Novel cerebrospinal fluid and blood tau biomarkers in Alzheimer's disease and other neurodegenerative diseases

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A mis padres y a mi hermana

"We choose to go to the moon in this decade and do the other things, not because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, because that challenge is one that we are willing to accept, one we are unwilling to postpone, and one which we intend to win, and the others, too".

John F. Kennedy

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ABSTRACT

Tau is a soluble and natively unfolded protein predominately expressed in neurons within the central nervous system (CNS). The major roles of tau, which include microtubule (MT) dynamics and stabilization, are tightly regulated by a complex array of posttranslational modifications (PTMs). In pathological conditions, these regulatory mechanisms are disrupted, leading to tau detachment from MT and its subsequent aggregation. As a result, the MT architecture collapses, contributing to neuronal dysfunction and death. Tau pathology is a common finding in several neurodegenerative disorders, which are generally classified under the umbrella term of tauopathies, including Alzheimer's disease (AD). Two of the most prominent events associated with tau pathology are abnormal phosphorylation and truncation, which decrease tau ability to bind MTs. From a fluid biomarker perspective, these two events are important, given that the resulting tau species are secreted into cerebrospinal fluid (CSF) and blood (plasma and serum), where they can be quantified. Among the tau species present in CSF, two of them have been clinically validated as biomarkers for Alzheimer's disease; phosphorylated tau at threonine 181 (p-tau181) and total-tau ([t-tau] meaning assays hypothetically measuring any tau variant). Measuring tau species in blood represents an exciting clinical alternative to CSF, as they are cost-effective and widely accessible. These biomarkers show very high performance in identifying brain pathology; however, we hypothesize that the full biomarker potential, in terms of disease staging and mechanistic understanding of tau protein in biofluid was yet to be uncovered.

The aim of this thesis was to identify and quantify novel tau species, both in terms of fragments and phosphorylations, and evaluate their biomarker

potential in CSF and blood in the context of AD *continuum* and other neurodegenerative diseases. In blood, we developed and validated two novel assays capable of measuring p-tau181 and phosphorylated tau at threonine 231 (p-tau231), demonstrating that that both biomarkers are highly specific for AD across all clinical stages. In addition, we showed that plasma p-tau181 and p-tau231 display a sequential increase during preclinical AD, where p-tau231 increases prior to overt amyloid plaque deposition in the brain. Furthermore, we demonstrated their strong association with gold standard definitive diagnosis of AD at post-mortem, ultimately proving that both biomarkers are clearly reflective of AD. We further extended these findings for plasma p-tau181 by demonstrating that even a decade before post-mortem, this biomarker is highly specific to AD pathology. We also showed that patients diagnosed with AD dementia based on clinical criteria with low levels of plasma p-tau181 are likely to have non-AD pathology.

Using a combination of exploratory and targeted mass spectrometry methods in soluble brain tissue extracts, from pathologically confirmed AD and control cases, we were able to identify phosphorylated tau at threonine 235 (p-tau235) as a highly specific phosphorylation in AD. Moreover, our results indicated the presence of sequential phosphorylation events starting with phosphorylation at threonine 231 and followed by serine 235. To demonstrate the biomarker potential of p-tau235, we developed an assay for its quantification in CSF. We found that p-tau235 has potential as a clinical biomarker for AD, and importantly, that in combination with p-tau231 it can successfully stage asymptomatic AD cases. Finally, we investigated the potential biomarker value of different non-phosphorylated (t-tau) assays targeting tau fragments of various lengths in AD and other neurodegenerative disorders and compared their performance with other novel p-tau species and clinically validated p-tau181 and t-tau assays in both CSF and blood. Our findings suggest that targeting N-terminal tau species provides an advantage in terms of early identification of AD, and most notably, allows the detection of AD pathology in blood.

In conclusion, the work included in this thesis has helped to achieve a long-sought goal in the field of fluid biomarkers: measuring multiple p-tau and t-tau species in blood, which now are being evaluated for clinical utility. This is expected to allow a worldwide implementation of fluid biomarkers, providing cost-effective and simple tools that can facilitate the diagnostic work-up in

primary care and memory clinics. In addition, the biomarkers presented here may serve as valuable tools in clinical trials, finding the best window of opportunity for novel compounds and helping to evaluate their effectiveness tackling disease.

Keywords: tau, phosphorylation, truncation, Alzheimer's disease, CSF, blood.

