Towards a better understanding of fluid biomarkers for Alzheimer's disease

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Towards a better understanding of fluid biomarkers for Alzheimer's disease

Emelie Andersson

DOCTORAL DISSERTATION

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Abstract:

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The aim of this thesis was to gain a better understanding of the neurobiological correlates behind the changes in CSF and blood biomarkers for AD, including the Aβ42/Aβ40 ratio, total tau (t-tau), phosphorylated tau (p-tau), and neurofilament light (NfL). This was studied using a translational approach, in which participants from the prospective and longitudinal Swedish BioFINDER study, as well as different AD mouse models, were included.

In paper I, we found that NfL in CSF, but not in blood, was increased in response to cerebral Aβ pathology and associated with compromised white matter microstructure in individuals with preclinical sporadic AD. Furthermore, in 5xFAD mice, NfL in CSF was increased before that in blood as cerebral Aβ pathology started to develop.

In paper II, we showed that CSF p-tau217, p-tau181, and t-tau were increased in response to Aβ pathology in preclinical sporadic AD, several years before pathological levels of aggregated tau could be detected in the brain with positron emission tomography (PET). Moreover, CSF t-tau was increased in response to Aβ pathology in 5xFAD mice, although this model does not form tau aggregates.

In paper III, we demonstrated that CSF and blood Aβ42/Aβ40 ratios were reduced with age in an *App* knock-in mouse model of preclinical AD, and that their initial decline was preceded by a limited cerebral deposition of Aβ in extracellular plaques. We also found the Aβ42/Aβ40 ratio in CSF tended to more strongly associate with measures of cerebral Aβ pathology than the corresponding ratio in blood.

In paper IV, by using 5xFAD mice, we showed for the first time that a low CSF Aβ42/Aβ40 ratio in AD may better reflect a high cerebral burden of soluble Aβ protofibrils, particularly enriched in Aβ42, rather than increased cerebral deposition of insoluble Aβ fibrils in extracellular plaques. We also found that elevated concentrations of NfL and t-tau in CSF collected from these mice to some degree may reflect neuronal injury and loss induced by soluble Aβ protofibrils in the brain.

Together, our findings provide new information on the temporal and correlative relationships between pathological processes in the brain and changes in clinically relevant fluid biomarkers in AD. This knowledge may be valuable for future human studies aiming at increasing the understanding of the disease, as well as for the use of these fluid biomarkers in clinical trials.

Key words: Alzheimer's disease, biomarker, cerebrospinal fluid, blood, amyloid-beta, tau, neurofilament light

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To my family

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Abstract

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The aim of this thesis was to gain a better understanding of the neurobiological correlates behind the changes in CSF and blood biomarkers for AD, including the Aβ42/Aβ40 ratio, total tau (t-tau), phosphorylated tau (p-tau), and neurofilament light (NfL). This was studied using a translational approach, in which participants from the prospective and longitudinal Swedish BioFINDER study, as well as different AD mouse models, were included.

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Together, our findings provide new information on the temporal and correlative relationships between pathological processes in the brain and changes in clinically relevant fluid biomarkers in AD. This knowledge may be valuable for future human studies aiming at increasing the understanding of the disease, as well as for the use of these fluid biomarkers in clinical trials.

Populärvetenskaplig sammanfattning

Idag uppskattas att ungefär 55 miljoner människor världen över är drabbade av en demenssjukdom, där Alzheimers sjukdom utgör majoriteten av fallen. Vid Alzheimers sjukdom börjar nervcellerna i de delar av hjärnan som styr minnet och andra kognitiva funktioner förtvina och dö i onormal utsträckning. Den bakomliggande orsaken till detta är inte helt kartlagd, men proteinet amyloid-β (Aβ) verkar spela en central roll vid utvecklingen av sjukdomen. Olika former av Aβ förekommer naturligt i den friska hjärnan, där Aβ40 och Aβ42 är vanligast. Vid Alzheimers sjukdom klumpar dessa emellertid ihop sig på ett onormalt sätt, vilket leder till att både mindre ansamlingar, så kallade oligomerer och protofibriller, och större senila plack som återfinns mellan nervcellerna bildas. Förekomsten av dessa sjukliga former av Aβ leder till skador på nervcellerna som gör att de får svårare att kommunicera med varandra. Ett annat protein som spela en viktig roll i sjukdomsförloppet är tau. Vid Alzheimers sjukdom modifieras tau på ett onormalt sätt genom så kallad fosforylering, vilket leder till att proteinet klumpar ihop sig till små nystan – eller fibriller – inne i nervcellerna. Denna ansamling av fibriller leder så småningom till att nervcellerna dör.

För att få en inblick i hur hjärnan mår kan nivåerna av olika ämnen – så kallade biomarkörer – undersökas i ryggvätska, det vill säga den vätska som omger hjärnan och ryggmärgen. Vid Alzheimers sjukdom sjunker kvoten mellan Aβ42 och Aβ40 (Aβ42/Aβ40) i ryggvätska jämfört med friska personer, samtidigt som nivåerna av både fosforylerat tau (p-tau) och den totala mängden tau (t-tau) ökar. Dessa förändringar kan upptäckas flera år innan minnesstörningar på grund av Alzheimers sjukdom utvecklas, vilket benämns som sjukdomens prekliniska fas. Även nivåerna av ett protein som kallas neurofilament light (NfL) är förhöjda i ryggvätska vid Alzheimers sjukdom, en förändring som tros återspegla skada och förlust av nervceller. Tack vare senare års utveckling av nya mätmetoder har forskningen visa att flertalet av dessa proteiner även är förändrade i blod. Vad som ligger bakom förändringarna av dessa vätskebaserade biomarkörer vid Alzheimers sjukdom är emellertid inte helt klarlagd.

I denna avhandling har vi haft som målsättning att undersöka sambanden mellan sjukliga förändringar i hjärnan och förändrade nivåer av biomarkörer i ryggvätska och blod vid Alzheimers sjukdom.

I det första delarbetet fann vi att nivåerna av NfL i ryggvätska var förhöjda hos kognitivt friska personer med tecken på onormal förekomst av Aβ i hjärnan, det vill säga i den prekliniska fasen av sjukdomen. Liknande förändringar i blod kunde däremot först observeras i samband med att lindriga minnesstörningar på grund av sjukdomen utvecklades. Vi fann även att höga nivåer av NfL i ryggvätska, men inte i blod, var förknippade med sjukliga förändringar i hjärnans vita substans hos kognitivt friska personer. Vidare studier i möss, som likt personer med Alzheimers sjukdom utvecklar senila plack i hjärnan, visade att nivåerna av NfL i ryggvätska ökade tidigare än i blod. Tillsammans talar dessa fynd för att NfL i ryggvätska är en mer tillförlitlig biomarkör för skada och förlust av nervceller i den prekliniska fasen av Alzheimers sjukdom i jämförelse med blod.

I det andra delarbetet fann vi att nivåerna av både p-tau och t-tau var förhöjda i ryggvätska hos personer med preklinisk Alzheimers sjukdom. Denna förändring var förknippad med ansamlingen av senila plack i hjärnan och skedde innan förekomsten av tau-fibriller i nervcellerna kunde påvisas med hjälp av PET-kameraundersökning. Vidare studier i möss kunde bekräfta att nivåerna av t-tau i ryggvätska ökar som svar på ansamlingen av senila plack i hjärna i frånvaro av tau-fibriller. Dessa resultat tyder på att förekomsten av senila plack i hjärnan leder till förhöjda nivåer av p-tau och t-tau i ryggvätska under det tidiga skedet av Alzheimers sjukdom, och att dessa förändringar föregår ansamlingen av tau-fibriller med flera år.

I det tredje delarbetet var målsättningen att få en bättre förståelse för det tidsmässiga sambandet mellan Aβ-relaterade förändringar i hjärnan och sänkt Aβ42/Aβ40 kvot i ryggvätska och blod vid preklinisk Alzheimers sjukdom. I möss som speglar detta tidiga skede av sjukdomen fann vi en tidsmässig sekvens av händelser där en begränsad ansamling av senila plack i hjärnan föregick sänkningen av Aβ42/Aβ40 kvoten i både ryggvätska och blod. Våra fynd talar även för att Aβ42/Aβ40 kvoten i ryggvätska på ett mer tillförlitligt sätt reflekterar Aβ-relaterade förändringar i hjärnan i det tidiga sjukdomsförloppet i jämförelse med motsvarande kvot i blod.

I det fjärde delarbete var vi intresserade av att vidare förstå den underliggande orsaken till varför Aβ42/Aβ40 kvoten i ryggvätska sjunker vi Alzheimers sjukdom. Genom studier i möss har vi för första gången kunna visa att en sänkt Aβ42/Aβ40 kvot i ryggvätska bättre återspeglar mängden Aβ som ansamlas i lösliga protofibriller än den som återfinns i olösliga senila plack i hjärnan. Fynden från denna studie tyder även på att förhöjda nivåer av NfL och t-tau i ryggvätska till viss del reflekterar skada och förlust av nervceller som orsakas av lösliga Aβ protofibriller.

Sammantaget bidrar fynden från avhandlingens fyra delarbeten med ny kunskap om sambanden mellan sjukdomsprocesser i hjärnan och förändrade nivåer av kliniskt relevanta vätskebaserade biomarkörer vid Alzheimers sjukdom.

List of abbreviations

Aβ	Amyloid-beta
AD	Alzheimer's disease
ADAM	A disintegrin and metalloprotease
ADL	Activities of daily living
AICD	APP intracellular domain
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	Beta-site APP cleaving enzyme 1
BDNF	Brain-derived neurotrophic factor
Cdk5	Cyclin-dependent kinase 5
CSF	Cerebrospinal fluid
CU	Cognitively unimpaired
CDR	Clinical dementia rating
DTI	Diffusion tensor imaging
ELISA	Enzyme-linked immunosorbent assay
ECL	Electrochemiluminescence
EOAD	Early-onset Alzheimer's disease
FA	Fractional anisotropy
FAc	Formic acid
FDA	Food and Drug Administration
FDG	Fluorodeoxyglucose
GSK-3β	Glycogen synthase kinase-3 beta
IP	Immunoprecipitation

ITC	Inferior temporal cortex
IQR	Interquartile range
MRI	Magnetic resonance imaging
MAPT	Microtubule-associated protein tau
MCD	Mild cognitive deficit
MCI	Mild cognitive impairment
MD	Mean diffusivity
MS	Mass spectrometry
MSD	Meso scale discovery
NDS	normal donkey serum
NfL	Neurofilament light
NFT	Neurofibrillary tangles
NIA-AA	National Institute on Aging and Alzheimer's Association
NMDA	N-methyl-D-aspartate
PB	Phosphate buffer
PET	Positron emission tomography
PP2A	protein phosphatase 2A
PSEN1	Presinilin-1
PSEN2	Presinilin-2
P-tau	Phosphorylated tau
SILK	Stable isotope labelling kinetics
Simoa	Single molecule array
TBS	Tris-buffered saline
TBSS	Tract-based spatial statistics
TBSX	Tris-buffered saline supplemented with 0.25% Triton X-100

Introduction

Alzheimer's disease

The history of Alzheimer's disease

In 1901, a 51-year-old woman named Auguste Deter was admitted to a mental asylum in Frankfurt. She had years before started to show signs of delusions, memory impairment, and disorientation, which had progressively worsened to the point where her husband could no longer provide care for her. At the mental asylum, she met Alois Alzheimer, a young psychiatrist and neuropathologist who had an interest in dementing disorders. Alois Alzheimer became fascinated by the unusual condition of his patient, and although he moved to another position at a psychiatric clinic in Munich in 1903, he continued to follow her case. After almost five years at the mental asylum in Frankfurt, Auguste Deter passed away, and her brain was sent to Alois Alzheimer for examination. At a conference shortly thereafter, he presented his striking histopathological findings of extensive atrophy together with what he described as numerous cortical "miliary foci" and peculiar intracellular neurofibrillary changes (1). A report of his clinical and histopathological observations was published in 1907 (2), and a few years later, the German psychiatrist Emil Kraepelin introduced the eponym Alzheimer's disease (AD) for the condition that Alois Alzheimer had described (1).

The "miliary foci" and neurofibrillary changes that were described by Alois Alzheimer in 1907 are today recognized as extracellular senile plaques and intracellular neurofibrillary tangles (NFTs), and together with neurodegeneration, constitute the main neuropathological hallmarks of AD. Although the components of these lesions remained unresolved for long, amyloid-beta (Aβ), generated from a protein known as amyloid precursor protein (APP), and the microtubule-binding protein tau could be identified in senile plaques and NFTs, respectively, during the mid 1980s (3-9). These proteins have since then been subject to intense investigation to understand underlying disease mechanisms, identify potential drug targets, and develop diagnostic and prognostic biomarkers.

Epidemiology

Dementia is a major cause of disability, dependency, and mortality in the aged population (10), today estimated to affect more that 55 million people globally (11). AD, a neurodegenerative disorder clinically characterized by progressive decline in memory and other cognitive domains (12), is recognized as the most common cause of dementia, accounting for about 60-70% of all cases (10). As a result of increased life expectancy, a tripling of the number of people affected by this devastating disease is anticipated by 2050, where the highest increase is projected to occur in low- and middle-income countries (11). In addition to the great suffering that AD and other dementias bring to the diseased people and their families, this will result in a high financial burden for the global society, which already today is estimated to be more than US$ 1 trillion annually (13).

The strongest risk factor for the development of AD is aging, and the majority of those who are affected by the disease are over 65 years of age when they experience their first symptoms (13). This late onset form of the disease is known as sporadic and accounts for about 95% of all AD cases (14). When the disease makes its debut before the age of 65, it is often referred to as early-onset AD (EOAD). For a minority of these cases, rare mutations in certain genes that are transmitted through inheritance are the underlying cause of the disease (15). This is known as familial AD and is characterized by symptom onset usually around the age of 40-55 (16).

Neuropathology

As described by Alois Alzheimer over 100 years ago (2), the presence of both extracellular Aβ plaques and intracellular NFTs in the brain is today required for an AD diagnosis (17). These key pathological characteristics are accompanied by neuroinflammation (18), as well as neuronal injury and loss (19) (Figure 1).

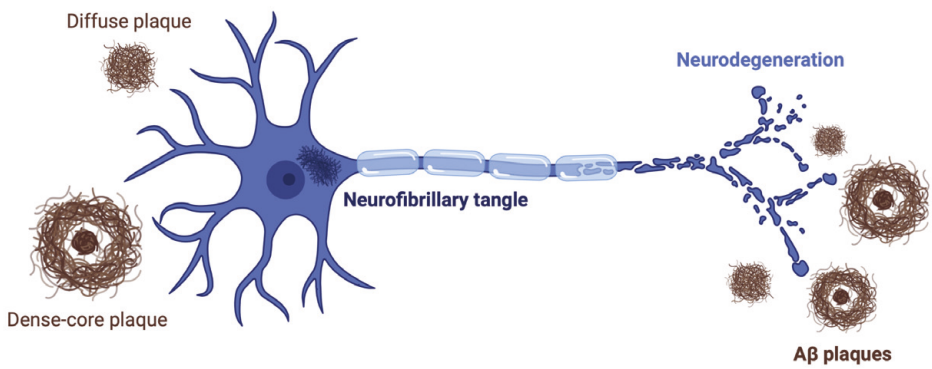

Figure 1: The illustration shows the neuropathological hallmarks of AD. Created with BioRender.

Amyloid plaques

Several types of extracellular Aβ deposits have been identified, of which dense-core plaques and diffuse plaques are predominant in the brain of AD patients (19). These are classified based on their staining with certain dyes, such as thioflavin S and Congo red, that specifically bind to amyloid fibrils (20).

Dense-core plaques are intensely stained with thioflavin S and Congo red, and are characterized by a core of tightly packed fibrillar Aβ that is surrounded by a halo of more loosely organized Aβ (20, 21). A subset of these has been defined as neuritic, as they associate with abnormal neuronal processes known as dystrophic neurites (19). These can be of axonal or dendritic origin (22), and contain either APP, abnormal tau protein, or both (23). Some of the dystrophic neurites that associate with neuritic plaques also contain neurofilament proteins (24-26), which are essential structural components of the neuronal cytoskeleton and are predominantly found within the axon (27). This suggests that cytoskeletal abnormalities are part of the neurodegenerative process. Neuritic plaques are also commonly surrounded by activated microglia cells, as well as reactive astrocytes, which are the principal drivers of neuroinflammation in the AD brain (28, 29). In addition, synaptic loss is usually observed in the vicinity of neuritic plaques (30).

