Tau fragments:

role as biomarkers and in the pathogenesis of Alzheimer's disease and other tauopathies

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Ai miei genitori

<<Ed elli a me: “Se tu segui tua stella,
non puoi fallire a glorïoso porto,
se ben m’accorsi ne la vita bella”>>

Canto XV, *Inferno*, *La Divina Commedia*, Dante Alighieri

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ABSTRACT

Tau protein is physiologically expressed in neurons, where it is involved in microtubule assembly and stability. Tau functions are rigorously regulated by a series of modifications, *e.g.* phosphorylation and dephosphorylation. When these mechanisms are dysregulated or other modifications occur, it leads to a group of diseases defined as "tauopathies", characterized by build-up of tau protein aggregates in neurons and glial cells (neurofibrillary tangles, astrocytic plaques, tufted astrocytes). Tauopathies include, among others, Alzheimer's disease (AD), frontotemporal dementia (FTD) and diseases characterized by frontotemporal lobar degeneration (FTLD) such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). Among the many post-translational modifications that tau can undergo, proteolytic processing is gaining increasing attention, as many studies have shown that cleavage of tau in brain is related to disease. It has also been consistently observed that tau in cerebrospinal fluid (CSF) consists of a series of fragments, with predominance of N-terminal and mid-region fragments compared to C-terminal ones.

The aim of this thesis was to identify and quantify specific tau fragments in CSF with novel targeted immunoassays, and assess their potential as biomarkers for different tauopathies. We identified two major pools of tau consisting of species cleaved at either amino acid (aa) 123 or 224, reflecting different mechanisms of tau processing in AD. While cleavage generating tau N-123 is part of the physiological tau turnover, the generation of tau N-224 was shown to have clinical relevance, being related to AD; N-224 tau showed significantly higher concentrations in AD CSF compared to control and was related to worsening cognitive

performance over time. Also, in the primary tauopathies PSP and CBD, N-224 tau did not correlate to total tau (t-tau) content, showing promise as a candidate biomarker for tauopathies other than AD.
Based on previous reports of tau cleavage by asparagine endopeptidase (AEP) at aa 368, we also developed a new immunoassay targeting tau fragments cleaved C-terminally of aa 368 (tau 368). Our results demonstrate that, although tau 368 is measurable in CSF and overall increased in AD, only a small portion of the total content of CSF tau ends at 368. Instead, most of tau 368 is retained in tangles, as shown by the decrease in the tau 368/t-tau ratio over the course of disease and immunohistochemical staining of tangles. Of potential clinical relevance, we also showed a strong negative association of the CSF tau 368/t-tau ratio and uptake of the tau PET tracer [^{18}F]GTP1, supporting the hypothesis that the ratio reflects underlying tau pathology and entrapment of tau 368 in tangles.
When applying the newly-developed immunoassays to CSF from a FTD cohort, we observed that none of the measures showed a significant difference between the likely FTLD-TDP-43 and likely FTLD-tau pathology groups. However, when normalised for t-tau, N-224 showed a significant difference between FTLD-tau and FTLD associated to TAR DNA-binding protein 43 (FTLD-TDP-43), suggesting that, although the novel measures do not have a superior diagnostic accuracy to the classic tau biomarkers, there are different patterns in fragment concentrations between pathological groups, and different profiles for each tauopathy.
Finally, since the N-224 fragment showed potential clinical relevance in the differential diagnosis of tauopathies, we aimed to identify the enzyme responsible for cleavage at aa 224. By using a fluorescence resonance energy transfer (FRET) peptide, containing a tau sequence which included aa 224, and high resolution mass spectrometry, we identified the enzyme responsible for cleavage as calpain-2. We confirmed the results in a gene knock-down SH-SY5Y cell model, where we measured a significant reduction in tau N-224 in the cell media after knock-down of the calpain-2 gene. These findings suggest that the calpain-2 pathway should be investigated as a possible target in the treatment of tauopathies.

Keywords: tau, fragments, Alzheimer's disease, tauopathy, cerebrospinal fluid

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SAMMANFATTNING PÅ SVENSKA

Över 50 miljoner människor världen över är drabbade av demens och detta har också en stor påverkan på familjer och samhälle. Även om orsakerna till demens fortfarande är okända, vet vi att om vissa komponenter i hjärnans nervceller slutar fungera korrekt så bidrar det till demensen.

En av dessa komponenter är proteinet tau, som vanligtvis hjälper till att bibehålla neuronernas funktion och struktur. Tau kan genomgå förändringar som leder till att proteinet aggregerar i nervcellerna i form av neurofibrillära nystan, vilket är kännetecknet för en grupp demenssjukdomar som kallas "tauopatier". Tauopatier inkluderar bland annat Alzheimers sjukdom (den vanligaste typen av demens), frontotemporal demens, progressiv supranukleär pares och kortikobasal degeneration. Dessa sjukdomar har en komplex sjukdomsbild och för närvarande är det bara undersökning av hjärnan vid obduktion som kan bekräfta om den kliniska diagnosen som gjordes medan patienten ännu levde, var korrekt. Studier av biomarkörer, dvs biologiska parametrar som avspeglar processer i kroppen, kan hjälpa oss att förstå orsaken till dessa sjukdomar och bidra till en säkrare klinisk diagnos.

Hittills har man bara kunnat analysera en form av tau i cerebrospinalvätskan (Csv), den vätska som omger hjärnan. Denna analys ger oss dock inte den fullständiga bilden eftersom det finns många olika former av tau. De högst förekommande formerna av tau består av den initiala (N-terminala) delen samt mittregionen av proteinet, medan former av tau som enbart består av slutdelen (C-terminala delen) förekommer i mycket längre koncentrationer. De olika formerna av tau i Csv bildar ett mönster och detta mönster skiljer sig mellan friska försökspersoner och försökspersoner med tauopatier, men även mellan personer med olika tauopatier.

Syftet med detta arbete var att identifiera och kvantifiera specifika former av tau i Csv och utvärdera deras potential som biomarkörer för olika tauopatier genom att etablera nya analysmetoder. Vi identifierade två former av tau där det ena var kopplat till normal omsättning av tau, medan den andra formen visade en relation till Alzheimers sjukdom, progressiv supranukleär pares och kortikobasal degeneration, vilket tyder på att detta fragment kan vara en ny biomarkör. Vi utvecklade också en ny, högkänslig metod för analys av en form av tau som innefattar den C-

terminala delen. Våra resultat visar att, även om detta fragment är mätbart i Csv, är det endast en liten del av den totala mängden tau som innehåller C-terminala delen. Majoriteten av C-terminalt tau stannar i hjärnan som en del av de neurofibrillära nystanen. Vid analys av flera olika varianter av frontotemporal demens observerade vi skillnader i mönstren i olika patientgrupper. Slutligen identifierade vi ett enzym som ansvarar för att generera den form av tau som vi sett relaterar till demens och som bör undersökas som ett möjligt terapeutiskt mål för behandling av tauopatier.

Dessa resultat tillhandahåller inte bara nya verktyg för att förbättra diagnosen av taupatier, utan också för förståelsen av hur dessa sjukdomar skiljer sig på molekylär nivå, vilket kan leda till nya behandlingssätt. Ytterligare studier behövs för att etablera möjliga tillämpningar i demensbehandlingen.

LIST OF PAPERS

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

I. **Cicognola C**, Brinkmalm G, Wahlgren J, Portelius E, Gobom J, Cullen NC, Hansson O, Parnetti L, Costantinescu R, Wildsmith K, Chen H, Beach TG, Lashley T, Zetterberg H, Blennow K, Höglund K. *Novel tau fragments in cerebrospinal fluid: relation to tangle pathology and cognitive decline in Alzheimer's disease.* Acta Neuropathologica, 2019 Feb;137(2):279-296.

II. Blennow K, Chen C, **Cicognola C**, Wildsmith K, Manser P, Sanabria Bohorquez S, Zhang Z, Xie B, Peng J, Hansson O, Kvartsberg H, Portelius E, Zetterberg H, Lashley T, Brinkmalm G, Kerchner G, Weimer R, Ye K, Höglund K. *Cerebrospinal fluid levels of a tau fragment ending at amino acid 368 correlate with tau PET: a candidate biomarker for tangle pathology in Alzheimer's disease.* Submitted to *Brain*.

III. Foiani MS, **Cicognola C**, Ermann N, Woollacott IOC, Heller C, Heslegrave AJ, Keshavan A, Paterson R, Ye K, Kornhuber J, Fox NC, Schott JM, Warren JD, Lewczuk P, Zetterberg H, Blennow K, Höglund K, Rohrer JD. *Searching for novel CSF biomarkers of tau pathology in frontotemporal dementia – an elusive quest*. Journal of Neurology, Neurosurgery and Psychiatry, 2019 Jul;90(7):740-746.

IV. **Cicognola C**, Satir T, Brinkmalm G, Matečko-Burmann I, Agholme L, Bergström P, Becker B, Zetterberg H, Blennow K, Höglund K. *Tauopathy-associated tau fragment ending at amino acid 224 is generated by calpain-2 cleavage.* Manuscript.

Papers not included in the thesis:

Leuzy A, **Cicognola C**, Chiotis K, Saint-Aubert L, Lemoine L, Andreasen N, Zetterberg H, Ye K, Blennow K, Höglund K, Nordberg A. *Longitudinal tau and metabolic PET imaging in relation to novel CSF tau measures in Alzheimer's disease.* European Journal of Nuclear Medicine and Molecular Imaging, 2019 May;46(5):1152-1163.

Cicognola C, Chiasserini D, Eusebi P, Andreasson U, Vanderstichele H, Zetterberg H, Parnetti L, Blennow K. *No diurnal variation of classical and candidate biomarkers of Alzheimer's disease in CSF.* Molecular Neurodegeneration, 2016 Sep 7;11(1):65.

CONTENT

ABBREVIATIONS

aa	amino acid
AChE	acetylcholinesterase
AD	Alzheimer's disease
AEP	asparagine endopeptidase
ALS	amyotrophic lateral sclerosis
APOE	apolipoprotein E
APP	amyloid precursor protein gene
Aβ	amyloid beta
bvFTD	behavioural variant of frontotemporal dementia
C9orf72	gene located on chromosome 9 open reading frame 72
CBD	corticobasal degeneration
CBS	corticobasal syndrome
CID	collision-induced dissociation
CNS	central nervous system
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EV	extracellular vesicle
FAD	familial Alzheimer's disease
FBS	frontal behavioural-spatial syndrome

FDG-PET	fluorodeoxyglucose-positron emission tomography
FRET	fluorescence resonance energy transfer
FTD	frontotemporal dementia
FTD-MND	frontotemporal dementia-motor neuron disease
FTLD	frontotemporal lobe degeneration
FTLD-NI	frontotemporal lobe degeneration-no inclusions
FTLD-U	frontotemporal lobe degeneration-tau negative
GRN	progranulin gene
HPLC	high-performance liquid chromatography
IHC	immunohistochemistry
IP	immunoprecipitation
iPSC	induced pluripotent stem cells
IWG	International Working Group
IWG-2	International Working Group-2
LBD	Lewy body dementia
LC	liquid chromatography
LPA	logopenic progressive aphasia
m/z	mass/charge ratio
MAPT	microtubule-associated protein tau gene
MCI	mild cognitive impairment
MCI-AD	mild cognitive impairment due to AD

MMSE	Mini Mental State Examination
MTBR	microtubule-binding region
MRI	magnetic resonance imaging
MS	mass spectrometry
MTs	microtubules
NDEVs	neuronally-derived extracellular vesicles
NFTs	neurofibrillary tangles
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke- Alzheimer's Disease and Related Disorders Association
NINDS-SPSP	National Institute of Neurological Disorders and Stroke-Society for Progressive Supranuclear Palsy
NMR	nuclear magnetic resonance
OND	other neurological diseases
PA	pathological aging
PDEVs	peripherally-derived extracellular vesicles
PET	positron emission tomography
PHFs	paired helical filaments
PiB	Pittsburgh compound B
PiD	Pick’s disease
PNFA	progressive non fluent aphasia
PPA	primary progressive aphasia

PPA-NOS	primary progressive aphasia-not otherwise specified
PRM	parallel reaction monitoring
PSEN1	presenilin 1 gene
PSEN2	presenilin 2 gene
PSP	progressive supranuclear palsy
PSPS	progressive supranuclear palsy syndrome
p-tau	phosphorylated tau
ROI	region of interest
RP-HPLC	reverse-phase high performance liquid chromatography
SFs	straight filaments
SILK	stable isotope labeling kinetics
Simoa	single molecule array
SUVR	standardized uptake value ratio
svPPA	semantic variant primary progressive aphasia
TDP-43	TAR DNA-binding protein 43
t-tau	total tau

1. INTRODUCTION

1.1 Tau protein

1.1.1 Function and structure

Tau is a neuronal protein expressed mostly in axons and, in minor part, in dendrites (Tashiro et al., 1997, Kempf et al., 1996, Binder et al., 1985). Tau binds to tubulin inducing its polymerization into microtubules (MTs) and, consequently, maintaining MTs stability and spacing (Kadavath et al., 2015, Chen et al., 1992). Due to its structural role, it is also involved in axon outgrowth and elongation and, therefore, in morphogenesis, polarity and plasticity of the neuron (Dawson et al., 2010, Dawson et al., 2001).

Tau is encoded in the *MAPT* (microtubule-associated protein tau) gene on chromosome 17 (Goedert et al., 1989). In the central nervous system (CNS), six different isoforms of tau are produced from the *MAPT* gene, following alternative mRNA splicing of exons 2, 3 and 10 (Fig. 1) (Goedert et al., 1989). The isoforms differ by the presence of highly preserved repetitive domains in the C-terminal (R) or amino acid (aa) inserts in the N-terminal (N). Each isoform can contain zero, one or two N-inserts or three to four R domains. In the adult human brain, all the six isoforms are expressed, with equal amounts of 3R and 4R tau. Ratios of 3R:4R other than 1:1 are associated to neurodegenerative diseases (Spillantini and Goedert, 1998, Spillantini et al., 1998).

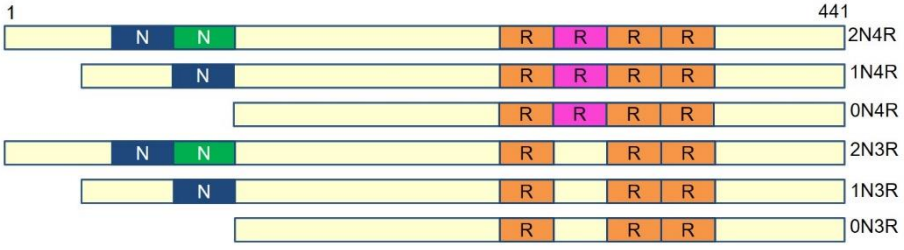

Figure 1. Tau isoforms. Each isoform can contain zero, one or two N-inserts (0N, 1N, 2N) and three to four R inserts (3R, 4R).

The foetal isoform of tau is the 0N3R, which, due to its lower microtubule stabilizing activity, allows for higher synaptic plasticity (Goode and Feinstein, 1994, Goedert and Jakes, 1990). In the adult brain, however, a small amount of foetal tau is still present, possibly for ongoing synaptic plasticity (Bullmann et al., 2009). The peripheral isoform of tau or "big tau" is encoded with two additional exons, 4 and 6, resulting in higher molecular weight, hence the name (Couchie et al., 1992, Goedert et al., 1992b).

Tau structure can be divided in four domains, with different functions: N-terminal, proline-rich, MT-binding region (MTBR) and C-terminal (reviewed in Arendt et al., 2016). When tau is bound to MTs through the MTBR (R-repeats), the N-terminal undergoes a conformational change and works as a spacer to keep MTs at the right distance from each other (Chen et al., 1992, Butner and Kirschner, 1991). The proline-rich region is involved in cell signalling, while the MTBR and C-terminal are involved in the polymerization of MTs and interactions with the plasma membrane (Goode et al., 1997, Brandt and Lee, 1993, Butner and Kirschner, 1991). Eighty percent of all tau is bound to MTs at any time point, but only for 40 ms at a time, through a "kiss and hop" mechanism that does not prevent the regular axonal transport mechanisms (Janning et al., 2014). In this way, tau also regulates the axonal transport mediated by kinesin and dynein-dynactin motor proteins (Janning et al., 2014).

All tau functions are regulated through rigorous phosphorylation and dephosphorylation mechanisms on serine (n=45), threonine (n=35) and tyrosine (n=5) residues. Tau is bound to MTs in the unphosphorylated state and detached in the phosphorylated state (Selden and Pollard, 1986, Lindwall and Cole, 1984, Selden and Pollard, 1983). This mechanism is responsible for the regulation of binding dynamics, subcellular distribution, axonal transport, delivery to dendritic spines and association with the plasma membrane (reviewed in Arendt et al., 2016).

1.2 Tauopathies: mechanisms behind disease pathogenesis

Tau can undergo a series of modifications responsible for a group of diseases defined as "tauopathies". Tauopathies are a group of sporadic or familial diseases characterized by build-up of tau protein aggregates in neurons and glial cells. Tauopathies include, among others, Alzheimer's disease (AD), frontotemporal dementia (FTD) and diseases characterized by frontotemporal lobar degeneration (FTLD) such as Pick's disease (PiD), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD).

For many years, hyperphosphorylation of tau was considered the main mechanism behind the formation of tau aggregates, through a multi-step process (Simic et al., 2016). However, there are many other post-translational modifications that tau can undergo in brain (proteolysis, acetylation, glycosylation methylation, etc.). Amongst these, proteolysis is gaining increasing attention due to its observed ability to enhance tau aggregation in neuronal cell models (Wang et al., 2007).