Sammanfattning på svenska

Tau är ett lösligt och naturligt oveckat protein som huvudsakligen uttrycks i neuroner i det centrala nervsystemet (CNS). Dess viktigaste funktioner inkluderar mikrotubuli (MT)-dynamik och -stabilisering. Dessa funktioner är reglerade via en komplex uppsättning posttranslationella modifieringar (PTM). I patologiska tillstånd är dessa reglermekanismer störda vilket kan leda till att tau frigörs från MT och bildar aggregat. Detta leder i sin tur till att MT-strukturen kollapsar med efterföljande neuronal funktionsstörning och nervcellsdöd. Tau-patologi är vanligt vid flera neurodegenerativa sjukdomar, vilka vanligtvis sorteras in under den övergripande beteckningen tauopatier. Alzheimers sjukdom (AD) tillhör denna grupp. Två betydande faktorer som utlöser tau-patologi är abnorm fosforylering och trunkering, som båda minskar taus förmåga att binda till MT. Ur ett biomarkörperspektiv är dessa faktorer essentiella eftersom de resulterande tau-formerna läcker ut till både likvor och blod, där de kan kvantifieras. Bland de tau-former som finns i likvor har två validerats kliniskt som biomarkörer för AD; tau fosforylerat på treonin-181 (p-tau181) och total-tau. Att mäta tau-former i blodprov är ett spännande kliniskt alternativ till likvor eftersom blodprov är mer allmänt tillgängliga och provtagningen billigare. Dessa biomarkörer är väldigt träffsäkra när det gäller att påvisa hjärnförändringar, men det återstår mycket arbete innan den fulla potentialen för tau-biomarkörer i kroppsvätskor är ordentligt utredd, till exempel vad beträffar indelning i sjukdomsstadier och förståelse av de patologiska mekanismerna som biomarkörerna återspeglar.

Syftet med detta arbete var att identifiera och kvantifiera nya tau-former – både fragment och fosforyleringar – samt utvärdera deras potential som likvor- och blodbiomarkörer för att studera utvecklingen av AD och andra neurodegenerativa sjukdomar. För blod utvecklade och validerade vi två nya analyser för att mäta p-tau181 och tau fosforylerat på treonin 231 (p-tau231). Analyserna visade att båda biomarkörerna har hög diagnostisk specificitet för AD i alla kliniska stadier. Dessutom visade vi också att plasma p-tau181 och p-tau231 ökade gradvis i den prekliniska fasen av AD. p-tau231 ökar till och med innan tydlig amyloid plack-patologi kan observeras i hjärnan. Dessutom påvisade vi en stark koppling till den definitiva diagnosen av AD vid obduktion, vilket betyder att båda biomarkörerna reflekterar neuropatologi vid AD. Vi

kunde dessutom demonstrera att plasma p-tau181 är mycket specifik för AD-patologi och att biomarkören ändrar sig redan ett decennium före obduktionen. Därutöver kunde vi också visa att patienter med AD-liknande demens enligt kliniska kriterier men normala plasma p-tau181-nivåer inte har AD-patologi i hjärnan.

Genom att använda en kombination av explorativa och riktade masspektrometrimetoder för analys av den lösliga komponenten av hjärnvävnadsextrakt – från patologiskt bekräftade AD- och kontrollfall – kunde vi identifiera tau fosforylerat på treonin 235 (p-tau235) som en mycket specifik fosforylering i AD. Dessutom indikerade resultaten att fosforyleringen sker sekventiellt, först fosforyleras treonin 231 och därefter serin 235. För att visa biomarkörpotentialen hos p-tau235 utvecklade vi en kvantitativ analysmetod för likvor. Vi fann att p-tau235 har potential som klinisk biomarkör för AD, men också att man framgångsrikt kan kombinera den med p-tau231 för att skilja ut asymtomatiska AD-fall. Slutligen undersökte vi det potentiella biomarkörvärdet av olika t-tau-analyser riktade mot tau-fragment av olika längd både i AD och andra neurodegenerativa tillstånd. Prestandan hos dessa tau-fragment jämfördes med andra nya p-tau-former samt de kliniskt validerade p-tau181- och t-tau-analyserna i både likvor och blod. Fynden tyder på att N-terminala tau-former är bättre biomarkörer när det gäller tidig identifiering av AD och de är en förutsättning för att påvisa AD-patologi i blod.