While neuritic plaques are considered to be closely associated with neuronal injury, diffuse plaques seem to be more benign, despite their abundancy in the AD brain. Diffuse plaques are assemblies of filamentous Aβ that are weakly stained by thioflavin S and Congo red (20). They are typically not associated with inflammatory cells or dystrophic neurites, although diffuse neuritic plaques can be found in the advanced stage of the disease (19, 31).

In AD, the deposition of Aβ as extracellular plaques tend to follow a predictable spatiotemporal pattern. The earliest Aβ deposits are found in neocortical association areas, primary in the medial parietal and frontal cortices. This is followed by involvement of other neocortical areas, where the primary motor and sensory cortices are the last to be affected. Finally, Aβ deposits can be detected in the brain stem and cerebellum (32).

Although Aβ predominantly deposits as extracellular plaques in the AD brain, it also accumulates intraneuronally (33). Furthermore, Aβ deposits are commonly found within cerebral blood vessels, which is known as cerebral amyloid angiopathy (34).

Neurofibrillary tangles and neuropil threads

Intracellular NFTs are primarily composed of filaments that are formed by misfolded tau protein (4-8, 35). NFTs initially appear as pretangles that are found within morphologically healthy neurons. These subsequently develop into mature tangles, which displace the nucleus and undertake the shape of the neuron in which

they reside. Eventually, the neurons die and the remnant of the mature tangles, called ghost tangles, are left in the extracellular space (36). In addition to NFTs, tau lesions known as neuropil threads are found in the AD brain. These are segments of dendrites and axons that contain filamentous tau (37).

Similar to extracellular Aβ plaques, the progression of NFTs in the AD brain follows a stereotypic spatiotemporal pattern. Neuropathological studies on postmortem brain tissue have shown that the first NFTs typically appear in the transentorhinal cortex from where they reach the entorhinal cortex (stages I-II). As the disease progresses, hippocampus becomes involved (stages III-IV), with NFTs further spreading to neocortical association areas (stages V-VI) (38). This spreading pattern is referred to as the "Braak staging scheme" and is supported by cross-sectional studies in which positron emission tomography (PET) imaging has been used to visualize tau filaments *in vivo* (39). However, individual tau patterns that do not converge with the Braak staging scheme have been described, and a recent neuroimaging study, indeed, suggested that four distinct spatiotemporal spreading patterns of tau filaments may occur in AD (40).

Neurodegeneration

In the AD brain, the accumulation of intracellular NFTs, rather than extracellular Aβ plaques, has shown to closely associate with grey matter atrophy and subsequent cognitive decline (41). Grey matter atrophy is usually observed in the medial temporal lobe early in the disease, where specifically the entorhinal cortex and hippocampus, two areas important for the formation of memories, are affected. As the disease progresses, the grey matter atrophy further extends along the parietal and frontal cortices (42), where the posterior cingulate, precuneus, and the temporoparietal association cortex becomes particularly involved (43). Areas of the motor cortex are usually spared until the very advanced stage of the disease.

The Alzheimer's disease continuum

The patterns of the neuropathological lesions described above is reflected by the symptoms that develop as AD progresses (42, 44). However, as a result of the development of imaging and fluid biomarkers that are able to measure AD-specific brain changes in living persons, it has been revealed that abnormal deposits of fibrillar Aβ may be present in the brain at least a decade before the first symptoms manifest (45-47). The time period during which pathological changes are present in the brain in the absence of symptoms is known as preclinical AD, and constitute the first of three phases along what is referred to as the AD continuum (48, 49).

When biomarker evidence of disease-specific brain changes coincides with impairment of cognitive abilities, a transition to the second phase of the continuum has occurred. This is known as mild cognitive impairment (MCI) due to AD, or

prodromal AD (48, 50). The symptoms usually begin as decline in episodic memory (51), and may be accompanied by executive dysfunction (52). Although these cognitive changes may be noticeable to family and friends, they are not yet severe enough to interfere with the ability to carry out activities of daily living (ADLs) (48).

AD dementia represents the last phase of the continuum, and is in addition to a disease-specific biomarker profile, characterized by cognitive decline that with different severity affects the ability to carry out ADLs (48, 53). In the mild stage, independency mostly remains, although assistance with some tasks may be needed. As the disease progresses, the problems with memory worsen, communication and execution of ADLs become more difficult, and changes in personality and behavior may occur. In the most severe stage, physical abilities are affected, eventually leading to difficulties with swallowing and consequently intake of food and drink. Complete assistance and care are necessary at this stage of the disease (48).

APP and Aβ

APP

APP is a type-1 single pass transmembrane glycoprotein characterized by a large extracellular/luminal N-terminal domain and a short cytoplasmic C-terminal domain (9, 54). The protein is encoded by a single gene located on chromosome 21 (9, 55, 56), and several isoforms that arise from alternative splicing of the pre-mRNA transcript have been identified (57), of which APP containing 695 amino acids (APP695) is the main isoform generated in neurons (58, 59). APP matures in the secretory pathway, where it during transit through the endoplasmic reticulum and Golgi apparatus is post-translationally modified by *N*- and *O*-glycosylation, tyrosine sulphation, and phosphorylation (60-63). Matured APP eventually reaches the plasma membrane, where the protein is either proteolytically processed or re-internalized into the endosomal-lysosomal pathway (64).

APP cleavage and generation of Aβ

Mature APP that has been transported to the plasma membrane via the secretory pathway is further processed in two canonical proteolytic pathways: the non-amyloidogenic pathway and the amyloidogenic pathway (64) (Figure 2).

In the non-amyloidogenic pathway, APP that has been internalized into the plasma membrane is cleaved within the Aβ domain (65-68), thereby preventing the generation of Aβ. This cleavage is performed by α-secretases (69), which in early studies were identified as membrane-bound zinc metalloproteinases (70). Several zinc metalloproteinases of the ADAM (a disintegrin and metalloprotease) family have been shown to possess α-secretase activity, including ADAM9 (71), ADAM10

(72), and ADAM17 (73). It has been proposed that ADAM10 exerts the main constitutive α-secretase activity in neurons (74, 75), while ADAM9 and ADAM17 have been implied to mainly perform regulated cleavage of APP (74, 76), a process that is defined by its dependency of protein kinase C. Cleavage by α-secretases results in the release of a large soluble ectodomain known as sAPPα and the generation of a membrane-bound truncated C-terminal fragment called α-CTF (64-68). The α-CTF is further processed by γ-secretase, which is a multi-subunit protease complex embedded within the membrane (77). This final step of the pathway liberates an extracellular truncated Aβ peptide termed p3 (78) and a cytosolic fragment known as APP intracellular domain (AICD) (79-81).

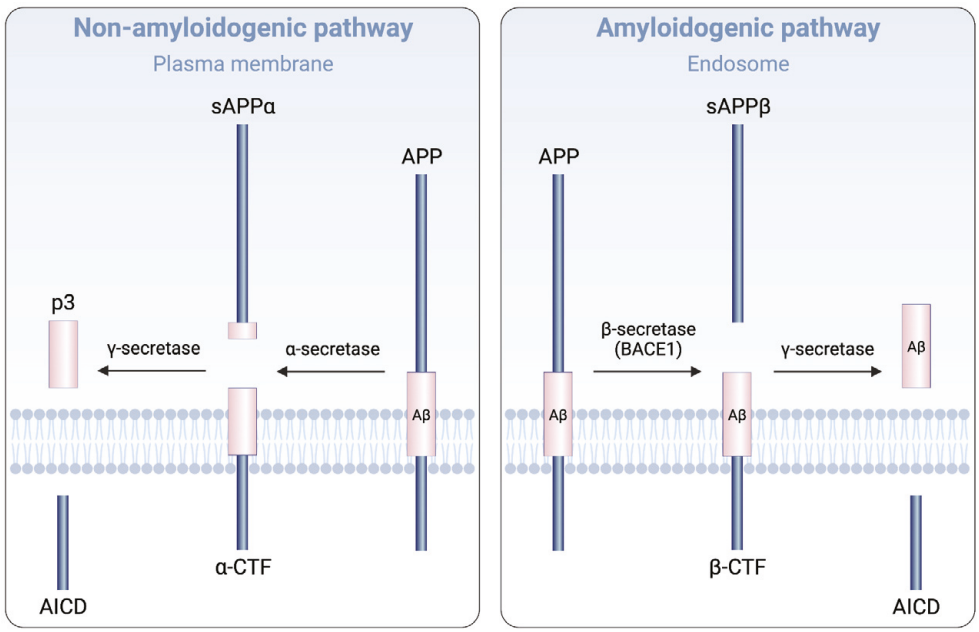

Figure 2. The illustration shows the cleavage pathways of the transmembrane protein APP**.** APP can be processed in two alternative pathways. In the non-amyloidgenic pathway (left image), which takes place at the plasma membrane, α-secretase cleaves APP within the Aβ domain, resulting in the release of sAPPα. The remaining membrane-bound fragment α-CTF is cleaved by γ-secretase, subsequently liberating p3 and AICD. In the amyloidogenic pathway (right image), which occurs in the endosomal-lysosomal system, APP is cleaved by β-secretase at the N-terminus of the Aβ domain, resulting in the release of sAPPβ. The remaining membrane-bound fragment β-CTF is subsequently cleaved by γ-secretase to generate Aβ and AICD. Created with BioRender.

APP that is not processed by α-secretases in the plasma membrane is rapidly internalized via clathrin-mediated endocytosis (82) and subsequently enters the endosomal-lysosomal system where amyloidogenic processing occurs (83, 84). In this pathway, APP is initially cleaved by β-secretase at the N-terminus of the Aβ domain, resulting in the shedding of a large soluble fragment known as sAPPβ (85). The β-secretase activity is exerted by beta-site APP cleaving enzyme 1 (BACE1) (86-91), which is a membrane-bound aspartyl protease that is highly expressed in the brain. Consistent with its acidic pH optimum, BACE1 has been found to be particularly enriched in endosomes (87), supporting that this cellular compartment is a major site for proteolytic processing of APP by the protease. Following cleavage by BACE1 and subsequent release of sAPPβ, the truncated C-terminal fragment of APP known as β-CTF remains bound to the membrane where it undergoes further processing by γ-secretase to generate Aβ and AICD. This occurs in a sequential manner, where γ-secretase initially cleaves β-CTF after amino acid 48 or 49, generating longer membrane-anchored Aβ peptides. These are subsequently processed every third amino acid, finally resulting in the release of mainly Aβ42 and Aβ40, respectively (92, 93).

Biochemical properties of Aβ

Soluble Aβ monomers are continuously generated in the healthy brain, and although their function during physiological conditions remains largely unknown, they have been implicated in the regulation of synaptic vesicle release (94), maintenance of neuronal glucose homeostasis (95), and regulation of neuronal expression of brain-derived neurotrophic factor (BDNF) (96), a protein that plays a role in synaptic plasticity (97). In AD, an imbalance between the generation and clearance of Aβ monomers are thought to cause their aggregation, consequently resulting in the formation of extracellular Aβ plaques (98). In comparison to Aβ40, whose generation is favoured in the amyloidogenic pathway, Aβ42 has a higher propensity to aggregate into oligomers, protofibrils, and fibrils (99). This is reflected in the brain of deceased AD patients, in which both Aβ peptides are commonly found in extracellular Aβ plaques, but where Aβ42 is the predominant species (100).

The molecular pathways by which Aβ aggregates in the AD brain are not completely understood, but *in vitro* studies have suggested that this is initiated by the assembly of two or more monomers into oligomers, a process known as primary nucleation. A portion of these soluble aggregates are subsequently converted to insoluble fibrils, whose surfaces are able to catalyze the generation of oligomers from monomers. This fibril-dependent generation of oligomers is referred to as secondary nucleation and has been suggested to be the predominate source of these soluble aggregates in the presence of fibrils (101, 102). Indeed, studies in both deceased AD patients (21, 103-105) and mouse models of the disease (105) have proposed that the halo of

more loosely organized Aβ surrounding the fibrillar centre of extracellular dense-core plaques is particularly enriched in Aβ oligomers.

Soluble oligomeric Aβ species that have a molecular weight greater than 75 kDa are defined as protofibrils (106, 107). These were initially described in 1997 by Walsh and colleagues (108), and have since then been identified in the AD brain, as well as in the brain from mouse models of the disease (109, 110). Small soluble Aβ oligomers (< 75 kDa) and protofibrils have been shown to be highly toxic to neurons, reflected by their ability to impair synaptic plasticity in mouse hippocampus (111-115), profoundly altered electrical activity of neurons in culture (116), induce synaptic and neuronal loss in both *in vivo* and *in vitro* model systems (103, 105, 114, 115, 117), and impair memory and leaning behavior in rodents (103, 115). Aβ fibrils, on the other hand, have been suggested to be more inert. These insoluble aggregates correlate poorly with clinical symptoms and neurodegeneration in humans (118, 119), and experimental studies have shown that their effect on synaptic plasticity is limited (103). Indeed, recent studies have suggested that soluble Aβ oligomers and protofibrils that are loosely organized in the periphery of extracellular Aβ plaques are sequestered by microglial cells into tightly packed insoluble fibrillar deposits as an attempt to protect surrounding neurons from damage (120, 121).

Tau

Tau is a microtubule-associated protein (122) that is predominantly found in the axonal compartment of neurons (123). In humans, the protein is encoded by the *MAPT* gene, which contains 16 exons and is found on chromosome 17 (124). Following transcription, the pre-mRNA undergoes alternative splicing of exon 2, 3 and 10, which give rise to six isoforms of the protein (125). These can be categorized as either three repeat (3R) or four repeat (4R) tau depending on the exclusion or inclusion of exon 10, respectively (124) (Figure 3).

Within the axon, tau plays an essential role in stabilizing the microtubule (122), an interaction that is mediated by the microtubule-binding domain of the protein (126). Under physiological conditions, this interaction is tightly regulated by post-translational modifications of tau, where phosphorylation has gained particularly much attention. More than 40 phosphorylation sites have been identified along the protein, of which many are found within the proline-rich domain (127).

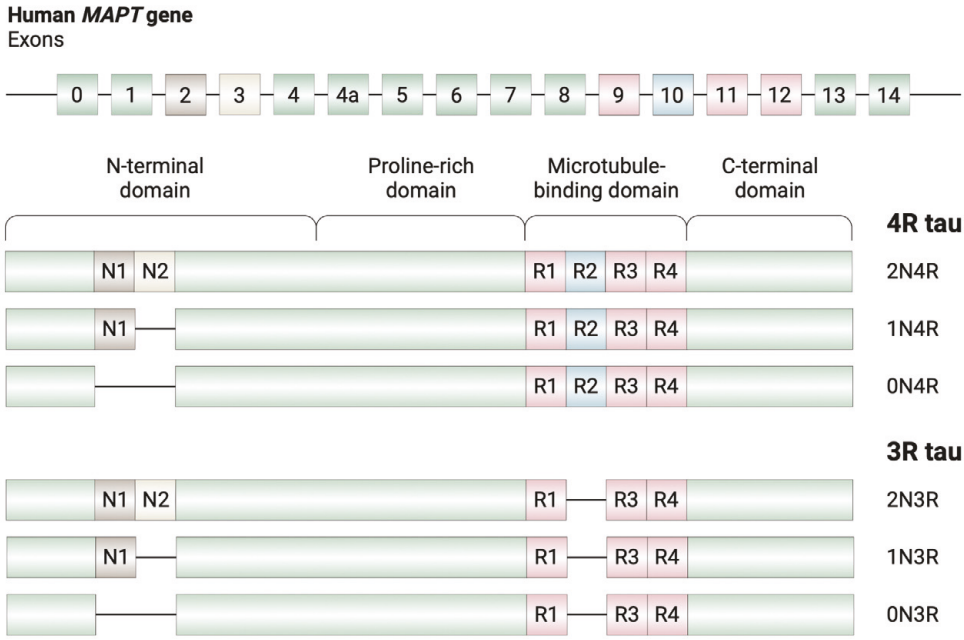

Figure 3. The illustration shows the isoforms of the microtubule-binding protein tau**.** Human tau is encoded by the *MAPT* gene, which contains 16 exons. Six isoforms of the protein have been identified, which are generated through alternative splicing of exon 2, 3, and 10. Inclusion of exon 10 gives rise to four repeat (4R) tau isoforms, while exclusion of this exon gives rise to three repeat (3R) tau isoforms. Created with BioRender.