1.2.1 Tau hyperphosphorylation, misfolding and aggregation

While phosphorylation is part of the physiological regulation of tau activity, kinase/phosphatase imbalance and consequent hyperphosphorylation result in detachment from MTs, MTs disassembly, and overall increase of unbound tau monomers (Mudher et al., 2017, Cowan and Mudher, 2013, Bancher et al., 1989). This leads to conformational changes, misfolding and, ultimately, aggregation into soluble oligomers, which later organize in insoluble paired helical and straight filaments (PHFs, SFs) and neurofibrillary tangles (NFTs) in AD and in tau inclusions in other tauopathies (Mudher et al., 2017, Cowan and Mudher, 2013, Bancher et al., 1989).

Depending on the disease, tau filaments can be composed of 3R tau, 4R tau or both, and adopt distinct conformations according to the patognomonic proportion between 3R and 4R tau, as observed in AD (3R and 4R filaments) compared to PiD (3R) at cryo-electron microscopy (cryo-EM) (Falcon et al., 2018, Fitzpatrick et al., 2017). *In vitro*, 3R isoforms assemble into twisted PHFs, while 4R isoforms assemble into SFs (Goedert et al., 1996). The cores of PHFs and SFs from AD brain consist of two identical

protofilaments arranged base-to-base and back-to-base, respectively (Crowther, 1991). This conformation creates a double-helical structure with alternating wide and narrow regions and specific periodicity in PHFs, while in SFs the two strands are more linear (Crowther, 1991). Different mutations on the *MAPT* gene also originate abnormal tau filaments with distinct morphologies (twisted ribbon-like, rope-like), depending on the nature of the tau mutation (Crowther and Goedert, 2000).

Although NFTs are considered the pathological hallmark of tau-associated disease, there is growing evidence that the prefibrillar oligomeric tau might represent the toxic form, while fibrillary tau might be a late stage manifestation and possibly a defensive strategy to decrease the amount of circulating oligomeric tau (reviewed in Cowan and Mudher, 2013, Gendreau and Hall, 2013). Also, phosphorylation has been questioned as the main cause for aggregation, as phosphorylation at specific sites has been shown as protective against aggregation (Sandhu et al., 2017, Schneider et al., 1999). Therefore, inhibition of tau phosphorylation is still debated as a possible target for therapy, as it might be counterproductive (Mandelkow and Mandelkow, 2012).

1.2.2 Tau proteolysis and fragmentation in brain

Several tau fragments and the proteases responsible for their cleavage have been identified in tauopathy brains (Fig. 2) (reviewed in Quinn et al., 2018). An N-terminal tau fragment of 20-22 kDa, spanning aa 26-230, has been shown to interact with amyloid beta (Aβ) peptides in synaptic mitochondria, causing mitochondrial dysfunction in AD (Amadoro et al., 2012). A shorter version of this fragment (17 kDa, aa 45-230) was observed in AD, PSP and CBD brain (Ferreira and Bigio, 2011, Garg et al., 2011). The fragment is cleaved *in vitro* by proteases of the calpain family, although it is not clear if calpain-1 or -2 is responsible for the cleavage (Garg et al., 2011, Park and Ferreira, 2005). Calpain-1 is also responsible for tau cleavage at aa 242, producing a 24 kDa C-terminal fragment present in AD and FTLD, which also showed seeding and propagation properties in vitro (Matsumoto et al., 2015). Cleavage by calpain-1 was also observed in AD brain at aa 323 and aa 326, with differences related to disease stage and tau conformation in vitro (Chen et al., 2018a). Another protease family, showing upregulated activity in AD and other tauopathies, is the caspase family. Caspase-3 is responsible for tau cleavage at aa 421 (Zhao et al., 2015, Rissman et al., 2004, Gamblin et al., 2003). Tau 1-421 (Tau-C) has been found associated with NFTs in AD and is increased in AD and FTLD brains compared to controls (Zhao et al.,

2015, Rissman et al., 2004, Gamblin et al., 2003). The same fragment also showed aggregation properties in vitro, while phosphorylation at p422 blocked the cleavage and could potentially be protective against aggregation (Sandhu et al., 2017, Yin and Kuret, 2006). Asparagine endopeptidase (AEP) is responsible for tau cleavage at aa 368, and tau fragments ending at aa 368 (1-368, 256-368) have been shown to trigger neuronal apoptosis and have increased ability to aggregate into PHFs (Zhang et al., 2014).

Several other tau fragments have been identified, although the responsible protease is still unknown. A proteomic study identified several tau fragments lacking part of the N-terminal (124-441, 224-441, among others) in AD and control brains (Derisbourg et al., 2015). The 124-441 tau fragment shows a stronger binding to MTs in neuroblastoma cell lines; this gain of function is ultimately supposed to impair synaptic plasticity and transport along the MTs (Derisbourg et al., 2015). Tau ending at aa 391 has been identified after pronase treatment of NFTs, and the same fragment showed increased aggregation properties in vitro (Yin and Kuret, 2006, Novak et al., 1993). A C-terminal tau fragment (tau35), with an unknown cleavage site between aa 182-194, was identified in FTLD brains and has been shown to cause alteration in the synaptic activity and motor function in a mouse model (Bondulich et al., 2016, Wray et al., 2008, Arai et al., 2004).

Brain protein extraction techniques have been used to enrich for tau filaments from NFTs and identify which fragment of the tau protein is their main constituent (Fig. 2). Tau belonging to tangles can be found, after several extraction steps, in the trypsin-resistant sarkosyl-insoluble pellet of brain homogenates (Taniguchi-Watanabe et al., 2016, Sahara et al., 2013, Arai et al., 2004, Hanger et al., 1998, Goedert et al., 1992a, Greenberg and Davies, 1990). The core of the filaments consists of C-terminal tau fragments spanning different lengths, according to the underlying disease and the prevalence of 3R or 4R tau (Taniguchi-Watanabe et al., 2016). Cryo-EM studies have shed light on the 3D structure and C-terminal sequences involved in NFTs formation in AD and PiD (Falcon et al., 2018, Fitzpatrick et al., 2017). While the core of the NFTs consists of C-terminal tau (aa 306-378 in AD, aa 254-378 in PiD), N-terminal tau is distributed around it in a disorganised structure defined as “fuzzy coat”, which is lost after pronase treatment (Fitzpatrick et al., 2017).

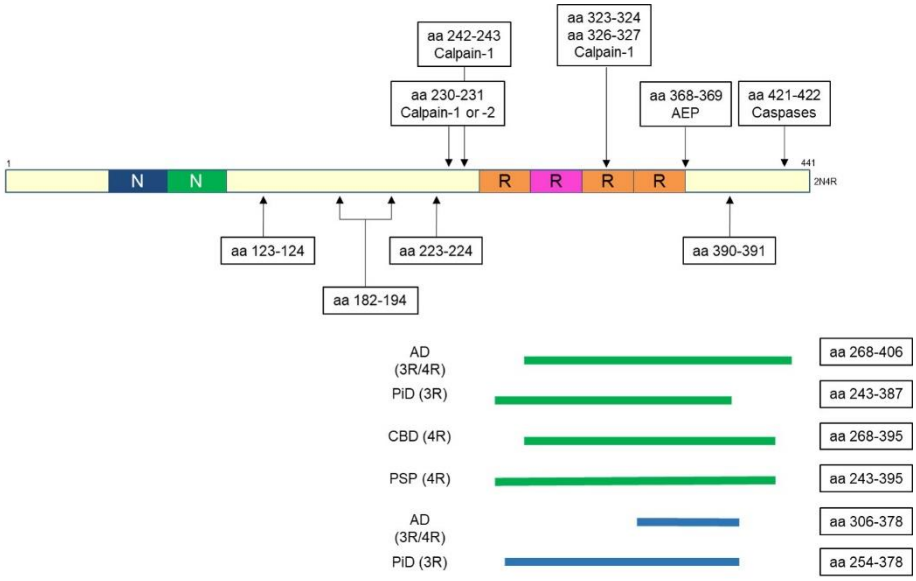

Figure 2. Tau proteolytic sites with known (top) and unknown proteases (bottom). In green:amino acid (aa) range of the tau species identified in sarkosyl-insoluble, trypsin-resistant pellet from brain homogenates (Taniguchi-Watanabe et al., 2016). In blue: range of the NFT tau filaments identified by cryo-EM (Falcon et al., 2018, Fitzpatrick et al., 2017).

1.3 Tauopathies: classification, clinical presentation and pathology

Tauopathies are classified into primary or secondary, depending on whether tau pathology is considered the major contributing factor to neurodegeneration or not, respectively (Williams, 2006). Primary tauopathies include FTD and diseases characterized by FTLD, while AD is considered a secondary tauopathy.

1.3.1 Alzheimer's disease

AD is the most common tauopathy, being responsible for up to 70% of the 50 million dementia cases worldwide. As the world population ages, AD cases are expected to double by 2030 and triple by 2050. Aging is considered the main risk factor for AD, and more than 95% of the affected are over 65 years old (late-onset AD) (World Health Organization, 2019). Early-onset AD is usually an autosomal dominant familial form of AD, which is clinically undistinguishable from late-onset, but it affects people of 40 to 50 years of age and generally has a faster progression (Tanzi, 2012, Reitz et al., 2011). The two forms have also genetic differences. The pathophysiology of early-onset AD is linked to mutations in the amyloid precursor protein (*APP*) gene and presenilin genes (*PSEN1* and *PSEN2*), which are involved in Aβ metabolism (Scheuner et al., 1996). In late-onset AD, only the gene encoding apolipoprotein E (*APOE*) has been linked to increased susceptibility to AD, but with no consistent model of transmission (Tanzi, 2012). *APOE* expresses three different isoforms, APOE ε2, APOE ε3 and APOE ε4. The presence of a single APOE ε4 allele increases the risk of late-onset AD of 2-3 fold, 5-fold if the allele is present in two copies (Corder et al., 1993).

Although aging is the main risk factor, it has been shown that AD neuropathology starts ~20 years earlier than the clinical onset (Fig. 3) (Sperling et al., 2011). Early symptoms of AD usually consist of short-term memory impairment, and are currently defined as mild cognitive impairment (MCI). If MCI is due to AD, the disease will insidiously and progressively evolve into dementia, characterized by impaired communication, disorientation, confusion, poor judgment, behavioural changes and, ultimately, inability to perform any daily activity (McKhann et al., 1984).

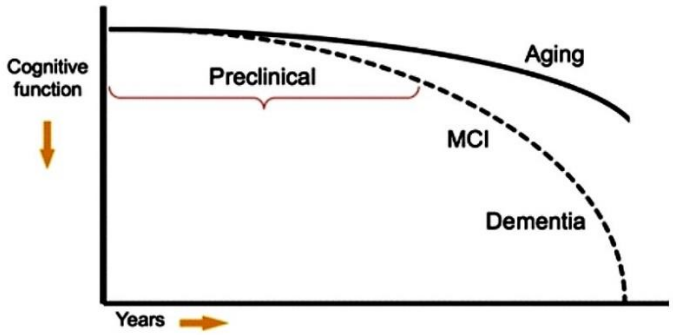

Figure 3. The continuum of AD: a long preclinical phase, where the neuropathology is already present, precedes the onset of early symptoms of AD, defined as MCI (Sperling et al., 2011).

One of the main neuropathological hallmarks of AD are Aβ plaques, composed of aggregates of Aβ peptides built up between nerve cells. Aβ spread can be classified with Thal staging: Aβ deposits are first found in the neocortex (phase 1); then, with disease progression, Aβ spreads to other cortical brain regions (phase 2), diencephalic nuclei, striatum, basal nuclei (phase 3), brain stem (phase 4) and cerebellum (phase 5) (Thal et al., 2002).

Tau load in the AD brain is described as a triad of NFTs (in the nerve cell soma), neuropil threads (in the dendrites) and neuritic component of the corona of the Aβ plaques (Braak and Braak, 1991). These formations represent the second main pathological hallmark of AD. The spreading of tau pathology in AD is classified by Braak staging; deposits are initially found in the transentorhinal cortex and hippocampus (stage 1-2), and then extend to temporal, frontal and parietal cortices (stage 3-4). Finally, sensory and motor areas are affected (stage 5-6) (Braak and Braak, 1991).

There is an open discussion in the scientific community on the interplay between Aβ and tau. Studies on mice support the hierarchical role of Aβ in causing hyperphosphorylation of tau, which in turn mediates toxicity in neurons (Terwel et al., 2008, Gotz et al., 2001, Lewis et al., 2001). Biomarkers studies also suggest an initiating role of Aβ, since it has been found decreased in cerebrospinal fluid (CSF) at least 5 to 10 years before conversion to AD dementia, whereas total tau (t-tau) and phosphorylated tau (p-tau) seem to be later markers (Buchhave et al., 2012). At the same time, the secondary role of tau has been questioned, since Aβ toxicity is critically dependent on the presence of tau, especially in the dendrite

(Ittner et al., 2010, Rapoport et al., 2002). Moreover, tau deposits are the first to appear and start in regions (transentorhinal cortex, hippocampus) directly responsible for the functions that are first impaired in AD, such as memory (Braak et al., 2006). Being still not clear if Aβ or tau initiate the AD cascade, it has been proposed that both of them target cellular processes synergistically and amplify each other's toxic effects (Ittner and Götz, 2011).

1.3.2 Frontotemporal dementia and frontotemporal lobar degeneration

FTD is a term that describes a group of early-onset dementias (<65 years in 75% of the cases) currently affecting 15-22/100000 of the population (Fig. 4) (Onyike and Diehl-Schmid, 2013). FTLD is a term used in neuropathology to define a heterogeneous group of disorders with selective degeneration of the frontal and temporal lobes (Fig. 4) (Neary et al., 1998). FTD represents the clinical counterpart of FTLD.

Clinically, FTD patients can present with two main syndromes: behavioural variant FTD (bvFTD) or primary progressive aphasia (PPA) (Neary et al., 1998). PPA can be further classified in semantic variant (svPPA) or progressive non fluent aphasia (PNFA). More recently, a third variant has been added to the group, defined as logopenic progressive aphasia (LPA), which is mostly associated with AD pathology and only in a minority of cases with FTLD (Lashley et al., 2015, Gorno-Tempini et al., 2004). bvFTD is characterized by changes in personality and behaviour (apathy, disinhibition, abnormal appetite, stereotypic behaviour), usually with insidious onset. While social cognition is affected (ability to interpret other people's mental state and emotions), memory can remain untouched by the disease. Semantic dementia is associated with severe anomia, *i.e.* the inability to name objects or understand word meaning. Patients with PNFA produce dysfluent and distorted speech (speech apraxia), without grammar structure (agrammatism). LPA patients can produce a grammatically correct speech, but they have trouble with word-finding and difficulty in understanding and retaining long sentences (Gorno-Tempini et al., 2011, Rascovsky et al., 2011).

FTD can also largely overlap with motor neuron diseases such as amyotrophic lateral sclerosis (FTD-MND/ALS) and parkinsonian disorders like progressive supranuclear palsy syndrome and corticobasal syndrome (PSPS, CBS) (Bak, 2010, Josephs et al., 2006, Neary et al., 1998). Motor symptoms in ALS are represented by dysarthria, dysphagia, muscle

atrophy and fasciculations of the tongue. The disease has a rapid progression, usually leading to death within 2–3 years. PSPS and CBS share the same symptoms as Parkinson's disease (rigidity, tremor, bradikynesia, gait impediment), but usually with unilateral onset and no response to levodopa treatment. PSPS is also characterized by gaze disturbances (especially vertical saccades) and loss of balance, while typical features of CBS are cortical sensory deficit, apraxia and alien hand syndrome.

Mutations on three main genes, namely *MAPT*, progranulin (*GRN*) and chromosome 9 open reading frame 72 (*C9orf72*), have been identified as responsible of the familial variants of FTD and associated to 10-20% of the sporadic variants (Rohrer et al., 2009).

FTLD can be classified based on the nature of the protein aggregates observed at histopathology. In 40% of all FTLD cases, tau inclusions are found in neurons or both neurons and glial cells (FTLD-tau). Tau-negative cases (FTLD-U), on the other hand, are classified based on the presence of ubiquitin inclusions associated or not with TAR DNA-binding protein 43 (TDP-43) accumulation. Only few cases have no discernible pathological inclusions (FTLD-NI). FTLD-tau can be further classified based on the predominance of specific tau isoforms: 3R-tau (*e.g.* PiD), 4R-tau (*e.g.* PSP, CBD) or 3R and 4R tau (*e.g.* tangle-only dementia) (Cairns et al., 2007).

Tau aggregates in FTLD can take different shapes than in AD and also involve glial cells (reviewed in Kovacs, 2016). In the pathological counterpart of PSPS, defined as PSP, filamentous tau aggregates are present in astrocytes (tufted astrocytes) and oligodendrocytes (coiled bodies), along with NFTs and pretangles in neurons. In CBD (the pathological counterpart of CBS), tau aggregation in the neuron is less fibrillar, while in astrocytes it accumulates in the distal part of the astrocytic processes (astrocytic plaques). In PiD, tau filaments are packed in spherical cytoplasmic inclusions (Pick bodies).

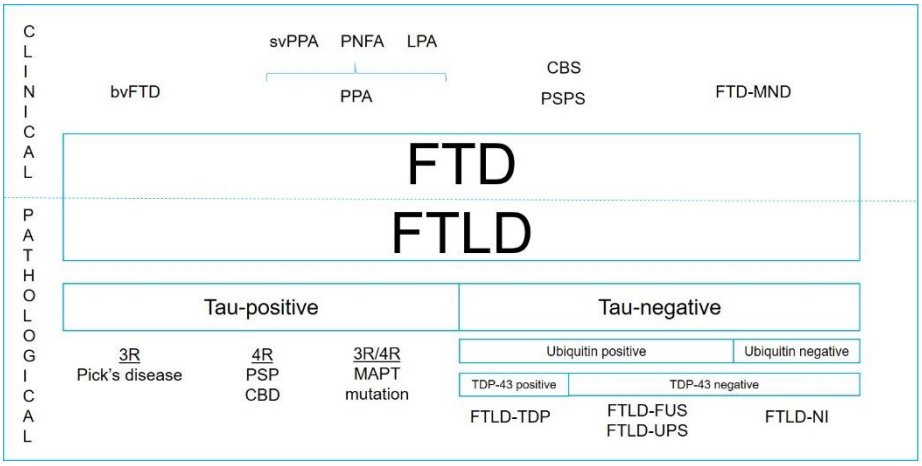

Figure 4. Classification of FTD and FTLD (adapted from Lashley et al., 2015, Rohrer, 2012).

