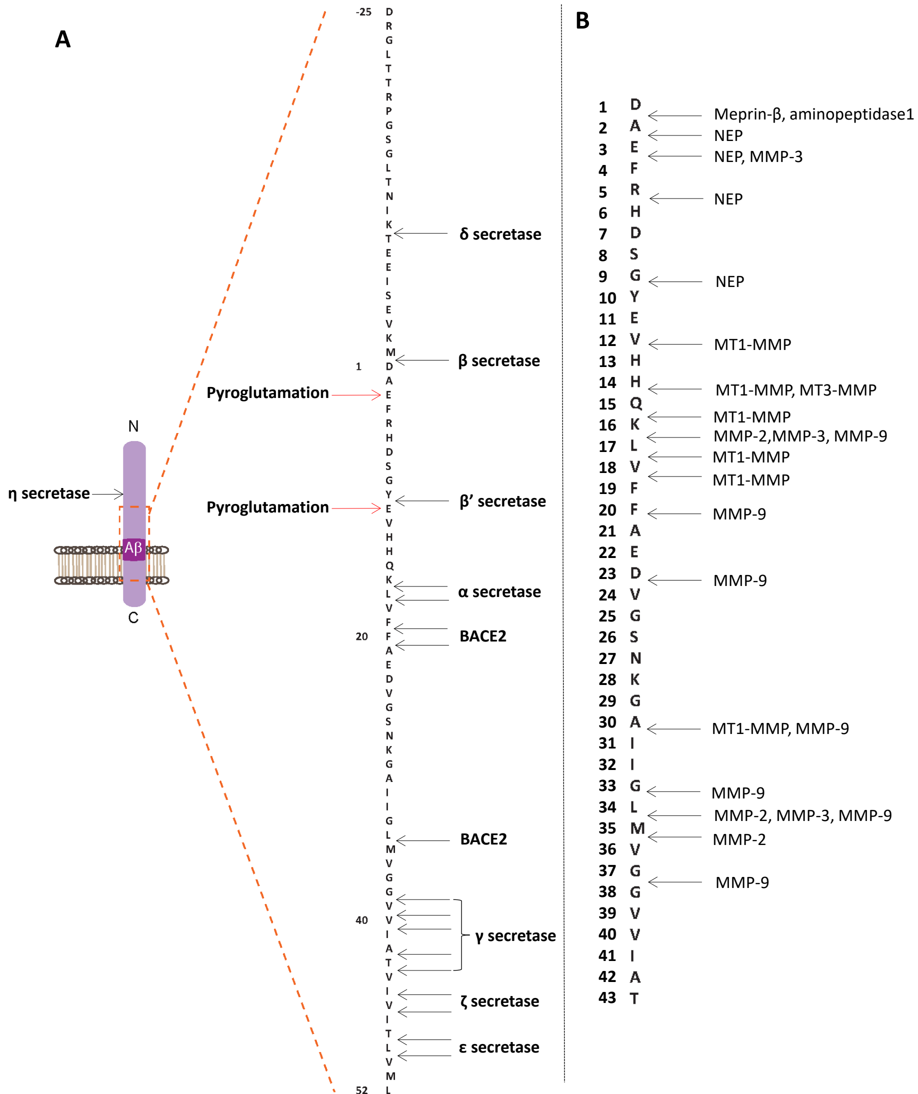


Figure 5. (A) On the left side of the APP/Aβ sequence, the sites for pyroglutamation are shown. On the right side, the cleavage sites of the major enzymes involved in the APP processing are shown. (B) The cleavge sites of the enzymes responsible for Aβ clearance are shown. NEP: neprilysin, MMP-2: Matrix metalloproteinase-2, MMP-3: Matrix metalloproteinase-3, MMP-9: Matrix metalloprotase-9, MT1-MMP: Membrane-Type 1 matrix metalloproteinase and MT3-MMP: Membrane-Type 3 matrix metalloproteinase.

The non-amyloidogenic pathway

In the non-amyloidogenic pathway (Figure 6), APP is initially cleaved by α-secretase followed by a second cleavage by γ-secretase, producing Aβ17-42 [99], thus precluding the formation of full-length Aβ. In an alternative pathway, both α- and β-secretase act on the same APP molecule, producing Aβ1-13 to Aβ1-16 (Figure 6) [119, 120]. The APP cleavage in the non-amyloidogenic pathway takes place on the cell surface [99, 107].

The amyloidogenic pathway

In the amyloidogenic pathway (Figure 6), APP is initially cleaved by β-secretase followed by cleavage by γ-secretase. Before the final γ-secretase cleavage (at aa 37, 38, 39, 40, 42 or 43), proteolysis starts with ε-secretase cleaving at aa 49 or 48, followed by ζ-secretase cleavage at aa 46 or 45. Two different stepwise processes can be followed, the Aβ40 processing pathway and the Aβ42proccessing pathway [99]. In the Aβ40 processing pathway, the initial cleavage of ε-secretase, creating Aβ49, followed by ζ-secretase creating Aβ46. Finally, γ-secretase can cut stepwise creating Aβ43, Aβ40, Aβ38, and Aβ37. In the Aβ42 processing pathway, the initial cleavage of ε-secretase, creating Aβ48, followed by ζ-secretase creating Aβ45, followed by the final stepwise cut by γ-secretase creating Aβ42 and Aβ39. The APP cleavage in the amyloidogenic pathway occurs within intracellular compartments [99].

Other secretases and post translational modifications

There are several enzymes that have been suggested to be involved in the generation of Aβ species, such as δ-secretase, η-secretase (Figure 5A) and meprin β (Figure 5B) [121-123]. δ-Secretase is an asparagine endopeptidase (AEP), while η-secretase is a matrix metalloprotease MT5-MMP. Although they both cut outside the Aβ sequence (Figure 5), they are involved in the production of APP/Aβ fragments, which may also contribute to AD pathology [121, 124]. Meprin β is zinc metalloprotease linked with AD by generating Aβ2-X peptides [125].

There are also several other Aβ peptides, including N-terminally truncated (*e.g.*, Aβ-X, Aβ3-X, Aβ4-X, Aβ5-X and Aβ11-X) and C-terminally truncated, including AβX-38, AβX-40 and AβX-42, that are generated from different enzymes such a combination of β-secretase or neprin-β and γ-secretase [115, 126]. The N-terminal aa of both Aβ3-X and Aβ11-X is glutamic acid, which can become cyclized, either by glutaminyl cyclase or spontaneously, in a reaction called pyroglutamation.

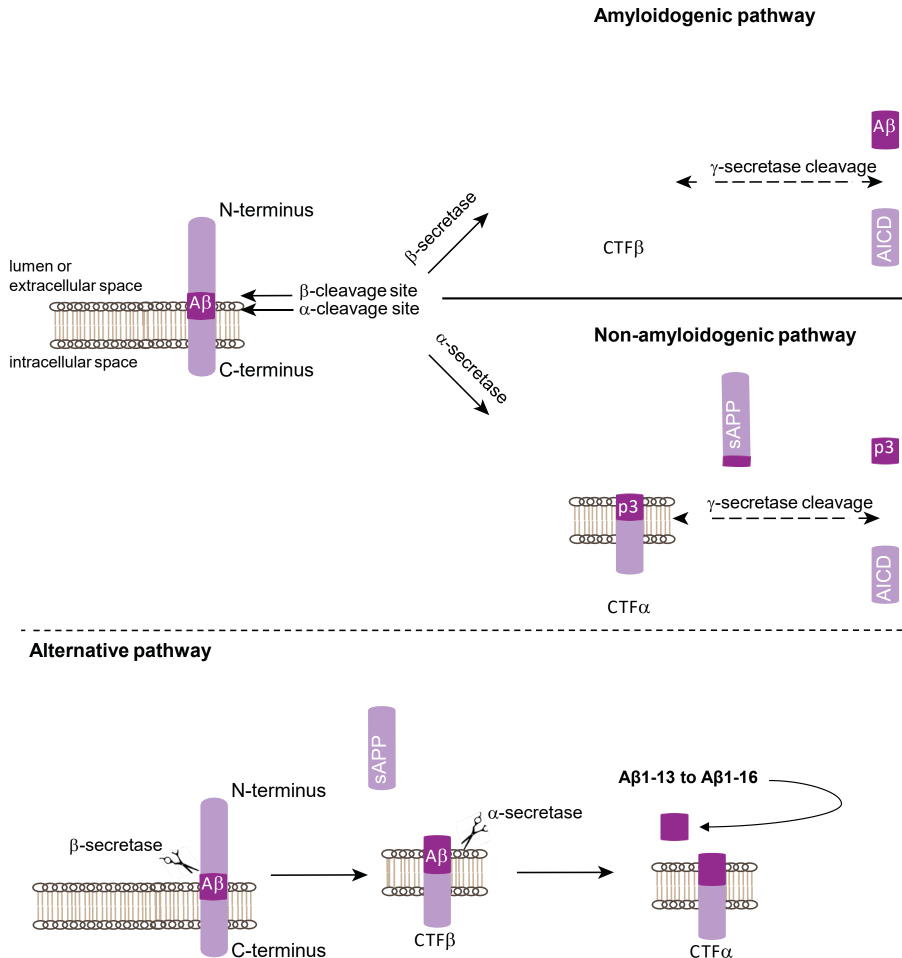


Figure 6. Illustration of the APP processing pathways. In the amyloidogenic pathway, β-secretase cleaves APP, producing sAPPβ and CTFβ, followed by a second cleavage by γ-secretase producing Aβ and AICD. In the non-amyloidogenic pathoway, α-secretase initially cleaves APP producing sAPPα and CTFα, and a second cleavage by γ-secretase produces p3 and AICD. In an alternative pathway, β-secretase cleaves APP, producing sAPPβ and CTFβ followed by α-secretase cleavage producing CTFa and Aβ1-13 to Aβ1-16. Illustration by Tugce M Satir.

1.5.3 Physiological roles of APP and APP cleavage products

Although Aβ and its precursor protein APP have been mainly linked to AD pathology, they may also have physiological functions. For example, APP has been described to be involved in axonogenesis, neuritic growth and neuronal adhesion [128]. Both sAPPα and sAPPβ are involved in neuronal differentiation [129], with their shared N-terminal domain to promote neurite and axonal outgrowth [130]. The C-terminal domain of sAPPα is neuroprotective and contributes to cognitive function [131]. AICD regulates transcription and interacts with adaptor proteins to regulate cell signalling [96, 132]. Aβ peptides are important for central nervous system viability during brain embryogenesis. Short Aβ peptides (produced by α- and β-secretase) may act as synaptic regulators [133]. Despite the neurotoxicity of aggregated Aβ40 and/or Aβ42, monomeric Aβ40 and Aβ42 can promote NPCs proliferation and neurogenesis [134].

1.5.4 Aβ clearance

Under physiological conditions, Aβ can be cleared from the brain in several ways. Astrocytes and microglial cells can endocytose Aβ [135], Aβ can be enzymatically degraded [136, 137] or cleared from the brain via the blood-brain-barrier [138], and finally, can be drained through the perivascular space [49]. Multiple Aβ-degrading enzymes are proposed to be involved in the Aβ clearance process including neprilysin (NEP, also able to degrade oligomeric Aβ), insulin-degrading enzyme (IDE, poor degradation of oligomeric Aβ), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), glutamate carboxypeptidase II, endothelin-converting enzyme (ECE), plasmin, angiotensin-converting enzyme (ACE) and cathepsin B[139]. The enzymes cleavage sites are shown in Figure 5.