In AD, tau gets abnormally hyperphosphorylated (128-130), which results in its detachment from the microtubule (131). Importantly, this also appears to promote aggregation of tau into filaments, particularly paired helical filaments (PHFs), which are the main component of NFTs (128-130). All six tau isoforms are found in PHFs in the AD brain, where the core of these aggregates is formed by two identical protofilaments comprising a part of the microtubule-binding region (129) (Figure 4).

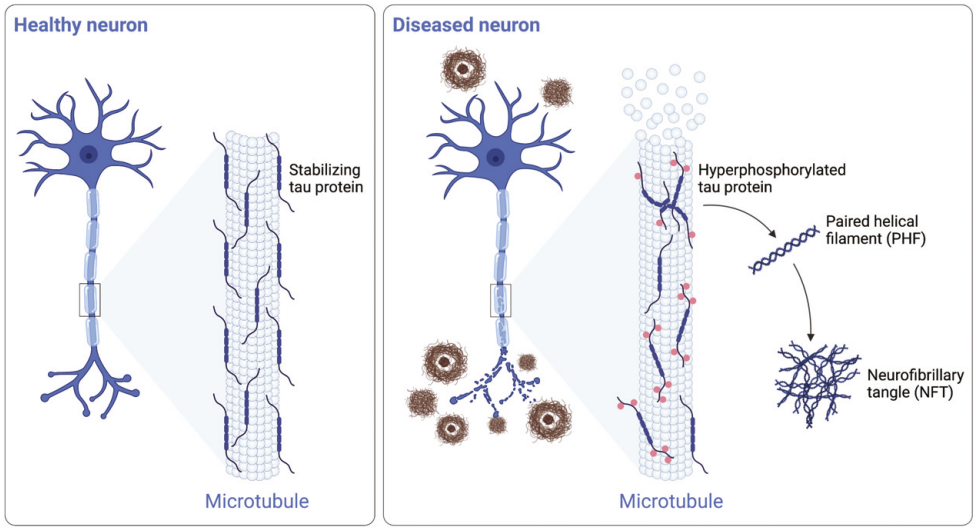

Figure 4. The illustration shows the role of tau in the healthy and diseased brain**.** In the healthy neuron (left image), tau is essential for stabilizing the microtubule within the axon. In the AD brain (right image), tau becomes abnormally hyperphosphorylated and detaches from the microtubule. The hyperphosphorylated state makes tau more prone to aggregate into PHFs and subsequently NFTs. Created with BioRender.

The link between Aβ and tau pathology

According to the amyloid cascade hypothesis, which was originally put forward by Hardy and Higgins (132) in 1992, Aβ initiates a pathological cascade that results in the formation of tau aggregates, ultimately leading to neuronal loss and cognitive decline (133). Indeed, neuroimaging studies in familial AD have shown that the development of Aβ pathology may precede the formation of tau filaments by several years (134). Other studies in sporadic AD have suggested that the spread of tau to neocortical regions may be accelerated by the regional presence of Aβ pathology (135), which is in line with findings that accumulation of neocortical tau almost exclusively occurs in individuals with Aβ pathology in the brain (136). Although the mechanistic relationship between Aβ and tau is not fully understood, experimental studies have shown that soluble Aβ oligomers isolated from the brain of deceased AD patients (137), as well as synthetic variants of such soluble aggregates (138), are able to induce hyperphosphorylation of tau in neuronal cell culture systems. This effect of soluble Aβ oligomers *in vitro* has been associated with tau-dependent disruption of the neuronal cytoskeleton and degeneration of neurites (137), supporting Aβ as an upstream driver of impaired tau metabolism. These findings are well in line with additional studies in mice showing that the burden and propagation of established tau pathology in the brain is enhanced when pathogenic Aβ is introduced (139-141). Further investigations have suggested that

Aβ pathology initially creates an environment that facilitates the formation of smaller tau aggregates in dystrophic neurites, and that these in turn are necessary for the formation of NFTs and neuropil threads at a later disease stage (142). Although the signaling pathways involved in Aβ-mediated alterations of tau metabolism have not been completely elucidated, kinases, such as glycogen synthase kinase 3β (GSK-3β) (143) and cyclin-dependent kinase 5 (cdk5) (144), and phosphatases, such as protein phosphatase 2A (PP2A) (145), have been suggested to play a role. These enzymes are important regulators of tau phosphorylation, and have been reported to be altered in the AD brain (146-148).

Genetic risk factors

As mentioned previously, familial AD accounts for a minority of all EOAD cases. The most common causes of this rare form of dementia are mutations in the *PSEN1* or *PSEN2* genes (149). These encode presenilin-1 and presenilin-2, respectively, which are transmembrane proteins that constitute the catalytic domain of γ-secretase complexes (150-153). Familial AD can also by caused by mutations in the *APP* gene. These mutations are commonly found around the cleavage sites of BACE1 and γ-secretase, increasing the overall production of Aβ or the Aβ42/Aβ40 ratio, respectively (154). A few mutations within the Aβ sequence of *APP* have also been identified, including the Arctic and Osaka mutations. These affect the aggregation properties of Aβ, where the Arctic mutation specifically enhances the formation of soluble protofibrils (106, 155). The identification of these rare mutations as an underlying cause of AD has laid the foundation for the development of numerous mouse models that are commonly used in the research field. By introducing human *APP* and/or *PSEN1/2* harboring such mutations, these mice develop Aβ pathology in the brain that resembles that observed in humans with AD (156).

In terms of sporadic AD, several genetic risk factors that increase the likelihood of developing the disease have been identified (157). Most notable is the polymorphic *APOE* gene, which encodes the cholesterol transporter apolipoprotein E (ApoE). The *APOE* gene has three main variants, which are known as ε2, ε3, and ε4 (158). Specifically, the *APOE* ε4 variant is strongly linked to the development of sporadic AD, where studies have reported a 3- to 15-fold increased risk depending on if you have inherited one of two copies of this gene variant (159).

Treatment

Cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) (160) and the N-methyl-D-aspartate (NMDA) receptor antagonist memantine (161) are the main medications used for the treatment of AD. These do not affect the underlying pathology of the disease, but are used to temporarily relieve symptoms in mild to

moderate AD dementia. Cholinesterase inhibitors are doing so by increasing the levels of the neurotransmitter acetylcholine, which is reduced in AD due to loss of certain neurons (160). Memantine, on the other hand, inhibits the effect of the neurotransmitter glutamate, which is released in excessive amounts in the AD brain, causing further damage to the neurons (161).

Many attempts have been made to develop drugs that in different ways target the underlying pathology of AD, but the majority of clinical trials that have been conducted have failed to show any clinical benefit of such drugs (162). However, in 2021, the Food and Drug Administration (FDA) granted accelerated approval to Aducanumab, a monoclonal antibody that targets and remove Aβ plaques in the brain. The clinical benefit of this disease-modifying therapy is however uncertain, and the process of accelerated approval requires this to be verified in a post-approval clinical trial.

Recently, a large phase III clinical trial in early AD with another monoclonal antibody known as Lecanemab reported promising results. By selectively targeting soluble Aβ protofibrils, the drug removed Aβ plaques in the brain and reduced cognitive decline by 27% compared with the placebo group (163). In January 2023, Lecanemab was granted accelerated approval by the FDA for treatment of MCI based on results from the phase II trial (164).

Fluid biomarkers for Alzheimer's disease

Biochemical changes in the brain can be reflected in the cerebrospinal fluid (CSF), which is a colourless liquid that occupies the cerebral ventricles, as well as the cerebral and spinal subarachnoid spaces within the central nervous system (165). CSF can be safely sampled by lumbar puncture (166), whereafter proteins that have reached this fluid compartment from the extracellular space of the brain can be measured by the use of different biochemical methods, such as immunoassays and mass spectrometry (MS) (167).

During the past two decades, several CSF biomarkers that can detect the key pathological hallmarks of AD have been developed (167), where Aβ42 (or the Aβ42/Aβ40 ratio), phosphorylated tau (p-tau), total tau (t-tau), and neurofilament light chain (NfL) have been extensively investigated. These have become important in the clinical work-up of AD, evaluation of disease-modifying therapies in clinical trials, and research aiming at increasing the understanding of the disease. Indeed, when the National Institute on Aging and Alzheimer's Association (NIA-AA) presented a new research framework for the definition of AD in 2018, these CSF biomarkers got a prominent role. In this framework, AD is not defined by its clinical presentation, but rather by its underlying pathology that in living persons is identified by biomarkers associated with either Aβ plaques (A), NFTs (T), or neurodegeneration (N) (168) (Table 1). However, the underlying pathological processes that cause changes in key CSF biomarkers for AD are not yet fully elucidated.

Table 1
Biomarker grouping by the ATN research framework

A: Aβ plaques or associated pathological state
CSF Aβ42 or Aβ42/Aβ40 ratio
Aβ-PET
T: NFTs or associated pathological state
CSF p-tau
Tau PET
N: Neurodegeneration or neuronal injury
CSF t-tau
CSF NfL
Anatomical magnetic resonance imaging (MRI)
Fluorodeoxyglucose (FDG)-PET

CSF and imaging biomarkers associated with Aβ plaques, NFTs, and neurodegeneration according to the ATN research framwork developed by the NIA-AA.

Although CSF collection is a safe procedure that is clinically implemented in many countries for the work-up of AD, it is not widely available outside of specialized clinics and can be regarded as invasive. Much effort has therefore been made to develop easily accessible blood-based biomarkers that reliably can detect AD-related pathological processes in the brain. Such biomarkers would facilitate implementation in clinical practice, and enable a more efficient screening of potential participants in clinical trials (169).

Aβ

The concentration of Aβ42 in CSF has consistently been found to be reduced in the prodromal and dementia stages of AD, where an approximate 50% decline has been reported in patients with AD dementia compared with cognitively healthy individuals (170). Reduced concentrations have also been found in the preclinical phase of the disease, where longitudinal studies have suggested that this drop occurs at least a decade before symptom onset (46, 47). This has made it possible to identify cognitively healthy individuals at high risk of developing AD dementia later in life, and has important implications for clinical trials and future clinical management, as disease-modifying therapies are likely most effective when introduced during this early phase of the disease (169).

In 2003, an autopsy study conducted by Strozyk and colleagues (171) showed that lower concentrations of ventricular CSF Aβ42, collected postmortem, associated with higher numbers of neuritic plaques in AD-affected brain regions, even in cognitively healthy individuals. These findings were later confirmed by Tapiola and colleagues (172), who reported an inverse association between the concentration of lumbar CSF Aβ42, collected antemortem, and the cerebral burden of neuritic plaques at autopsy. Since then, PET ligands that specifically bind to fibrillar Aβ in the brain have been developed, which have enabled *in vivo* visualization of plaque pathology throughout the AD continuum (173). Similar to the autopsy studies, the concentration of Aβ42 in CSF has been found to inversely associate with Aβ-PET retention, where a high concordance between the two measures has been reported (174-176). On the basis of these findings, it has been proposed that the concentration of Aβ42 in CSF is reflective of the burden of fibrillar Aβ in the brain, and that its decline in AD is caused by deposition of Aβ42 into these insoluble aggregates, thereby reducing the availability of this Aβ peptide to reach the CSF. Nevertheless, both cross-sectional (174-178) and longitudinal (47, 179, 180) studies have shown that the concentration of Aβ42 in CSF reaches a plateau early in the disease, although the burden of fibrillar Aβ in the brain continues to increase. These findings point toward a more complex relationship between cerebral Aβ pathology and reduced concentrations of Aβ42 in CSF during the course of AD than previously described.

In addition to Aβ42, the concentration of Aβ40 can be reliably measured in CSF. Although Aβ40 also is found in fibrillar deposits in the brain (100), the concentration of this Aβ peptide remains unchanged in CSF along the AD continuum (170). Nevertheless, the use of Aβ40 in ratio with Aβ42, *i.e.*, the CSF Aβ42/Aβ40 ratio, has shown higher concordance with Aβ-PET compared with CSF Aβ42 alone (177, 178, 181, 182). The reason for this improved performance is not completely understood, but it is possible that the ratio provides correction for individual variability in total Aβ production or preanalytical confounding factors (169). Similar to CSF Aβ42, a reduced CSF Aβ42/Aβ40 ratio has been found already in preclinical AD (183, 184). Notably, the decline of both these measures has been reported to occur before Aβ-PET becomes abnormal (178, 185-188).

In contrast to CSF, measurement of the concentration of Aβ42 in blood has proven challenging, with initial studies showing inconsistent results (189). However, the use of recently developed ultrasensitive immunoassays (183, 184, 190, 191) and high-precision immunoprecipitation MS (IP-MS) methods (192-194) have demonstrated more promising results. Particularly, the Aβ42/Aβ40 ratio and the Aβ40/Aβ42 ratio in plasma have shown to predict cerebral Aβ pathology with moderate to high accuracy (183, 191-195), with the use of IP-MS being superior to immunoassay-based measurements (195). Similar to CSF, the Aβ42/Aβ40 ratio in plasma declines already in the preclinical phase of the disease (183, 184), a change that may occur before a significant burden of fibrillar Aβ in the brain, as measured by Aβ-PET, is reached (193). However, the use of the Aβ42/Aβ40 ratio in plasma to identify Aβ status may be limited, considering that only a 10-15% decline is observed in individuals with cerebral Aβ pathology (184, 191-193).

Tau

Different soluble tau species can be reliably measured in CSF, where t-tau and tau phosphorylated at threonine 181 (p-tau181) localized in the proline-rich domain of the protein have been commonly studied (170).

The concentration of t-tau in CSF is increased in AD (170), and has been suggested to reflect its passive release into the extracellular space as a result of neuronal injury and loss. Indeed, its concentration is also elevated in other neurodegenerative disorders (196), as well as in acute brain disorders such as stroke (197) and traumatic brain injury (198, 199). Studies in humans using stable isotope labelling kinetics (SILK) have however suggested that the increased concentration of CSF t-tau in AD, at least to some degree, may reflect elevated production and active release of the protein in response to Aβ pathology (200). Mechanisms beyond passive release have also been implied in mouse models of AD, in which increased concentrations

of CSF t-tau in response to Aβ pathology have been demonstrated long before the presence of modest region-specific neurodegeneration (201).

The concentration of p-tau181 in CSF is also increased in AD (170), but in contrast to t-tau, the change is disease-specific (197, 202, 203), supporting that this biomarker reflects abnormal tau metabolism rather than neuronal injury and loss. The development of Tau PET tracers that specifically bind to PHFs in the brain has made it possible to study the association between the burden of these tau aggregates and CSF tau biomarkers along the AD continuum (204). Such studies have revealed that the concentration of p-tau181 and t-tau in CSF may be elevated already in preclinical AD, despite the absence of an abnormal burden of PHFs in the brain (205). Indeed, the two CSF tau biomarkers have shown to only moderately associate with the cerebral burden of these tau aggregates, mainly in the symptomatic stage of the disease (41, 205-207). In addition, measures of grey matter atrophy (205) and cognition (41, 205) have been demonstrated to be more closely linked to the burden of PHFs in the brain than to the concentrations of p-tau181 and t-tau in CSF. These findings support that the two CSF tau biomarkers may reflect pathological processes beyond aggregation of tau, particularly in the early stage of the disease. Such pathological processes may be linked to the deposition of Aβ in the brain (208, 209), but further investigations are needed to fully understand the complex relationships between Aβ pathology, altered CSF p-tau and t-tau concentrations, and tau aggregation.

In recent years, several studies investigating phosphorylation sites upstream of threonine 181 in the proline-rich domain of the tau protein have emerged. In the context of the work of this thesis, tau phosphorylated at threonine 217 (p-tau217) has been shown to distinguish AD dementia from cognitively healthy individuals, as well other dementias, with higher accuracy than p-tau181 (210-212). In further comparisons between the two CSF p-tau isoforms, p-tau217 has shown the strongest association with the burden of both tau aggregates and Aβ pathology in the brain (210). Similar to p-tau181, the concentration of p-tau217 in CSF is elevated in preclinical AD. However, it has been suggested that the initial change of p-tau217 may occur prior to that of p-tau181 (208, 209).