1.4 Tauopathies: the role of biomarkers in the diagnosis

1.4.1 Alzheimer's disease diagnosis

AD dementia and MCI are currently diagnosed clinically, following the diagnostic criteria from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (Albert et al., 2011, McKhann et al., 2011, McKhann et al., 1984). Following these criteria, AD can only be confirmed post-mortem with histopathology.

Research diagnostic criteria for AD were presented in 2007 by the International Working Group (IWG) (Dubois et al., 2007). The criteria were refined in 2014 (IWG-2), including the use of biomarkers in the diagnosis (Dubois et al., 2014). IWG-2 criteria for AD require the presence of an appropriate clinical AD phenotype (typical or atypical) and a pathophysiological biomarker consistent with the presence of AD pathology (Dubois et al., 2014). Other classifications, including both imaging (magnetic resonance imaging, MRI; positron emission tomography, PET) and CSF biomarkers, have been proposed, such as the A/T/N. "A" refers to the Aβ biomarkers (amyloid-PET or CSF $A\beta_{1-42}$); "T," to tau biomarkers (CSF p-tau, or tau-PET); and "N" to biomarkers of neurodegeneration or neuronal injury ($[^{18}F]$fluorodeoxyglucose (FDG)-PET, structural MRI, or CSF t-tau) (Jack et al., 2016). In the latest research framework from 2018, AD diagnosis is not defined by the cognitive impairment associated to the disease, but by the underlying pathologic processes documented at postmortem or with in vivo biomarkers (Jack et al., 2018). Although premature for the application in clinical settings, the new framework is shifting the definition of AD "from a syndromal to a biological construct" (Jack et al., 2018).

1.4.1.1 AD CSF biomarkers

CSF is produced by the choroid plexus (a network of blood vessels in the four brain ventricles) and through passive diffusion from the brain parenchyma (Fig. 5). Due to its anatomical distribution and proximity to the parenchyma, CSF can closely reflect brain pathological processes.

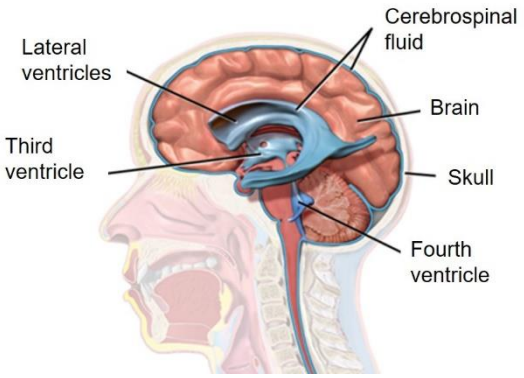

Figure 5. The cerebrospinal fluid is produced by blood filtration in the choroid plexus, a network of blood vessels present in the four ventricles of the brain.

Classic CSF biomarkers for AD diagnosis are represented by the Aβ peptide 1-42 ($A\beta_{1\text{-}42}$ or $A\beta_{42}$), p-tau and t-tau. $A\beta_{42}$ is present at lower concentrations in AD CSF, due to its accumulation in the cortical plaques (Tapiola et al., 2009, Fagan et al., 2006, Strozyk et al., 2003). T-tau levels reflect the intensity of neuronal and axonal degeneration and damage in the brain, but the increase is not specific for AD, being present in stroke, brain trauma and Creutzfeldt-Jakob disease (CJD) (Ost et al., 2006, Zetterberg et al., 2006, Hesse et al., 2001, Otto et al., 1997). CSF levels of p-tau reflect both the phosphorylation state of tau and the formation of NFTs in the brain (Buerger et al., 2006, Hampel et al., 2005). Several studies have reported strong correlations between the levels of t-tau and p-tau in patients with AD and in healthy elderly individuals, but not in CJD or stroke (Hampel et al., 2004, Riemenschneider et al., 2003, Sjogren et al., 2001). Normal p-tau levels could therefore be used to discriminate AD from other types of dementia (Koopman et al., 2009). Along with these core biomarkers, the CSF $A\beta_{42/40}$ ratio seems to better reflect brain amyloid production, being superior than $A\beta_{42}$ alone (Lewczuk et al., 2017, Dumurgier et al., 2015). A recent metanalysis, comprising 15699 patients

with Alzheimer's disease and 13018 controls, proved the strong association of core biomarkers positivity with AD and therefore recommended the use of CSF biomarkers in clinical practice and clinical research (Olsson et al., 2016).

1.4.1.2 AD imaging biomarkers

Structural MRI is used for measurements of brain volume and atrophy, which in AD is especially pronounced in the entorhinal cortex and hippocampus, even in preclinical phases (Scahill et al., 2002, Dickerson et al., 2001, Fox et al., 1996).

[^{18}F]FDG–PET measures the glucose metabolism in specific brain areas; in patients with pathologically confirmed AD, a progressive reduction in glucose metabolism has been reported to occur years in advance of clinical symptoms (Mosconi et al., 2009).

The most established tracer for *in vivo* imaging of brain amyloid, Aβ peptide aggregation and neuritic plaque formation is Pittsburgh compound B ([^{11}C]-PiB) (Klunk et al., 2004). [^{11}C]-PiB retention is increased in the cortical and subcortical brain regions of AD patients compared with the same regions in healthy controls, and the uptake correlates well with levels of Aβ in AD brain tissue at autopsy (Ikonomovic et al., 2008, Klunk et al., 2005).

The development of a tau-specific tracer represents a huge challenge, since tau is located both in the intra- and extra-cellular space. First generation tau tracers ([^{18}F]AV-1451, [^{18}F]THK-5317, [^{18}F]THK-5351 and [^{18}F]PBB3) lacked sufficient specificity and selectivity and showed off-target binding to monoamine oxidase (MAO) A and B (reviewed in Okamura et al., 2018). Second generation compounds ([^{18}F]RO6958948, [^{18}F]MK-6240, [^{18}F]GTP-1, [^{18}F]PI2620) are currently being tested and show promise as specific tau tracers (reviewed in Schöll et al., 2018).

Comparing CSF and PET-based measures of tau may provide complementary information. It seems that CSF t-tau and p-tau behave as biomarkers of "disease state", since they appear to be increased from prodromal AD and plateau afterwards. In contrast, tau-PET is a biomarker of "disease stage", since tracer binding increases through mild to moderate dementia (La Joie et al., 2018, Mattsson et al., 2018, Mattsson et al., 2017, Blennow and Hampel, 2003).

1.4.2 Frontotemporal dementia diagnosis

Frontotemporal dementia (FTD) is an umbrella term that describes a group of clinical syndromes. Originally it was used only to describe the behavioural variant of FTD, but now it also covers PPA and its variants, plus forms overlapping with motor neuron disease and parkinsonisms (Neary and Snowden, 2013, Josephs et al., 2006, Gorno-Tempini et al., 2004, Neary et al., 1998). bvFTD and PPA are currently diagnosed clinically following the latest criteria from 2011 (Gorno-Tempini et al., 2011, Rascovsky et al., 2011).

However, controversy arises for overlapping forms. In these cases, behavioural and cognitive symptoms can occur before, after or simultaneously with motor ones, and an initial diagnosis might have to be revised (Woollacott and Rohrer, 2016). ALS combined with FTD has its own set of criteria, which have been recently updated (Strong et al., 2017). Diagnostic criteria for PSP were published in 2003 and are still applied in clinical settings (Litvan et al., 2003); however, more recently, diagnostic criteria for CBD have changed the view on PSP, rebranding it a subtype of CBD (PSP syndrome, PSPS) (Armstrong et al., 2013). Along with PSPS, three other phenotypes are now considered part of CBD: corticobasal syndrome (CBS), frontal behavioural-spatial syndrome (FBS) and progressive non fluent aphasia (PNFA) (Armstrong et al., 2013).

1.4.2.1 FTD CSF biomarkers

CSF biomarkers $A\beta_{42}$, t-tau and p-tau have also been evaluated as biomarkers for FTD. A number of studies consistently shows that t-tau and p-tau are lower in FTD than in AD and Aβ is normal (Irwin et al., 2013, Irwin et al., 2012, Bian et al., 2008, Grossman et al., 2005). Hence, AD core biomarkers are currently mostly used to exclude an underlying AD pathology or diagnose AD with atypical presentations, since they can discriminate AD from FTD with high accuracy (Santangelo et al., 2015, Irwin et al., 2013, Toledo et al., 2012). Even in patients expressing a *MAPT* mutation, CSF tau levels are significantly lower than in AD patients (Karch et al., 2012). As for TDP-43 pathology, p-tau/t-tau ratio could successfully distinguish TDP-43 subjects within an FTLD group (Borroni et al., 2015).

CSF biomarkers have also been evaluated in parkinsonisms. In PSP, there has been no observed consistent elevation of CSF t-tau or p-tau compared to healthy controls (Hall et al., 2012, Sussmuth et al., 2010). In another study, CSF tau concentrations in PSP were extremely low, being lower

than both AD and control ones (Wagshal et al., 2015). However, tau concentrations in CSF could help distinguish early CBS from early PSP, and core AD biomarkers are useful to detect AD-like syndromes within the CBS spectrum (Borroni et al., 2011, Urakami et al., 2001).

One hypothesis for the low tau concentrations observed in diseases of the FTD spectra is that tau is sequestrated in neurons and glia, and significant extracellular tau pathology is absent (Grossman et al., 2005). Being a very heterogeneous disease, there is great need to find FTD-specific biomarkers. Alternative CSF measures are being taken into consideration for immunoassay and mass spectrometry (MS) analysis (Del Campo et al., 2018, Hu et al., 2010, Mattsson et al., 2008).

1.4.2.2 FTD imaging biomarkers

MRI is routinely used in the diagnosis of FTD, as group-specific patterns of grey matter atrophy allow differential diagnosis from AD with high specificity (81%) (Meeter et al., 2017, Harper et al., 2016). In general, FTD is associated with frontal and temporal lobe atrophy, with differences among the FTD subtypes (Schroeter et al., 2008, Schroeter et al., 2007). bvFTD has usually an asymmetrical, right-side predominant atrophy of the frontotemporal lobe, while in svPPA there is more antero-inferior temporal lobe involvement (Rohrer and Fox, 2009). In PNFA, the inferior frontal lobe and insula are affected, mostly on the left hemisphere (Rohrer and Fox, 2009). LPA is characterised by left temporo-parietal involvement (Rohrer and Fox, 2009). CBS can be distinguished from the asymmetric (predominantly left) pattern of brain atrophy involving the frontal cortex and striatum, while in PSPS lower brain structures (midbrain, pons) are also involved, more than the frontal cortex (Boxer et al., 2006). Distinctive signs of midbrain atrophy in PSPS are visible at T2-weighed MRI as the "hummingbird sign" (sagittal view) and "Mickey Mouse sign" (axial view) (Sonthalia and Ray, 2012). MRI and, particularly, diffusion tensor imaging (DTI) appear promising for early detection of motor cortex and pyramidal tracts atrophy in MND; however, imaging correlates of FTD associated to MND have not been investigated extensively (Kassubek et al., 2012, Rohrer, 2012).

[^{18}F]FDG–PET is also used in the diagnostic work-up of FTD. Glucose hypometabolism is often asymmetrical, and distinct pattern can be identified for each variant (Diehl-Schmid et al., 2007, Ishii et al., 1998). [^{18}F]FDG–PET can also be used to predict possible conversion to AD, PSPS or CBS (Cerami et al., 2017).

[^{11}C]-PiB-PET can be used for differential diagnosis with AD, being bvFTD, svPPA and PNFA mostly PiB-negative. Most LPA cases, instead, have underlying AD pathology and show a [^{11}C]-PiB binding pattern similar to AD (Rabinovici et al., 2008).

In tau-PET studies, the efficiency of the tracer uptake seems to depend on which tau isoform (3R, 4R or both) characterises the pathology. [^{18}F]AV-1451 showed increased uptake in the temporal cortex, frontal cortex, and basal ganglia in patients with FTD with *MAPT* mutation, which is a 3R/4R tauopathy (Smith et al., 2016). However, in 4R conditions such as PSP, there was no correlation between [^{18}F]AV-1451 binding and post-mortem tau pathology (Marquié et al., 2017). Recent studies with [^{18}F]THK-5351 look promising for detection of CBS and PSPS (Ishiki et al., 2017, Kikuchi et al., 2016).

1.5 Evidence for tau fragmentation in cell models and biological fluids: the quest for new biomarkers

An *in vivo* model, using stable isotope labeling kinetics (SILK) in the CNS and induced pluripotent stem cell (iPSC)-derived neurons, showed that most tau in cell media lacks the MTBR and C-terminal part (Sato et al., 2018). Interestingly, 4R tau isoforms and phosphorylated tau species have faster turnover rates than 3R isoforms and unphosphorylated tau, suggesting that the cell metabolizes aggregation-prone tau species differently than other forms of tau (Sato et al., 2018). Also, cells expressing FTD-related mutations show less extracellular tau compared to cells overexpressing wild-type tau (Karch et al., 2012). Other studies on iPSC-derived neurons also show that, although most intracellular tau is full-length, the majority of extracellular tau is C-terminally truncated and released both actively by living neurons and passively by dead cells (Kanmert et al., 2015). The small amount of C-terminal tau present, on the contrary, is released only after cell death (Kanmert et al., 2015). Interestingly, the secretion of N-terminal tau fragments appears to be stimulated by Aβ exposure (Sato et al., 2018, Kanmert et al., 2015). Similarly, N-terminal tau in the extracellular space has been shown to increase Aβ levels in human cortical neurons (Bright et al., 2015).

Most of the data on CSF tau come from studies performed with commercially available immunoassays using antibodies with epitopes directed to the mid-region of the protein (HT7, BT2, AT120). However, several studies suggest that tau is present as different fragments in CSF, with N-terminal and mid-region tau representing the most abundant variants (Chen et al., 2018b, Sato et al., 2018, Hansson et al., 2017, Russell et al., 2017, Barthelemy et al., 2016a, Barthelemy et al., 2016b, Amadoro et al., 2014, Meredith et al., 2013, Borroni et al., 2009, Borroni et al., 2008, Portelius et al., 2008, Sjogren et al., 2001, Johnson et al., 1997). Already in 1997, an immunoprecipitation (IP) study combined with western blot showed that CSF tau consisted primarily of a band migrating at 26-28 kDa, with additional smaller fragments in AD CSF (Johnson et al., 1997). A 20-22 kDa N-terminal fragment of tau, which was already shown to cause neurotoxic synaptic activity and interact with Aβ, was also found in CSF and discriminated with high sensitivity diseases characterized by mnesic disability (Amadoro et al., 2014).

Reverse-phase high performance liquid chromatography (RP-HPLC) was also used to enrich and concentrate tau prior to western blot analysis (Meredith et al., 2013). Multiple N-terminal and mid-domain fragments of tau, with sizes ranging from 20 kDa to 40 kDa, were detected in CSF, while full-length tau and C-terminal fragments were not detected. To quantify levels in AD and controls, in-house enzyme-linked immunosorbent assays (ELISAs) were developed to detect regions of the protein outside the t-tau standard assay region (Meredith et al., 2013). N-terminal (aa 9-198, aa 9-163) assays measures significantly higher concentrations in AD compared to control samples, while the ELISA specific for a more C-terminal region (aa 159-335) could not detect the protein in CSF. SILK studies showed that CSF tau consists of fragments of full-length brain tau, cleaved at the end of the mid-domain between aa 222 and 225, while C-terminal tau is not detectable (Sato et al., 2018). Similar results were also observed in CSF with ultrasensitive single-molecule array (Simoa)-based assays, showing significant differences in N-terminal tau between AD and AD-MCI versus controls (Chen et al., 2018b). It should be noted that the regular assays used for measuring CSF tau do not measure fragments specifically; the antibodies used bind to epitope regions of several aa, and if the epitope is present the assays are not able to differentiate between fragments or full-length tau.

MS has also been helpful to characterize tau species and their aa sequence in detail. In a first study from 2008, 19 fragments of tau were detected in human CSF (Portelius et al., 2008). Subsequently, different groups have detected tau peptides spanning from the N- to the C-terminus of the protein in CSF, showing an expansion of the mid-region tau pool (and in minor part of the N-terminal pool) in AD compared to controls (Russell et al., 2017, Barthelemy et al., 2016a, Barthelemy et al., 2016b). In Lewy body dementia (LBD) and PSP, tau peptides are overall less abundant, but the CSF profile is very similar between diseases (Barthelemy et al., 2016b).

Another IP-western blot study, using tau antibodies directed to the mid-region and C-terminus of tau, also showed fragments of different size (33 kDa, 55 kDa), representing a C-terminally truncated and an extended tau form, respectively (Borroni et al., 2009). The diseases investigated (AD, PSP, CBS, PD, FTD, LBD) showed differences in the 33/55 kDa fragment ratio; PSP, in particular, had a lower 33/55 kDa ratio than the other neurodegenerative diseases, suggesting a different pathophysiological mechanism (Borroni et al., 2009, Borroni et al., 2008). However, later studies have attributed this behavior to an assay artefact (Kuiperij and Verbeek, 2011). Other newly developed assays directed to N-terminal and

mid-region tau fragments also show extremely low CSF concentrations in PSP compared to AD and control groups (Wagshal et al., 2015).