1.5.5 Aβ aggregation

Aβ aggregation is a progressive process, as showing in Figure 7. Aβ contains two hydrophobic parts, the C-terminus of the full length Aβ1-42, and the Aβ16-20 part consisting of the KLVFF aa sequence. The initial β-hairpin is formed by the folding of the C-terminal region onto the KLVFF [24, 140-142]. Later, two β-hairpins will form the first dimer and continue forming different oligomers [140, 142-144]. The oligomerization eventually will lead to the formation of β-sheets structures, and in turn to the formation of protofibrils [142, 145-147]. Finally, two or more protofibrils will twist around each other forming fibrils [141, 142, 147]. So far, all forms of Aβ are soluble. Aβ aggregates can been characterized either as 'soluble' or 'insoluble', depending on whether the Aβ aggregates will stay in the aqueous phase after

high-speed centrifugation or not [148]. To extract the insoluble Aβ aggregates (consisting of amyloid plaques), detergent and harsh acids (like 70% formic acid) are required. Recently, it has been that proposed that pyroglutamation together with Aβ1-40 deposition are critical events in initiating the formation and maturation of diffuse plaques into cored plaques, with increased neurotoxicity and eventual development into AD [149]. The short Aβ peptides produced by α- and β-secretase (Figure 6) do not contain hydrophobic parts and do not aggregate [133, 150].

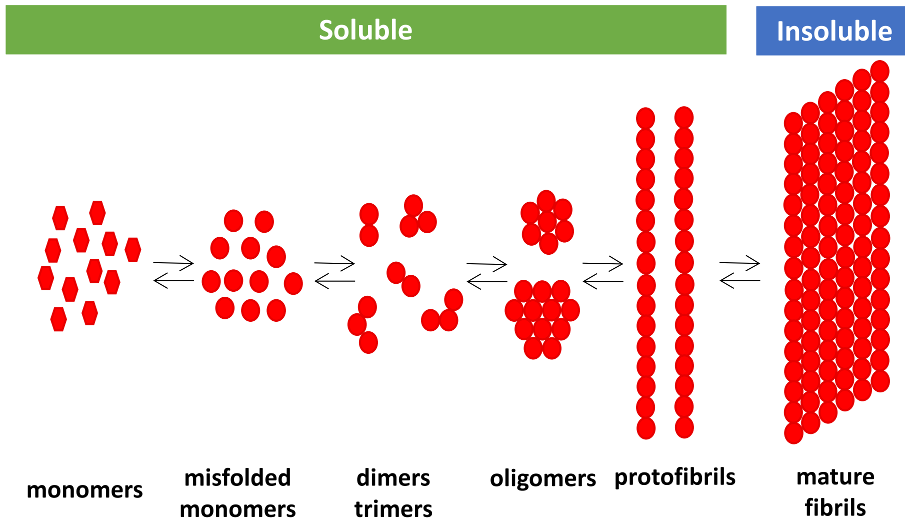


Figure 7. Initially, monomers become misfolded. The misfolded monomers will then form dimers, oligomers and finally protofibrils. Protofibrils can aggregate further, creating mature fibrils that are insoluble.

1.5.6 **Aβ neuropathology**

Aggregated Aβ depositions can have different morphology. Amyloid plaques in the extracellular space of parenchyma can form diffuse and cored amyloid plaques. Studies have identified AβX-40 as the major component of the core of an amyloid plaque, while AβX-42 is localized in diffuse amyloid aggregates [149, 151-153]. Recently it was proposed that pyroglutamation together with Aβ1-40 deposition are critical events in initiating the formation and maturation of diffuse plaques into cored plaques, with increased neurotoxicity and eventual development into AD [149]. The insoluble Aβ aggregates forming the cored plaques are also known as congophilic plaques. They can be stained by Congo red and thioflavin-S because of their β-sheet structure. On the other hand, the majority of diffuse plaques are not visible

with the above mentioned staining techniques, instead anti-Aβ antibodies are used for these plaques.

Aβ deposition and spread in AD is well characterized and is divided into five phases, or more commonly known Thal phases [20]. In the first phase, Aβ is only present in neocortex, with allocortical brain regions to be added in phase two. In phase three, Aβ is also observed in the diencephalic nuclei, the striatum, and the cholinergic nuclei of the basal forebrain. In phase four, Aβ is present in several brainstem nuclei, and phase five is characterized by Aβ deposition in cerebellum.

Apart from the parenchyma, Aβ depositions may also be observed in the vessel walls; a characteristic of CAA, where Aβ1-40 and Aβ4-40 are the major forms [149, 154]. The leptomeningeal vessels are affected first, followed by parenchymal vessels, with increased severity as mentioned above (see 1.2.1).

1.6 Therapeutics

Up to today, there is no treatment that can cure AD or any other type of dementia. A main focus for several years was the development of β- and γ-secretases inhibitors [155]. However, the γ-secretase inhibitors have not been successful due to lack of efficacy and/or side effects [156]. Despite the fact that β-secretase inhibition was successful and Aβ production was reduced, side effects, such as worsening in cognition, led to discontinuation of these studies [157, 158]. Furthermore, α-secretase activators have been proposed as potential treatments for AD, with positive efficacy results [159, 160]. Recently there have been some promising results using treatments with antibodies [161]. One of these is aducanumab [161, 162], which is currently pending for FDA (US Food and Drug Administration) approval and further considered for an application to the European Medicines Agency (EMA). Another antibody with positive results and currently in phase 3 is BAN2401. BAN2401 is a humanized version of mAb158, which is an antibody that targets Aβ protofibrils [163]. However, there are drugs that ease the symptoms for the patients. Memory symptoms can be treated with cholinesterase inhibitors and memantine [164]. Moreover, patients can also receive treatment for behaviour and sleep changes.

2 AIM

2.1 General aim

The general aim of this thesis was to investigate the differences between Aβ deposits in human brain tissue in different neurodegenerative dementias (toxic Aβ), as well as in patients with amyloid pathology but not cognitive impairment (non-toxic Aβ).

2.2 Specific aims

Paper I: Investigate the unique 'lake-like' appearance of the Aβ deposition in the presubiculum in comparison with the neighbouring entorhinal region.

Paper II: Explore the amyloid signature in AD patients with and without CAA pathology.

Paper III: Compare the Aβ peptide pattern in AD, DS and control patients, and investigate possible differences in the amyloid deposition and aggregation between the disease groups.

Paper IV: Investigate potential differences in synaptic degeneration and Aβ peptide pattern between AD and PA, as well as find any possible correlation between them.

Paper V: Characterize Aβ peptides present in DLB and PDD and investigate any qualitative or quantitative differences in the amyloid pathology between them.

3 MATERIALS AND METHODS

The focus in this thesis is on analysis of Aβ peptides in post-mortem human brain tissue. Several approaches were used, all of them centering on sample preparation and mass spectrometry.

3.1 Sample collection

CSF was used for method development and for quality controls through different experiments. It was acquired from de-identified samples from the Clinical Neurochemistry laboratory at the Sahlgrenska University Hospital/Mölndal, upon ethical approval by the regional ethical committee. The CSF was collected according to a standardised procedure, with collection of 12 mL by lumbar puncture (LP) through the L3/L4 or L4/L5 interspace of the spinal discs. The CSF was collected in polypropylene tubes, centrifuged at 2000×g at 4 °C for 10 min, to remove any debris, aliquoted in new tubes and stored at -80 °C until further use.

Brain samples were acquired from either the Queen Square Brain Bank for Neurological Disorders (QSBB), UCL, London, or from the Netherlands Brain Bank (NBB), Amsterdam, The Netherlands. All subjects gave their informed consent to donate their post mortem brains for scientific research, with ethical permissions approved by regional ethical committees. After the arrival of the brain to the brain bank, one hemisphere was fixed in formalin to be used for neuropathological diagnosis and immunohistochemical experiments. The other hemisphere was coronally sliced and, fast-frozen on brass plates at -80 °C. The tissue was then stored at -80 °C until further use

3.2 Ethical considerations

Working with human material is a sensitive subject. Before the start of the experimental work, hypotheses were defined and detailed study plans were designed to ensure proper handling of the tissue. The studies were conducted so that the tissue consumption was kept as low as possible and to ensure the safety of involved laboratory personnel. All samples handled were previously de-identified.

The experimental work followed the Helsinki declaration for “Ethical principles for medical research involving human subjects” and “Good research practice” by the Swedish Research Council. All ethical permissions from their site were submitted to Gothenburg’s ethical vetting board, which issued the permission to handle the material (Dnr: 012-15).

3.3 Sample preparation

3.3.1 Protein extraction from brain tissue

In this thesis was a physical method, more specifically mechanical force (with metal beads), used to homogenize and lyse cells from human brain tissue. To avoid any protein degradation, a protein inhibitor cocktail was used (cOmplete™ Protease Inhibitor Cocktail, Roche) and all homogenization was performed at low temperature (on ice). The extraction buffer [Tris Buffered Saline (TBS)] used contained tris(hydroxymethyl)aminomethane [$NH_2C(CH_2OH)_3$] (Tris base) to prevent protein denaturation and to stabilize the pH, and low salt (NaCl) concentration to prevent nonspecific protein interactions. Depending on the downstream analysis [such as liquid chromatography (LC) and mass spectrometry (MS), see 3.4.1 and 3.4.2], EDTA and salts had to be eliminated [in this case by immunoprecipitation (IP), see 3.3.3].

After the tissue lysis in TBS, centrifugation of the homogenate was performed to remove, for example, insoluble cell debris. After centrifugation, the supernatant contained all the water soluble species, while the pellet contained membrane bound and water insoluble species. Depending on the nature of the protein/peptides of interest, an additional cell fractionation and/or subcellular structure separation might be needed. This can be achieved by the use of different detergent/buffer solutions and density gradient methods.

In this work, a protocol for detection of Aβ peptides from brain tissue was optimized. After the initial centrifugation, soluble Aβ peptides, oligomers and fibrils can be found in the supernatant (water soluble fraction, or TBS fraction). To isolate the Aβ peptides from the plaques, 70% formic acid (FA) was used to dissolve the amyloid plaques, accompanied by a short step of mechanical force. To further enhance this procedure, a short sonication step was added to further dissolve the amyloid plaques. After centrifugation, the supernatant contained Aβ peptides and water insoluble proteins (FA fraction), while the remaining pellet contained lipids and cell debris.

After collection of the TBS and FA fractions, the samples were stored at -80 °C until further use. TBS fraction samples were directly stored without any further handling, while the FA fraction samples must be dried in a vacuum centrifuge before storage at -80 °C. The reason behind drying the FA fraction was to have a manageable sample volume in the downstream analysis.

3.3.2 Brain section slices

To analyse Aβ peptides in a specific brain region, 10 µm thick sections were cut from fresh-frozen tissue using a cryostat (Leica). It is crucial to keep a low temperature when handling frozen tissue to avoid tissue thawing and ultimately protein degradation. The tissue slices were stored at -80 °C pending further use.

3.3.3 Immunoprecipitation

Working with complex samples, such as tissue homogenates, plasma, or CSF, is challenging since the analyte of interest may be present at a low concentration compared with the total protein/peptide content. One way to reduce the complexity of the sample prior to MS analysis is to use IP.

IP is an affinity purification method where a specific protein is isolated and concentrated out of a complex sample. IP is based on an antibody coupled to a solid surface. In this work was tube-based micron-size magnetic beads used, also known by the trade mark Dynabeads. The workflow is described in Figure 8. The magnetic beads used in this thesis were pre-coated with sheep anti-mouse IgG (Figure 8A). Initially the magnetic beads were incubated with the anti-Aβ antibody allowing binding of the antibody to the beads (Figure 8B). After this first incubation, the above complex was further incubated with the sample of interest (Figure 8C). During this incubation, the anti-Aβ antibody binds the Aβ present in the sample. In this step it is crucial to select the appropriate detergent and the amount used, based on the analyte of interest. Separation of Aβ from the rest of proteins was achieved by applying a magnetic field and removing the supernatant (Figure 8D). After the separation, the unbound proteins have been washed away (Figure 8E). By using FA to lower the pH, the Aβ peptides dissociates from the beads complex (Figure 8F). Finally, by applying a magnetic field again, the FA fraction, containing the Aβ peptides, was removed (Figure 8G) and ready to be used for downstream applications without the beads (Figure 8H).