Recent development of novel assays has made it possible to reliably measure the concentrations of p-tau181 and p-tau217 in human blood samples (213-216). Similar to CSF, the concentrations of these p-tau isoforms in plasma start to increase before symptom onset (213-215, 217), and can with high accuracy discriminate the disease from other neurodegenerative disorders (214-216, 218). This is a pioneering advancement for AD diagnostics, given that the global use of current CSF and imaging biomarkers is limited due to the invasiveness, high cost, and restricted availability. However, studies on these plasma biomarkers remain outside of the scope of this thesis and will not be discussed in further detail.

Neurofilament light chain

NfL has emerged as a promising fluid biomarker of neuroaxonal injury and loss in several acute and chronic neurological disorders, including AD (219). NfL is a neurofilament protein that is expressed exclusively in neurons (27). It belongs to the family of intermediate filaments, which are essential components of the cell cytoskeleton, and are defined according to their diameter of approximately 10 nm that is "intermediate" between the diameters of actin filaments (~7 nm) and microtubules (~25 nm). These have been classified into six groups, in which NfL, together with neurofilament heavy chain (NfH), neurofilament medium chain (NfM), and α-internexin, constitute class IV intermediate filament proteins (220).

During maturation of neurons in the central nervous system, NfL interacts with NfH, NfM, and α-internexin to form highly organized heteropolymer protein complexes that provide structural stability to large myelinated axons (221). Similar assemblies are found in the peripheral nervous system, with the exception that α-internexin is replaced by a class III intermediate filament protein known as peripherin (222). Within myelinated axons, NfL is essential for radial growth and effective propagation of nerve impulses (223, 224), and has been suggested to play a role in both ER distribution (225) and regulation of mitochondrial dynamics (226). Recent studies have also shown that NfL is an integral component of synapses, where it is involved in maintaining the structural integrity and function of dendritic spines, as well as in the regulation of specific receptors important for neurotransmission (227, 228).

In the healthy brain, low concentrations of NfL are released into the extracellular space from where it reaches the CSF, as well as the circulatory system. These concentrations are markedly elevated in response to neuroaxonal damage, regardless of the cause (27). In AD, increased concentrations of NfL in both CSF (229-234) and blood (229, 235-237) have been reported in the symptomatic stages of the disease, in which they have found to be associated with poor cognitive performance (231, 235, 236) and accelerated brain atrophy (231, 232, 235, 236). For NfL in CSF, associations with white matter abnormalities have also been shown (231, 238). In addition, a few studies have revealed increased concentrations of NfL in CSF already in cognitively health individuals with signs of Aβ pathology in the brain (232, 233). In one study, such changes were associated with brain atrophy at this early disease stage (232). Notably, for sporadic AD, comparable studies investigating NfL in blood have not been able to demonstrate such changes before symptom onset (236, 239). This is in contrast to familial AD, where longitudinal studies have shown that increased concentrations of NfL in blood precede expected symptom onset by 15-20 years (240-242).

As the concentrations of NfL in CSF and blood are increased in a broad range of neurological disorders, the diagnostic use of these biomarkers in AD is limited (243). Nevertheless, they may be important tools to track the intensity of ongoing

neurodegeneration, and are likely to have a value in the evaluation of disease-modifying therapies in clinical trials where assessment of the effect on neurodegenerative processes could be of importance. However, to optimize their use in such settings, further understanding about the pathological processes that these biomarkers reflect along the AD continuum, particularly in the preclinical phase of the disease, are needed.

Aims of the thesis

The general aim of this thesis was to explore the association between pathological processes in the brain and changes in key fluid biomarkers of AD along the disease continuum. More specifically, we aimed to:

- compare the performance of NfL in CSF and blood as biomarkers of neurodegeneration by investigating their associations with disease-related brain changes along the AD continuum in humans, as well as in a mouse model of the disease (paper I).
- investigate the temporal relationship between cerebral development of fibrillar Aβ plaques, altered p-tau and t-tau in CSF, and accumulation of insoluble tau aggregates in the brain along the AD continuum in humans, as well as in a mouse model of the disease (paper II).
- investigate the temporal and correlative relationship between Aβ pathology in the brain and altered Aβ42/Aβ40 ratios in CSF and blood in a mouse model of preclinical AD (paper III).
- investigate the association between key features of cerebral Aβ pathology and changes in CSF Aβ42/Aβ40 ratios, NfL, and t-tau in a mouse model of AD to deepen our understanding of the pathological processes that these fluid biomarkers reflect (paper IV).

Methodological considerations

This section will provide an overview of the methods applied in this thesis. For more detailed descriptions, please see the method section of the individual papers.

Experimental studies

Ethical approval

The animal experimental procedures were carried out in accordance with Swedish animal research regulations and approved by the committee of animal research at Lund University (ethical permit number: 7482/2017).

Animals

5xFAD

The research field has long relied on first-generation mouse models that overexpress human *APP* and *PSEN1* transgenes under the control of certain promotor elements (156). One such model is the 5xFAD, which was used in **paper I, II, and V**. These mice harbor five mutations known to cause familial AD in humans; the Swedish (K670N/M671L), Florida (I716V) and London (V717I) mutations in the *APP695* isoform transgene, and the M146L and L286V mutations in the *PSEN1* transgene (244). The combined presence of these mutations increases the generation of Aβ in the brain, particularly Aβ42 (244-248). This results in a phenotype that resembles some of the key pathological characteristics of AD, including cerebral Aβ pathology, neuroinflammation, synaptic and neuronal loss, and cognitive deficits. More specifically, extracellular Aβ plaques start to accumulate in subiculum and the deep cortical layers around two months of age, rapidly spreading to other brain regions with age. This is accompanied by reduced levels of pre-and postsynaptic markers from around 4 months (244), and by 9 months, significant neuronal loss is apparent in the regions where amyloidosis is most severe (244, 249).

As one of few models with previously confirmed neurodegeneration (244, 249), 5xFAD mice were chosen for experimental studies in **paper I** and **II**, in which

different fluid biomarkers of neuronal injury and loss were investigated in association with cerebral Aβ pathology over time. Furthermore, it has previously been shown that Aβ peptides of different length, most notably Aβ42 and Aβ40, to a significant degree deposit in the brains of 5xFAD mice (244). This was an important characteristic for the experimental studies in **paper IV**, where we aimed to gain a better understanding of the pathological processes that reduced CSF Aβ42/Aβ40 ratios in AD reflect.

App$^{NL\text{-}F/NL\text{-}F}$ and App$^{NL/NL}$ knock-in

To overcome the issue with *APP*-overexpression in first-generation mouse models, several knock-in mouse models that express endogenous levels of *APP* while producing pathogenic human Aβ have recently been introduced to the field (250-253). These may provide a better tool to gain further insight into the dynamics of fluid biomarkers in relation to cerebral Aβ pathology during preclinical AD. Thus, in **paper III** where we aimed to investigate changes in CSF and blood Aβ and their relation to cerebral amyloidosis in the very early disease stage, slowly progressing *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice were chosen as a model system. In these mice, the Aβ sequence of the endogenous *APP* gene has been humanized and two mutations linked to familial AD have been introduced: the Swedish (KM670/671NL) and Beyreuther/Iberian (I716F) (250). This results in increased production of Aβ in the brain, where the Beyreuther/Iberian mutation particularly favors the generation of Aβ42 over Aβ40 (245, 250, 254) (Figure 5). Consequently, extracellular Aβ plaques accumulate in cortex and hippocampus in an age-dependent manner, starting from around 6 months. *App*$^{NL/NL}$ knock-in mice that only harbor the Swedish mutation and show no sign of extracellular Aβ plaques in the brain, even at advanced age, were used as controls (250).

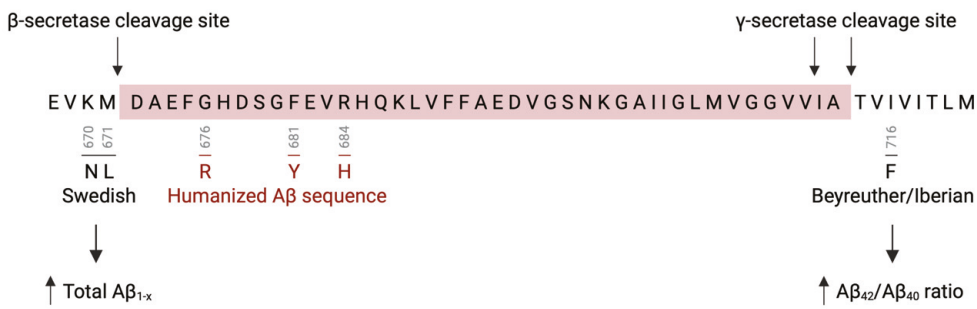

Figure 5. The illustration shows the construct of the *APP* gene in *App* knock-in mice. In *App*$^{NL/NL}$ and *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice, the endogenous *APP* gene has been humanized by the replacement of three amino acids within the Aβ domain. The generation of Aβ is accelerated due to the introduction of the Swedish (KM670/671NL) mutation alone or in combination with Beyreuther/Iberian (I716F) mutation.

3xTg

First-generation *APP*-overexpressing 3xTg mice that demonstrate a relatively slow disease progression were used as an additional model system in **paper III**, with the aim to compare with $App^{NL\text{-}F/NL\text{-}F}$ knock-in mice in regards to the dynamics of certain fluid biomarkers. Together with endogenous expression of human *PSEN1* harboring the M146V mutation, 3xTg mice overexpress human *APP695* with the Swedish (KM670/671NL) mutation, as well as human $MAPT_{(4R0N)}$ with the P301L mutation. This results in cerebral deposition of intracellular Aβ from 3 months and accumulation of extracellular Aβ plaques from 6 months (255), although there have been reports on phenotypic drift in the development Aβ pathology in various colonies (256).

Sample collection and preparation

CSF

In **paper I-IV**, CSF was collected from the cisterna magna, with acquired volumes ranging from 5-15 µl depending on factors such as mouse strain, age, and sex. Previously published protocols have described the use of either a glass capillary (257) or a needle in combination with a polypropylene pipette tip (258) for penetration of the overlying dura mater and subsequent sample collection in mouse models of AD. For analysis of Aβ, which is a sticky protein, the latter method has in some instances been suggested to be preferable due to the assumption that less protein binds to the surface of polypropylene pipette tips in comparison with glass capillary tubes. However, in a pilot study using fresh human CSF, we did not find any differences in the concentrations of Aβ following uptake of the fluid into the two different collection systems. Furthermore, using a glass capillary tube resulted in a consistently larger collection volume from the mouse cisterna magna, as well as a notably higher number of samples that were free from blood contamination. Thus, to ensure a high success rate, and thereby also reducing the number of animals, glass capillary tubes were chosen for collection in our studies. The procedure was carried out terminally under isoflurane anesthesia, and all samples were collected between 9 AM and 1 PM to minimize potential diurnal fluctuations in the investigated CSF biomarkers (259, 260).

Blood

In **paper I and III**, blood was terminally collected from the left ventricle of the heart and allowed to clot for 2 h at room temperature. Subsequently, the samples were centrifuged at 2000 x *g* for 20 minutes and the supernatant, that is the serum, was collected and stored at -80°C.

Brain tissue

In **paper I-IV**, brains were removed following transcardial perfusion with ice-cold 0.1 M phosphate buffer (PB). Left hemispheres were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 48 h at 4°C and then immersed in 30% sucrose solution for 48 h at 4°C. Following fixation, brains were serially cut into 30 μm sagittal sections with a Leica SM2010R sliding microtome and kept in an antifreeze solution containing 30% sucrose and 30% ethylene glycol in PB at -20°C. Right hemispheres were dissected into different regions and stored at -80°C until use.

Biochemical analysis of CSF and blood samples

CSF and serum samples collected from mice were analyzed using single molecule array (Simoa) technology (Quanterix), which enables ultra-sensitive protein detection in small sample volumes.

For analysis of NfL in paper **I** and **IV**, a homebrew kit was used to transfer the monoclonal antibodies and calibrators from the NF-light ELISA kit provided by UmanDiagnostics AB onto the Simoa platform (261). The assay, which originally was developed for detection of NfL in human samples, has shown to perform well also for measurement of NfL in mice (262), as the sequence to which the monoclonal antibodies are directed is fully conserved between the two species (263). In **paper II and IV**, the Simoa® Mouse Tau Discovery Kit (Quanterix) was used to measure the concentration of CSF t-tau. Furthermore, the concentrations of human Aβ42 and Aβ40 in CSF and serum in **paper III**, as well as in CSF in **paper IV**, were measured using the Simoa® Aβ42 and Aβ40 Advantage Kits (Quanterix). All measurements were performed in singlicates on a Simoa HD-1 Analyzer (Quanterix).

Fluorescent staining

In **paper I-III**, fibrillar dense-core Aβ plaques were identified in sagittal brain sections by staining with 0.01% thioflavin S in 50% ethanol for 10 minutes. Stained specimens were mounted on glass slides and coverslipped with SlowFade^{TM} Gold Antifade Mountant (Invitrogen).

Immunofluorescence staining was performed in **paper III-IV** for detection of Aβ42 and Aβ40 deposits in the collected brains. Free-floating sagittal sections were treated with 88% formic acid (FAc) to enhance antigen retrieval, permeabilized in tris-buffered saline (TBS) supplemented with 0.25% Triton X-100 (TBSX) to improve antibody access to intracellular Aβ, and blocked with 5% normal donkey serum (NDS) in TBSX to prevent non-specific binding of the antibodies. The sections were then incubated with anti-Aβ42 (Invitrogen, 1:1000 dilution) or anti-

Aβ40 (IBL, 1:100 dilution) primary antibodies followed by appropriate Alexa-fluorophore-conjugated secondary antibodies. Stained specimens were mounted on glass slides and coverslipped with ProLong™ Diamond Antifade Mountant (Invitrogen).

Image acquisition and analysis

In **paper I, II, and IV**, fluorescence images of the brain regions of interest were acquired using a 10x objective lens on an inverted Olympus IX70 fluorescence microscope. The stained area was quantified using the Fiji software by applying an automated threshold that was maintained for all images analyzed. Furthermore, in **paper IV**, images for qualitative assessment of the distribution of the investigated Aβ peptides within extracellular plaques were captured using a 40x objective on a Leica SP8 laser scanning confocal microscope.

In **paper III**, whole brain sections were imaged using a 10x objective lens on an Operetta® CLS™ High Content Analysis System (PerkinElmer). Manual segmentation of cortex and hippocampus was performed, and the stained area in these regions was quantified using the Fiji software by a similar procedure as described for **paper I, II, and IV**.

Biochemical analysis of brain tissue

Homogenization

In **paper III and IV**, cortex from the right hemisphere was homogenized at 10% (w/v) in TBS (50 mM tris-HCl, 150 mM NaCl, pH 7.6) supplemented with protease and phosphatase inhibitors (Thermo Scientific) using the FastPrep-24™ Classic bead beater grinder and lysis system (MP Biomedicals). The homogenized cortical brain tissue was aliquoted and stored at -80°C until use.

Analysis of Aβ in soluble and insoluble brain tissue extracts

To extract soluble and insoluble forms of Aβ, prepared cortical brain tissue homogenates were thawed on ice and centrifuged at 14 000 x *g* for 30 min at 4°C. The supernatant was collected as the TBS-soluble fraction, which is enriched in Aβ monomers, oligomers, and protofibrils. To extract TBS-insoluble fibrillar Aβ species, the remaining pellet was resuspended at 10% (w/v) in ice-cold 70% FAc supplemented with protease and phosphatase inhibitors (Thermo Scientific), sonicated on ice, and centrifuged at 14 000 x *g* for 1 h at 4°C. The supernatant was collected and subsequently neutralized in 1M Tris-base at room temperature. The TBS- and FAc-soluble extracts were aliquoted and stored at -80°C until use.

The concentrations of human Aβ42 and Aβ40 in the TBS- and FAc-soluble fractions were measured using the V-PLEX Aβ Peptide Panel 1 (6E10) or the V-PLEX Human Aβ42 Peptide (6E10) kit provided by Meso Scale Discovery (MSD). All samples were measured in singlicates, as these electrochemiluminescence (ECL) immunoassays have shown low intra-plate coefficient of variance in previous analyses performed in our laboratory.