Preliminary studies on plasma also show that fragments spanning from the N-terminal (aa 6-18) to the mid-region (aa 194-198), measured by Simoa assay, are significantly increased in AD and MCI due to AD (Chen et al., 2018b). Serum ELISAs measuring tau cleaved at aa 152 (Tau-A) and at aa 421 (Tau-C), showed an inverse correlation of the levels of the fragments to the cognitive function and a good separation between AD and MCI cohorts (Henriksen et al., 2015, Inekci et al., 2015, Henriksen et al., 2013). However, a prospective analysis did not show any predictive power of Tau-A and Tau-C for cognitive outcomes (Neergaard et al., 2018).

Taken together, all these studies reinforce the hypothesis that tau is cleaved at multiple sites and the C-terminal is retained at brain level. This process is especially amplified in AD, where the aggregation-prone C-terminus builds up the core of the tangles. In other primary tauopathies, although fragmentation is present, N-terminal and mid-region tau are either not secreted in CSF or undergo uptake by the glia. These findings have major implications for the development of new tau biomarkers for neurodegenerative diseases: measuring tau fragments outside the mid-region might add to the information from traditional tau immunoassays and provide tools for differential diagnosis between AD and primary tauopathies, as well as among primary tauopathies.

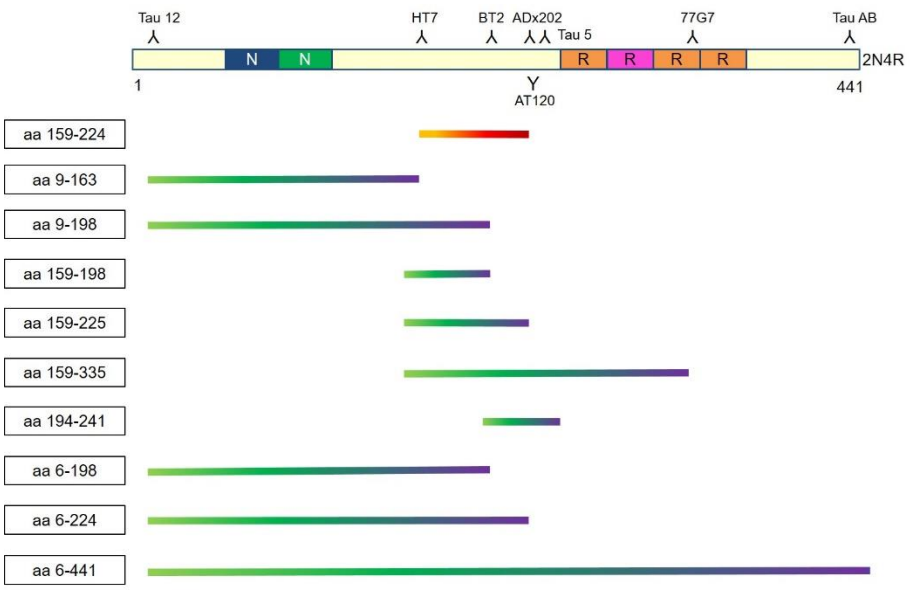

Figure 6. Tau regions covered by standard (in yellow-red) and novel (green-purple) immunoassays (Chen et al., 2018b, Wagshal et al., 2015, Meredith et al., 2013). Antibodies are shown over of their epitope region, which consists of several amino acids (aa); if the epitope is present, the assay cannot really assess the real length of the fragments, as the epitope might also be part of full-length tau.

2. AIM

2.1 General aim

To identify and characterise different tau fragments present in CSF and brain and evaluate their biomarker potential and role in the pathogenesis of AD and primary tauopathies.

2.2 Specific aims

- Paper I: to evaluate the role of newly identified tau fragments (N-123, N-224) as biomarkers and in the pathology of AD

- Paper II: to evaluate the potential of tau 368 as AD biomarker and its relationship with uptake of the [^{18}F]GTP1 tau-PET ligand.

- Paper III: to evaluate the potential of newly identified CSF tau species (N-123, N-224, N-mid, tau 368) as biomarkers for tau pathology in FTD.

- Paper IV: to identify the enzyme responsible for tau cleavage at amino aa 224.

3. METHODS

3.1 Cerebrospinal fluid collection

CSF can be sampled below the terminal part of the spinal cord through lumbar puncture (Fig. 7). In our studies (Paper I, II, III), lumbar puncture was performed following the guidelines for standardized CSF testing preanalytical procedures (Vanderstichele et al., 2012, Blennow et al., 2010). CSF was collected from the L3/L4 or the L4/L5 vertebral inter-space, in the morning. The first 12 mL of CSF were transferred to a polypropylene tube and immediately transported to the local laboratory and centrifuged (10 min at 1800 x g at +20˚C). The supernatant was mixed (to avoid possible gradient effects), aliquoted in polypropylene tubes and stored at –80˚C pending analysis.

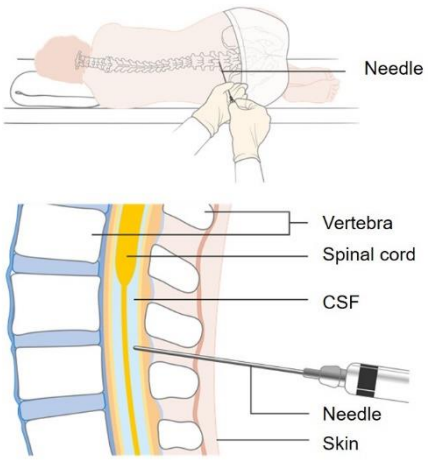

Figure 7. Lumbar puncture. CSF is tapped between vertebrae L3-L4 or L4-L5, with the patient in lying or sitting position.

3.2 Brain protein extraction

Brain protein extraction is a multi-step process used to isolate proteins from different cell compartments based on their solubility (Fig. 8). Several protocols can be used according to the protein of interest. For tau, sections of cryopreserved brain tissue are usually first homogenized in an aqueous buffer (*e.g.* tris buffer saline, TBS), containing protease inhibitors. The homogenate is then centrifuged or ultracentrifuged, and the supernatant is used to analyse the soluble fraction of brain proteins. Both supernatant and pellet can undergo other extraction steps, usually using stronger detergents (sarkosyl, Triton X-100) to extract the insoluble protein fraction. The final pellet can also be treated with trypsin to examine tangle-related tau species (Taniguchi-Watanabe et al., 2016).

The TBS fraction was of particular interest to us due to its alleged closer resemblance with the CSF milieu (Paper I, II, IV). Radioimmunoprecipitation assay (RIPA) buffer was also used to extract the whole soluble protein fraction and part of the insoluble (Paper I).

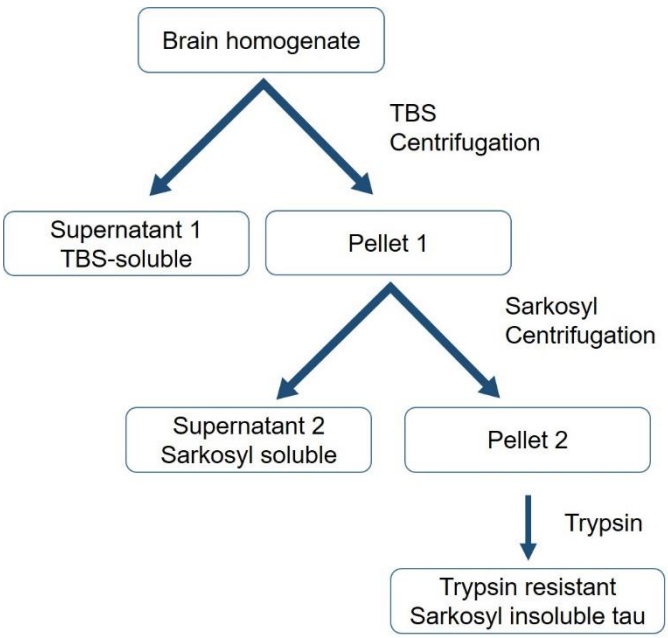

Figure 8. Brain tau protein extraction. Example of a possible protocol with multi-step extraction.

3.3 Liquid chromatography-mass spectrometry

Liquid chromatography (LC) separates the individual components of a complex matrix based on their different chemical properties. The sample is injected in the instrument and a mobile (liquid) phase is used to pass it over a stationary (solid) phase, causing the sample components to separate due to the different proportion between the two phases. The solid phase is selected based on the characteristics of the proteins or peptides to separate (*e.g.*, hydrophobicity, size, or charge). The most commonly used separation method for LC-MS is reversed-phase (RP) chromatography, in which the stationary phase consists of particles with alkyl chains of different length (*e.g.*, C4, C8, or C18). In an aqueous mobile phase, proteins and peptides bind to the stationary phase by hydrophobic interaction. By gradually increasing the concentration of organic solvent in the mobile phase, the analytes elute from the stationary phase in order of increasing hydrophobicity. The instrument will measure the UV absorbance of the different components of the sample coming out of the column and generate a chromatogram. LC is often coupled to MS to reduce the complexity of the analyte mixture infused into the mass spectrometer at a given time, and to increase the effective concentration of each infused analyte.

In our experiments (Paper I, II, IV) we used a nano-LC instrument. A first trap column (C18) was used for desalting and sample clean-up, avoiding contamination of the mass spectrometer. Separation was then performed on another, longer, C18 column, connected to the mass spectrometer via a nano-electrospray interface.

MS is used to identify compounds from a sample based on their molecular mass. Mass spectrometers consist of three basic parts: an ion source, one or more mass analysers, and a detector. The ion source transforms the analyte molecules into ions, which are then separated using magnetic and/or electric fields in the mass analyser, and detected at their specific mass/charge ratio (*m/z*). Many mass spectrometers are also capable of multi-stage experiments, called tandem mass spectrometry or MS/MS. Two different proteins could produce peptide ions of the same mass, but differ based on their unique fragmentation pattern (peptide fingerprinting); in MS/MS, selected ions are isolated in the mass analyser and subjected to fragmentation, followed by mass analysis and detection of the generated fragment ions. Fragment ion spectra can be used to derive structural information and identify proteins by database searching. The ability to identify, quantify, and characterize large numbers of

proteins from biological samples has enabled a discovery-type, hypothesis-generating approach to studying biological systems, defined as proteomics.

In our case (Paper I, II, IV), we used electrospray ionization (ESI), where a high voltage is applied on a liquid sample, creating a spray of ionized droplets. ESI is the best interface for LC, since they both work with liquid samples. As mass analyser, we used a hybrid quadrupole/Orbitrap (Q-Exactive, Thermo Fisher®) (Fig. 9). The quadrupole acts as a mass filter, isolating selected precursor ions based on *m/z*. These ions are accumulated in a collision cell and fragmented by the emission of a neutral gas (collision-induced dissociation, CID). The fragment ions are then analysed in the Orbitrap, which works as a mass analyzer. The Orbitrap is an electrostatic ion trap, in which ions oscillate along a central spindle electrode, with frequencies inversely proportional to the square-root of their *m/z*. Detector electrodes located at the outside walls record an image current of the ions, and Fourier transformation is used to resolve the frequencies of the individual ion types and determine their *m/z* values. For discovery work, selection of precursor ions for fragmentation is usually guided by which ions are detected above a set intensity threshold, through so-called data-dependent acquisition.

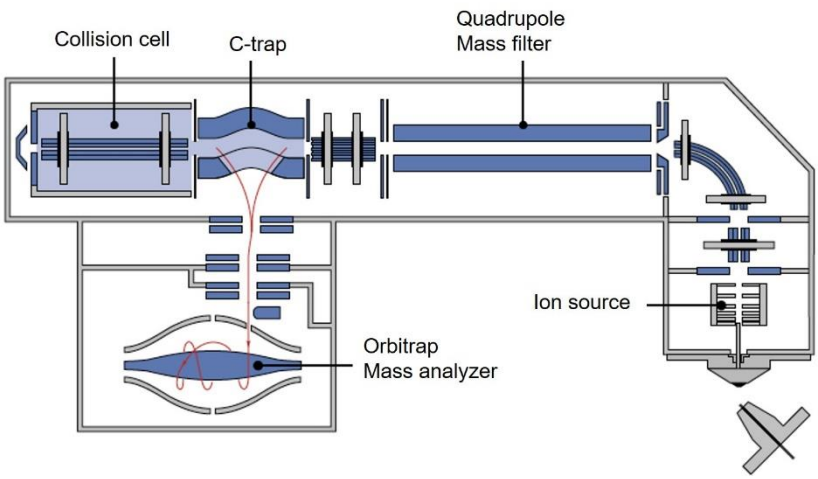

Figure 9. Schematic representation of a Q-Exactive instrument (courtesy of Thermo Fisher®)

With this system, MS spectra are therefore collected at two stages: first from the quadrupole (MS), then from the Orbitrap (MS/MS). The collection of the information from both the precursor ion and the fragment ions is defined as parallel reaction monitoring (PRM). After acquisition, various software are used for processing and analysis of MS data, and protein/peptide identification and quantification.

3.4 Immunoprecipitation

Immunoprecipitation (IP) is a method used to isolate a specific analyte from a complex sample matrix (*e.g.*, brain tissue homogenate, CSF, etc.) using antibodies immobilized on beads, usually agarose or paramagnetic (Fig. 10). Antibodies against a specific substrate can be initially free in the sample or cross-linked to beads. With free antibody, the immunocomplex antibody-antigen is retrieved and analysed; this method is preferred when the analyte of choice is known to be low in concentration in the sample or the antibody has low affinity. With cross-linking, instead, the antibody will not elute together with its antigen, and only the substrate of interest will be in the final sample. After the bead-antibody complex is incubated with the sample, this undergoes several washing steps, using a magnet to retain the beads. Finally, the analyte is eluted. IP can ultimately be considered as a small-scale purification process, aiming to isolate just enough material to be analysed with other methods, such as MS (IP-MS).

In our experiments, we used paramagnetic beads (Dynabeads®, Invitrogen) conjugated with sheep anti-mouse or sheep anti-rabbit IgGs, acting as secondary antibodies. Primary mouse or rabbit antibodies against tau were crosslinked to the secondary antibodies on the surface of the beads. (Paper I, II, IV).

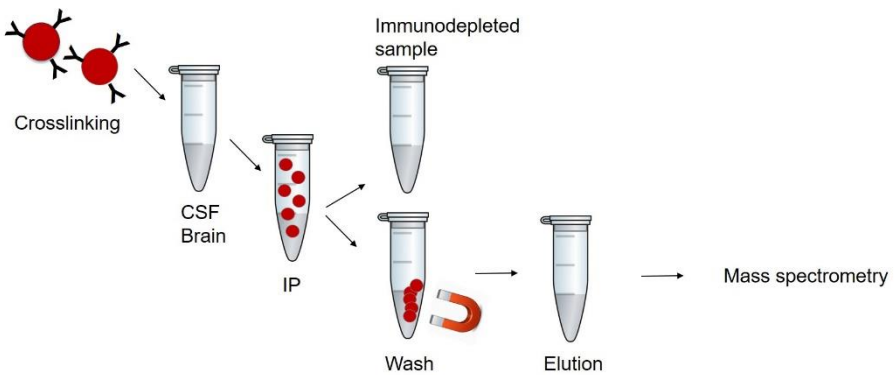

Figure 10. Immunoprecipitation. Antibodies conjugated to magnetic beads are used to enrich a specific analyte from a complex matrix.

3.5 Antibody-based assays

3.5.1 Enzyme-linked immunosorbent assay

The purpose of an enzyme enzyme-linked immunosorbent assay (ELISA) is to determine the quantity of a molecule of interest (antigen) in a biological sample. The antigen can be recognized either when immobilized onto a solid support, usually a 96-wells polystyrene microtiter plate, or on the surface of an antibody coating the plate (capture antibody). The first approach is used in direct and indirect ELISA (Fig. 11a, b). In direct ELISA, the antigen is adsorbed on the plate and binds to the primary antibody conjugated with horseradish peroxidase (HRP) (Fig. 11a). The HRP reacts with a chromogen and the reaction can be measured by colorimetric detection. Indirect ELISA requires an additional step where the primary antibody is bound by a HRP-labeled secondary antibody (Fig. 11b). Sandwich ELISA is more specific, due to the recognition of the antigen onto a capture antibody by a primary antibody, rather than on the support material (Fig. 11c). The primary antibody is either recognized by a secondary antibody conjugated to HRP or is bound directly to biotin, which ultimately binds to streptavidin linked to HRP (Fig. 11d). The biotin-streptavidin complex is used as an enhancement step for better detection.

In our studies, core AD biomarkers were measured with commercially available ELISA kits (INNOTEST® $A\beta_{1-42}$, hTAU Ag and PHOSPHO-TAU 181P, Fujirebio) (Vanmechelen et al., 2000, Andreasen et al., 1999, Blennow et al., 1995). We also developed in-house ELISA assays to measure N-123, N-mid and x-224 tau (Paper I, III, Figure 12a, c, d).