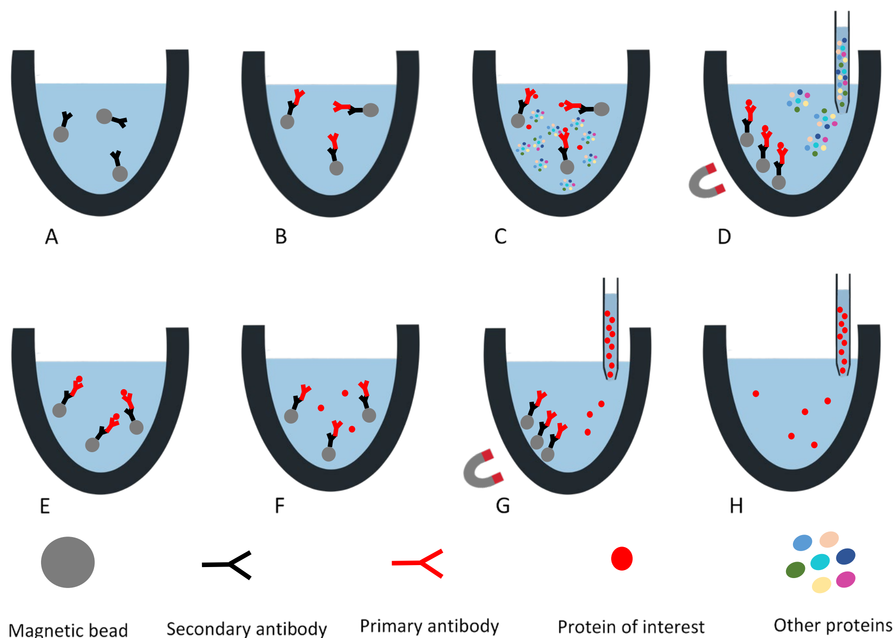


Figure 8. Immunoprecipitation workflow.

3.3.4 **Immunohistochemistry**

Immunohistochemistry (IHC) is a staining method which in this project was used to visualize Aβ in tissue. There are two distinct parts of IHC, the sample preparation step and the sample-staining step. There are many different approaches, but here the IHC in frozen tissue using the avidin-biotin-complex will be described.

First, the tissue sections used for IHC were thawed and dried at room temperature (RT). To minimize protein degradation, the drying step should be short; preferably conducted in a desiccator. Second, the tissue has to be fixed to the slide to avoid any detachment during the following steps. For the fixing process, either a crosslinker (most often a formaldehyde-based solution) or alcohols can be used, depending on the downstream analysis. In the next step, elimination of the endogenous peroxidase activity was required (since the avidin-biotin-complex is used) which was accomplished by incubating the section in methanol and 0.3% hydrogen peroxide. An antigen retrieval step using 70% FA was essential for the antibody to be able to reach the desired target. Before incubation with the anti-Aβ antibody, blocking (using milk or serum) was required to avoid unspecific binding. This was

followed by incubation with a biotinylated secondary antibody, which was a required step for the avidin-biotin-complex detection system. Next, an avidin-biotinylated horseradish peroxidase (HRP) complex was incubated with the tissue samples followed by chromogenic detection. Finally, the tissue was incubated with di-aminobenzidine (DAB), where it reacted with the HRP to form a brown precipitate at the site of antibody binding. After the end of the staining, the slides could be further counterstained with hematoxylin (nuclei visualization), to help identify the cell's morphology. Washes were used to avoid any residue transfer between the different steps. A schematic representation is shown in Figure 9.

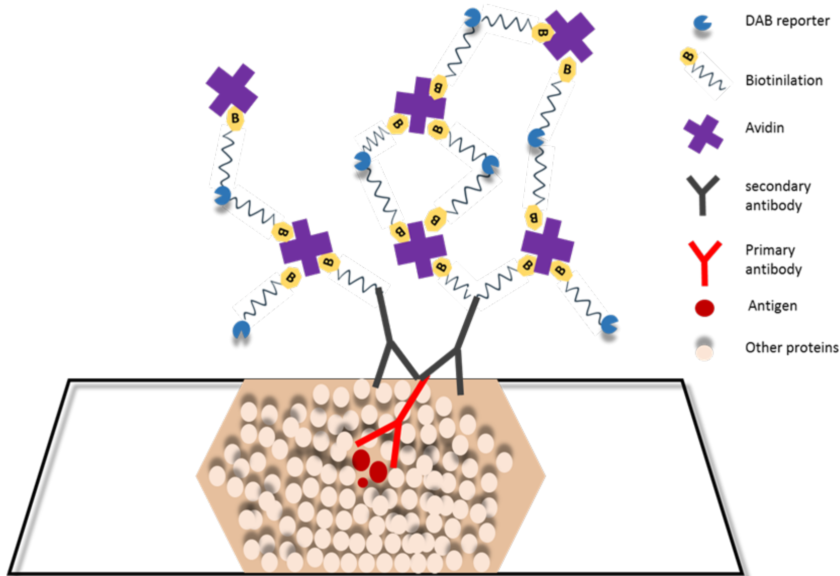


Figure 9. Immunohistochemistry complex.

3.3.5 Laser capture microdissection

Laser capture microdissection (LCM) is a method used to isolate a specific population of interest from tissue (or cells) with the help of a laser. LCM is appropriate for both genomics and proteomics downstream analysis. The sample consists of the stained tissue section (that has previously been prepared as described above), which is placed in a specific thin membrane (polyethylene naphthalene) slide.

The specimen is placed under a microscope, which can be either an optical or fluorescent microscope, depending on the staining technique used. Using a

software interface, the user can mark the area of interest (Figure 10A). After defining the area of interest, the laser moves around and cuts out the pre-marked area automatically. The cut tissue will then be transported to a tube by either gravity, or by a laser pressure catapult (Figure 10 B and C), leaving holes in the tissue (Figure 10 D). In the first configuration, an upright microscope is used (LEICA) and the laser beam cuts the sample from above by moving a dichroic mirror, and by taking advantage of the gravity the cut-out piece is captured into a tube placed below the sample. In the second procedure an alternative configurations used where the laser pulse cuts around the sample and by generating a photonic force, the material slides upwards to a specific adhesive cap tube (ZEISS).

Figure 10. Laser capture microdissection. (A) Selection and cutting of the desired area. (B) The cut piece will fly into the cap of a tube, where all pieces will be selected (C). In the end the tissue is left with holes in the areas that have been removed (D). Panel B was adapted from Zeiss.

In the current work, amyloid plaques have been stained and isolated mainly by a LEICA LCM system. Although the above procedure can be semi-automated, it is time consuming, since for sufficient sample collection 300-500 amyloid plaques needs to be collected which typically takes 60-90 min.

3.4 Analytical methods

Proteomics is defined as the study of the proteome (all proteins in an organism or in a defined compartment such as an organ) and it was first introduced by Peter James in 1997 [165]. To study the proteome, MS is nowadays the method of choice replacing the gel-based methods. MS is currently an analytical technique that is used to analyse a compound by measuring the mass-to-charge ratio (*m/z)* of produced ions (as described below).

In this work two different mass spectrometry methods were used, MALDI-TOF-MS and LC-ESI-MS with tandem MS (MS/MS).

3.4.1 Liquid chromatography

Liquid chromatography (LC) is an analytical method, where a complex mixture can be separated based on chemical properties. LC requires two components, a liquid mobile phase and a stationary phase. Typically, the stationary phase is a porous solid that can been packed into a column. Today, the packing particles of the stationary phase are very small and the system pressure is relative high in the column, the system is therefore referred to as high-performance liquid chromatography (HPLC). Based on the stationary phase, LC can be categorized into normal phase and reverse phase (RP) chromatography. The mobile and stationary phase should have different polarities. In normal phase chromatography the stationary phase is more polar than the mobile phase, while in RP the mobile phase is more polar than the stationary. In this work, RP-HPLC was used.

In RP-HPLC the stationary phase consists typically of surface-modified silica; for peptide analysis, octadecyl carbon chains (C18) are typically bound to the silica. A common way to operate is to use a gradient, also referred to as gradient elution chromatography. By adjusting the amount of water or organic solvent, the retention time of a specific compound can increase or decrease depends on its hydrophobicity. In a mixture, all the different components are retained to different degrees in the column depending on their polarity, structural characteristics, and interaction with the mobile phase. In order to dissociate less polar compounds and elute them from the column, a gradual decrease of the mobile phase polarity is applied.

RP-HPLC, which was used in the present work, can readily be coupled on-line to MS. In this way, the sample is introduced directly to the inlet of the mass spectrometer, as described below. By using this approach, complex samples can be handled, allowing identification of thousands of proteins in a single analysis.

3.4.2 Mass spectrometry

Mass spectrometry is an analytical method that separates ions based on their *m/z*. MS is a sensitive technique that can be used to identify and quantify proteins, peptides, and lipids and also provide structural information. All mass spectrometers consist of three main parts, an ion source, an analyser, and a detector, as shown in Figure 11.

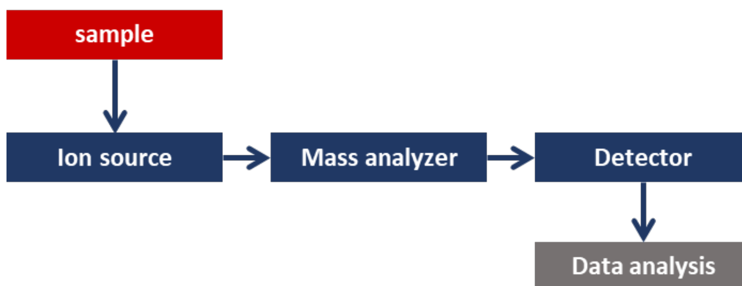


Figure 11.Mass spectrometry principle.

Ionization

Protein and peptide analysis by MS became feasible after the almost simultaneous development of two different soft ionization techniques; electrospray ionization (ESI) [166, 167] and matrix-assisted laser desorption/ionization (MALDI) [168, 169]. ESI is softer than MALDI, but also more sensitive to contamination. However, ESI is well suited for direct coupling to an LC system, providing both an additional separation step as well as sample cleanup. In ESI, gas-phase ions are created in an electrostatic field that is applied between a liquid containing emitter and the inlet of the mass spectrometer (Figure 12). The ionization procedure in MALDI is different. Here, the analyte is mixed with a UV-absorbing matrix and spotted onto a metal target plate, which is inserted into the mass spectrometer. The sample spot is then irradiated with laser pulses, causing matrix and analyte molecules to desorb. A portion of the desorbed analyte molecules are ionized, thus becoming gas-phase ions.

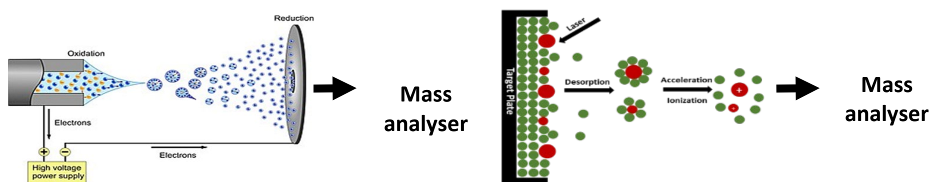


Figure 12. ESI and MALDI ionization techniques. Figure panel for ESI was adapted from Wikipedia.

Mass analyzers

There are several types of mass analyzers and in this thesis were a quadrupole, orbitrap, and time-of-flight (TOF) analyser used.

A quadrupole consists of four rods with hyperbolic inner surface, arranged in parallel and employs a superposition of direct current (DC) and radio frequency (RF) potentials. The opposite rods are connected electrically in pairs, with the two pairs having opposite potentials, thus creating a quadrupolar field. The ions are entering the opening between the rods and travel parallel to them (Figure 13).

Only ions within a specified m/z range will have stable trajectories and pass through the quadrupole and finally reach the detector on the far side. A mass spectrum can be acquired by scanning the ratio of DC and RF potentials and monitoring the abundance of the detection ions. Although its resolution is comparative poor, the quadrupole is relatively sensitive and has a high dynamic range, and it can act as a well-defined m/z filter, making it ideal for tandem analysis (see below).