Isolation of brain soluble Aβ protofibrils

The concentration of brain soluble Aβ protofibrils and their content of certain Aβ peptides were measured in **paper IV**. Prepared cortical brain tissue homogenates were thawed on ice and centrifuged at 16 000 x *g* for 1 h at 4°C. The TBS-soluble fraction was then collected and divided into two aliquots. The first aliquot was used to determine the total concentration of brain soluble Aβ protofibrils using an in-house sandwich ECL immunoassay on the MSD platform. This immunoassay uses the mouse monoclonal antibody mAb158, which is the murine version of Lecanemab and selectively binds to Aβ protofibrils in solution (109, 264, 265), for antigen capture, and biotinylated mAb158 and Streptavidin SULFO-TAG (MSD) for antigen detection. The second aliquot was used to measure the concentrations of Aβ42 and Aβ40 in the brain soluble Aβ protofibrils. Briefly, Aβ protofibrils were isolated from the TBS-soluble extracts by immunoprecipitation using mAb158. Following monomerization, the concentrations of Aβ42 and Aβ40 in the isolated Aβ protofibrils were measured in duplicates using the V-PLEX Aβ Peptide Panel 1 (6E10) kit provided by MSD.

Statistical analysis

In the experimental studies conducted in **paper I-IV**, the sample size in the different age groups was small, and reliable assumptions regarding the distribution of the investigated fluid biomarker concentrations and measures of cerebral Aβ pathology could therefore not be made. Thus, to study the effect of age on these variables, rank-based nonparametric tests that do not require any assumption about the underlying distribution were applied. These included the Jonckheere-Terpstra trend test in **paper I and II** and the Kruskal-Wallis H test in **paper III and IV**. If a statistical significance was found, *post hoc* analysis for group comparisons were done using the Mann-Whitney U test. In **paper I and II**, corrections for multiple comparisons were made using the Bonferroni method. Furthermore, the Mann-Whitney U test was used to compare fluid biomarker concentrations between 5xFAD mice and non-transgenic littermates in **paper I and II**.

In **paper I-III**, correlations between fluid biomarkers and measures of cerebral Aβ pathology in the whole study population were analyzed using Spearman's rank-

ordered correlation coefficient. Meng's Z-test for correlated correlations was performed for comparisons between correlation coefficients in **paper I and III**.

In **paper IV**, simple and multiple linear regression analyses adjusted for sex were performed to study associations between each CSF biomarker (as outcome variable) and measures of cerebral Aβ pathology (as predictor variables). To meet the assumptions of linear regression, the predictors were Log10-transformed. For models with the same outcome variable, bootstrap procedures (n = 5000 iterations) were used to compare the adjusted R^2 between different regression models. Moreover, mediation analyses were conducted using a bootstrap method for estimation of mediation effects.

Statistical analyses were performed in IBM SPSS Statistics and R.

Clinical studies

Ethical approval

The study procedures were approved by the Regional Ethics Committee in Lund. All participant or their relatives gave written informed consent.

Study population

In **paper I** and **II**, study populations originating from the prospective and longitudinal Swedish BioFINDER study were included. This is a well-defined cohort that includes a clinically relevant population of patients with MCI and AD dementia, as well as cognitively healthy elderly. These are uniquely examined with world-leading technological platforms for fluid biomarker assessment and discovery, and brain imaging such as magnetic resonance imaging (MRI) and PET.

In **paper I**, 478 cognitively unimpaired (CU) individuals, 227 patients with MCI, and 113 patients with AD dementia with available baseline measurements of CSF and plasma NfL were included in the study. Study participants who were CU or diagnosed with MCI were categorized based on Aβ and neurodegeneration status, as defined as normal (–) or abnormal (+) CSF Aβ42/Aβ40 ratios (cutoff < 0.091) and cortical thickness in AD-susceptible temporal regions (cutoff < 2.25), respectively. All participants with AD dementia had abnormal CSF Aβ42/Aβ40 ratios, but information about cortical thickness was not available for this diagnostic group.

In **paper II**, 131 participants without or with cognitive impairment due to AD who had undergone Tau PET imaging with ^{18}F-flortaucipir and lumbar puncture for CSF

biomarker analyses were included in the study. For patients with cognitive impairment, an abnormal CSF Aβ42/Aβ40 ratio (< 0.1) was an inclusion criterion. All participants were grouped by Aβ status (based on the CSF Aβ42/Aβ40 ratio) and the Clinical Dementia Rating (CDR) global score into $A\beta^-$ CU (CDR, 0), $A\beta^+$ CU (CDR, 0), $A\beta^+$ with mild cognitive deficits (CDR, 0.5), and $A\beta^+$ AD dementia (CDR, 1 to 3).

CSF and blood sample collection

CSF was collected by lumbar puncture and centrifuged at 2000 x *g* for 10 minutes at 4°C. Blood was drawn into EDTA-containing tubes within 15 minutes of CSF sampling and centrifuged at 2000 x *g* for 10 minutes at 4°C to obtain the plasma. CSF and plasma samples were aliquoted into polypropylene tubes and stored at −80°C until biochemically analyzed.

Biochemical analysis of CSF and blood samples

In **paper I**, the concentration of NfL in CSF was measured using the NF-light ELISA kit (UmanDiagnostics AB, Umeå, Sweden). Moreover, an in-house assay, in which a homebrew kit was used to transfer the monoclonal antibodies and calibrators from the NF-light ELISA kit onto the Simoa platform (261), was used to measure the concentration of NfL in plasma. CSF Aβ42, Aβ40, and p-tau concentrations were analyzed using fully automated EUROIMMUN immunoassays.

In **paper II**, ECL assays developed by Eli Lilly and Company on the MSD platform were used to measure the concentrations of p-tau217 and p-tau181 in CSF. The concentrations of Aβ42, Aβ40, and t-tau were analyzed using fully automated EUROIMMUN immunoassays.

Imaging

In **paper I**, ^{18}F-flutemetamol PET scans for visualization of fibrillar Aβ in the brain were available in 244 CU individuals and 138 patients with MCI. The Aβ-PET signal in a global neocortical composite region was used, and the standardized uptake value ratio (SUVR) was defined as tracer uptake in a volume of interest normalized to a composite reference region. Furthermore, 478 CU individuals and 227 patients with MCI had undergone diffusion tensor imaging (DTI), from which parameter maps for fractional anisotropy (FA) and mean diffusivity (MD) were obtained.

In **paper II**, Tau PET with ^{18}F-flortaucipir was completed in all participants, where the signal from three regional measures was used: entorhinal cortex, inferior temporal cortex (ITC), and a composite region corresponding to Braak stage V-VI. Furthermore, 121 participants had undergone Aβ-PET with ^{18}F-flutemetamol, where the signal in a global neocortical composite region was used.

Statistical analysis

In **paper I**, the concentrations of NfL in CSF and plasma were compared between diagnostic groups stratified by Aβ and neurodegeneration status using univariate general linear models adjusted for age and sex, and the obtained *p*-values were corrected for multiple comparisons using the Bonferroni method. SPM12 multilinear regression models adjusted for age, sex, and the time interval between fluid collection and Aβ-PET acquisition were performed to test voxel-wise associations between CSF and plasma NfL concentrations and Aβ-PET SUVR. Associations between the two fluid biomarkers and DTI metrics were analyzed using tract-based spatial statistics (TBSS).

In **paper II**, differences in tau biomarkers between groups were investigated in linear regression models, including age and sex as covariates. Non-linear polynomial spline models were used to detect Aβ-PET thresholds for when tau biomarkers were increased. Mediation analyses were conducted using a bootstrap method for estimation of mediation effects.

Statistical analyses were performed in IBM SPSS Statistics and R.

Summary of key results

NfL in CSF and blood differentially detect neuroaxonal injury and loss in early AD (paper I)

In this study, we compared the performance of NfL in CSF and blood as biomarkers of neuroaxonal injury and loss at different stages of AD. This was investigated using a translational approach, in which participants from the Swedish BioFINDER study, as well as relevant mouse models, were included.

In the cohort derived from the Swedish BioFINDER study, we found that CU individuals with Aβ pathology (A+) in the brain had significantly elevated concentrations of NfL in CSF, both in the absence (N–) and presence (N+) of cortical grey matter atrophy, which continued to rise along the AD continuum. In contrast, significantly altered concentrations of NfL in plasma were observed only in patients with MCI and AD dementia (Table 2).

Table 2
CSF and plasma NfL concentrations in diagnostic groups stratified by A/N status

	CU				MCI				ADD
	A–N– *n* = 300	**A+N–** *n* = 118	**A–N+** *n* = 31	**A+N+** *n* = 29	**A–N–** *n* = 68	**A+N–** *n* = 79	**A–N+** *n* = 27	**A+N+** *n* = 53	**A+** *n* = 113
CSF NfL (pg/ml)	870 (437)	1112 (775)[a]	1292 (712)	1446 (864)[a]	1284 (1207)[b]	1292 (705)[b]	2238 (1803)[b]	1832 (1635)[b]	1827 (1591)[b]
Plasma NfL (pg/ml)	20 (10)	28 (66)	29 (19)	32 (18)	24 (17)[a]	27 (17)[b]	38 (48)[b]	30 (18)[b]	42 (26)[b]

Data are given as mean (SD). Univariate general linear models adjusted for age and sex were used for group comparisons. Statistical analyses were performed on log-transformed CSF and plasma NfL concentrations. *P*-values were corrected for multiple comparisons using the Bonferroni method.

[a]$p < 0.00125$ versus CU A–N–, [b]$p < 0.000125$ versus CU A–N–.

We also studied the trajectories of NfL in CSF and serum in 5xFAD mice and non-transgenic littermates. In 5xFAD mice, the concentrations of NfL in both fluid compartments were elevated in an age-dependent manner. Similar results were obtained for the concentration of NfL in CSF, but not in serum, in non-transgenic littermates. Importantly, at 2 months, the concentration of NfL in CSF was around 15-times higher in 5xFAD mice when compared with age-matched non-transgenic littermates. For NfL in serum, on the other hand, elevated concentrations were found from 4 months of age (Figure 6)

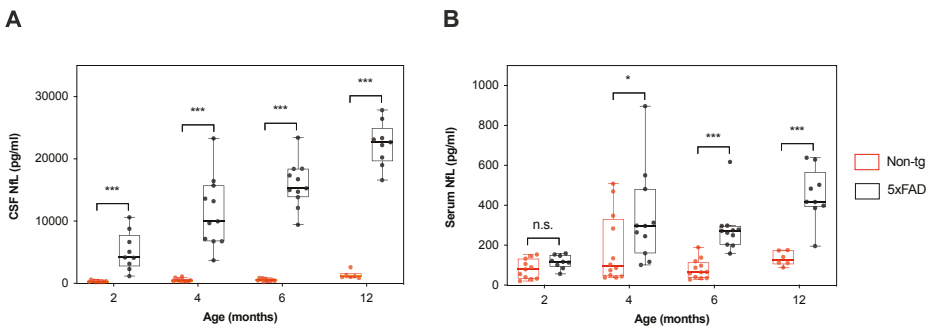

Figure 6: Scattered boxplots showing CSF and serum NfL concentrations in 5xFAD mice and non-transgenic littermates at different time points. (A) The concentration of NfL in CSF was higher in 5xFAD mice compared with age-matched non-transgenic littermates at all assessed time points. (B) The concentration of NfL in serum was higher in 5xFAD mice compared with age-matched non-transgenic littermates at 4, 6, and 12 months of age.

We were next interested in whether the concentrations of NfL in CSF and blood were associated with the deposition of fibrillar Aβ in the brain. In CU individuals derived from the Swedish BioFINDER study, we found that a high concentration of NfL in CSF was associated with a high burden of fibrillar Aβ, as determined by Aβ-PET, in widespread cortical regions. For the concentration of NfL in plasma, on the other hand, such associations were much more restricted. No associations between the concentration of NfL in any of the two fluid compartments and the deposition of cerebral Aβ fibrils were found in patients with MCI (Figure 7).

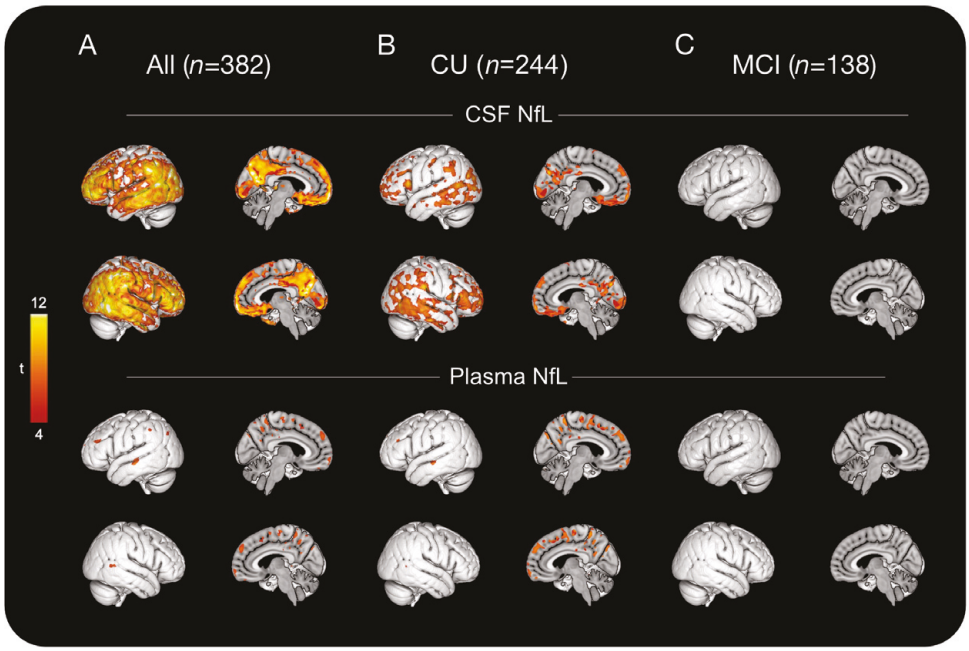

Figure 7: Images showing voxel-wise associations between Aβ-PET and NfL concentrations in CSF and plasma across (A) the whole study population, (B) CU subjects, and (C) patients with MCI.

The associations between the concentration of NfL in the two fluid compartments and the deposition of fibrillar Aβ in the brain were further studied in 5xFAD mice. We found that the concentrations of NfL in both CSF and serum positively correlated with the burden of cortical Aβ fibrils, as assessed by thioflavin S staining. Notably, and consistent with our findings in the cohort derived from the Swedish BioFINDER study, the correlation was significantly greater for the concentration of NfL in CSF in comparison with serum (Figure 8).

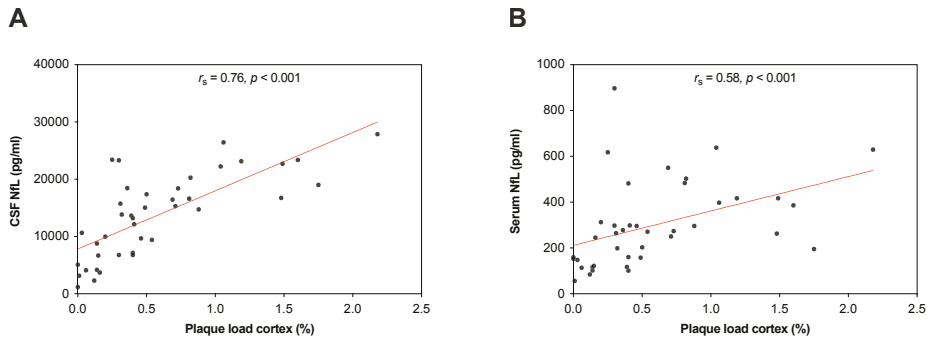

Figure 8: Scatterplots showing positive correlations between the burden of cortical thioflavin-S positive plaques and the concentrations of NfL in (A) CSF and (B) serum in 5xFAD mice.