Sammanfattningsvis har arbetena som ingår i denna avhandling bidragit till att uppnå ett länge eftersökt mål inom området för biomarkörer i kroppsvätskor: mätning av flera p-tau- och t-tau-former i blod. Analysmetoderna utvärderas nu för potentiell tillämpning i kliniken. De bör kunna implementeras världen över och vara kostnadseffektiva och förhållandevis enkla verktyg som underlättar den diagnostiska utvärderingen både i primärvården och på minneskliniker. Slutligen borde dessa biomarkörer vara värdefulla verktyg i kliniska prövningar, till exempel för att bestämma den optimala tidpunkten för att sätta in nya behandlingar och för att bidra till utvärderingen av deras effektivitet för att bekämpa sjukdom.

Nyckelord: tau, fosforylering, trunkering, Alzheimers sjukdom, likvor, blod

LIST OF PAPERS

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

I. Thomas K. Karikari, Tharick A. Pascoal, Nicholas J. Ashton, Shorena Janelidze, Andréa Lessa Benedet, **Juan Lantero-Rodriguez**, Mira Chamoun, Melissa Savard , Min Su Kang, Joseph Therriault, Michael Schöll, Gassan Massarweh, Jean-Paul Soucy, Kina Höglund, Gunnar Brinkmalm, Niklas Mattsson, Sebastian Palmqvist, Serge Gauthier, Erik Stomrud, Henrik Zetterberg, Oskar Hansson, Pedro Rosa-Neto, Kaj Blennow. "*Blood phosphorylated tau 181 as a biomarker for Alzheimer's disease: a diagnostic performance and prediction modelling study using data from four prospective cohorts*". The Lancet Neurology. 2020. 19(5):422-433.

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ABBREVIATIONS

A-	Amyloid pathology-negative
A+	Amyloid pathology-positive
Aβ	Amyloid-β
$A\beta_{1-40}$	Amyloid-β (1-40)
$A\beta_{1-42}$	Amyloid-β (1-42)
$A\beta_{1-42/40}$	Amyloid-β ratio (1-42/1-40)
AD	Alzheimer's disease
AND	Acute neurological condition
APOE	Apolipoprotein E (gene)
ApoE	Apolipoprotein E (protein)
APP	Amyloid precursor protein
Asp	Aspartic acid
AUC	Area under the curve
BSA	Bovine serum albumin
bvFTD	Behavioural variant frontotemporal dementia
CAA	Cerebral amyloid angiopathy
CBD	Corticobasal degeneration
CBS	Corticobasal syndrome
Cdk5	Cyclin-dependent kinase 5
CID	Collision-induced dissociation
CJD	Creutzfeldt-Jakob's disease
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
CTE	Chronic traumatic encephalopathy
CU	Cognitively unimpaired
CI	Cognitively impaired
CV	Coefficient of variation
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EEG	Electroecenphalography
ELISA	Enzyme-linked immunosorbent assay

ESI	Electrospray ionization
fAD	Familial Alzheimer's disease
fCJD	Familial Creutzfeldt-Jakob's disease
FDG	Fluorodeoxyglucose
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobar degeneration
GFAP	Glial fibrillary acidic protein
Glu	Glutamic acid
GSK-3β	Glycogen synthase kinase 3β
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IP	Immunoprecipitation
Kd	Dissociation constant
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LOD	Lower limit of detection
m/z	Mass-to-charge ratio
MAP	Microtubule-associated protein
MAPT	Microtubule-associated protein tau
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MSA	Multiple system atrophy
MT	Microtubule
MTBR	Microtubule-binding region
NfL	Neurofilament light
NFT	Neurofibrillary tangle
PD	Parkinson's disease
PET	Positron emission tomography
PHF	Paired helical filaments
PiD	Pick's disease
PPA	Primary progressive aphasia
PrPc	Cellular prion protein
PrPsc	Scrapie prion protein
PRR	Proline-rich region
PSEN	Presenilin

PSP	Progressive supranuclear palsy
P-tau	Phosphorylated tau
P-tau181	Phosphorylated tau at threonine 181
P-tau199	Phosphorylated tau at serine 199
P-tau202	Phosphorylated tau at serine 202
P-tau205	Phosphorylated tau at threonine 205
P-tau212	Phosphorylated tau at threonine 212
P-tau214	Phosphorylated tau at serine 214
P-tau217	Phosphorylated tau at threonine 217
P-tau231	Phosphorylated tau at threonine 231
P-tau235	Phosphorylated tau at serine 235
P-tau396	Phosphorylated tau at serine 396
P-tau404	Phosphorylated tau at serine 404
PTM	Posttranslational modification
Q	Quartile
RGP	Resorufin-D-galactopyranoside
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RP	Reverse phase
r_S	Spearman's rank correlation
S or Ser	Serine
sAD	Sporadic AD
SBG	Streptavidin β-galactosidase
sCJD	Sporadic Creutzfeldt-Jakob's disease
SD	Standard deviation
SF	Straight filament
Simoa	Single molecule array
SUVR	Standardized uptake value
T or Thr	Threonine
T-	Tau pathology-negative
T+	Tau pathology-positive
TBI	Traumatic brain injury
TBS	Tris-buffered saline
TDP43	TAR DNA-binding protein 43
T-tau	Total-tau
Tyr	Tyrosine