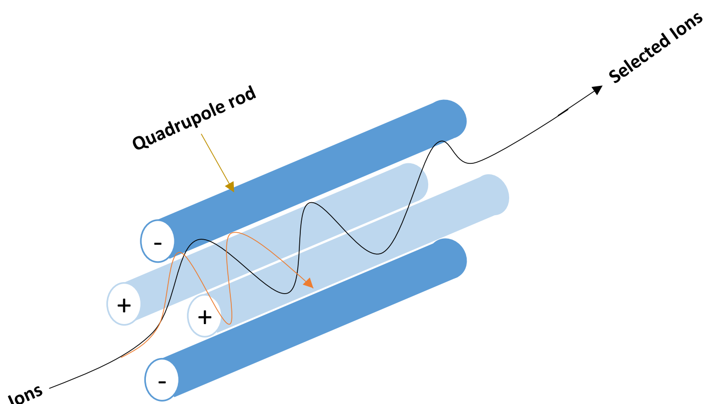


Figure 13. Quadrupole schematic.

The orbitrap has a very high resolution and excellent mass accuracy. The separation is based on an electric field between the inner and outer electrode in the orbitrap's cavity, where the trapped ions are orbiting around the central electrode as well as moving along its axis. The m/z separation is in this case obtained by measuring the m/z dependent frequency of the ion movement along the axis of the central electrode (Figure 15).

In TOF, the ions will accelerate toward ground potential (Figure 16). Exiting the acceleration region, all ions with the same charge will have the same kinetic energy, but different velocities depending on their mass. The ions then travel in a field-free region toward the detector, and are thus separated according to the time difference between a start signal and the pulse generated when an ion hits the detector. This requires a well-defined start signal, making MALDI well suited as an ion source. To improve mass resolution when using a TOF analyser, energy focussing devices are utilised. Delayed extraction, which is particularly important in combination with MALDI, compensates for the initial velocity spread of the desorbed ions by applying the extraction voltage shortly after the laser pulse hits the target. Delayed extraction also improves the sensitivity by reducing fragmentation due to collisions in the MALDI ion source. In addition, focusing can also be obtained by using a reflector; the combination of these devices allows for isotopic resolution up to and above 10 kDa.

Detection

Based on the way the signal is recorded, detectors can be divided into two main categories; those that record impacting ions and those that detect an image current. Typical impact detectors produce electron cascades after each impact, which in turn are amplified to a measurable electric signal. In the image current detector the ions do not hit the detector; instead, ion close to the detector electrode give rise to an image current in the electrode which is subsequently amplified. This method is utilized in certain trapping analyzers, such as the orbitrap, and a transient signal is recorded for a pre-specified time. Since the ions' oscillation frequencies are related to their m/z's, the latter can be obtained through Fourier transformation.

Tandem mass spectrometry

In order to increase the analytical ability, two or more mass analyser can be combined, known as tandem MS or MS/MS. Both mass analysers can be the same (*e.g.*, triple quadrupole, TOF/TOF) or different, (*e.g.*, quadrupole–orbitrap instrument). Initially the molecules are ionized (as described above) and enter in the first mass analyser (MS1), where the ions are separated by their m/z. After the ions exit MS1, ions with a specific m/z are selected for fragmentation (in this case collision-induced dissociation). The produced fragment ions enter the second mass analyser (MS2), where they are separated by their m/z and finally detected. The introduction of a fragmentation step makes it possible to identify ions since part of their internal structure can be determined. For example, without MS/MS, ions with

very similar m/z and retention time would not be distinguishable. The general principle is shown in Figure 14.

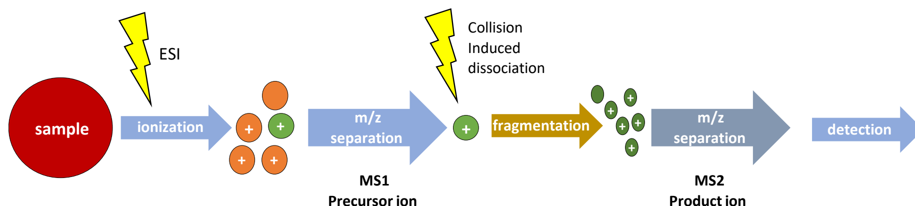


Figure 14. Schematic of tandem MS.

To increase sensitivity, a small number of peptide ion can be selected for analysis, a procedure known as targeted proteomics. The sample is typically, spiked with known amounts of heavy-isotope labelled forms of the selected peptides so that absolute quantification can be obtained.

3.4.3 Instrumentation

Many different mass spectrometers can be produced by combining different ion sources and one or more analysers. In these studies, two different mass spectrometers were used, a Q Exactive, which is an ESI hybrid quadrupole–orbitrap instrument (Thermo Fisher Scientific) and an ultrafleXtreme, which is a MALDI TOF/TOF instrument (Bruker Daltonics).

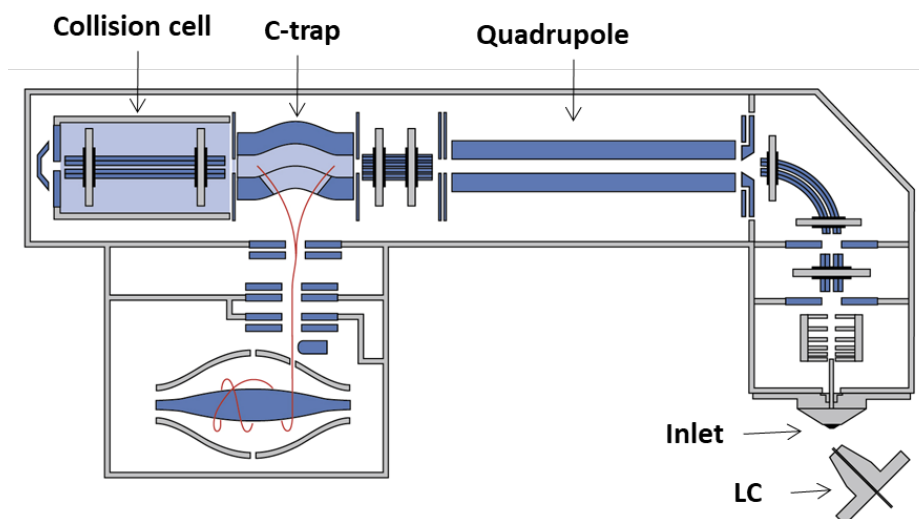


Figure 15.Q Exactive instrumentation set up. Adapted from Thermo Fisher Scientific.

ESI-quadrupole–orbitrap-MS

In the Q Exactive (Figure 15) the quadrupole is only used as an analyzer when operating in MS/MS mode. Precursor ions are selected using the quadrupole and then fragmented in the collision cell before entering the orbitrap for the ion separation and detection by the image current detector.

MALDI-TOF

The instrumentation set up of MALDI-TOF is shown in Figure 16. In order to ionize the proteins and peptides of interest, the sample has to be mixed with an appropriate matrix. It is crucial to select the appropriate matrix in order to get a good crystallisation of the sample. Here, a double layer method was used, and a first layer (seed layer) was spotted to the MALDI target, followed by the sample/matrix mixture. The matrix used for Aβ analysis was α-cyano-4-hydroxycinnamic acid.

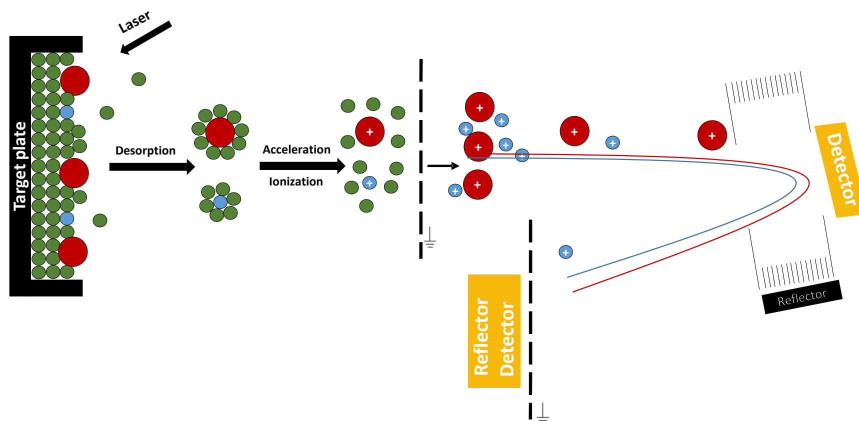


Figure 16. MALDI-TOF schematic.

3.5 Data analysis and evaluation

Depending on instrument and aim of the analysis, different software was used.

3.5.1 MALDI-TOF

Acquired data was internally calibrated using five Aβ peptides, spanning the *m/z* of interest, using Flexanalysis 3.4 (Bruker). Peak lists created using Bruker's built-in SNAP function, were exported to an in-house developed software for further analysis. Samples were analyzed in duplicates and peak

areas were normalized to the average area of either all or the most abundant peptides, depending on the experiment. For some samples the isotopic envelopes of Aβ1-40 and Aβ3-42 were overlapping. To overcome this problem, a manual evaluation was performed.

3.5.2 LC-quadrupole-orbitrap

The Mascot search engine (Matrix Science) is a data base search software matching acquired data with an already existing theoretical database. For larger peptides, such as Aβ, isotope and charge deconvolution needs to be perform before data analysis. This was performed using Mascot Distiller (Matrix Science).

Two quantitative analysis software were required to analyse data acquired from LC-ESI-MS. One of them is PEAKS Studio, which has a built-in deconvolution procedure, which automatically validates search results. For peptides with poor mass accuracy (>5 ppm) or search score, manual evaluation of the MS/MS spectra was performed.

The other one is Skyline that was used to obtain quantitative data from full MS measurements, from data dependent acquisition. To ensure that the correct peptide peak was selected, only peptides that were previously identified by PEAKS (MS/MS spectra acquired in the same run) were used. Peak areas were selected manually for each peptide in each sample.

3.5.3 Statistical analyses

Statistical analysis was performed using GraphPad Prism. In all the studies, non-parametric tests were used. To compare two groups the Mann-Whitney U-test was used, while to compare three or more groups Kruskal-Wallis was used. Statistical significance was given when comparisons gave p-values ≤0.05. For association analysis, Spearman's correlation was used.

4 RESULTS AND DISCUSSION

PAPER I: The presubiculum is preserved from neurodegenerative changes in Alzheimer's disease

The anatomical area of the presubiculum is characterized by the presence of a single large evenly distributed 'lake-like' Aβ deposit with minimal tau deposition or accumulation of inflammatory markers. In parallel, the neighbouring entorhinal cortex has severe neuronal loss, cored amyloid plaques, frequent NFT's, activated microglia and activated astrocytes. The aim of this study was to investigate the pathological differences behind the unique 'lake-like' appearance of Aβ deposition in the presubiculum in comparison with the neighbouring entorhinal region.

To achieve this, several techniques were used. Initially, the amyloid morphology of the presubiculum was investigated by immunohistochemical methods in patients having three different cerebral amyloidosis, SAD, and two types of FAD, familial British dementia (FBD) and familial Danish dementia (FDD). Like in AD, both FBD and FDD exhibit structures containing amyloid peptides, amyloid-Bri (ABri) and amyloid-Dan (ADan), respectively. In order to determine the possible protection against neurodegeneration and neuroinflammation, quantitative evaluation of the total tau pathology, the number of neurofibrillary tangles and levels of microglial activation were compared between presubiculum and entorhinal cortex in SAD and FAD patients. Laser capture microdissection was utilized to isolate the amyloid positive deposition in presubiculum and entorhinal cortex from SAD and FAD patients. The tissue isolated was further analysed by MALDI-TOF-MS to

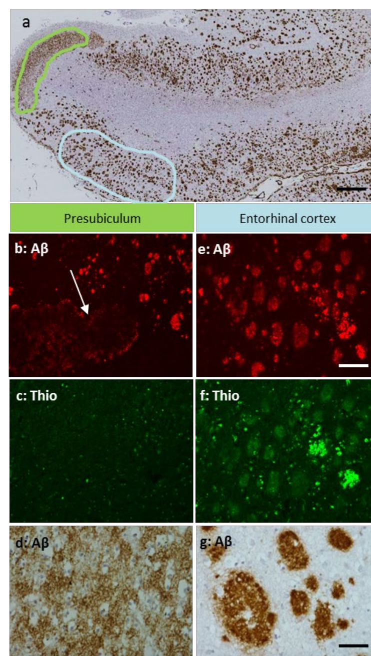


Figure 17. Immunostaining of Aβ with Congo red (b,e), thioflavin (c, f) and antibodies (d,g) in the presubiculum (b,c,d) and the entorhinal cortex (e,f,g).

determine the Aβ peptide pattern and by LC-ESI-MS to identify possible alterations in protein expression and/or pathways responsible for the differences between the two neighbouring regions.