NfL is particularly enriched in large myelinated axons, and its elevated concentrations in CSF and blood observed in AD and other brain disorders are thought to mainly reflect degeneration of these nerve cell projections (219). Since large myelinated axons are found within the subcortical white matter, we were next interested in studying how the concentrations of NfL in the two fluid compartments associated with microstructural white matter abnormalities in the BioFINDER study population. These abnormalities were measured by two metrics derived from DTI: FA and MD, where lower FA and higher MD are typically seen in relation to more severe white matter damage in brain disorders (266). We found that high concentrations of NfL in CSF were associated with low FA and high MD in widespread white matter regions in the whole study population, CU subjects, and patients with MCI. In contrast, high concentrations of NfL in plasma were associated with low FA only in patients with MCI, while no association with MD was observed in any of the diagnostic groups. Notably, the negative association with FA in patients with MCI was less widespread compared with that found for the concentration of NfL in CSF (Figure 9).

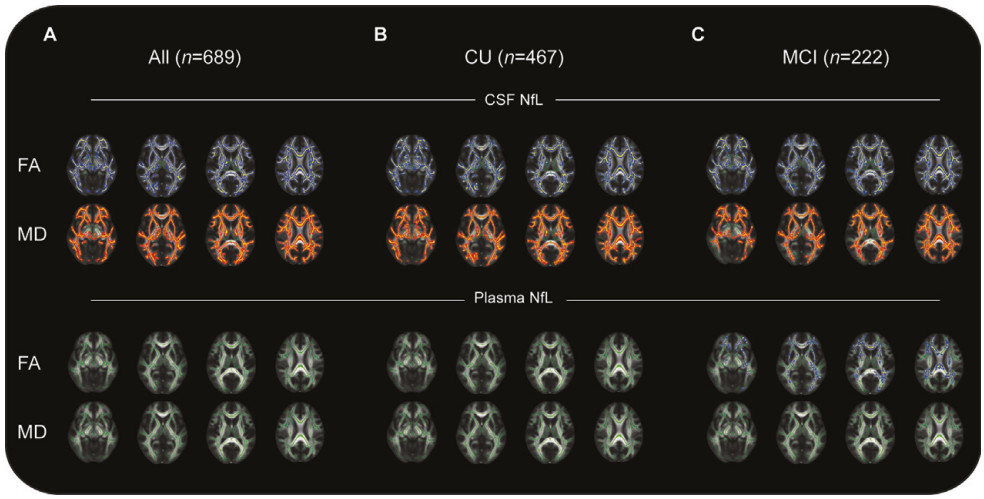

Figure 9: Images showing the associations between CSF and plasma NfL concentrations and FA and MD from DTI across (A) the whole study population, (B) CU subjects, and (C) patients with MCI. NfL in CSF was negatively associated (blue) with FA and positively associated (red) with MD in all investigated groups. NfL in plasma was negatively associated with FA in patients with MCI, but with a smaller spatial extent compared with NfL in CSF.

In summary, our findings suggest that the concentration of NfL in CSF more reliably may reflect neuroaxonal injury and loss than the corresponding measure in blood in the asymptomatic stage of sporadic AD.

Aβ pathology is associated with increases in soluble and phosphorylated tau that precede tau aggregation in AD (paper II)

In this paper, we studied the temporal relationships between cerebral development of fibrillar Aβ plaques, altered p-tau and t-tau in CSF, and accumulation of insoluble tau aggregates in the brain along the AD continuum in humans, as well as in relevant mouse models.

We first set out to investigate whether the concentrations of p-tau217, p-tau181, and t-tau in CSF were changed before insoluble tau aggregates, as measured by Tau PET, started to accumulate in the brain. In a cohort derived from the Swedish BioFINDER study, we found that CU individuals who were $A\beta^+$ (CSF Aβ42/Aβ40 ratios < 0.1) had higher concentrations of all the investigated CSF tau biomarkers when compared with those who were $A\beta^-$. These concentrations continued to rise as the disease progressed. In contrast, no change in Tau PET retention was found at this preclinical stage of the disease. Instead, a significant burden of insoluble tau aggregates was first observed in $A\beta^+$ individuals with mild cognitive deficits (MCD), which increased further in $A\beta^+$ patients with AD dementia (Figure 10).

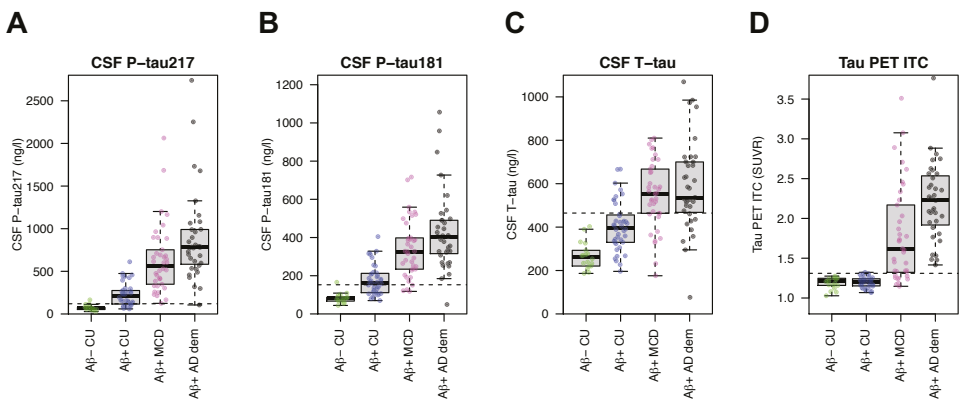

Figure 10: Scattered boxplots showing CSF p-tau and Tau PET biomarkers by Aβ and cognitive impairment. (A-D) Tau biomarkers are shown by group ($A\beta^-$ CU, $A\beta^+$ CU, $A\beta^+$ MCD, $A\beta^+$ AD dementia). Tau PET was sampled in inferior temporal cortex (ITC).

We further studied the concentrations of p-tau and t-tau in CSF and the burden of insoluble tau aggregates in the brain in relation to cerebral deposition of fibrillar Aβ, as measured by Aβ-PET. We found that the concentrations of p-tau217, p-tau181, and t-tau in CSF were elevated early in the disease process, before the threshold for abnormal levels of fibrillar Aβ in the brain was reached. The burden of insoluble tau aggregates, on the other hand, was increased at a later stage when deposition of fibrillar Aβ had already manifested. Notably, the increased burden of insoluble tau aggregates was only found in individuals with high CSF p-tau concentrations (Figure 11).

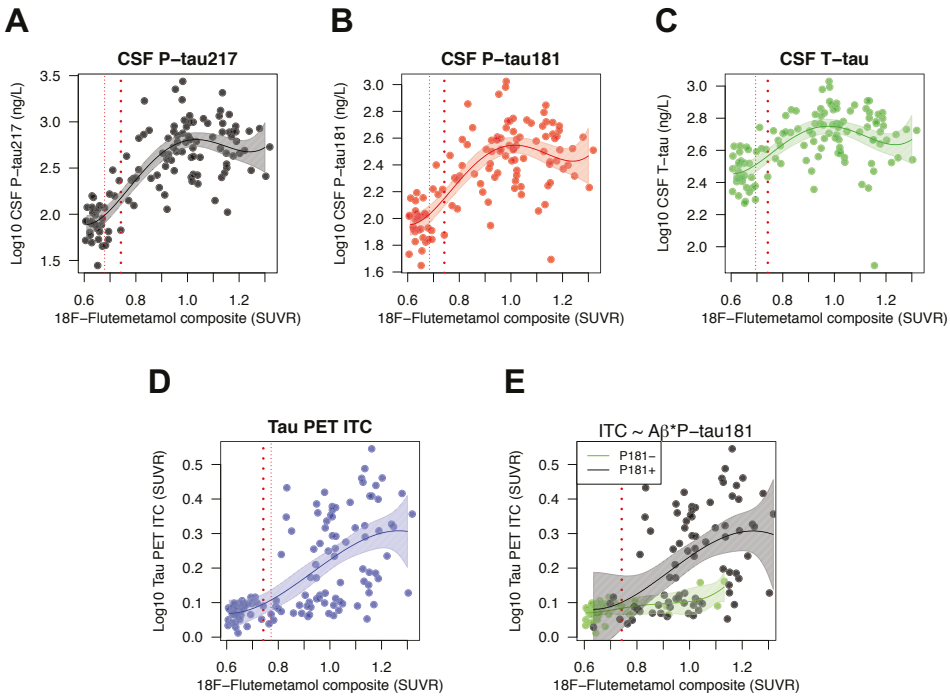

Figure 11: Scatterplots showing the levels of CSF tau and Tau PET by continuous Aβ-PET load. (A-C) CSF tau and (D) Tau PET measures in relation to global cortical ^{18}F-flutemetamol. The solid lines are fits from spline models of tau biomarkers on ^{18}F-flutemetamol. The thick dotted line shows an a priori ^{18}F-flutemetamol threshold. The thin dotted lines indicate the ^{18}F-flutemetamol level where tau biomarkers are significantly increased from. (E) Spline models for Tau PET fit separately in individuals with negative and positive CSF p-tau181, showing that the associations between ^{18}F-flutemetamol and Tau PET were only present in individuals with positive CSF p-tau levels. All biomarkers were log10 transformed to facilitate the fit of the spline models.

To follow up our finding that increased burden of insoluble tau aggregates seems to require changes in both the deposition of fibrillar Aβ and CSF p-tau, we tested whether increased concentrations of CSF p-tau statistically mediated the effect of Aβ-PET on Tau PET. Indeed, we found that both p-tau217 and p-tau181 fully mediated the relationship between the two PET measures (80% and 68% mediation, respectively) (Figure 12), suggesting that deposition of fibrillar Aβ may be linked to the formation of insoluble tau aggregates through increased release of soluble phosphorylated tau.

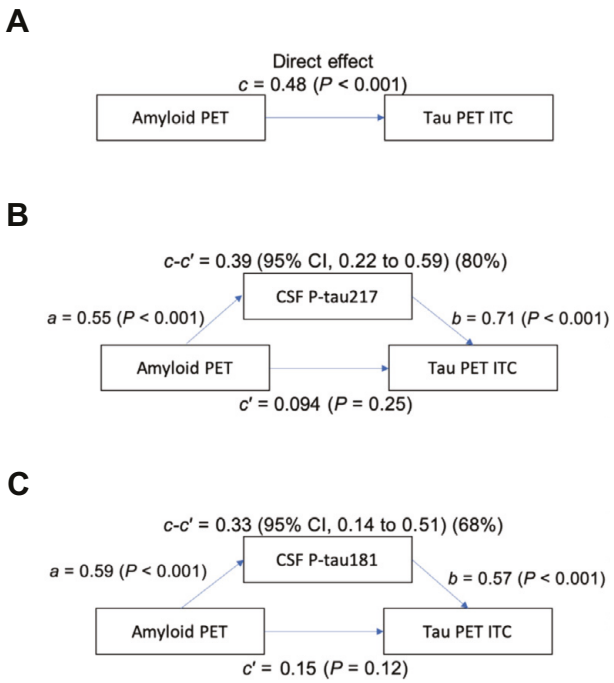

Figure 12: Images showing CSF p-tau biomarkers as statistical mediators of the relationship between Amyloid PET and Tau PET. Mediation analysis of the relationship between Aβ-PET, CSF p-tau biomarkers, and Tau PET in ITC. Aβ-PET is the global cortical ^{18}F-flutemetamol uptake. The direct effect (*c*) on Tau PET is shown in (A). Analyses are shown with (B) CSF p-tau217, and (C) CSF p-tau181 as mediators. The mediated effect is designated *c*-*c*′. The remaining effect of Aβ-PET on Tau PET after adjusting for the mediator is designated *c*′. The direct effect of Aβ-PET on the mediator is *a*, and the direct effect of the mediator on Tau PET is *b*. CSF p-tau217 and p-tau181 mediated a large part of the relationship between Aβ-PET and Tau PET. The analyses included individuals who were CU or who had mild cognitive deficits, to focus the analyses on the effects of Aβ on tau in early stages of AD.

To corroborate our findings in humans, we studied changes in the concentration of t-tau in CSF over time in 5xFAD mice. We found that the concentration of this CSF biomarker increased in response to cerebral Aβ pathology, starting from 4 months of age (Figure 13). Notably, this occurred independent of insoluble tau aggregates, which do not form in the brains of these mice (244).

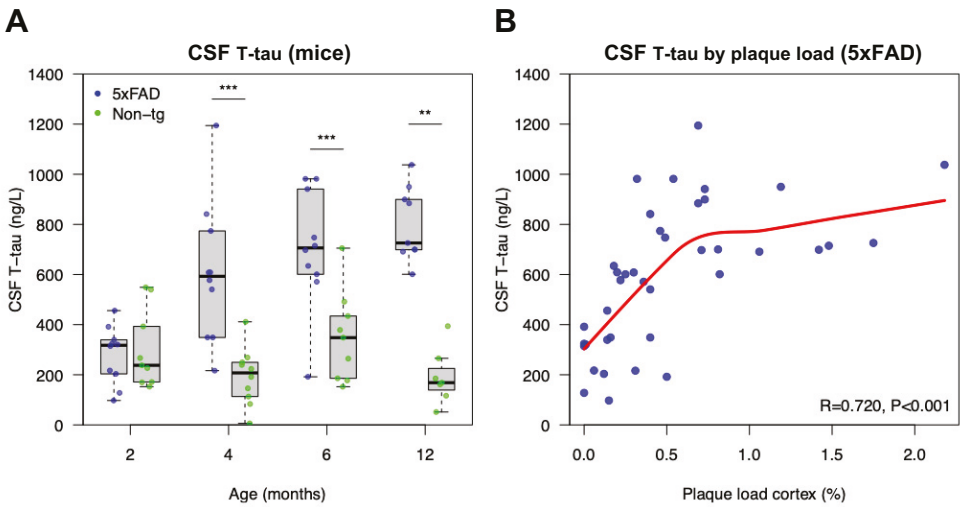

Figure 13. (A) Scattered boxplots showing that the CSF t-tau concentrations in 5xFAD mice were higher than those in age-matched non-transgenic littermates at 4, 6, and 12 months of age. (B) Scatterplot showing a positive correlation between the burden of cortical thioflavin S-positive plaques and the concentration of CSF t-tau in 5xFAD mice.

Together, our findings from this paper suggest that the concentrations of p-tau and t-tau in CSF are elevated in response to Aβ pathology in the early disease stages of AD, before Tau PET becomes abnormal, and that these alterations in soluble tau metabolism may constitute a link between the development of fibrillar Aβ plaques and the formation of insoluble tau aggregates in the AD brain.

Cerebral deposition of Aβ precedes reduced CSF and serum Aβ42/Aβ40 ratios in an *App* knock-in mouse model of AD (paper III)

In this study, we explored the temporal and correlative relationships between Aβ pathology in the brain and altered Aβ42/Aβ40 ratios in CSF and blood using the $App^{NL\text{-}F/NL\text{-}F}$ knock-in mouse model of preclinical AD.

We found that the Aβ42/Aβ40 ratio in CSF from $App^{NL\text{-}F/NL\text{-}F}$ knock-in mice was reduced from 12 months, while the corresponding ratio in serum started to decline somewhat later, at 16 months (Figure 14).

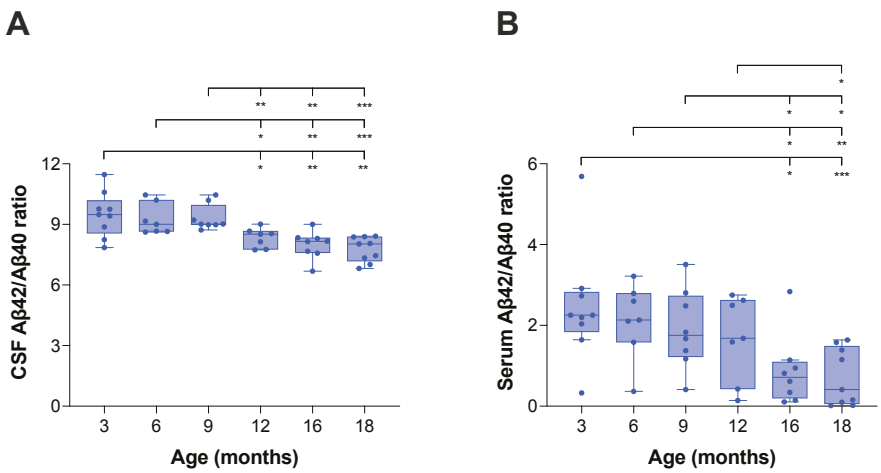

Figure 14: Scattered boxplots showing CSF and serum Aβ42/Aβ40 ratios in $App^{NL\text{-}F/NL\text{-}F}$ knock-in mice at different time points. (A) The Aβ42/Aβ40 ratio in CSF was significant reduced from 12 months while (B) a significant reduction of the Aβ42/Aβ40 ratio in serum was observed from 16 months.