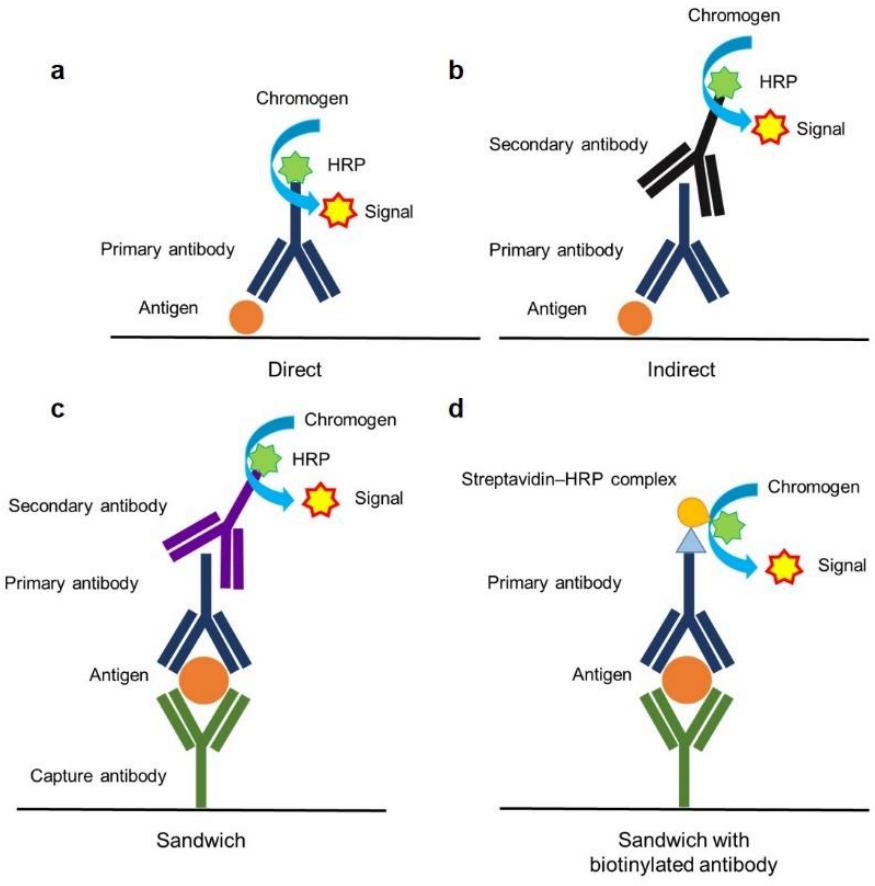

Figure 11. a) Direct ELISA: the antigen is adsorbed on the plate and binds to the primary antibody conjugated with HRP. b) Indirect ELISA: the primary antibody is bound by a HRP-labeled secondary antibody. c) Sandwich ELISA: the plate is coated with capture antibody that binds to the antigen. The primary antibody binds to antigen on the surface of the capture antibody. The detection is performed either with a secondary antibody conjugated to HRP or through a reaction between biotin on the primary antibody and a streptavidin-HRP complex (d).

3.5.2 Single-molecule array

Single-molecule array (Simoa) is an immunoassay technique developed by Quanterix® that follows the same principles of a sandwich ELISA, but with up to 1000-fold higher sensitivity. The first step consists in conjugating the capture antibody to paramagnetic beads. When mixed with the sample, the ratio bead:analyte will be around 10:1 and it will concentrate the antigen. The beads are then washed to avoid unspecific binding and incubated in sequence with the biotinylated detection antibody and β-galactosidase-labelled streptavidin (SBG). With this method, each bead that has captured a single protein molecule is labelled with an enzyme. Each single bead is loaded in an individual well with resorufin β-D-galactopyranoside (RGP) substrate and sealed with oil. If the analyte is present and an immunocomplex is formed, the substrate will become fluorescent. The concentration of the protein of interest in the test sample is determined by counting the number of wells containing both a bead and fluorescent product, relative to the total number of wells containing beads. This means that the concentration is determined digitally rather than by measurement of a total analog signal like in regular ELISA assays, which results in higher sensitivity and lower background signal. For this reason, Simoa assays have also been defined as digital ELISAs.

In our studies, we developed in-house Simoa assays to measure N-224 tau and tau 368 (Paper I, II, III, IV).

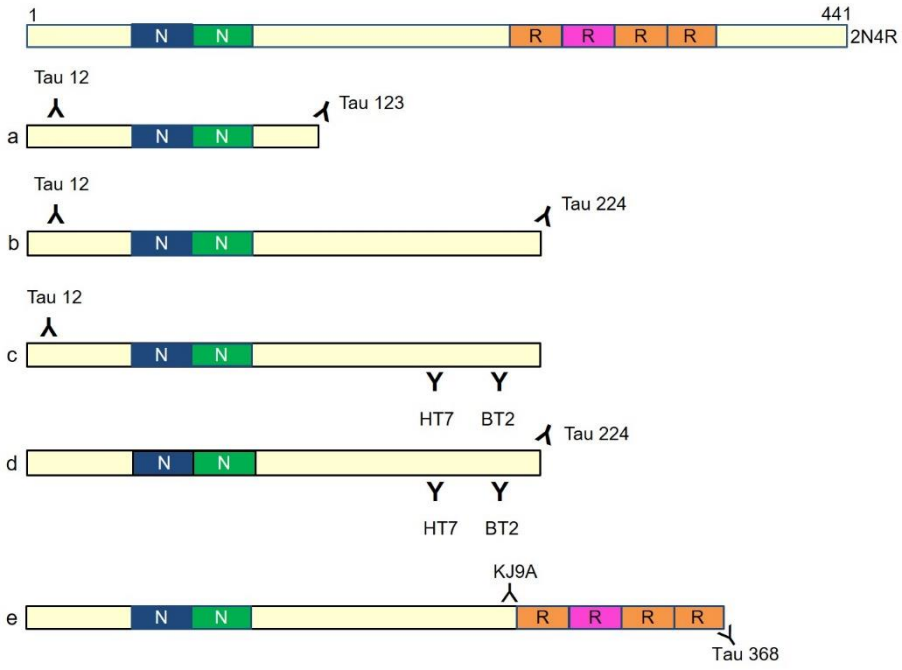

Figure 12. Overview of the in-house developed tau immunoassays. a, c, d: ELISA assays; b, e: Simoa assays. a) N-123: Tau12 has its epitope between aa 9-18, Tau 123 specifically binds aa 123, therefore the assay exclusively measures fragments ending at 123. b) N-224: Tau 224 specifically binds aa 224. c) N-mid: HT7 (aa 159-163) binds both phosphorylated and non-phosphorylated tau, BT2 (aa 194-198) only non-phosphorylated tau. d) x-224: the assay measures fragments starting at different distance from the N- terminal but all ending at aa 224 e) Tau 368: KJ9A has an unknown epitope on the C-terminal, Tau 368 specifically binds to aa 368.

3.5.3 Immunoassay validation

Method validation is required to determine the usefulness and quality of an assay. The aim of a method validation is to verify that the assay fulfils the requirements for its intended use. Validation should be performed before running any test, otherwise data cannot be interpreted. For our methods, we performed the validation following published guidelines (Andreasson et al., 2015). The lower limit of quantification (LLOQ) and upper LOQ (ULOQ) are determined by analysing the deviation from the true value of each calibrator point. The coefficient of variation (CV%) for the back calculated concentrations of the data from the calibrator curve is usually set to be <20% at LLOQ and ULOQ. The lower Limit of Detection (LOD) is determined by analysing 16 duplicates of the blank and by adding 3 standard deviations (SD) to the mean blank signal (16 replicates/one plate). Precision, defined as "the closeness of agreement between independent test results obtained under stipulated conditions", is measured by analysing samples at a known high and low concentration in 5 duplicates at 5 different occasions (ISO, 1994). Precision is ultimately defined by the calculated SD and the CV% using one-way ANOVA. The CV% usually is set to be <20%, but it can be adjusted based on the principle of "fit for purpose". "Fit for purpose" means that if the difference in the concentration of a given analyte between a disease and control group is far over 20%, then 20% is an acceptable variability; if the difference is not so wide, the assay might lose discrimination power and one would have to establish a lower accepted CV%.

3.5.4 Immunohistochemistry

Immunohistochemistry (IHC) is an antibody-based staining technique used on tissue slices (Fig. 13). Similarly, to an ELISA, IHC uses antibodies to retrieve specific antigens within a tissue. The immunocomplex can then be visualized as a chromogenic or fluorescence reaction under a microscope. Tissue sections are embedded in paraffin or cryomedia, sliced with a microtome and mounted on glass slides. Prior to the staining, the slice is dehydrated with increasing concentrations of EtOH and cleared from paraffin with xylene. To ensure an effective and specific immunocomplex formation, samples are heated for antigen retrieval and enzymatic activity, and reactive sites are blocked.

In our studies (Paper I, II), paraffin-embedded brain tissue slices were incubated with anti-tau primary antibodies and a biotinylated secondary antibody. Avidin-biotin complex (ABC) and diaminobenzidine (DAB) were used to develop the chromogenic reaction viewed at a light microscope (Fig. 13).

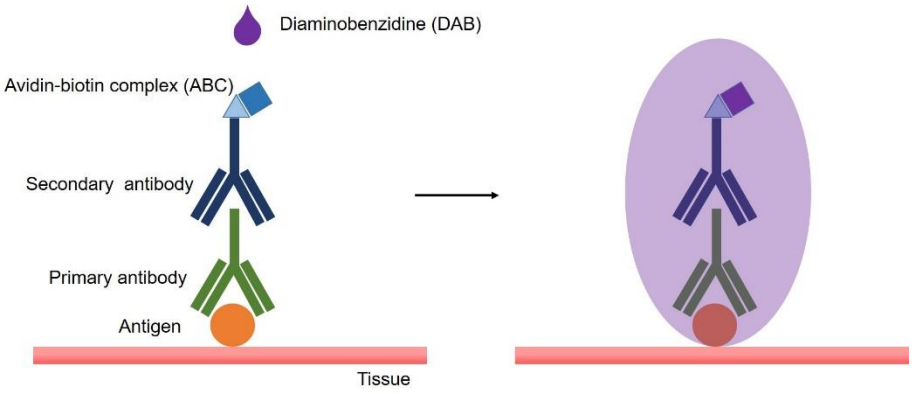

Figure 13. Immunohistochemistry. Tissue slices are incubated with a specific primary antibody, a biotinylated secondary antibody and ABC. The addition of DAB produces a chromogenic reaction.

3.6 Extracellular vesicles isolation

Extracellular vesicles (EVs) are a heterogeneous group of cell-derived membranous structures, present in biological fluids. There is growing evidence that EVs are involved in intercellular communication, and are under investigation as carriers for biomarker molecules (van Niel et al., 2018). Generally, EV subgroups can be divided (based on the centrifugation speed at which they are isolated) in apoptotic bodies (2000 x g), microvesicles (10000-20000 x g) and exosomes (100000-150000 x g) (Szatanek et al., 2015).

In Paper I, we have isolated EVs from serum, applying a particle precipitation method previously described (ExoQuick®), with some alterations (Mustapic et al., 2017, Fiandaca et al., 2015) (Fig. 14). After precipitation, IP was performed on the samples using biotinylated antibodies against neuronal cell-adhesion markers (L1CAM). The antibody-vesicle complex was pulled out using streptavidin bound to the surface of resin-agarose beads. The presence of target molecules on the vesicles was confirmed with immunoelectron microscopy. Concentration of particles in EV isolates was measured with nanoparticle tracking analysis (NTA) using ZetaView PMX 110 according to the manufacturer´s instructions (Particle Metrix®). IP samples were centrifuged, and the pellet was lysed and tested for biomarkers with ELISA and Simoa assays.

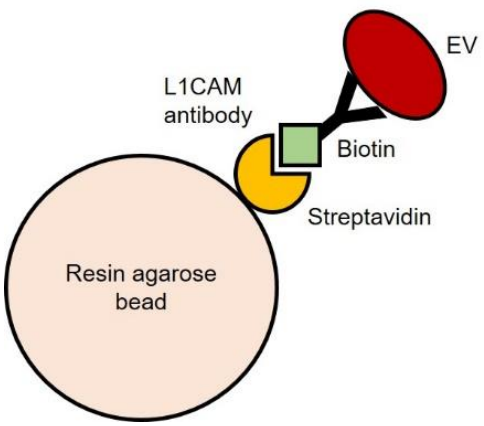

Figure 14. Extracellular vesicles isolation. After particle precipitation with ExoQuick®, samples are immunoprecipitated with biotinylated antibodies against L1CAM and streptavidin bound to the surface of resin-agarose beads.

3.7 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is based on the transfer of energy between a donor and an acceptor molecule in close proximity. The donor is always a fluorophore, while the acceptor can be either a fluorophore or a quencher (Fig. 15). When two fluorophores come close enough to each other, excitation of the donor by an energy source triggers an energy transfer towards the acceptor, which in turn emits fluorescence that can be measured with a reader. When using a donor and a quencher, having the two molecules in close proximity will result in a loss of signal. This second option is used in proteolytic cleavage studies where the fluorescence is produced when the quencher is divided from the donor (Fig. 15).

This last method was used in Paper IV to identify the enzyme responsible for tau cleavage at aa 224.

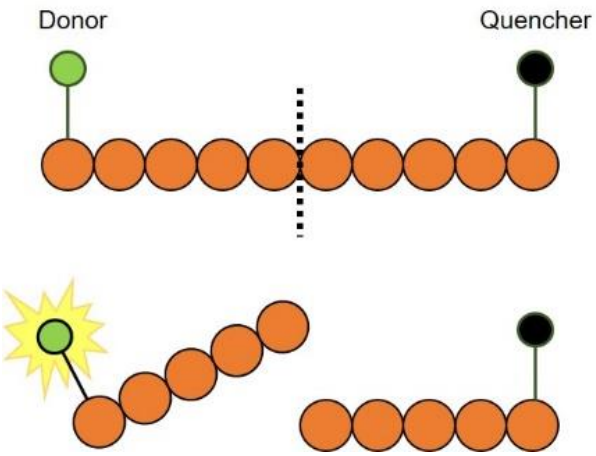

Figure 15. Fluorescence resonance energy transfer: a donor and a quencher molecule are in close proximity will result in a loss of signal; after proteolytic cleavage, the donor is divided from the quencher and will produce a fluorescent signal.

3.8 Gel protein electrophoresis and western blot

Gel protein electrophoresis is a technique used to separate proteins in a complex matrix based on their size and charge. Proteins can either be denatured with strong detergents (*e.g.* sodium dodecyl sulfate, SDS) prior to electrophoresis and studied in their unfolded state, or kept in their native conformation (native gel electrophoresis). After separation, the gel can be treated in different ways; a common procedure to visualize proteins on gel is by staining (e.g., Coomassie blue). Electrophoresis is also a separation technique used prior to MS analysis: big proteins can be trypsin-digested within the gel, extracted and identified at MS.

The process of transferring proteins from a gel to a membrane, and probing it with antibodies directed to a target protein, is defined as western blot. Western blot is fundamentally an immuno-based assay for protein identification. Similarly to an ELISA, western blot can involve one or more (primary and secondary) antibodies for detection, conjugated with HRP. When the membrane is incubated in a chemiluminescent substrate, HRP on the detection antibody will cleave it and produce a signal. The reaction can be observed on a camera reader where the protein of interest will appear as a band, localized at a specific molecular weight (MW). Although, to some extent, the intensity of the signal at band level is a measure of protein concentration, western blot is considered a semi-quantitative technique.

In Paper IV, we used native gel electrophoresis, western blot and in-gel tryptic digestion to identify the enzyme responsible for tau cleavage at aa 224.

3.9 Statistical analysis

GraphPad Prism v7.02 (GraphPad Software, La Jolla, CA, USA) was used to perform Mann-Whitney U and Kruskal-Wallis tests for group comparisons, ROC curve analysis and linear and non-linear regression analyses. Significant thresholds were set to $p < 0.05$. When required, p-values were adjusted for multiple comparisons using Dunn's test. Correlation was tested using the Spearman rank correlation coefficient. When non-parametric tests were used, linear regression lines were added to the figures as guidance. SPSS v25.0 (IBM Corporation, Armonk, NY, USA) was used for generalized linear model analyses. Log transformation was applied to linear regression analyses when data were not normally distributed. Age and sex were included as covariates.

4. MATERIALS

4.1 Cerebrospinal fluid samples

4.1.1 Pools

Pools were prepared mixing leftover de-identified CSF aliquots from clinical routine analyses (Paper I, II).

4.1.2 Discovery/pilot cohort

CSF from non-AD subjects (n=20) was from patients referred to the hospital for evaluation of psychiatric or neurological symptoms, with basic (cell count, albumin ratio, IgG index) and core ($A\beta_{42}$, t-tau, and p-tau) CSF biomarkers within normal ranges. Patients with AD (n=20) had a clinical diagnosis along with biomarker positive CSF (Paper I, II).

4.1.3 Validation/clinical cohort

CSF samples belonged to subjects characterized as AD (n=46), according to the NINCDS-ADRDA clinical criteria (McKhann et al., 1984) and IWG-2 biomarker criteria (Dubois et al., 2014). The control group (n=50) consisted of healthy volunteers (Paper I, II).

4.1.4 Longitudinal cohort

Samples were collected from 16 AD, 38 MCI, 20 MCI due to AD (MCI-AD) and 21 other neurological diseases (OND) subjects (Paper I). Subjects were clinically diagnosed with AD according to the NINCDS-ADRDA criteria (McKhann et al., 1984) and Petersen's MCI criteria (Petersen, 2004), with CSF biomarkers ($A\beta_{42}$, t-tau, p-tau) used as support. Cognitive assessment at follow-up was performed through Mini Mental State Examination (MMSE) every six months for ~2.3 years.

4.1.5 Biomarker stability cohort

CSF samples were collected during a six-month multi-centre study on 51 patients with AD on continuous treatment with acetylcholine esterase (AChE) inhibitors, as previously described (Hoglund et al., 2012, Blennow et al., 2007) (Paper I). AD was diagnosed following NINCDS-ADRDA criteria. MMSE was performed at baseline and at six months follow-up.

Treatment with AChE inhibitors at stable doses had to be ongoing for at least three months prior to the study.

4.1.6 PSP and CBS cohorts

CSF was collected from subjects diagnosed with probable or definite PSP (n=31) according to the criteria from the National Institute of Neurological Disorders and Stroke and Society for Progressive Supranuclear Palsy (NINDS-SPSP) (Litvan et al., 2003) or probable CBS (n=15) according to the criteria from Armstrong et al. (Armstrong et al., 2013) (Paper I).

4.1.7 FTD cohort

CSF was collected from 86 subjects (66 FTD, 20 controls). FTD patients met consensus diagnostic criteria for either bvFTD (n=21, one patient of whom with MND) or PPA (n=45) (Gorno-Tempini et al., 2011, Rascovsky et al., 2011). In the PPA cohort, 11 had semantic variant, 16 (two of whom had associated PSP) had non-fluent variant, 15 had logopenic variant and three did not meet criteria for any of the three variants, named PPA-not otherwise specified (PPA-NOS) (Gorno-Tempini et al., 2011). Ten patients were found to have a pathogenic mutation: *MAPT* (n=4), *GRN* (n=3) and *C9orf72* (n=3) (Paper III).