Morphological differences between the presubiculum and the entorhinal cortex were investigated in all SAD and FAD cases. In the presubiculum, a diffuse 'lake-like' Aβ deposition was observed, while large numbers of 'mature' cored amyloid plaques were present in entorhinal cortex (Figure 17). Similar results were observed in FBD and FDD patients.

Tau deposition and NFT frequency were more pronounced in entorhinal cortex compared with the presubiculum in both SAD and FAD patients (Figure 18). Neuroinflammation was investigated by analysis of Iba1, CD68 and CR3-43. Iba1 detection was similar in the presubiculum and the entorhinal cortex in FAD, while the microglia density in presubiculum was higher compared with the entorhinal cortex. CD68 and CR3-43 staining showed that the activated microglia density was reduced in presubiculum compared with entorhinal cortex in both SAD and FAD patients.

The Aβ peptide pattern was different between the presubiculum and the entorhinal cortex, with the same differences found in both SAD and FAD patients (Figure 19). In presubiculum, only Aβ1-42 and Aβ4-42 were identified, while in entorhinal cortex AβX-42 (where X=1,2,4,5,8,9) together with the pyroglutamate-modified forms of Aβ3-42 (pGlu Aβ3-42) and pGlu Aβ11-42. To validate these findings, immunohistochemical staining with different anti- Aβ antibodies was performed.

By explorative LC-ESI-MS were in total 2385 proteins identified. Increased protein amounts were observed in presubiculum compared with the entorhinal cortex, especially proteins involved in biosynthetic and biogenetic processes as well as metabolic changes. On the other hand, the amounts of proteins involved in cell organisation and cell signalling were decreased in presubiculum compared with the entorhinal cortex. Part of the proteins identified have previously been associated with AD, including proteins associated with amyloid processing and fibrillation, tau accumulation, and inflammation.

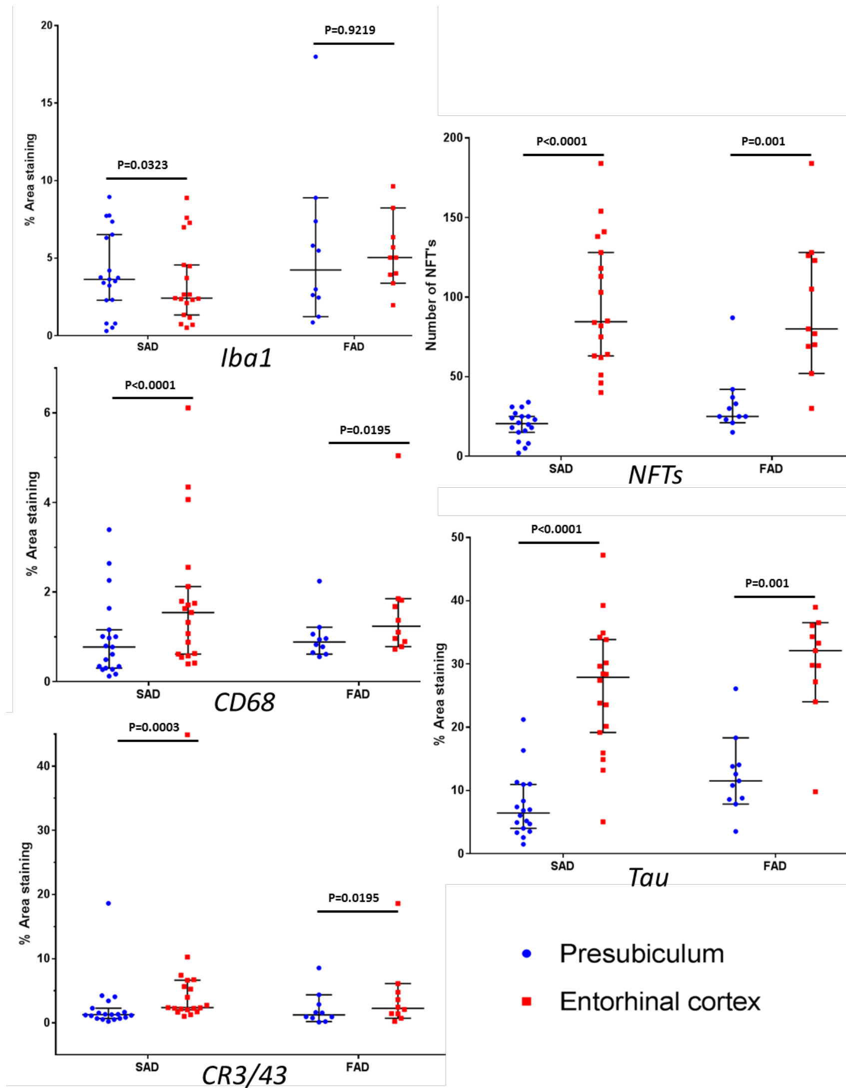
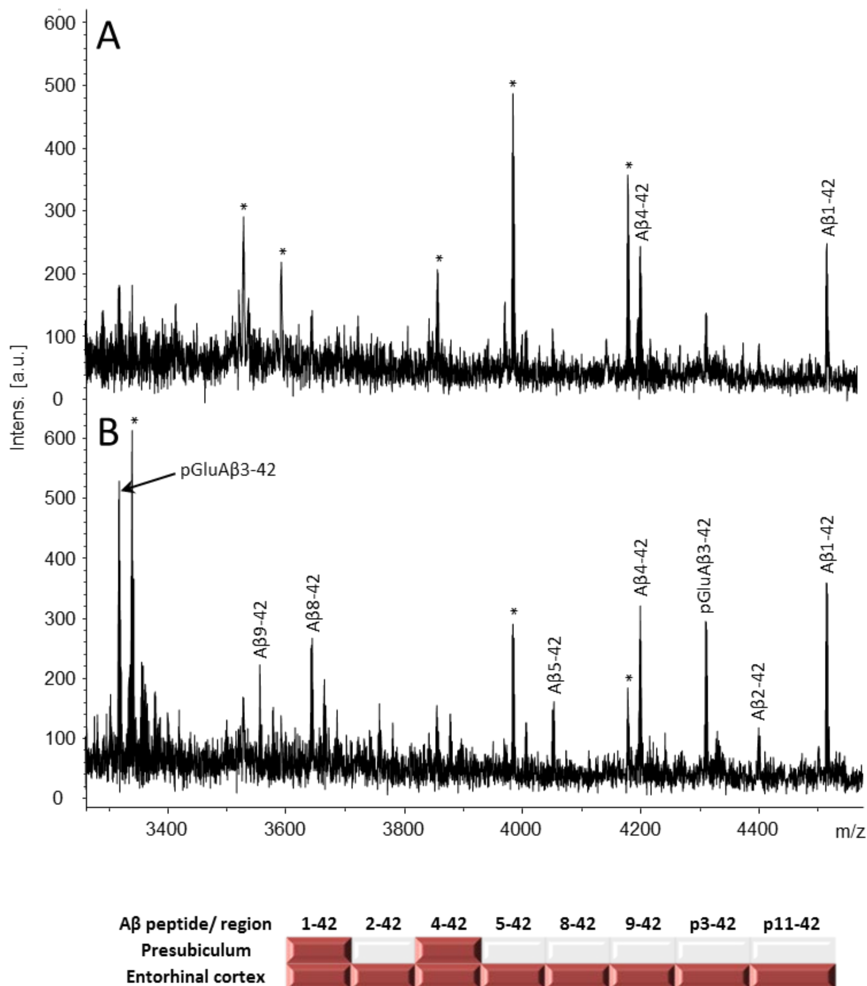


Figure 18. Scatter plots of microglia markers (Iba1, CD68 and CR3/43), NFTs and Tau in presubiculum and entorhinal cortex.



Aβ peptide/ region	1-42	2-42	4-42	5-42	8-42	9-42	p3-42	p11-42
Presubiculum								
Entorhinal cortex								

Figure 19. MALDI spectra showing the Aβ peptide pattern in (A) presubiculum and (B) entorhinal cortex. The asterisks in the spectra show non Aβ peaks. The table below shows the different Aβ peptides found in presubiculum and entorhinal cortex.

Morphological differences between the presubiculum and entorhinal cortex were observed in all cerebral amyloidosis examined (AD, FBD and FDD). Furthermore, the ‘lake-like’ structures in the presubiculum, was observed in all cerebral amyloidosis, apparently unaffected by the differences in the amino acid sequence, indicating that the observed morphology is likely due to local tissue factors, rather than processing of different proteins.

Although, no modified Aβ peptides were identified in the presubiculum using MALDI-TOF-MS, the staining of tissue using a pGlu-specific antibody was positive, indicating a possibly low concentration of these peptides (lower than the limit of detection for MALDI-TOF-MS). The presence of N-truncated and pGlu-modified Aβ peptides in entorhinal cortex, but not in presubiculum, may suggest their requirement for amyloid fibril formation.

Microglia assist the clearance of Aβ and is typically located around the amyloid plaques. Furthermore, increased Aβ deposition in AD patients is accompanied by microglia reduction. The absence of microglia in presubiculum suggests that these cells are not involved in the pre-amyloid removal.

PAPER II: A distinct brain beta amyloid signature in cerebral amyloid angiopathy compared to Alzheimer's disease

Cerebral amyloid angiopathy (CAA) is the second most common form of dementia after AD. Both diseases are characterized by extracellular depositions of Aβ, either in the vascular wall (in CAA) or as amyloid plaques (in AD). The aim in this project was to explore the Aβ peptide signature in AD patients with or without CAA pathology. To achieve this, immuno-histochemically characterized fixed tissue from the occipital lobe of AD patients ranging from no CAA to severe CAA was analysed. Figure 20 shows the Aβ pathophysiology of A) an AD patient without CAA pathology (AD/CAA-), B) an AD patient with CAA (AD/CAA+), and C) an AD patient with severe CAA (CAA+). The Aβ plaques were extracted from the tissue using 70% FA, followed by IP and then MALDI-TOF-MS and LC-ESI-MS.

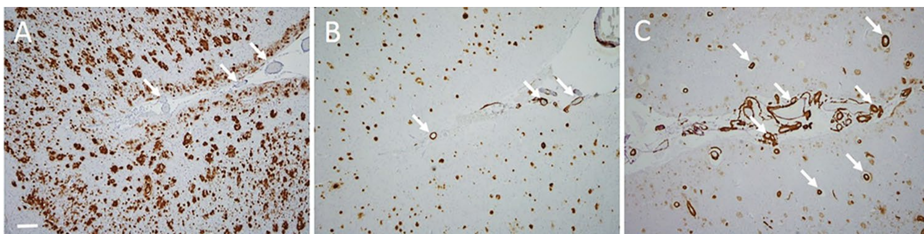


Figure 20. Immunohistochemical characterization of Aβ in the occipital cortex for cases with (A) AD/CAA−, (B) AD/CAA+ and (C) CAA+. Arrows show the presence or absence of Aβ in the vessels.

As shown in Figure 21, MALDI-TOF-MS analysis showed distinct Aβ peptide patterns in AD patients without CAA (A), with CAA (B) and severe CAA (C) pathology. Most of the Aβ peptides were identified in all groups (Figure 20 D). In AD cases with CAA, the most abundant Aβ peptides ended at aa 40 of which Aβ1-40 and Aβ2-40 were significantly increased compared with AD with no CAA. This was in contrast to AD subjects with no CAA where the most abundant Aβ peptides ended at aa 42 of which Aβ1-42, Aβ2-42 as well as pGlu Aβ3-42, and pGlu Aβ11-42 were significantly increased compared with CAA subjects. Although there is a general increase of AβX-40 in AD with CAA pathology, two subjects showed a more 'AD without CAA' like pathology (higher Aβ1-42 and Aβ4-42). Moreover, two CAA positive cases had a unique

pattern with increased amounts of AβX-37/38/39. This fact could potentially show a different CAA contribution in patients with AD.