Altered Aβ42/Aβ40 ratios in CSF and serum were preceded by cerebral deposition of Aβ in extracellular plaques, as assessed by immunohistochemistry and thioflavin S staining. More precisely, a few isolated extracellular Aβ plaques first appeared in cortical brain regions at 6 months, whereafter the burden in both cortex and hippocampus increased in an age-dependent manner. The initial decline of the Aβ42/Aβ40 ratio in CSF and serum at 12 and 16 months, respectively, occurred once the burden of extracellular Aβ plaques in the two brain regions started to become more pronounced (Figure 15). The development of extracellular Aβ plaques

coincided with elevated concentrations of insoluble and soluble Aβ42 extracted from cortical brain homogenates. These measures were significantly changed from 9 and 12 months, respectively.

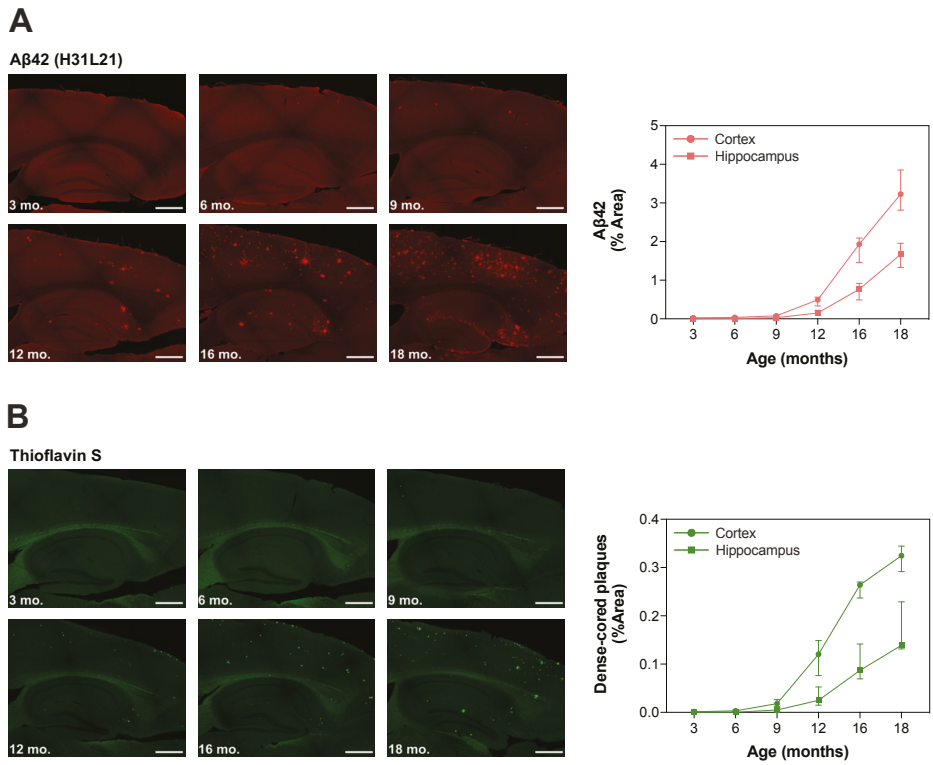

Figure 15: Images showing cerebral Aβ42 immunoreactivity and thioflavin S-positive fibrillar dense-core plaques in *App*$^{NL-F/NL-F}$ knock-in mice at different time points. Minor initial deposition of extracellular Aβ plaques in cortical brain regions was observed from 6 months of age. The burden of cortical and hippocampal (A) Aβ42 immunoreactivity and (B) thioflavin S-positive fibrillar dense-cored plaques were increased in an age-dependent manner. Scale bars: 500 μm.

We further found that the Aβ42/Aβ40 ratio in both CSF and serum negatively associated with the burden of extracellular plaques in cortex and hippocampus. However, the correlations tended to be greater when the Aβ42/Aβ40 ratio was measured in CSF compared with serum (Figure 16). Similar associations were found with the concentrations of soluble and insoluble Aβ42 extracted from cortical brain homogenates.

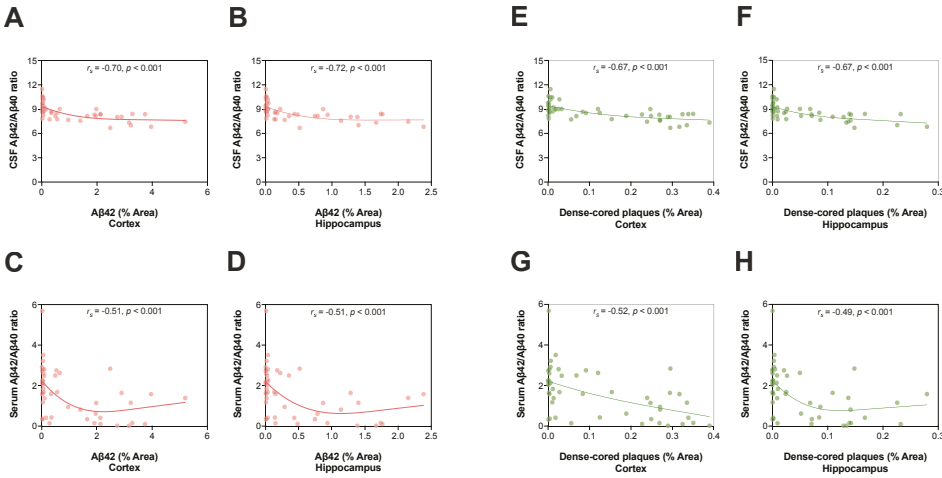

Figure 16: Scatterplots showing correlations between CSF and serum Aβ42/Aβ40 ratios and cerebral Aβ plaque burden and in *App*$^{NL-F/NL-F}$ knock-in mice**.** The Aβ42/Aβ40 ratios in CSF and serum negatively correlated with (A-D) Aβ42 immunoreactivity and (E-H) thioflavin S-positive fibrillar dense-core plaques in cortex and hippocampus.

In summary, our findings suggest a temporal sequence of events in which initial deposition of extracellular Aβ plaques is followed by a decline in CSF and blood Aβ42/Aβ40 ratios once the Aβ plaque pathology is somewhat more widespread but still relatively moderate. Furthermore, the Aβ42/Aβ40 ratio in CSF may more reliably reflect cerebral Aβ pathology than the corresponding ratio in blood in the early stages of AD.

Brain soluble Aβ protofibrils link Aβ plaque pathology to changes in key CSF biomarkers for AD (paper IV)

The Aβ42/Aβ40 ratio and the concentrations of NfL and t-tau in CSF are changed in preclinical AD, but their neurobiological correlates are not fully understood. In this study, we used 5xFAD mice to investigate the associations between these CSF biomarkers and measures of cerebral Aβ pathology, including Aβ42/Aβ40 ratios in extracellular plaques, insoluble fibrillar deposits, and soluble protofibrils. We were particularly interested in which of these measures of cerebral Aβ pathology was the strongest independent predictor of each of the studied CSF biomarkers.

The CSF Aβ42/Aβ40 ratio was reduced in an age-dependent manner as a result of decreased concentrations of Aβ42, while Aβ40 remained unchanged (Figure 17).

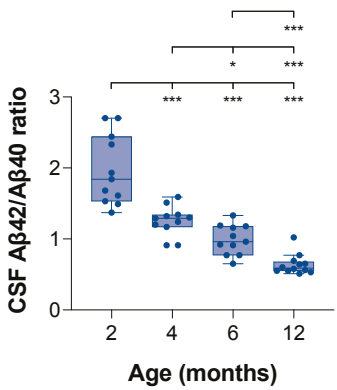

Figure 17: Scattered boxplots showing the CSF Aβ42/Aβ40 ratio in 5xFAD mice, which was reduced in an age-dependent manner.

Reduced CSF Aβ42/Aβ40 ratios coincided with increased cortical deposition of both Aβ42 and Aβ40, mainly in extracellular plaques, as determined by immunohistochemical studies. The relative deposition of Aβ42 was higher than that for Aβ40, reflected by increased Aβ42/Aβ40 immunoreactivity with age. Similarly, we found that the Aβ42/Aβ40 ratio in brain soluble protofibrils increased in an age-dependent manner, where the concentration of Aβ42 was about twenty times higher than Aβ40 in these soluble aggregates at 12 months. In contrast, the Aβ42/Aβ40 ratio in insoluble fibrillar deposits did not change with age (Figure 18).

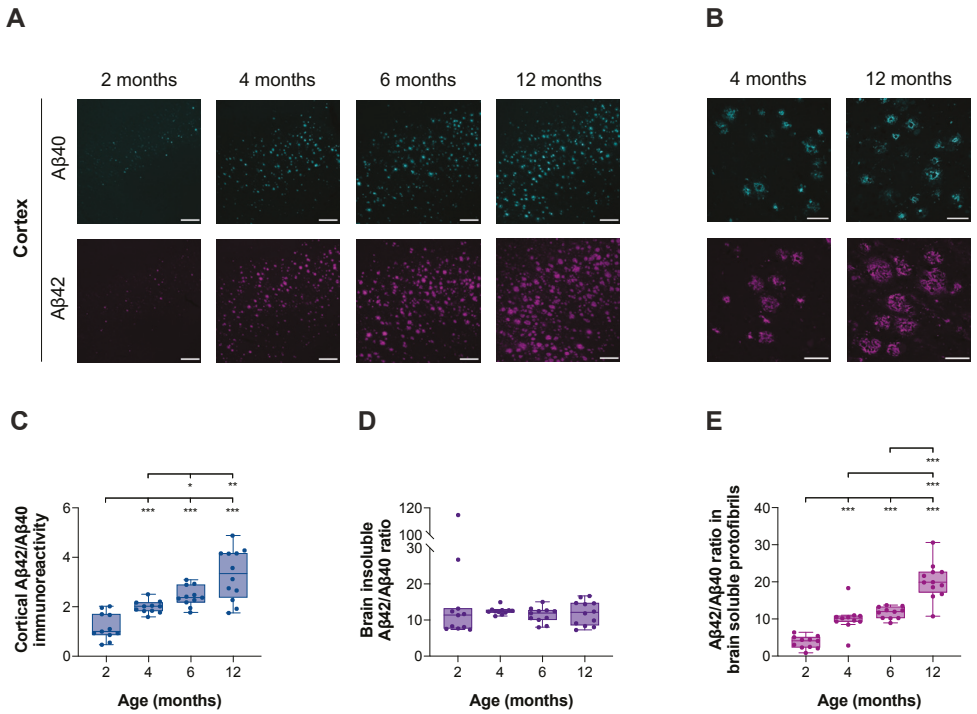

Figure 18: (A) Representative images of the cortical burden of Aβ42 and Aβ40 immunoreactivity in 5xFAD mice. Scale bar: 200 μm. (B) Aβ42 appeared to be more evenly distributed within the plaques while Aβ40 was more abundant around the core. Scale bar: 50 μm. (C) Scattered boxplots showing that the relative cortical deposition of Aβ42 was higher than that for Aβ40, resulting in significantly increased cortical Aβ42/Aβ40 immunoreactivity with age. (D) Scattered boxplots showing that the brain insoluble Aβ42/Aβ40 was not affected by age, while (E) the Aβ42/Aβ40 ratio in soluble protofibrils was increased in an age-dependent manner.

We next investigated the associations between the Aβ42/Aβ40 ratio in CSF and the different measures of cerebral Aβ pathology. In simple linear regression models, the Aβ42/Aβ40 ratio in CSF inversely associated with the corresponding ratio in extracellular plaques and soluble protofibrils, while no association with the Aβ42/Aβ40 ratio in insoluble fibrillar deposits was found. We also built a multiple linear regression model, in which all three measures of cerebral Aβ pathology were included as predictors of the CSF Aβ42/Aβ40 ratio. Interestingly, we found that a high Aβ42/Aβ40 ratio in soluble protofibrils was the strongest independent predictor of a low CSF Aβ42/Aβ40 ratio when compared with the Aβ42/Aβ40 ratio in extracellular plaques and insoluble fibrillar deposits (Figure 19A). Furthermore, mediation analysis revealed that the Aβ42/Aβ40 ratio in brain soluble protofibrils fully mediated the relationship between the corresponding ratio in extracellular plaques and CSF (Figure 19B).

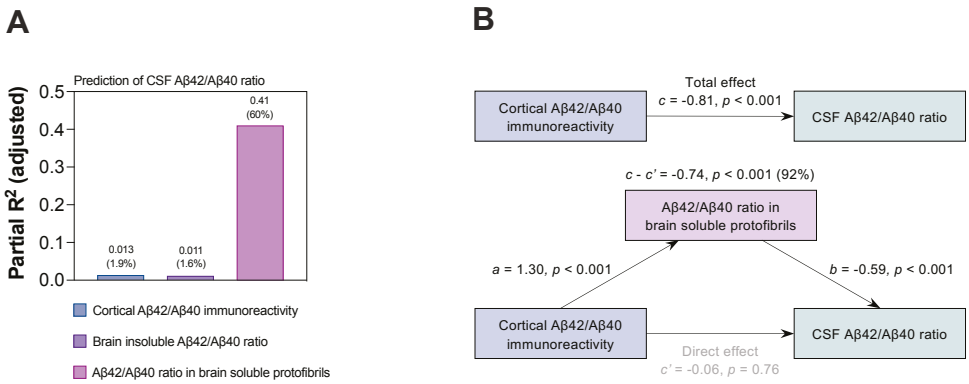

Figure 19: Associations between the CSF Aβ42/Aβ40 ratio and measures of cerebral Aβ pathology. (A) In the multiple linear regression model, lower CSF Aβ42/Aβ40 ratios independently associated with higher Aβ42/Aβ40 ratios in soluble protofibrils (partial R^2 = 0.41, β = -0.76, p < 0.001) and, to a very minor extent, lower brain insoluble Aβ42/Aβ40 ratios (partial R^2 = 0.011, β = 0.20, p < 0.05). Bars represent partial R^2 for each predictor in the model. The percentage within each bracket represents the ratio between the partial R^2 and the total R^2 of the model. (B) Mediation analysis revealed that 92% of the direct effect of cortical Aβ42/Aβ40 immunoreactivity on the CSF Aβ42/Aβ40 ratio was explained by the Aβ42/Aβ40 ratio in soluble protofibrils.

Soluble Aβ oligomers and protofibrils have in numerous studies been suggested to instigate neuronal damage in AD (267). However, it is not known to what degree the formation of these soluble aggregates relates to changes in CSF NfL and t-tau, which are established biomarkers of neurodegeneration and were shown in **paper I and II**, respectively, to be increased in response to Aβ pathology in 5xFAD mice. In simple linear regression models, both these CSF biomarkers positively associated with the Aβ42/Aβ40 ratio in extracellular plaques and soluble protofibrils, while no association with the Aβ42/Aβ40 ratio in insoluble fibrillar deposits was found. Multiple linear regression models further revealed that a high Aβ42/Aβ40 ratio in soluble protofibrils was the strongest independent predictor of a high CSF concentration of NfL and t-tau, respectively, when compared with the corresponding ratio in extracellular plaques and insoluble fibrillar deposits (Figure 20A and C). In these models, all three measures of cerebral Aβ pathology were included as predictors of each of the CSF biomarkers. In addition, the Aβ42/Aβ40 ratio in brain soluble protofibrils fully mediated the association between the Aβ42/Aβ40 ratio in extracellular plaques and each of the two CSF biomarkers (Figure 20B and D).