4.1.8 [^{18}F]GTP1 PET study

CSF measurements were obtained from participants enrolled in a longitudinal study on [^{18}F]GTP1 uptake, namely GN30009 (NCT02640092) (Paper II). The cohorts consisted of individuals with prodromal AD, mild or moderate AD dementia and healthy volunteers.

4.2 Brain samples

4.2.1 Nederlands Brain Bank

Samples were collected from the superior parietal lobe of control brains (Braak 0-I), four to seven hours post mortem and stored at -80˚C pending homogenization (Paper I, IV).

4.2.2 Banner Sun Health Institute

All brain lysates were made from cryosectioned fusiform gyrus from 81 AD and 33 controls and classified by Braak stages (Paper I).

4.2.3 Queen Square Brain Bank

Immunohistochemical analysis of the tau antibodies was carried out on formalin-fixed paraffin-embedded tissue sections from the frontal cortex of pathologically diagnosed AD cases and neurologically normal controls (Paper I, II).

4.3 Compliance to ethical requirements

De-identified left-over samples from clinical routine analyses were used for preparing CSF pools and in the discovery/pilot cohorts (Paper I, II), following a procedure approved by the Ethics Committee at University of Gothenburg (EPN 140811). CSF from the validation/clinical cohorts (Paper I, II) was collected at the Memory Clinic at Lund University (Dnr 695/2008, 2013/494 and 2014/467). Samples from the longitudinal cohort (Paper I) were collected at the Center for Memory Disturbances of the University of Perugia with approval from the local ethics committee (Prot. N. 19369/08/AV). All patients from the biomarker stability cohort (Paper I) gave informed consent and the study was approved by the ethics committee at Lund University, Uppsala University and at Karolinska Institute (Stockholm, Sweden). PSP and CBS patients (Paper I) were diagnosed at the Department of Neurology, Movement Disorders Unit, Sahlgrenska University Hospital, Gothenburg, Sweden. Sample collection was approved by the Regional Ethical Board at the University of Gothenburg (n. 460-13, 462-95, 210-95). In Paper III, the use of CSF samples was approved by the Queen Square NRES Committee (12/LO/1504, 16/LO/0465-190270, 14/LO/1758-155836). In the [^{18}F]GTP1 uptake longitudinal study GN30009 (Paper II) collection of baseline CSF samples was approved by the independent review board Copernicus Group (G0097, QUI1-15-580).

Ethical declaration from the Nederlands Brain Bank (Paper I, II, IV) can be found online at https://www.brainbank.nl/media/uploads/file/Ethical-declaration.pdf. Brain from the Banner Sun Health Institute (Paper I) belonged to subjects enrolled and annually assessed in the Arizona Study of Aging and Neurodegenerative Disorders, then autopsied in the Brain and Body Donation Program (Beach et al., 2015). Immunohistochemical analysis (Paper I, II) was carried out on brains that were donated to the Queen Square Brain Bank for Neurological Disorders, UCL Institute of Neurology, University College London (18/LO/0721-246790).

5. RESULTS AND DISCUSSION

5.1 Paper I

The aim of this study was to characterize tau fragments in brain and CSF using IP-MS. Once we identified specific fragments ending at aa 123 and aa 224, we developed antibodies directed to these cleavage sites and used them for immunohistochemistry and in targeted immunoassays against tau fragments of different length ending at aa 123 or 224 (N-123, N-224, x-224). Assays were applied to soluble brain fractions from pathologically confirmed subjects (81 AD patients, 33 controls), CSF from three cross-sectional and two longitudinal cohorts (a total of 133 AD, 38 MCI, 20 MCI-AD, 31 PSP, 15 CBS patients and 91 controls) and neuronally- and peripherally-derived extracellular vesicles (NDEVs, PDEVs, respectively) from serum of four AD patients and four controls.

5.1.1 Identification of novel fragments in brain and CSF

IP-MS analysis with anti-tau antibodies (Tau 12, HT7, BT2) showed the presence of several tau fragments in brain extract and CSF (Fig. 16). In brain tissue, tryptic peptides covered the whole tau protein, from the N-terminus (acetylated aa 2) to the C-terminus (aa 441). In CSF, analyses were performed without tryptic digestion as well, to observe the naturally occurring endogenous peptides. The two major tau pools detected were composed of peptides ending at aa 123 and aa 224, respectively, suggesting prominent proteolytic cleavage of the protein at these sites (Fig. 16, top part). In CSF, the abundance of fragments dropped significantly after aa 221, and no tryptic peptides were detected C-terminally of aa 254 (Fig. 16, bottom part).

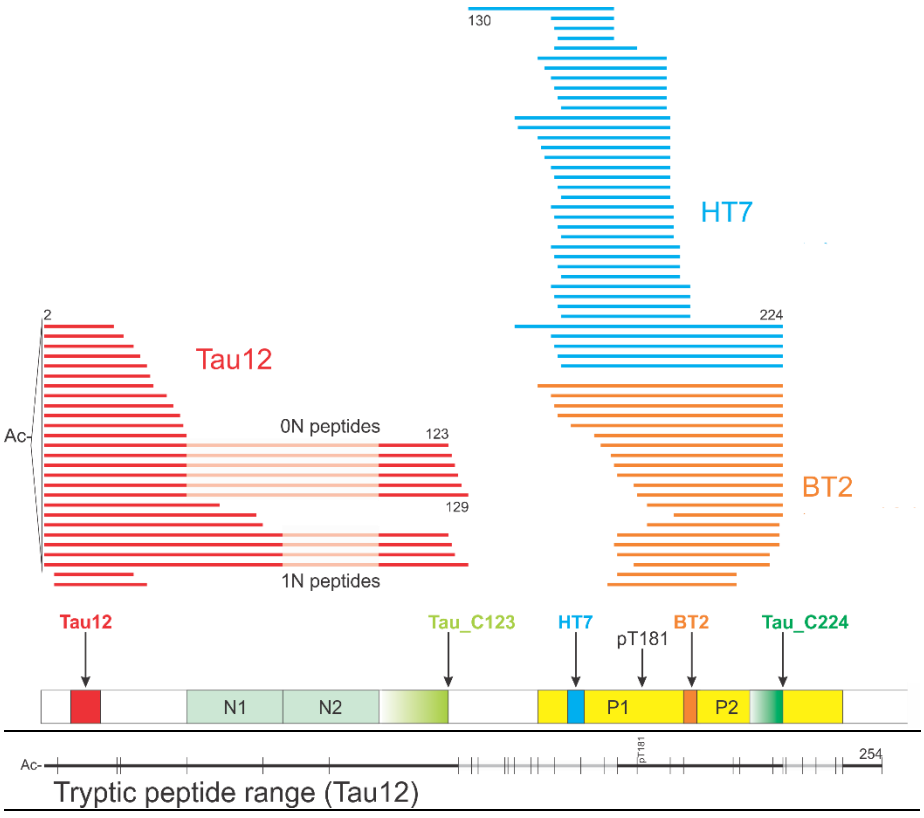

Figure 16. Top: endogenous tau species found with N-terminal and mid region specific antibodies (Tau 12 in red, HT7 in blue, BT2 in orange). Alignment and numbering refers to the 2N isoform; for peptides originating from the 0N and 1N isoforms, 2N sequence portions not included are dimmed. The protein schematic structure shows the respective antibody epitopes and T181. Bottom: in black, range (aa Ac-2 to 254) of tryptic peptides detected in CSF with Tau12 (regions with no coverage are dimmed). Vertical lines indicate possible tryptic cleavage sites.

5.1.2 Tau fragments ending at aa 224 in AD

Immunohistochemical staining showed positivity to the anti-tau 224 antibody in neurofibrillary tangles and neuropil threads in AD, while in control brain the antibody showed only faint cytoplasmic staining.

Data from AD brain extracts showed an apparent decreasing trend for the N-224 tau fragment over the course of disease (Braak III to VI), with lower concentration in AD brain compared to control, although not statistically significant when normalized to total protein content ($p=0.06$).

In CSF, the N-224 tau fragment was significantly higher in AD compared to control in all cohorts (discovery and validation cohorts, $p<0.001$; longitudinal cohort, $p=0.002$), in MCI-AD compared to stable MCI ($p=0.002$) and in MCI vs. OND ($p<0.0001$) (Fig. 17a, b). N-224 tau did not correlate as well to t-tau in AD compared to controls ($r=0.62$ and $r=0.75$, respectively), and the two groups had a skewed distribution (Fig. 17c). X-224 tau tightly correlated with t-tau in AD and controls, due to the overlapping region of interest of the assays (aa 159-240).

In the longitudinal cohort, the magnitude of cognitive decline was related to the initial concentration of the fragment. The MMSE score decreased significantly over years from baseline for the highest quartile (Q4) of N-224 concentrations at baseline (adj. $R^2=0.34$, $\beta=-0.58$, $p=0.0002$), while the same was not observed for t-tau and p-tau ($p>0.05$) (Fig. 17e).

In terms of stability and predictive power, levels of N-224 tau in AD subjects were unaffected by AChE inhibitor treatment at six months follow-up ($p = 0.78$), and concentrations were not significantly different across drug treatments ($p=0.08$). N-224 tau levels did not differ significantly depending on number of *APOE* ε4 copies ($p=0.48$), sex ($p=0.22$), or age ($p=0.08$).

The N-224 tau fragment was significantly higher in NDEV lysates compared to PDEV lysates ($p=0.03$) in both AD and control groups, but differences between AD and control NDEV were not significant (Fig. 17d).

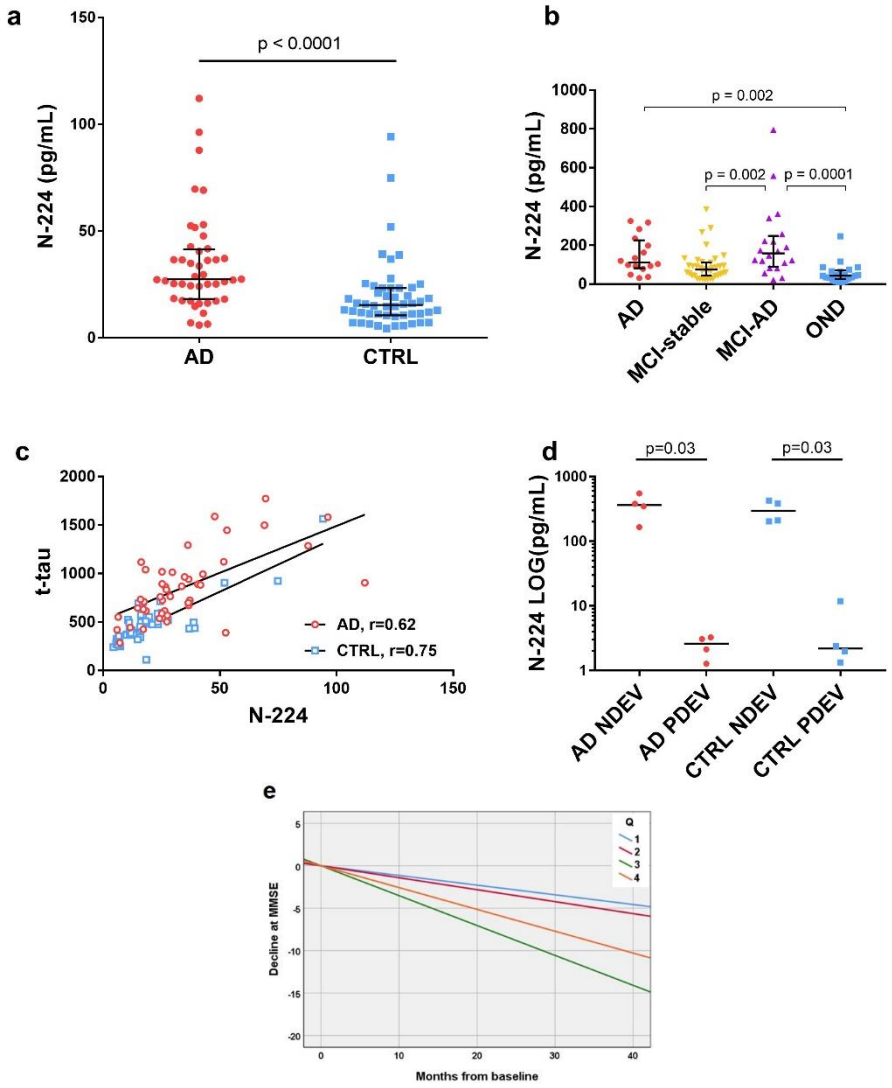

Figure 17. N-224 tau. a) concentration range of N-224 fragments in AD and controls from the validation cohort (lines representing median, bars representing interquartile range). b) concentration range of N-224 fragments in AD, MCI, and MCI-AD and OND (lines representing the median, bars representing interquartile range). c) correlation of N-224 fragment to t-tau and linear regression in AD and controls from the validation cohort. d) scatter dot plots of N-224 tau concentrations in serum NDEV and PDEV (lines representing median). e) disease progression over time in AD, MCI-AD and MCI cohorts, divided in quartiles (Q) based on the concentrations of N-224 tau at baseline, as measured by change in MMSE scores. Higher levels of CSF N-224 tau at baseline are related to faster decline.

5.1.3 Tau fragments ending at aa 224 in PSP and CBS

When comparing AD to PSP and CBS, we observed that all tau biomarkers concentrations, including N-224 tau, were significantly decreased in PSP and CBS, being even lower than in controls in most cases (Fig. 18a). Interestingly, CSF N-224 tau concentrations did not significantly correlate to t-tau in both PSP (r=0.48) and CBS (r=0.39) (Fig. 18b).

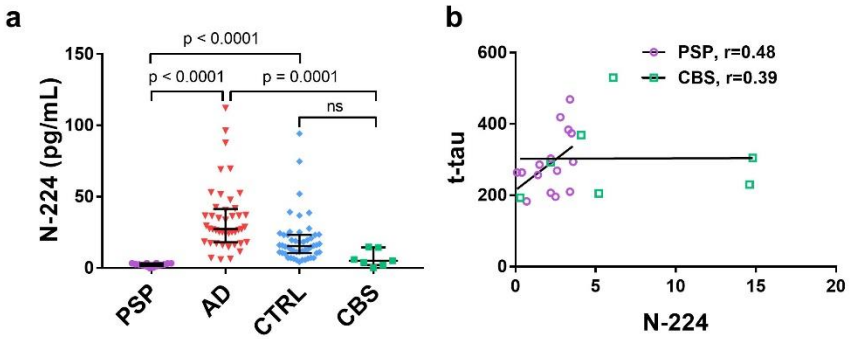

Figure 18. PSP and CBS cohort. a) concentration of N-224 tau in PSP and CBS (Aβ negative subjects only) compared to AD and controls from the validation cohort (lines representing the median, bars representing interquartile range). b) correlation to t-tau and linear regression in PSP and CBS cohorts (Aβ negative subjects only).

5.1.4 Tau fragments ending at aa 123

Immunohistochemical data on N-123 tau showed only a faint positivity of neuronal staining with anti-tau 123 and mostly in the cytoplasm.
In brain extract, there was no significant difference in the concentration between AD and controls ($p=0.5$) and no visible trend along Braak stages.

CSF concentrations of tau N-123 were significantly higher in AD compared to control subjects in the discovery cohort ($p<0.001$), but not in the validation cohort where an opposite behavior was observed, with higher concentrations in controls vs. AD ($p=0.001$) (Fig. 19a). Baseline levels of the N-123 fragments were significantly higher in AD vs. OND ($p=0.03$), MCI-AD vs. MCI ($p=0.01$) and MCI-AD vs. OND ($p=0.001$) (Fig. 19b). N-123 concentrations in CSF did not correlate with those of t-tau (Fig. 19c). Subjects in the highest quartile (Q4) of N-123 baseline concentrations showed worse cognitive outcome (adj. $R^2=0.36$, $\beta=-0.61$, $p=0.0001$) (Fig. 19e).

Data from serum EVs do not corroborate the brain- or disease-specificity of the fragment, which showed no significant difference between NDEV and PDEV or between AD and control groups (Fig. 19d).