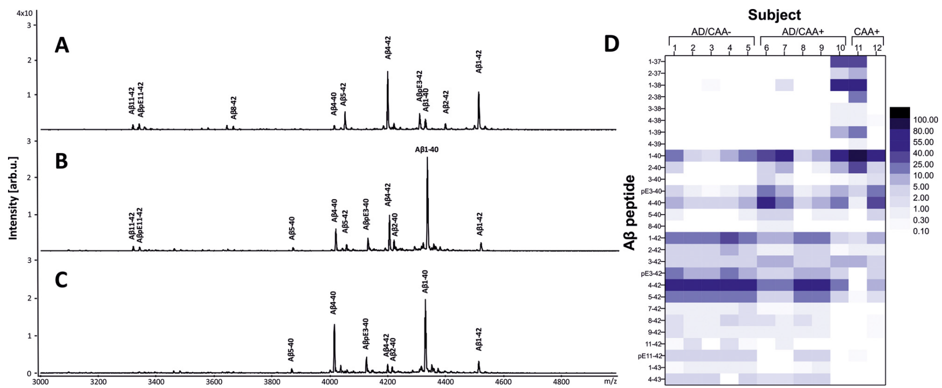


Figure 21. MALDI spectra showing the Aβ peptide pattern in (A) AD/CAA−, (B) AD/CAA+ and (C) CAA+. In (D) a heat map shows the relative abundance of all peptides measured by MALDI.

Further analysis by LC-ESI-MS identified 126 endogenous Aβ peptides in total, including oxidized and pyroglutamate-modified forms. Sixty of the peptides were only detected in the combined CAA-positive group.

The results are in line with previously published immunohistochemistry data showing that the most common Aβ peptides in plaques are N-terminally truncated and ending at aa 42 [AβX-42(X=1,2,3,4,5,7,8,9,11)], while AβX-40(X=1,2,3,4,5,8) is the main constituent of the amyloid deposits in the vessel wall. By using IP-MS, it was shown that not only the full length Aβ1-40 and Aβ1-42 differ between AD patients with and without CAA pathology, but there are also N-terminally truncated Aβ peptides ending at aa 40 and 42 that differ between these groups. There are several possible explanations for the differences between the groups. The environment of parenchymal tissue compared with the vessel wall might differ causing different preferentiation in the aggregation process. Difference in the local production might lead to different final peptide concentration. Moreover, differences in how Aβ peptides drain from the brain between different cases is also possible. In conclusion, there are several different Aβ patterns that can be observed in the human brain. Although, this finding needs to be validated in larger cohorts, this information may be used not only to improve our understanding of Aβ pathology in the brain, but also be useful in the development of pathology-specific biomarkers.

PAPER III: Characterization of monomeric and soluble aggregated Aβ in Down's syndrome and Alzheimer's disease brains

The aim of this paper was to compare the Aβ peptide pattern in AD, DS and control subjects, and investigate to possible differences in the amyloid deposition and aggregation between the samples.

To achieve this, 150 mg of cortical frozen tissue was used and Aβ peptides were extracted in a serial extraction by TBS, to extract soluble monomeric Aβ and protofibril/oligomeric Aβ, and FA to extract the Aβ in the plaques. After extraction, IP was performed either by a combination of the antibodies 6E10 and 4G8, to extract all monomeric and some aggregated Aβ peptides, or mAb158 to specifically extract protofibrilly/oligomeric Aβ. MS was finally used to identify and quantify all the Aβ peptides and compare the Aβ patterns between AD, DS and controls.

When investigating the monomeric Aβ peptides (by using 6E10+4G8 antibodies), a total of 76 Aβ peptides were identified by LC-ESI-MS/MS including Aβ1-X, Aβ2-X, Aβ3-X, Aβ4-X, Aβ5-X, Aβ11-X and APP/Aβ(-X to 15) (X= number aa extending N-terminally of position 1 of the Aβ sequence), as well as pGlu-modified peptides. The Aβ composition was different between the TBS and FA fractions, with higher levels of more hydrophobic peptides in the FA fraction, while the TBS fraction had a larger proportion of shorter and more hydrophilic peptides (Figure 22).

The Aβ peptide pattern was generally similar for AD and DS, with increased levels of the peptides in both AD and DS compared with controls. The only exception were the APP/Aβ(-X to 15) peptides which were most abundant in DS followed by controls and then AD (Figure 22). Moreover, the levels of AβX-40 and AβX-34 were increased in DS compared with AD. Despite the extra *APP* copy and the increased levels of AβX-40 and AβX-34 in DS compared with AD (Figure 23), the total Aβ load was similar for AD and DS.

The presence of shorter and more hydrophilic peptides (aa≤34), at higher levels in TBS compared with the FA fraction, may be explained by the fact that these peptides are less prone to aggregation. In line with this, the increased levels of the longer and more hydrophobic Aβ peptides (aa≥37) in the FA fraction can been explained by the fact that these peptides have a higher aggregation potency and/or inefficient clearance.

Figure 22. (A) All Aβ peptides identified by LC-ESI-MS and (B) their relative abundance in all groups and fractions.

Although the TBS and FA fractions had different Aβ compositions, there were no major differences found in the peptide patterns and levels of Aβ peptides between AD and DS patients. This indicates a similar pathway of Aβ production, degradation and accumulation in AD and DS patients. The exception was the APP/Aβ(-X to 15) peptides (Figure 23 A) (only found in the TBS fraction), which were more abundant in DS compared with controls, while in AD their amounts were even lower than in controls. This difference suggests difference in the enzyme activity of BACE1 and/or α-secretase activities between AD and DS patients. Differences between AD and DS were also observed for AβX-34 (Figure 23 B) and AβX-40 (Figure 23 C). Although both AD and DS had higher amounts of these peptides compared with controls, the amounts of these peptides were increased in DS compared with AD. A possible explanation for the increase of AβX-40 could be the CAA vessel contribution. However no pathophysiological information for CAA pathology was available making the interpretation problematical. The increased amounts of AβX-34 in DS compared with AD might be due to an increase of BACE2 activity as *BACE2* is also located on chromosome 21.

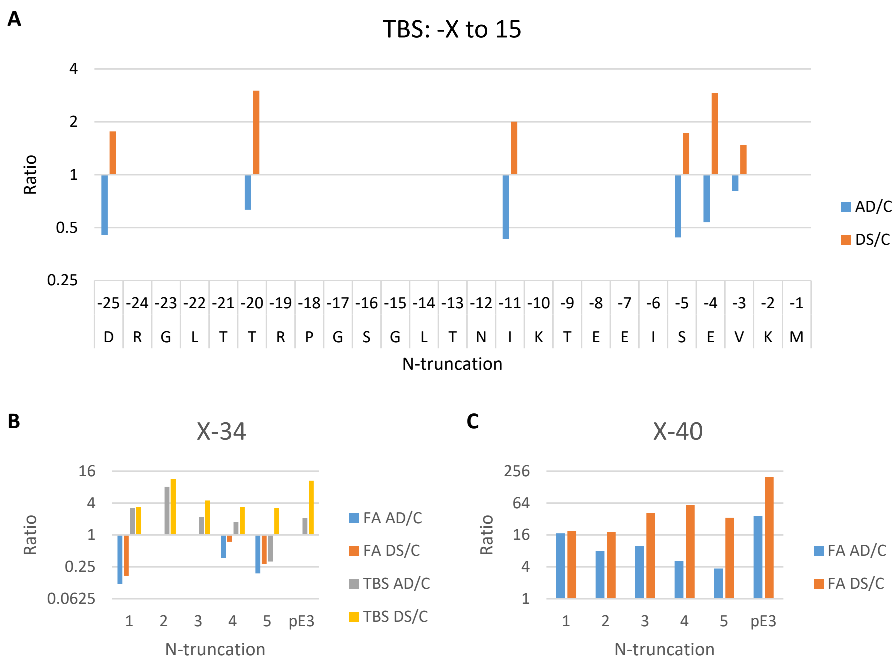


Figure 23. Aβ peptides with different abundance in AD and DS.

The use of the protofibril/oligomer-specific mAb158 antibody revealed the Aβ composition of the protofibrils/oligomers. When the protofibrils were investigated, Aβ1-40, Aβ1-42, and Aβ4-42 were identified as the main constituents, with higher levels in AD and DS compared with controls (Figure 24). A higher relative Aβ1-42 signal was observed when using mAb158 antibody compared with samples IP'd with 6E10 and 4G8. This indicates that the protofibrils/oligomers are enriched with peptides ending at aa42. This result combined with the results in the monomeric characterization of Aβ in AD and DS show minor differences between SAD and DS. This result hint that monitoring DS patients from early age might contribute to our understanding of plaque formation and finally neurodegeneration in sporadic AD. This also implies the possibility that treatment with clinical benefits in sporadic AD will also be beneficial for patients with DS.

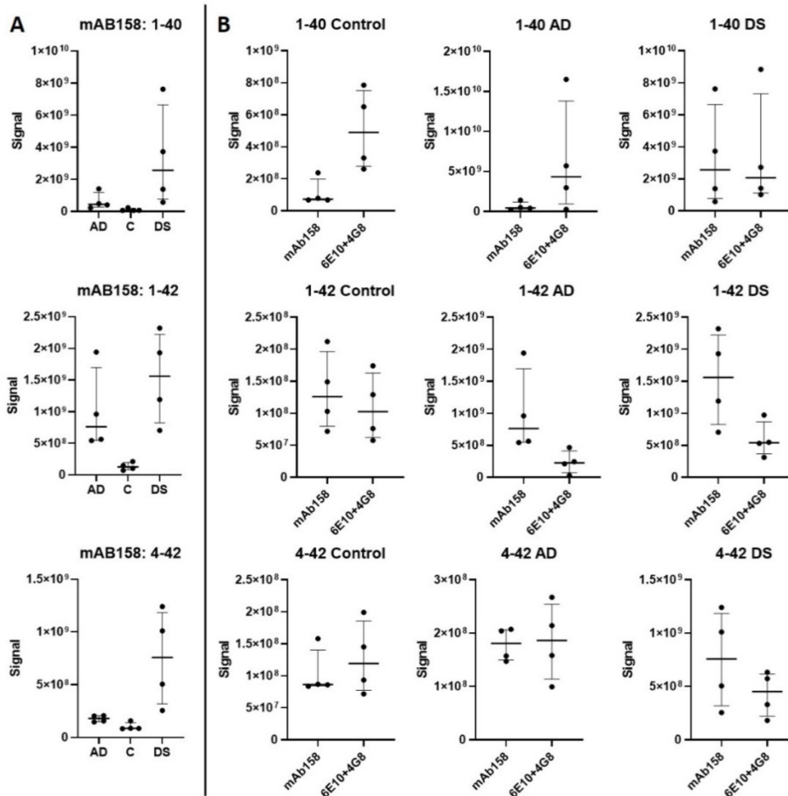


Figure 24. (A) Aβ peptides in the TBS fraction IP'd using mAb158, showing that the protofibrils/oligomers consist of Aβ1-40, Aβ1-42 and Aβ4-42. (B) Comparison of Aβ1-40, Aβ1-42 and Aβ4-42 in the TBS fraction IP'd with mAb158 or 6E10+4G8. Higher relative Aβ1-42 signals were observed when using mAb158 in both AD and DS.

PAPER IV: Amyloid pathology and synaptic loss in pathological aging

The condition where patients are amyloid positive but cognitively unimpaired at time of death is referred to as pathological ageing (PA). The aim of this project was to investigate potential differences in synaptic degeneration and Aβ peptide pattern, as well as to find any possible correlation between AD and PA.