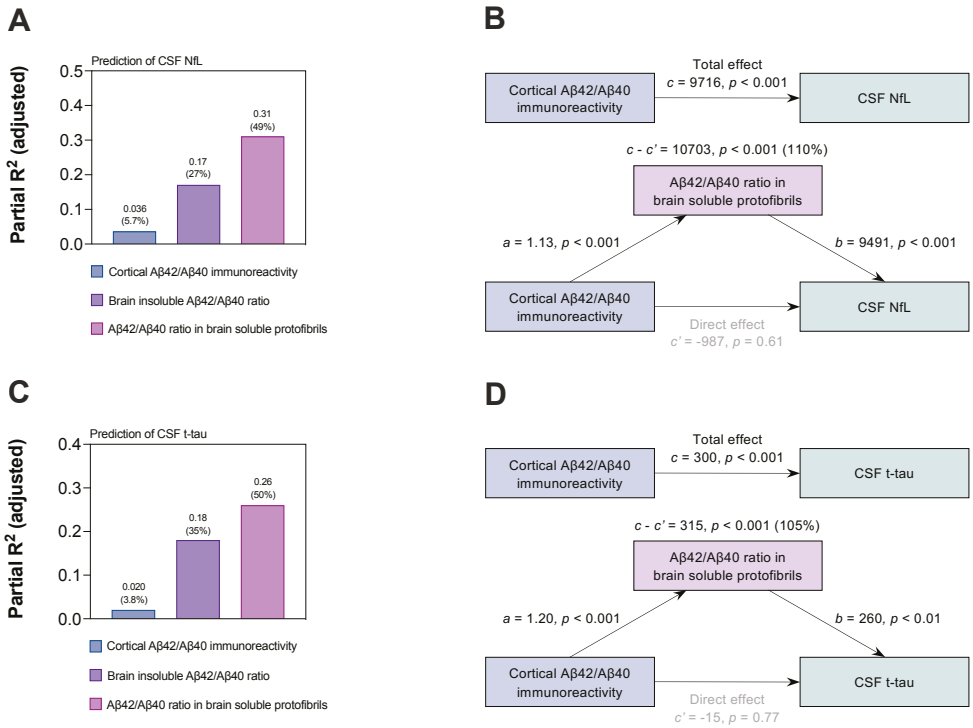

Figure 20: Associations between CSF biomarkers of neurodegeneration and measures of cerebral Aβ pathology. (A) Multiple linear regression analysis showed that higher concentrations of CSF NfL independently associated with higher Aβ42/Aβ40 ratios in soluble protofibrils (partial R^2 = 0.31, β = 0.80, p < 0.001) and lower brain insoluble Aβ42/Aβ40 ratios (partial R^2 = 0.17, β = -0.36, p < 0.01). Bars represent partial R^2 for each predictor in the model. The percentage within each bracket represents the ratio between the partial R^2 and the total R^2 of the model. (B) Mediation analysis revealed that the Aβ42/Aβ40 ratio in soluble protofibrils fully mediated the effect of cortical Aβ42/Aβ40 immunoreactivity on CSF NfL (110% mediation). (C) Multiple linear regression analysis showed that higher concentrations of CSF t-tau independently associated with higher Aβ42/Aβ40 ratios in soluble protofibrils (partial R^2 = 0.26, β = 0.64, p < 0.01) and lower brain insoluble Aβ42/Aβ40 ratios (partial R^2 = 0.18, β = -0.40, p < 0.01). Bars represent partial R^2 for each predictor in the model. The percentage within each bracket represents the ratio between the partial R^2 and the total R^2 of the model. (D) Mediation analysis revealed that the Aβ42/Aβ40 ratio in soluble protofibrils fully mediated the effect of cortical Aβ42/Aβ40 immunoreactivity on CSF t-tau (105% mediation).

In summary, our findings suggest that soluble Aβ protofibrils may constitute a link between Aβ plaque pathology and reduced CSF Aβ42/Aβ40 ratios in AD. Furthermore, increased concentrations of NfL and t-tau in CSF may to some degree reflect neuronal injury mediated by soluble Aβ oligomers during the course of the disease.

Discussion and future perspectives

In the work presented in this thesis, we have used a translational approach to explore the associations between changes in key fluid biomarkers for AD and pathological processes in the brain along the disease continuum.

In **paper I**, we showed that the concentration of NfL in CSF was increased in cognitively healthy individuals with signs of Aβ pathology in the brain, and continued to increase along the continuum of sporadic AD. The corresponding measure in blood, on the other hand, was elevated only in the symptomatic stages of the disease. These temporal differences were further corroborated in 5xFAD mice, and are in line with previous studies that have investigated the trajectories of NfL in either CSF (232, 233) or blood (236, 239) derived from distinct study populations. We also found that in cognitively healthy individuals, as well as in 5xFAD mice, the concentration of NfL in CSF showed a greater association with fibrillar Aβ in the brain than NfL in blood. Furthermore, only the concentration of NfL in CSF associated with cerebral white matter abnormalities in cognitively healthy individuals. Together, these findings suggest that NfL in CSF more reliably may reflect neuroaxonal injury and loss than the corresponding measure in blood in the preclinical phase of sporadic AD. This knowledge may have implications for the use of these two measures in clinical trials evaluating disease-modifying therapies. In clinical trials focusing on the symptomatic phases of sporadic AD, measurement of NfL in blood could be an easily accessible and cost-effective choice to reveal positive effects of such therapies on downstream neurodegenerative processes. However, in primary prevention trials focusing on the asymptomatic phase of the disease, NfL in CSF may be the preferable biomarker to track such changes.

Although it is believed that the concentration of NfL in the two investigated fluid compartments reflects passive release of this protein due to neuroaxonal injury and loss, it is possible that additional mechanisms may play a role. Indeed, we found that the concentration of NfL in CSF was elevated in cognitively healthy individuals, as well as in very young 5xFAD mice, in response to Aβ pathology despite the absence of significant neurodegeneration. Similar to what has been described for tau (200), it is tempting to speculate that pathogenic Aβ may cause alterations in the active release of NfL from neurons, which is consequently reflected by increased concentrations of this protein in CSF at the early phase of the disease. Investigating such mechanisms in future studies could provide a more detailed understanding of the underlying cause as to why NfL is changed in CSF

and blood along the AD continuum. The later change and weaker association with AD-related pathological processes observed for NfL in blood could possibly be explained by confounding factors that affect the measurement of the protein in this fluid compartment, but not in CSF. Such factors may be attributed to matrix effects, renal clearance, and degradation in the liver or by circulating enzymes (268). However, more studies are needed to fully understand the reasons for these differences.

We next turned our investigation to another protein found to a large extent in neuronal axons, namely tau. It is thought that deposition of Aβ in the brain initiates a pathological cascade that results in the formation of intracellular tau aggregates, ultimately leading to neuronal loss and cognitive decline (133). However, the link between Aβ pathology and tau aggregation is not entirely understood. In **paper II**, we demonstrated that the concentrations of the phosphorylated tau isoforms p-tau217 and p-tau181, as well as t-tau, were increased in CSF in response to Aβ pathology in preclinical sporadic AD, despite the absence of insoluble tau aggregates in the brain, as measured by Tau PET. These pathological alterations in the metabolism of soluble tau started very early in the disease process, before the threshold for an abnormal burden of cerebral Aβ fibrils was reached. Notably, the trajectory of CSF t-tau was similar in 5xFAD mice, in which the concentration of this biomarker started to increase shortly after extracellular deposition of fibrillar Aβ in the brain was initiated. Further elevated concentrations were observed in response to more advance Aβ pathology, although it is known from previous studies that this model does not form insoluble tau aggregates in the brain, even at an advanced age (244). Together, these findings suggest that phosphorylation and release of soluble tau is affected by early Aβ pathology, and that these alterations seem to precede significant formation of insoluble tau aggregates in the brain. This elaborates our knowledge from previous experimental studies (269), as well as SILK studies in humans (200), in which similar associations between Aβ and changes in the metabolism of soluble tau have been demonstrated. Furthermore, CSF p-tau and Tau PET may reflect different aspects of altered tau metabolism along the AD continuum, which is important to considered in the context of the ATN framework.

Another important finding from **paper II** was that insoluble tau aggregates started to accumulate in the brain once abnormal deposition of fibrillar Aβ had become established, and interestingly, only in individuals with high concentrations of p-tau in CSF. Indeed, we found that both p-tau217 and p-tau181 in this fluid compartment fully mediated the association between the burden of fibrillar Aβ and insoluble tau aggregates in the brain, as measured by Aβ-PET and Tau PET, respectively. These findings suggest that increased neuronal phosphorylation and release of soluble tau may constitute an important link between the deposition of fibrillar Aβ and the formation of insoluble tau aggregates in the AD brain. Interestingly, in a recent study in *App* knock-in mice that do not develop NFT pathology, both p-tau217 and

p-tau181 were found within dystrophic neurites surrounding extracellular Aβ plaques (270). Considering that such lesions have previously been implicated in the formation of insoluble tau aggregates (142), it is possible that increased concentrations of CSF p-tau217 and p-tau181 in early AD are accompanied by phosphorylation of these p-tau isoforms in dystrophic neurites, subsequently contributing to the development of NFT pathology later in the disease. It would be very interesting to investigate the association between p-tau isoforms in CSF and dystrophic neurites in the presence of Aβ in future experimental studies.

Although our findings in **paper II** have provided important novel insights on the temporal relationship between Aβ pathology and the release, phosphorylation, and aggregation of tau in the AD continuum, the molecular mechanisms by which Aβ may alter the metabolism of soluble tau reflected in CSF remain to be elucidated. In mice with progressive Aβ pathology, p-tau217 has been shown to co-localize with the active form of GSK-3β at postsynaptic sites in the brain (270). This is a kinase that is known to regulate tau phosphorylation, and whose function is altered in AD (143). In future studies, it would be very interesting to further elucidate the signalling pathways that GSK-3β and other disease-related tau kinases and phosphatases are implicated in, and which effect they have on p-tau isoforms in CSF. Such mechanisms would also be important to study in relation to changes in p-tau isoforms in blood, which similar to CSF are increased in response to Aβ pathology in preclinical AD (213-215, 217) and can with high accuracy discriminate AD from other neurodegenerative diseases (214-216, 218). These types of mechanistic studies would not only provide further knowledge about the pathological processes that different p-tau isoforms in CSF and blood reflect, but could potentially also lead to the identification of novel drug targets that upon alteration may slow down or stop the formation of tau aggregates at an early disease stage.

In **paper III**, we turned our focus to fluid biomarkers of Aβ pathology. We demonstrated that the Aβ42/Aβ40 ratio in both CSF and blood was reduced with age in *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice, findings that are well in line with the observed decline of the corresponding ratios in preclinical sporadic AD (183, 184, 271). Notably, the Aβ42/Aβ40 ratio in CSF dropped at an earlier time point than in blood, and occurred after initial deposition of extracellular fibrillar Aβ plaques was detected in the brain. These observations are interesting, considering that studies in humans have suggested that the Aβ42/Aβ40 ratio in the two fluid compartments may change before the threshold for an abnormal burden of Aβ fibrils in the brain, as measured by Aβ-PET, is reached (178, 193). Our findings raise the possibility that the sensitivity of Aβ-PET to detect fibrillar Aβ in the brain in early preclinical AD may be limited, and encourage continued search for fluid biomarkers that could be reflective of the initial amyloidogenic phase of the disease.

Our studies in *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice also revealed that the Aβ42/Aβ40 ratio in the two fluid compartments associated with the burden of cerebral Aβ pathology in

a negative manner, and that such associations tended to be greater for the measures in CSF compared with blood. These findings are well in line with a recent study performed in a human cohort consisting of mainly cognitively healthy individuals, in which the Aβ42/Aβ40 ratio in CSF was reported to be a better predictor of the burden of fibrillar Aβ in the brain, as measured with Aβ-PET (193). Together, these findings from mice and humans suggest that the Aβ42/Aβ40 ratio in CSF may be a more reliable biomarker of cerebral Aβ pathology than the corresponding ratio in blood in the preclinical phase of AD.

The prevailing hypothesis as to why the Aβ42/Aβ40 ratio is reduced in CSF and blood in AD proposes that cerebral deposition of Aβ42 in fibrillar aggregates results in reduced availability of this Aβ peptide to reach the two fluid compartments (167). However, in *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice, we found that Aβ42/Aβ40 ratios in CSF and blood did not further change following their initial decline, although the burden of fibrillar Aβ in the brain continued to increase. These findings are consistent with cross-sectional (174-178, 193) and longitudinal (47, 179, 180) studies in humans, and suggest that the impact of cerebral Aβ fibrils on the two fluid measures may be limited.

To gain further understanding of the underlying pathological events that altered CSF Aβ42/Aβ40 ratios in AD reflect, we continued to study the association between this fluid biomarker and key features of cerebral Aβ pathology in **paper IV**, using 5xFAD mice. Similar to what we found in *App*$^{NL\text{-}F/NL\text{-}F}$ knock-in mice, and what has previously been reported in other *APP*-overexpressing transgenic mouse models (201, 272), the CSF Aβ42/Aβ40 ratio in 5xFAD mice was reduced with age. This change coincided with elevated cerebral Aβ42/Aβ40 ratios in extracellular plaques and soluble protofibrils, while the corresponding ratio in fibrillar deposits remained unaltered. Interestingly, the strongest independent predictor of a low CSF Aβ42/Aβ40 ratio was attributed to a high Aβ42/Aβ40 ratio in soluble protofibrils. We also found that this measure mediated the association between the Aβ42/Aβ40 ratio in extracellular plaques and the corresponding ratio in CSF. Together, these findings suggest that the CSF Aβ42/Aβ40 ratio may reflect the cerebral burden of soluble Aβ protofibrils, rather than deposits of insoluble fibrillar Aβ, and propose a novel hypothesis as to why this CSF biomarker is reduced in AD. Previous studies have shown that a very limited amount of soluble Aβ aggregates reaches the CSF in patients with AD (273-275), suggesting that they are retained in the brain, potentially due to their sticky properties. It is possible that during their formation through secondary nucleation mechanisms, monomeric Aβ42, which easily diffuses into the CSF, is widely consumed in the brain, consequently resulting in reduced Aβ42/Aβ40 ratios in CSF. Together with mechanistic validation of our reported findings, such associations would be important to investigate in future studies. For these purposes, treatment with substances that selectively remove soluble Aβ protofibrils from the mouse brain, in combination with SILK studies, could provide valuable information on how to interpret altered CSF Aβ42/Aβ40 ratios in research

settings, as well as in response to disease-modifying therapies evaluated in clinical trials.

In **paper IV**, we further studied if the investigated measures of cerebral Aβ pathology could independently predict changes in established CSF biomarkers of neurodegeneration, including NfL and t-tau, which in **paper I** and **II** were showed to be elevated in 5xFAD mice over time. We found that a high cerebral Aβ42/Aβ40 ratio in soluble protofibrils was the strongest independent predictor of high concentrations of NfL and t-tau in CSF, and that this measure of cerebral Aβ pathology mediated the association between the Aβ42/Aβ40 ratio in extracellular plaques and each of the two CSF biomarkers. Increased concentrations of NfL and t-tau in AD may thereby to some degree reflect neurodegenerative processes induced by soluble Aβ protofibrils, and corroborate previous findings suggesting that soluble Aβ aggregates are the primary neurotoxic Aβ species in the AD brain (103, 105, 111-117, 276, 277).

Concluding remarks

In conclusion, the work presented in this thesis suggests the following:

- In preclinical sporadic AD, NfL in CSF may be a more reliable biomarker of neuroaxonal injury and loss than the corresponding measure in blood.
- Development of Aβ pathology in the brain associates with increased concentrations of p-tau and t-tau in CSF in the early stages of AD, before a significant burden of insoluble tau aggregates can be detected in the brain.
- The altered phosphorylation and release of soluble tau reflected in CSF in the early stages of AD may constitute a link between the development of fibrillar Aβ plaques and the accumulation of insoluble tau aggregates in the brain.
- Deposition of cerebral Aβ fibrils in extracellular plaques may precede the initial decline of the Aβ42/Aβ40 ratio in CSF and blood in the earliest stage of AD.
- The Aβ42/Aβ40 ratio in CSF may more reliably reflect Aβ pathology in the brain than the corresponding ratio in blood in the early stage of AD.
- A reduced CSF Aβ42/Aβ40 ratio may be an indirect measure of enhanced formation of soluble Aβ protofibrils, rather than the deposition of fibrillar Aβ in extracellular plaques, in the AD brain.
- Elevated CSF concentrations of NfL and t-tau in AD may to some degree reflect neurotoxic processes induced by soluble Aβ protofibrils.

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