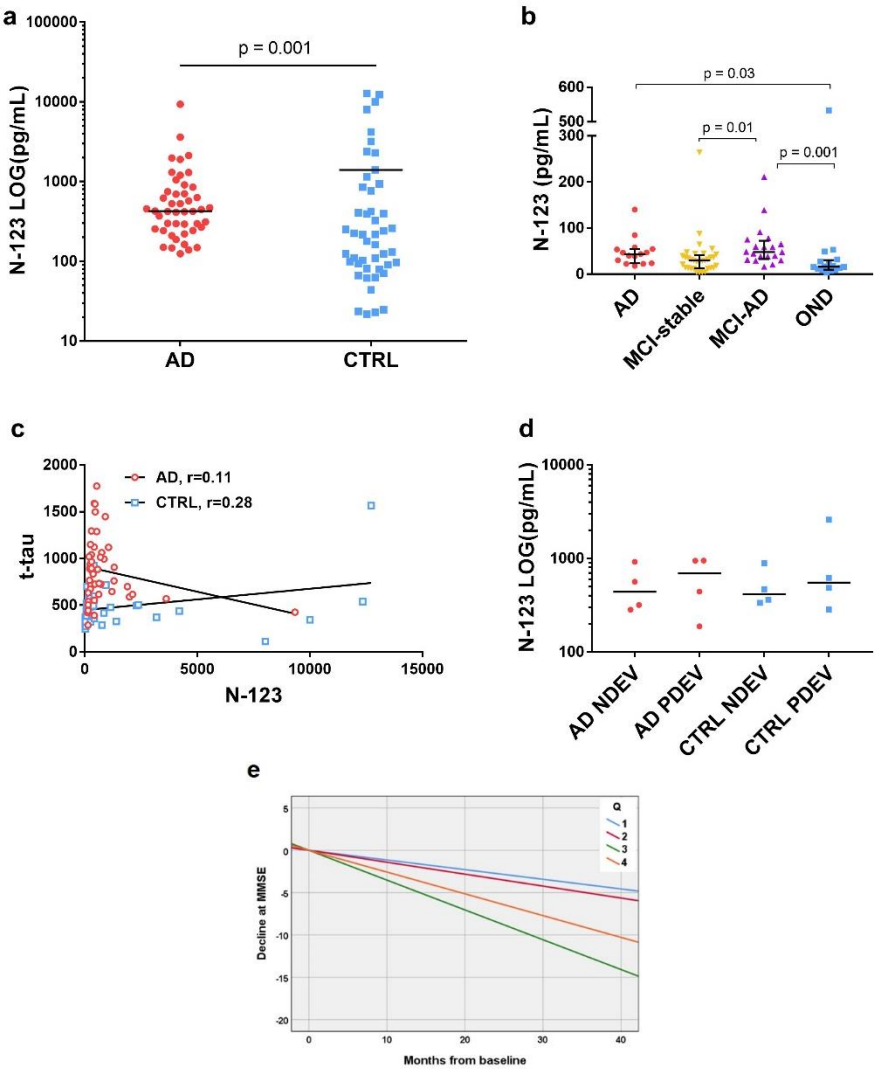

Figure 19. N-123 tau. a) concentration range of N-123 fragments in AD and controls from the validation cohort (lines representing median, bars representing interquartile range). b) concentration range of N-123 fragments in AD, MCI, and MCI-AD and OND (lines representing the median, bars representing interquartile range). c) correlation of N-123 fragment to t-tau and linear regression in AD and controls from the validation cohort. d) scatter dot plots of N-123 tau concentrations in serum NDEV and PDEV (lines representing median). e) disease progression over time in AD, MCI-AD and MCI cohorts, divided in quartiles (Q) based on the concentrations of N-123 tau at baseline, as measured by change in MMSE scores. Higher levels of CSF N-123 tau at baseline are related to faster decline.

5.1.5 Discussion

These findings suggest that the 224-fragment is deposited in AD brain and colocalized with the tangles. The lower concentrations in brain extracts at more severe stages of disease can be explained by the technique used for protein extraction: RIPA buffer extracts all the soluble and part of the insoluble tau, but not tau aggregated into tangles. N-224 might be trapped in the "fuzzy coat" of tau filaments and therefore less likely to be extracted with this technique in samples from later stages of disease, where more tau is sequestrated in the tangle load (Fitzpatrick et al., 2017).

In CSF, tau N-224 is increased in AD and shows only a sub-optimal correlation to the concentrations of t-tau, suggesting that N-224 might be a new biomarker representing the product of a different metabolic route affecting the N-terminus of the protein, as opposed to the mid-region pool. Also, higher concentrations of N-224 at baseline could be predictive of worse cognitive outcome. Results from EV isolation show that N-224 is a brain-specific biomarker.

The aggregated results suggest that, in the CNS, there is a disease-related upregulated cleavage at this site, which leads to enhanced release from neurons of the N-terminal fragment and, ultimately, higher CSF concentration of the fragment and a deposition into tangles. At a tangle level the 224-cleaved tau might therefore be an indirect marker of ongoing tangle formation. Another potential explanation for the results is that the production of the fragment is enhanced in the fuzzy coat of tangles.

The different behaviour of tau biomarkers in primary tauopathies can be referred to an overall lower concentration of CSF tau, however it is not known whether this is due to reduced tau production, reduced secretion into the CSF, or uptake by the glia. The lack of correlation of N-224 tau to p-tau and t-tau suggests a different underlying disease mechanism compared to AD, which does not include cleavage of N-terminal tau.

Our findings also suggest that N-123 tau is not present in the tangles, while there is an active secretion of the fragment in CSF. N-123 tau does not have a clear diagnostic role, but it might represent a general marker for tau metabolism occurring in CNS and peripheral nervous system, a hypothesis reinforced by its presence in the PDEV. However, no indications have been found on how this relates to the disease state and/or stage, although higher CSF levels at baseline are related to higher

rate of cognitive decline at follow-up as measured by decline at MMSE. It is possible that there is an intrinsic balance between tau fragmentation and stability and that the N-123 fragment is a marker of physiological processes within a given range of concentrations.

In conclusion, among the several fragments of tau present in CSF, there are two major pools consisting of tau species cleaved at either aa 123 or 224, and the two fragments reflect different mechanism of tau processing in AD. While cleavage generating tau N-123 could be part of the normal function of tau turnover, the generation of tau N-224 is related to AD. In support of this hypothesis is the lack of consistent differences of CSF N-123 tau levels comparing AD and control, while fragments ending at aa 224 are significantly higher in AD CSF compared to control and relate to the decrease in cognitive performance over time. Moreover, N-224 tau in primary tauopathies shows great promise for elucidating the role of differential tau processing in AD compared to other tauopathies.

5.2 Paper II

Emerging evidence suggests that tau proteolysis is involved in the formation and spreading of tau aggregates, but no biomarkers reflecting tangle pathology are yet available. In this study, we evaluated the role of a newly identified C-terminal fragment of tau (tau 368), cleaved by AEP, as a biomarker of tangle pathology (Zhang et al., 2014).

IP-MS and IHC were used to evaluate the presence of tau 368 species in CSF and tangles, respectively. A novel Simoa assay for quantification of tau 368 in CSF was developed and evaluated in a pilot (AD=20, controls=20) and in a clinical cohort (AD=37, controls=45), where the IWG-2 biomarker criteria was applied. Moreover, tau 368 was measured in CSF from a third cohort of 11 healthy volunteers and 38 amyloid PET-positive prodromal, mild or moderate AD patients with paired [^{18}F]GTP1 scans.

5.2.1 Tau 368 in brain and CSF

Anti-tau 368 in AD brain tissue showed staining of the filamentous structures in neurofibrillary tangles at IHC.

IP-MS of CSF using the polyclonal tau antibody KJ9A confirmed the presence of endogenous tau species ending at aa 368.

In the pilot study, tau 368 was significantly increased in AD compared to controls ($p<0.001$). T-tau and tau 368 showed a positive correlation both in the AD and control group ($p=0.01$, $r=0.6$, for both). Tau 368/t-tau ratio was significantly decreased in AD compared to controls ($p<0.001$).

In the clinical study cohort, CSF concentrations of tau 368 were significantly increased in AD compared to controls ($p=0.01$) (Fig. 20a). Conversely, tau 368/t-tau ratio was decreased in AD compared to controls ($p<0.0001$) (Fig. 20b). T-tau and tau 368 showed a positive correlation within the AD group ($p=0.002$, $r=0.57$) as well as the control group ($p<0.001$, $r=0.9$) (Fig. 20c).

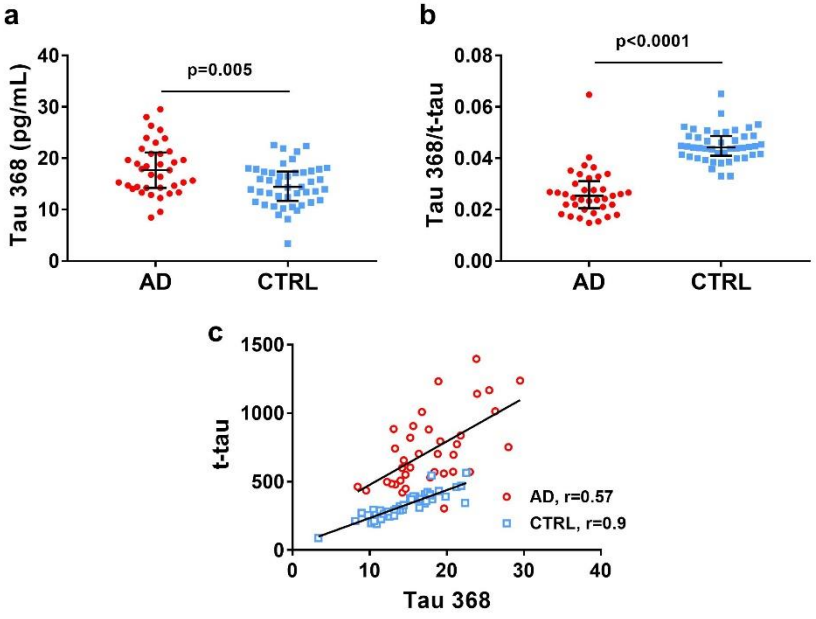

Figure 20. Tau 368. a) concentration range of tau 368 in AD and controls from the clinical study cohort (lines representing median, bars representing interquartile range). b) tau 368/t-tau ratio in AD and controls from the clinical study cohort (lines representing median, bars representing interquartile range). c) correlation of tau 368 to t-tau and linear regression in AD and controls from the clinical study cohort.

5.2.2 Tau 368 and [^{18}F]GTP1 uptake at PET

[^{18}F]GTP1 standardized uptake value ratio (SUVR) increased step-wise with disease severity (p<0.001), and was elevated in amyloid positive healthy volunteers. CSF levels of the tau 368 fragment alone did not correlate with [^{18}F]GTP1 SUVR (r=0.06, p=0.69). However, the ratio of tau368/t-tau in CSF decreased with increasing disease severity (p<0.001) (Fig. 21a). Tau 368/t-tau ratio was negatively correlated to [^{18}F]GTP1 SUVR in the temporal meta-ROI (r= -0.73, p<0.001) (Fig. 21b).

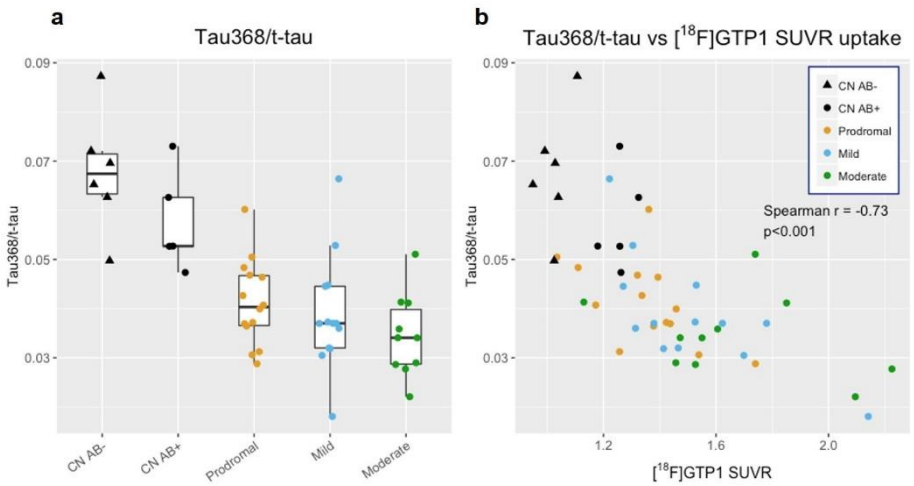

Figure 21. Tau 368 and [^{18}F]GTP1 uptake. a) tau 368/t-tau ratio in CSF decreases with disease severity (CN=cognitively normal). b) [^{18}F]GTP1 uptake and tau 368/t-tau ratio in CSF are negatively correlated.

5.2.3 Discussion

We demonstrated that tau 368 can be quantified in CSF, with increased concentrations in AD compared to controls. The decrease in the tau 368/t-tau ratio in AD compared to controls suggests that, although tau 368 is overall increased, only a small proportion of the whole CSF tau ends at aa 368.

Our hypothesis is that in AD brain there is a different tau fragmentation pattern, which creates fragments ending at aa 368, containing the MT binding domain. These fragments have an increased propensity for aggregation and tangle formation, and are therefore retained in AD brain, as shown at IHC, resulting in less tau 368 excreted into CSF.

Of potential clinical relevance, we also showed a strong negative association (r=-0.73) of the CSF tau 368/t-tau ratio and [^{18}F]GTP1 SUVR, further supporting that the ratio reflects underlying tau pathology and entrapment of tau 368 in tangles.

5.3 Paper III

The aim of the study was to evaluate the role of classical tau CSF biomarkers (p-tau, t-tau) and newly discovered CSF tau species (N-123, N-mid, N-224 and tau 368, non-phosphorylated tau) in the detection of tau pathology in FTD, as currently no specific FTD biomarkers are available. Immunoassays targeting tau biomarkers were applied to CSF from 86 subjects, 66 with a clinical diagnosis within the FTD spectrum and 20 controls.

5.3.1 Comparison of likely AD- versus likely FTLD-pathology subjects

Since patients with bvFTD or PPA (particularly LPA) may have underlying AD pathology rather than FTLD pathology, FTD patients were first grouped based on their CSF $A\beta_{42}$ concentrations into those likely to have AD pathology ($A\beta_{42}$<550pg/ml, n=21) and those with FTLD pathology ($A\beta_{42}$>550pg/ml, n=45). The atypical AD group consisted of 14 LPA, three with non fluent variant PPA, one with semantic variant PPA, one with PPA-NOS and two with bvFTD.

T-tau and p-tau concentrations were significantly higher in the AD group compared to controls and the FTLD group ($p<0.05$). The AD group also showed significantly higher concentrations than controls for: N-mid, N-224 and non-phosphorylated tau ($p<0.05$) (Fig. 22a, b, c). No significant differences were seen for tau N-123 or tau 368.

The FTLD group showed significantly higher concentrations than controls for t-tau, N-mid, N-224 and non-phosphorylated tau, but not for other tau fragments (Fig. 22a, b, c).

None of the novel immunoassays showed a significant difference between the AD and FTLD group.

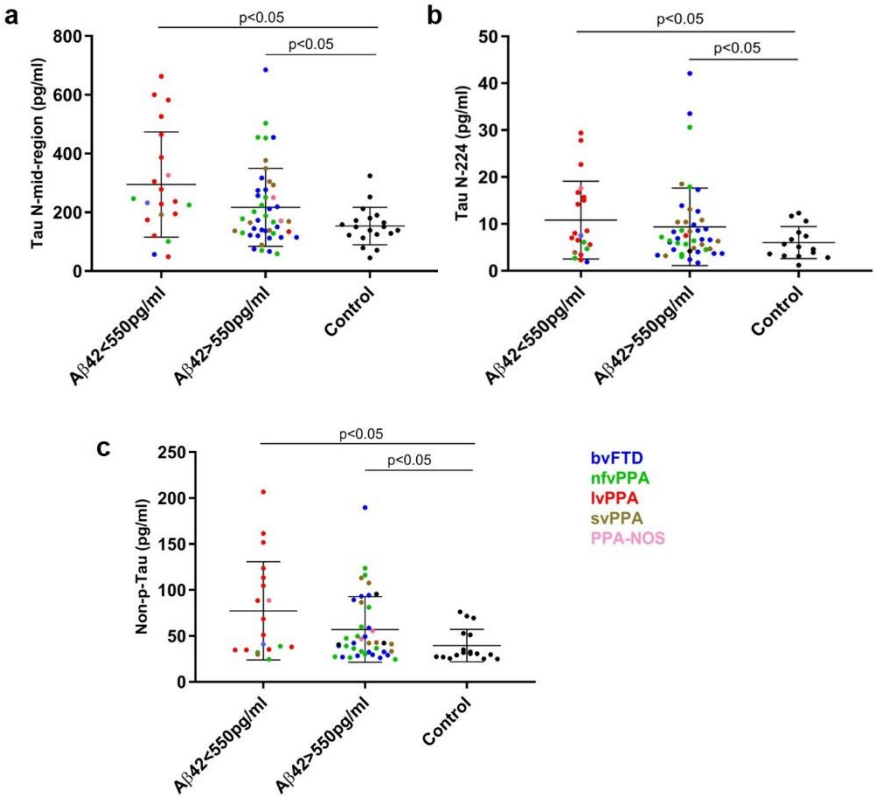

Figure 22. Biomarkers with significant differences to controls in subjects with likely AD- and likely FTLD pathology. a) N-mid, b) N-224 tau, c) non-p-tau).

5.3.2 *Comparison of likely FTLD-tau versus likely FTLD-TDP-43 pathology subjects*

Seven individuals, namely *MAPT* mutation carriers, PSP patients and one patient with bvFTD who was found at post-mortem to have CBD, were attributed to the likely FTLD-tau pathology group. The FTLD-TDP-43 group consisted of 18 subjects, namely *GRN* and *C9orf72* mutation carriers and subjects with a clinical diagnosis of svPPA or MND.

CSF t-tau concentrations were significantly higher in the FTLD-tau and FTLD-TDP-43 groups compared to controls ($p<0.05$) (Fig. 23a). N-mid, N-224 and non-phosphorylated tau showed higher concentrations in the FTLD-TDP-43 group compared to controls ($p<0.05$), while only N-mid and N-224 tau were significantly higher in the FTLD-tau group compared to controls (Fig. 23b, c). No significant differences were seen for tau N-123 and tau 368. None of the measures showed a significant difference between the FTLD-TDP-43 and FTLD-tau groups.

Since the p-tau/t-tau ratio has been shown to improve the discrimination between tau and TDP-43 pathology, we performed a sub-analysis normalising tau markers for t-tau (Meeter et al., 2018, Borroni et al., 2015, Hu et al., 2013). The p-tau/t-tau ratio was significantly lower for both the FTLD-TDP-43 and the FTLD-tau group compared to controls ($p<0.05$), but no significant difference between FTLD-tau and FTLD-TDP-43 groups was present (Fig. 23d). ROC curve analysis measuring the ability of the p-tau/t-tau ratio to differentiate probable FTLD-tau from FTLD-TDP-43 showed a sensitivity of 61.1% and specificity of 85.7%.

Of the novel tau species, both tau x-368 and tau N-224 had a significantly different ratio in the FTLD-tau group compared to controls ($p<0.05$) (Fig. 23e, f). For tau 368, the ratio was also lower in the FTLD-TDP-43 group compared to controls ($p<0.05$), but there was no difference between the FTLD-tau and FTLD-TDP-43 groups. N-224 tau showed a significantly higher ratio for FTLD-tau compared to the FTLD-TDP-43 group ($p<0.05$). Sensitivity and specificity of N-224/tau ratio in differentiating between FTLD-tau and FTLD-TDP-43 groups was 61.1% and 57.1%, respectively. No significant differences were seen in the other novel tau measures.