To achieve this, IP-MS was used to analyse five synapse-associated proteins (RBM3, synaptophysin, SNAP-25, synaptotagmin-1, synaptotagmin-7) and Aβ peptides. Post mortem brain tissue from three different brain regions was used. The tissue was homogenized followed by a serial extraction using TBS and FA. The TBS fraction was used to analyse the five synapse-associated proteins, while Aβ peptides were analysed in the FA fraction. Separate IPs were performed for each analyte. Targeted mass spectrometry was used for synapse-associated proteins, while Aβ peptides were analysed by both MALDI-TOF-MS and LC-ESI-MS/MS.

The synaptic protein panel was first tested in a pilot cohort of AD, FAD, PA and control subjects. The occipital lobe was the only brain region analysed in this explorative study. No difference between PA and controls or between AD and FAD patients was observed. SNAP-25, synaptotagmin-7, and RBM3 showed significantly decreased concentration in AD (and FAD) compared with PA.

Further investigation was performed in a validation cohort (Figure 25). This study included analyses of both synaptic dysfunction and Aβ pathology, in the same samples from AD and PA individuals. Three brain regions were examined, occipital lobe, frontal lobe and cerebellum. Synaptophysin, RBM3 and SNAP-25B concentration were lower in AD compared with PA in both occipital and frontal lobe. Furthermore, in AD patients was synaptotagmin-7 significantly lower only in the frontal lobe, while synaptotagmin-1 was significantly lower only in the occipital lobe. In this study, the SNAP-25 concentration in AD patients was lower compared with PA only in frontal lobe, but not in occipital lobe (as shown in the pilot study). No difference in the concentration of any synapse-associated proteins was found in the cerebellum. Between the different synapse-associated proteins the strongest positive correlations was found between SNAP-25B, RBM3 and synaptophysin in all brain regions.

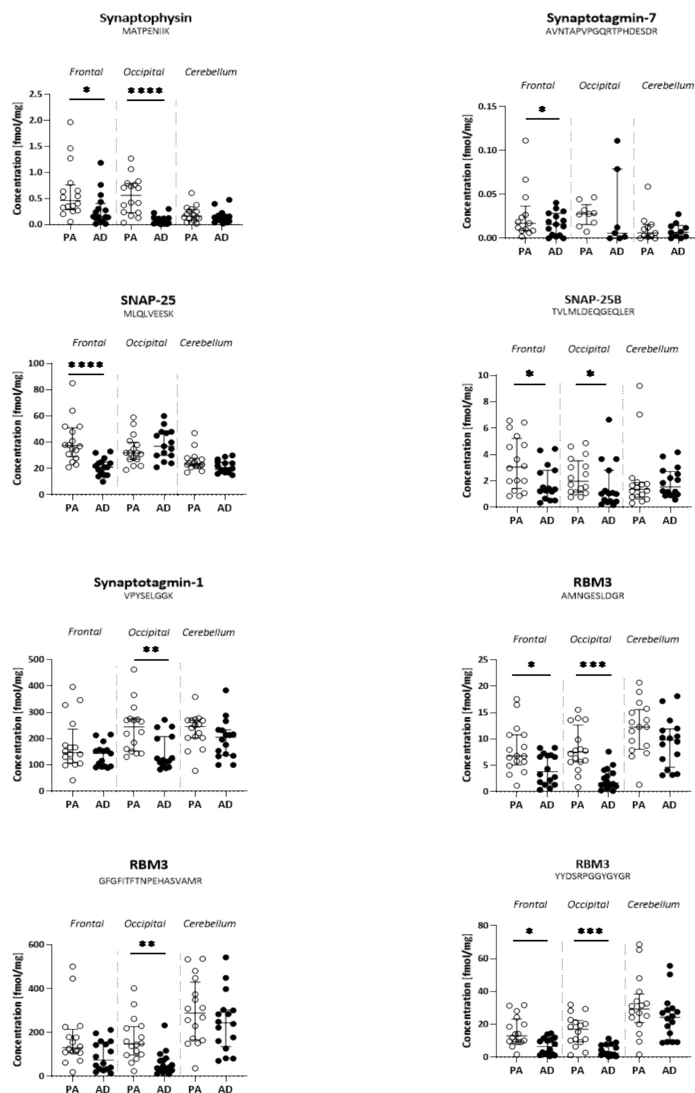


Figure 25. Scatter plots showing the differences in abundance between PA and AD for five synaptic proteins in frontal lobe, occipital lobe and cerebellum.

Aβ immunohistochemistry showed an increased Aβ load in the frontal lobe of AD patients compared with controls. IP-MALDI-TOF-MS analysis showed that the Aβ peptide pattern did not differ between frontal and occipital lobe, while fewer peptides where detected in the cerebellum (Figure 26). Aβ1-40 and Aβ4-40 had a general significantly higher relative abundance in AD compared

with PA, in both frontal and occipital lobe. In cerebellum only Aβ4-40 showed higher relative abundance in AD compared with PA. The same tendency was observed for several AβX-40 peptides in AD compared with PA. On the contrary, in PA, higher relative abundance was observed for Aβ1-42 (frontal lobe) and Aβ4-42 (occipital lobe) compared with AD. A similar tendency was also observed for other AβX-42 peptides. The relative abundance of pGlu Aβ3-40 and pGlu Aβ3-42 was higher in AD compared with PA. Referring to relative abundance in MALDI-TOF results means that a specific peptide is increased/decreased in a specific sample in relation to the other peptides in the same sample, although this wouldn't directly translate into absolute amounts in comparison with other samples. A specific Aβ37/38/39 peptide pattern was observed (similar to previous studies) and correlated to CAA pathology. Similarly, the levels of Aβ1-40, Aβ4-40 and Aβ5-40 correlated with CAA pathology in both frontal and occipital lobe. In general, all synapse-associated proteins were positively correlated with the relative abundance of the AβX-42 peptides, while a negative correlation was observed with the relative abundance of AβX-40 peptides in AD patients. In PA patients, there was also a negative correlation between the relative abundance of pGlu Aβ3-42 and the synapse-associated proteins.

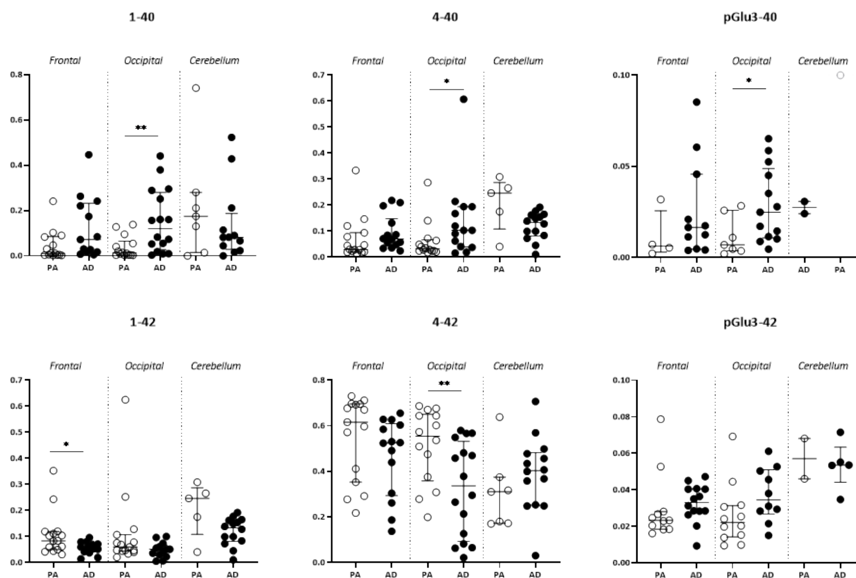


Figure 26. Scatter plots of the major Aβ peptides measured by MALDI-TOF-MS for PA and AD in frontal lobe, occipital lobe and cerebellum.

Synaptic deterioration pathology is an early indicator of AD. This study shows an overall decrease in the concentration of synapse-associated proteins in AD (and FAD) compared with PA (and controls), indicating synapse dysfunction and loss, in AD patients. As no synaptic loss was observed in PA patients, these results confirm the absence of cognitive decline in PA patients, supporting the hypothesis that cognitive impairment is connected with synaptic dysfunction.

Previous studies have identified AβX-40 as the major component of the core of amyloid plaques, mostly found in AD, while AβX-42 is localized in diffuse amyloid aggregates, found in both AD and PA. This can explain the increase abundance of AβX-40. At the same time, increase of Aβ1-40 (and AβX-40 in general) could also be explained by CAA pathology. The relative abundance of pGlu Aβ3-40 and pGlu Aβ3-42 was higher in AD compared with PA, and they correlated negatively with the synaptic protein levels. Possibly the cell toxicity caused by pyroglutamate-modified Aβ peptides is the reason behind the decreased amounts of synaptic proteins. The correlation between the increased relative abundancies of Aβ1-40, Aβ4-40, pGlu Aβ3-40 and the decreased synaptic protein concentrations, indicates that the formation of the cored plaques might drive the toxic pathology and symptoms present in AD.

PAPER V: Exploring amyloid beta peptides in Parkinson's disease dementia and dementia with Lewy bodies

Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB) are two neurodegenerative diseases sharing many similarities and there is an open debate whether they are different diseases, or if they are different stages of the same disease. The aim of this study was to characterize Aβ peptides present in DLB and PDD post-mortem brain tissue in both frontal and occipital lobes, to elucidate if there were any qualitative or quantitative differences in the amyloid pathology between DLB and PDD.

To achieve this, clinical and neuropathological characteristics were examined to explore qualitative differences between PDD and DLB patients. Furthermore, Aβ peptide analysis by IP-MS was performed to evaluate their abundance in PDD and DLB. Prior to IP, a serial extraction was performed for tissue from both frontal and occipital lobe, but only the FA fraction was used for analysis.

Clinical evaluation of the patients showed that DLB patients had significantly shorter disease duration and died at a younger age compared with PDD patients. No significant differences were found regarding the age of the first motor symptoms, the age of the first cognitive symptoms, the age at visual hallucinations and the MMSE scores. Furthermore, no difference was found between PDD and DLB based on their neuropathological characteristics (brain weight, post mortem delay, ABC score, CAA score and *APOE* genotype).

By MALDI-TOF-MS analysis, 32 Aβ peptides (including pyroglutamate-modified forms) were detected, most of them found in both PDD and DLB patients (Figure 27). On average, Aβ4-42 was the most intense peak, followed by Aβ1-42, Aβ5-42, and pGlu Aβ3-42. When comparing the Aβ peptide patterns between the two disease groups, no significant differences were observed between PDD and DLB in any of the two brain regions. Furthermore, LC-ESI-MS was used to identify any possible differences that MALDI-TOF-MS could not. In this analysis, 84 Aβ peptides (including pyroglutamate-modified forms), with a range of N- and C-terminally truncated species were identified. Similarly to the MALDI measurements, there was no difference in the pattern between the brain regions or between the patient groups. However, the amounts of several Aβ peptides were significantly higher in DLB compared

with PDD. Some individuals also showed a distinct Aβ peptide pattern of AβX-37/38/39 which previously has been correlated with CAA pathology.

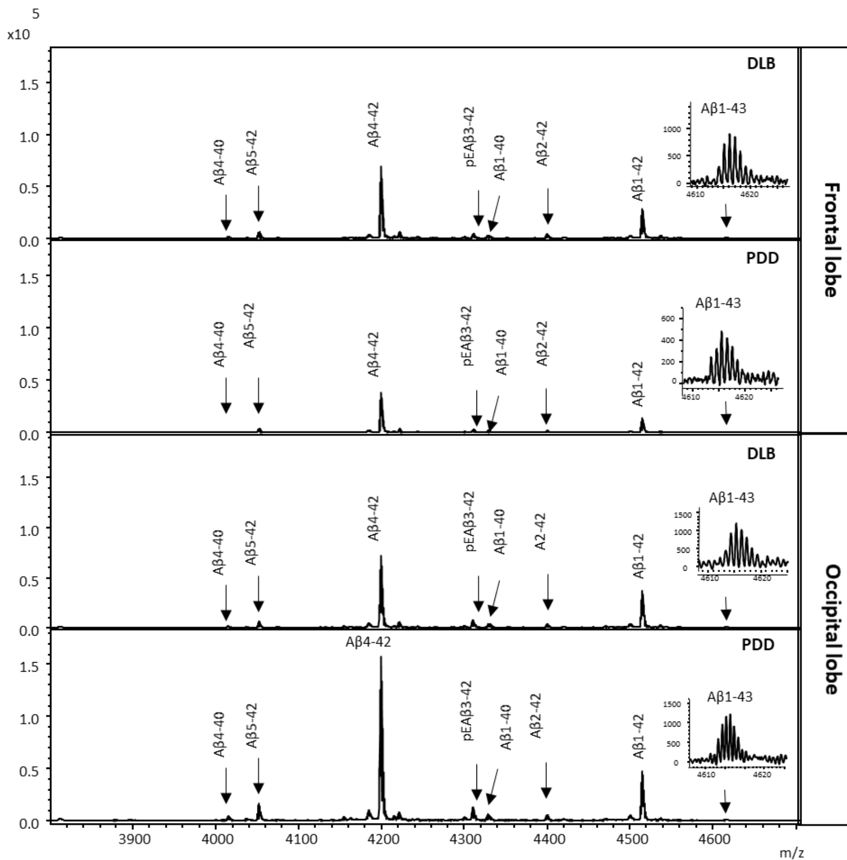


Figure 27. MALDI spectra showing the Aβ peptide pattern for DLB and PDD in both frontal and occipital lobes.

Apart from the significantly shorter disease duration and the significantly younger age of death in DLB compared with PDD, no significant difference was found in clinical and neuropathological characteristics. In the same line, no difference in the Aβ peptide pattern was found, except for the higher abundance of some longer Aβ species (including AβX-40 and AβX-42). As no pattern difference was observed between DLB and PDD, this might indicate a similar molecular mechanism of the Aβ production in DLB and PDD. However, the increased amounts of some of the Aβ peptides in DLB compared with PDD, indicate either increased Aβ production or decreased clearance.

Unfortunately, the only information on α-synuclein pathology was the relative crude Braak staging, which did not allow for any possibility to correlate for the severity of α-synuclein pathology to the Aβ peptide pattern. The Aβ peptide pattern of AβX-37/38/39, is more often seen in patients with high CAA score, indicating a possible link between them.

In this study, it was not possible to detect any Aβ-related qualitative difference between DLB and PDD, but the Aβ load was higher iv DLB. From the Aβ pathology, we cannot rule out that DLB and PDD are the same disease, with (slightly) different progression. Moreover, it indicates that both the DLB and PDD patient groups have a significant AD contribution that most likely contributes to cognitive decline.

5 CONCLUSIONS

Amyloid pathology has been the centre of dementia research since amyloid plaques and neurofibril tangles were identified as the hallmarks of AD. Aβ peptides are one of the major component of amyloid plaques and the accumulation of Aβ starts decades before the first symptoms appear. Today Aβ is widely used as a biomarker reflecting the plaque pathology and as a therapeutic target. Despite the extensive research on Aβ peptides, we still do not fully understand exactly how and why the accumulation of Aβ peptides and amyloid plaques formation contribute to cognitive decline. The aim of this work was to reveal the complex nature of Aβ and its relation to dementia. Homogenates of human brain tissue gave a unique opportunity to investigate amyloid pathology. The use of different antibodies allowed the isolation and subsequent identification of different forms of Aβ. MS was utilized to identify more than hundred different Aβ peptides, of which some have not previously seen reported in brain tissue.

Neuropathological studies have shown that the Aβ accumulation (and plaque formation) is not ubiquitous throughout the brain. The regions that are affected first are the cortical areas, while cerebellum is affected last. Previous studies have shown that patients that have amyloid pathology in the cerebellum are the patients that are most severally affected. However, no differences were identified between the frontal and occipital lobes regarding either the Aβ peptide pattern or the relative amounts of Aβ (papers V and IV). Yet, an increase in AβX-37/38/39 peptides was observed in the occipital lobe in papers II, III, IV and V, possibly due to increase CAA contribution. Cerebellum which is the last region affected exhibited a minimum amount of Aβ peptides, mostly including Aβ1-42 and Aβ4-42.

In paper II, a distinct Aβ peptide pattern was observed between AD patients without and with CAA pathology. The different Aβ peptide patterns in AD with and without CAA shows also that the level of CAA contribution can differ. Since most of the DS patients also have widespread plaque pathology, the Aβ peptide profile in AD was compared to DS (paper III). Despite the extra copy of *APP* in DS patients, no major differences were found between AD and DS, with exception of APP/Aβ (-X to 15), AβX-40 and AβX-34, with higher abundance in DS compared with AD. In the same samples, the Aβ peptide profile of protofibrils/oligomers were investigated, showing that Aβ1-40, Aβ1-42, and Aβ4-42 as the main constituents, with higher levels in AD and DS compared with controls. These results hint that monitoring DS patients from

early age might contribute to our understanding of plaque formation and finally neurodegeneration in sporadic AD. This also implies the possibility that treatment with clinical benefits in sporadic AD may also be beneficial for patients with DS. Finally, Aβ deposition in DLB and PDD patients (sharing amyloid and α-synuclein pathology), was investigated to identify possible differences between them. However, no difference regarding the Aβ peptide pattern between DLB and PDD was found, although the Aβ load was significantly higher in DLB compared with PDD. The Aβ peptide pattern of both DLB and PDD showed a higher abundance of AβX-42 peptides in contrast to AD where AβX-40 peptides are the major forms. We cannot rule out that DLB and PDD are the same disease, with (slightly) different progression, but we can assume that the DLB and PDD possibly are a continuum of AD and PD diseases.

Aβ peptide accumulation has also been connected with synaptic loss, with pyroglutamate-modified Aβ peptides having increased cell toxicity. In paper IV, the relation between different Aβ peptides and synaptic proteins was tested. Increased synaptic loss was correlated with increased relative abundance of AβX-40 peptides as well as the pGlu Aβ3-40/42. Despite the fact that there is no morphological differences between the plaques present in PA and AD, the Aβ peptide pattern is different as well as the degree of synaptic loss. PA had increased AβX-42 accumulation compared with AD, while the opposite pattern is observed for AβX-40. Furthermore, the synaptic loss is more pronounced in AD compared with PA. It has previously has been shown that Aβ42 is the major form in diffuse plaques and the diffuse part of cored plaques, while Aβ40 is the major form of the core of cored plaques. Moreover, AD patients have more cored plaques than PA, a fact that can explain the increase of AβX-40 peptides in AD patients. Data from paper I also show that the presubiculum, a region that shows a 'lake-like' Aβ deposit, minimal tau deposition and/or accumulation of inflammatory markers, has Aβ1-42 and Aβ4-42 in a lower amounts than the neighbouring entorhinal region. The entorhinal region had increased levels of AβX-42 peptides, together with the presence of pyroglutamate-modified Aβ peptides. Based on these results, the hypothesis is that the accumulation of pyroglutamate-modified Aβ together with AβX-40 seems to be what drives the synaptic loss and finally the cognitive decline.

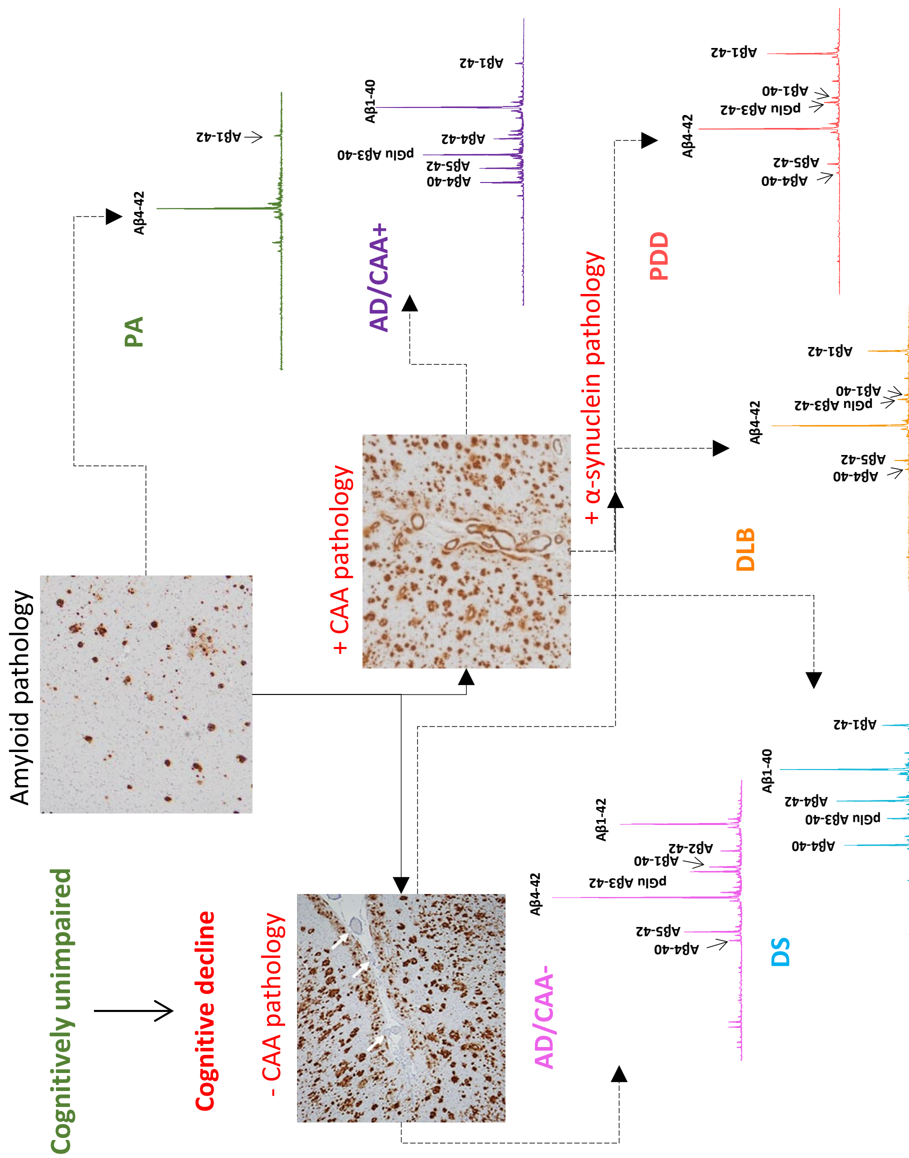


Figure 28. Overview of the thesis conclusion.

In conclusion, Figure 28 shows the Aβ deposits in different types of dementia, as well as in cognitively unimpaired patients with amyloid pathology. This thesis demonstrates that even though the deposits of Aβ were similar across the different dementias investigated (except for CAA), the data presented indicate that there are different toxic (and non-toxic) Aβ assemblies in the different diseases. For CAA there is a distinct increase of AβX-40 peptides and for DS the amounts of APP/Aβ(-X to 15) differed from AD. Moreover, Aβ1-40, Aβ1-42, and Aβ4-42 peptides were identified as the main components of protofibril/oligomer Aβ composition. It has become clear that the contribution of Aβ can differ between the different types of dementia, raising the question whether or not more Aβ biomarkers are needed for a more accurate diagnosis.

6 FUTURE PERSPECTIVES

Although Aβ has been in the centre of dementia research for decades, it becomes more and more obvious that several other factors have a role in disease progression. To answer these questions more extensive studies of neuropathologically well characterized patients have to be performed. Simultaneous examination of Aβ peptide pattern and different tau forms, together with genomic, transcriptomic, proteomic, metabolomics and lipidomics profiles, will give a more complete picture of the pathology of each dementia type. Moreover, studies that translate the Aβ peptide pattern in the brain into an Aβ profile in CSF or plasma for different type of dementias may provide more information of Aβ processing and supplement the Aβ biomarkers used today. With a better understanding of the different pathologies, earlier and more specific diagnoses may be possible, and subsequently lead to more successful treatments, acting on the pathology before it become irreversible.

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