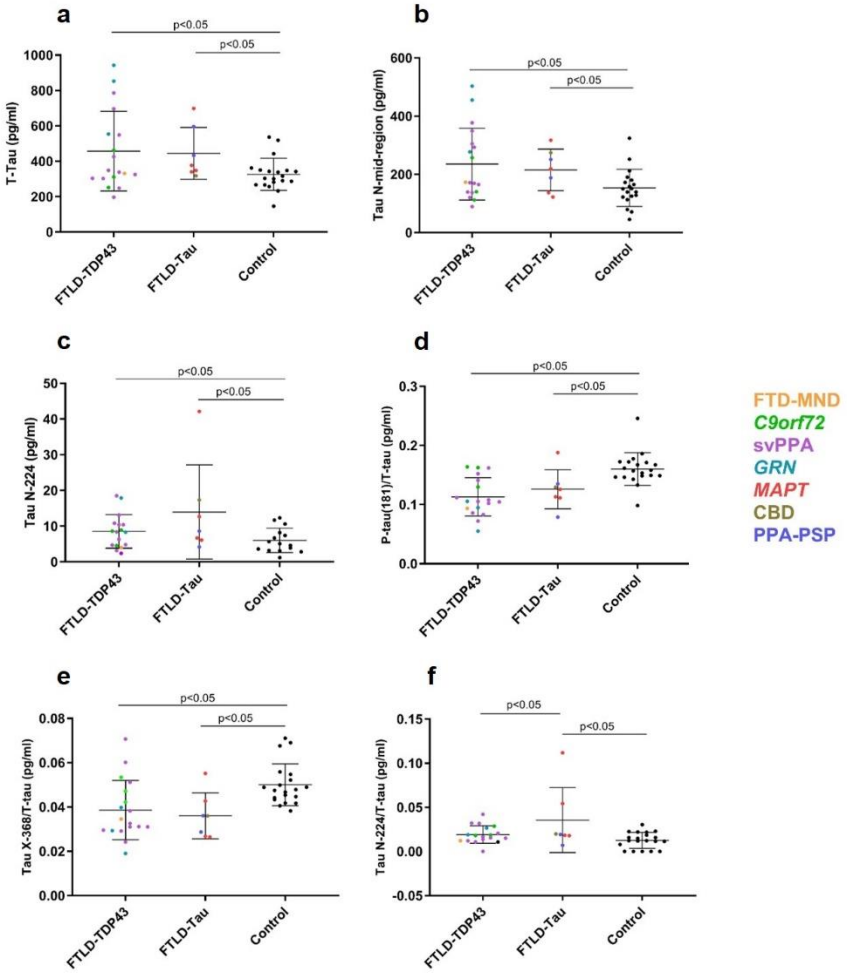

Figure 23. a, b, c) biomarkers with significant differences to controls in subjects with TDP-43 and tau pathology (a: t-tau; b: N-mid; c: N-224). d, e, f) biomarker ratios with significant differences to controls in subjects with TDP-43 and tau pathology (d: p-tau/t-tau; e: tau 368/t-tau; f: N-224/t-tau).

5.3.3 Discussion

Several tau measures (t-tau, p-tau, N-mid, N-224, non-phosphorylated tau) discriminated clinically diagnosed FTD subjects with underlying AD pathology from controls and, with the exception of p-tau, showed increased concentrations in FTD compared to controls. None of the measures showed a significant difference between the FTLD-TDP-43 and FTLD-tau groups, but, when normalised for t-tau, N-224 showed a significant difference between FTLD-tau and FTLD-TDP-43, although it separated the groups with sensitivity and specificity of only <65%. A similar sensitivity (61.1%) and higher specificity (85.7%) was found for the p-tau/t-tau ratio.

Although the novel measures do not have a superior diagnostic accuracy to the classic tau biomarkers, we observe different patterns in the concentrations of the fragments between the pathological groups, suggesting different profiles for each tauopathy. Identifying other fragments specific to FTD will require further studies.

5.4 Paper IV

The N-224 tau fragment showed potential clinical relevance in the differential diagnosis of tauopathies (Paper I), but the protease responsible for cleavage at aa 224 had not been identified at the time of the study. Hyperactivation of calpains (mainly calpain-1 and -2) has consistently been found in AD brains in previous studies (Kurbatskaya et al., 2016, Rao et al., 2014, Chesser et al., 2013, Gao et al., 2013, Chen et al., 2008, Vosler et al., 2008, Lewis et al., 2000, Grynspan et al., 1997, Yang and Ksiezak-Reding, 1995, Nixon et al., 1994). Other studies have also shown tau cleavage from calpain-1 and -2 in the sequences adjacent to aa 224 (Matsumoto et al., 2015, Ferreira and Bigio, 2011, Garg et al., 2011, Park and Ferreira, 2005). In this paper, our aim was to identify the enzyme responsible for cleavage of tau at aa 224, with a focus on calpain-1 and -2.

5.4.1 Calpain-1 vs. calpain-2

We first assessed the proteolytic activity of calpain-1, calpain-2 (from lysates of CAPN2-transfected cells) and brain protein extracts, combining them with a custom tau peptide (aa 220-228), engineered with FRET technology, as substrate. The substrate contained the aa 224 site and, if cleavage occurred, the proteolytic activity would be observed as progressive development of fluorescence, measured as relative fluorescence units (RFU). Brain protein extracts were obtained with two different protocols. In protocol 1, 100 mg of brain were extracted sequentially with TBS and sarkosyl detergent. In protocol 2, 200 mg of samples were extracted with TBS only. Samples from both protocols were then ultracentrifuged for 20 minutes at 200000 x g. In this experiment, only the soluble fraction of four out of six brain extracts, namely CTRL 1 (TBS), AD 2 (TBS), CTRL 2 (TBS) and CTRL 3 (TBS), and calpain-2 (crude and purified cell lysate) cleaved the substrate (Fig. 24a, b, c, d). Calpain-1 did not show any proteolytic activity (Fig. 24c, d).

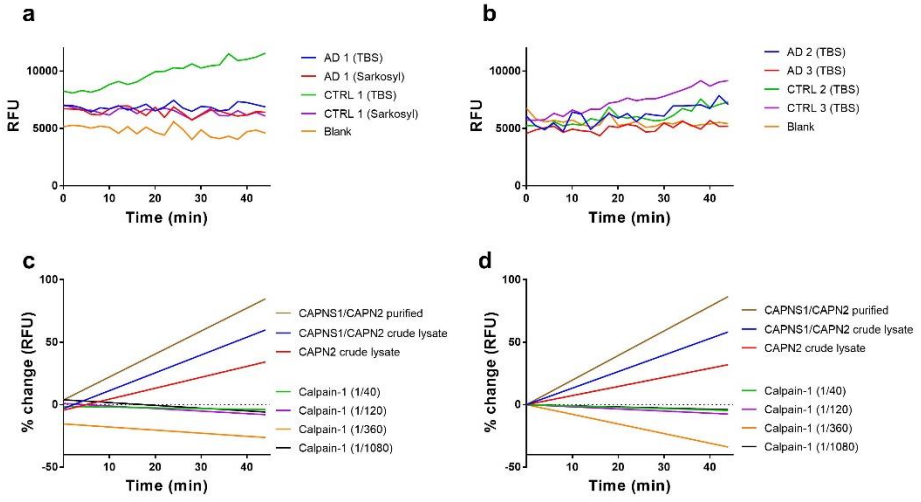

Figure 24. Development of fluorescence from cleavage of the FRET substrate over time. a) Brain fractions extracted with protocol 1. b) Brain fractions extracted with protocol 2. Y-axes show relative fluorescence units (RFU), x-axes show time in minutes. c, d) Linear (c) and non-linear (d) model of change (%) in RFU from baseline to endpoint of the measurement.

5.4.2 Native gel overlay with FRET substrate and MS analysis of proteolytically-active bands

Brain extracts showing cleavage of the substrate, namely CTRL 1 (TBS) and CTRL 3 (TBS), were run on a native 10% Tris-Glycine gel. The gel was then overlaid with a Tris buffer solution containing the FRET substrate (Fig. 25, lane 1). The gel bands showing fluorescence (corresponding to proteolytic activity) were cut out and trypsinated in-gel for subsequent MS analysis. Among several peptide hits, peptide identification revealed presence of the calpain-2 catalytic and regulatory subunit (CAPN2, CAPNS1, respectively), but no calpain-1 catalytic subunit (CAPN1). Calpain-2 depletion from brain extract and chemical inhibition resulted in loss of proteolytic activity (Fig. 25, lane 2 and 4). A calcium inhibitor (EDTA), interfering with the activation mechanism of the enzyme, did not suppress the activity, suggesting that it is not possible to stop the activation of the enzyme, once it has occurred, by depleting calcium (Fig. 25, lane 3).

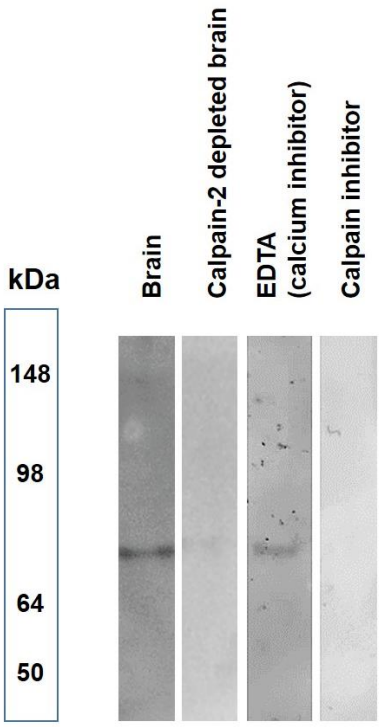

Figure 25. Brain extract on native gel overlaid with FRET substrate. Calpain-2-depleted brain extract (lane 2) and brain extract combined with calpain inhibitor (lane 4) show no cleavage of the FRET substrate. Calcium inhibitor EDTA (lane 3) does not affect the cleavage activity. Molecular weight markers are indicated to the left of the panel.

5.4.3 CAPN2 knock-down in a SH-SY5Y cell model and effects on N-224 tau in cell-conditioned media

To investigate how knock-down of calpain 2 affects the cellular processing of tau at aa N-224, calpain 2 was targeted using a siRNA against *CAPN2*. We then assessed the effects of the knock-down by measuring N-224 tau in SH-SY5Y cell-conditioned media. Calpain-2 protein was successfully knocked down in SH-SY5Y cells, as shown by a reduction in optical density (OD) of 93% compared to the control at western blot (Fig. 26a). Cell-conditioned media showed a reduction in the concentration of N-224 tau of 75% compared to the control, as measured by a neoepitope-specific immunoassay (Fig. 26b). To test for a possible compensation by *CAPN1*, we measured the gene expression of *CAPN1* and *CAPN2* in *CAPN2*-siRNA treated cells. After siRNA treatment, mRNA levels of *CAPN2* significantly decreased as compared to the scrambled control, while *CAPN1* expression remained stable.

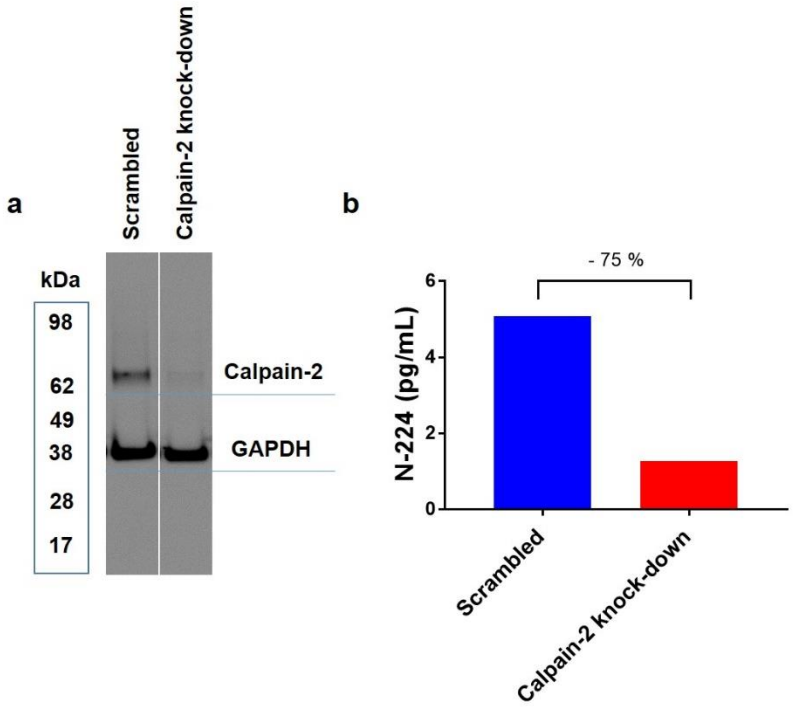

Figure 26. CAPN2 knock-down in SH-SY5Y cell model. a) Western blot on lysate from SH-SY5Y cells with anti-calpain-2 antibody: after knock-down, the band representing the CAPN2 product shows a decrease in OD of 93% (lane 2) compared to scrambled control (lane 1). Molecular weight markers are indicated to the left of the panel. b) N-224 tau concentrations in cell media from scrambled and calpain-2 knock-down cells: in cell-conditioned media from CAPN2 knock-down cells, N-224 concentrations show a decrease of 75% compared to scrambled control.

5.4.4 Discussion

We have shown that calpain-2 cleaves tau between aa 220 and aa 228 and *CAPN2* knock-down reduces significantly the amount of tau ending at aa 224 in cell-conditioned media. Since N-224 tau has shown potential as a biomarker for tauopathies, these findings encourage further investigation of the calpain-2 pathway for discovery of new targets in tauopathy treatment.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

With the studies included in thesis, we have confirmed that tau in CSF is composed by a series of fragments, as suggested previously (Sato et al., 2018, Meredith et al., 2013). Namely, we have identified new N-terminal fragments ending at aa 123 and aa 224, and developed targeted immunoassays against them, as well as against the tau 368 fragment generated by cleavage from AEP (Zhang et al., 2014). We have applied the immunoassays to clinical cohorts representing primary and secondary tauopathies, along with cohorts belonging to the clinical FTD spectra. N-224, in particular, appears as a promising biomarker for the characterization of tauopathies, being not correlated to t-tau in tauopathies other than AD (PSP, CBS). This led us to investigate the genesis of this fragment further, and to the identification of calpain-2 as the enzyme responsible for cleavage at this site. Tau 368 concentrations in CSF, interestingly, are inversely correlated to the uptake of a tau PET tracer ([^{18}F]GTP1) binding to pathological tau. These results are in line with previous findings of a lack of C-terminal tau fragments in CSF; also, this fits with the model that describes the core of tangles as composed by C-terminal tau (Sato et al., 2018, Fitzpatrick et al., 2017).

The main conclusions of this work, and the respective future perspectives, can be summarized as follows:

- *N-224 shows promise as a CSF biomarker for the characterization of tauopathies.*
  Although not superior to t-tau and p-tau in terms of diagnostic power in AD, N-224 tau is very low in primary tauopathies and its levels are unrelated to the ones of t-tau and p-tau. These findings still need to be replicated in an independent clinical cohort, but adding N-224 to the panel of AD biomarkers might help in the differential diagnosis of atypical AD and FTD cases. No specific biomarkers for primary tauopathies are available, and the gold standard for diagnosis of AD and primary tauopathies is still histopathology at post mortem; ideally, we would need longitudinal CSF samples collected during the lifetime of the subjects, and pathological confirmation at post mortem.

- *Tau 368 may be used in combination with PET in a multimodal approach to AD diagnosis.*
  In the case of [^{18}F]GTP1, the strong negative correlation of the uptake to tau 368 in CSF suggests that the tracer effectively binds to C-terminal/pathological tau structures. Since tau PET tracers are not yet used outside research settings, combining CSF with PET measures may help validate the efficacy of new tracers. The advantage of adding an antibody-based measure to an imaging one, is knowing the exact epitope on tau which is interested by the binding. This information is not available for tau PET tracers, since they bind to a 3D structure. Thus, immunoassays might function as an indirect internal control for the quality of the PET tracer.

- *The calpain-2 pathway should be further investigated in primary tauopathies and as therapeutic target.*
  We have identified calpain-2 as the enzyme responsible for cleavage at aa 224, in a neuroblastoma cell model (SH-SY5Y). We are currently performing nuclear magnetic resonance (NMR) experiment visualize the effects of calpain-2 on tau structure, and check if cleavage at aa 224 could promote misfolding and aggregation of C-terminal tau. Immunofluorescence studies with calpain-2 specific antibodies also show positive staining in 50 to 75% of the neuronal tau inclusion and in 10-25% of glial cells in PSP and CBD (Adamec et al., 2002). These findings, taken together with the role of N-224 tau as a biomarker in primary tauopathies, encourage the investigation of the calpain-2 pathway in PSP and CBD brain. One initial step could be to co-stain with anti-tau 224 and anti-calpain-2 antibodies, to check the proximity of the structures interested by the staining. Moreover, a calpain-1- and calpain-2-specific inhibitor drug has recently been tested in a phase 1 clinical trial in an AD cohort (Lon et al., 2019). Analysing N-224 tau in CSF before and after treatment and extending the trial to PSP and CBS subjects could shed light on the role of the calpain-2 pathway as a therapeutic target.

In conclusion, this thesis highlights the complexity of tau species in AD and primary tauopathies, and the need to characterize subjects affected by these diseases with different tau measures. Although the aim is always to find one gold standard biomarker, it is increasingly evident that the study of tau cannot be reduced to a single parameter. The N-terminal, secreted in CSF, and the C-terminal, reflecting *in vivo* pathology, are both fundamental in the study of tauopathies.

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