VaD	Vascular dementia
vCJD	Variant Creutzfeldt-Jakob's disease

1. INTRODUCTION

1.1 TAU PROTEIN

1.1.1 Isoforms and structure

Tau protein was originally identified in 1975 by Weingarten and colleagues when examining porcine brains [1]. In humans, tau protein is encoded on chromosome 17 by one gene located at locus 17q21.31 [2-4]. Comprised by 16 exons, the tau gene, called Microtubule-Associated Protein Tau (*MAPT*), undergoes alternative mRNA splicing, which results in the expression of different isoforms. In the central nervous system (CNS), alternative splicing of exons 2, 3 and 10 can lead to the formation of six isoforms of the protein [5]. Exons 2 and 3 determine the presence or absence of two N-terminal amino acid sequences (0N, 1N or 2N), whereas exon 10 is responsible for the number of microtubule binding repeats on the C-terminal side (3R when exon 10 is absent or 4R when exon 10 is present) (*Fig. 1*). 3R and 4R isoforms are expressed in seemingly identical amounts in adult brain [6-8]. Interestingly, 3R isoforms have lower affinity for microtubules (MT) than 4R counterparts [6], which may provide the necessary flexibility to allow neuronal plasticity. In contrast, 1N is the most prevalent isoform accounting for approximately half of total tau (54%), followed by 0N (37%) and 2N (9%) [6-8]. It has been suggested that the N-terminal inserts are related to the cellular distribution of the isoform [9]. CNS specific tau isoforms are mainly expressed in neurons, predominantly in axons [10] but also to a lesser extent in other locations such as the soma and the dendrites [11-13]. CNS specific tau has been also found in glial cells [14] and extracellularly in the interstitial fluid [15]. In the peripheral nervous system, splicing of two other exons, 4A and 6, leads to the generation of a high molecular weight isoform, commonly referred to as “big tau” [16, 17].

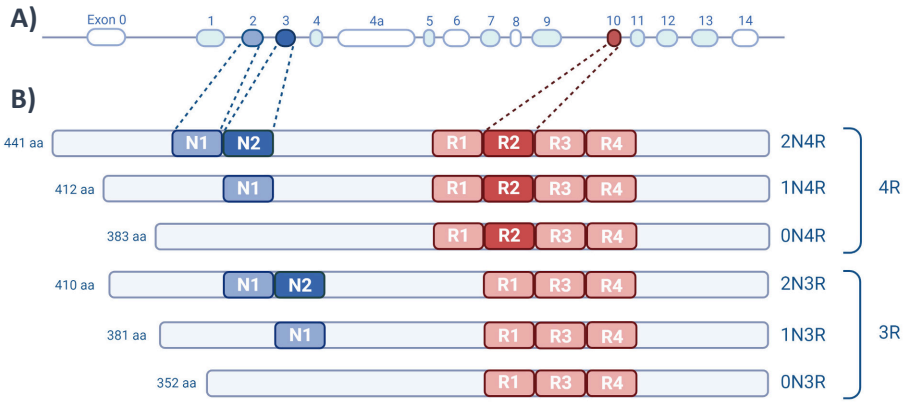


Figure 1. *Tau protein gene and six CNS specific tau isoforms.* ***A)*** *Tau gene is comprised by 16 exons. In CNS tau, exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutively expressed.* ***B)*** *Alternative splicing of exons 2, 3 and 10 result in the six CNS specific tau isoforms: 2N4R, 1N4R, 0N4R, 2N3R, 1N3R and 0N3R. Created with BioRender.com.*

Tau is widely described as natively unfolded and highly soluble protein [18, 19], thus not prone to aggregation. Despite this, tau seems to have a tendency to display a hairpin-like conformation, in which the N-terminal and C-terminal fold towards the core of the protein [20]. Based on the association or not with the MT, tau sequence is classically subdivided into two different domains: N-terminal projection domain (1-198aa, 2N4R numbering) and MT-assembly domain (199-441aa). Another common way to subdivide tau sequence relies on function and amino acid composition: N-terminal region (1-150aa), proline rich region or PRR (151-245aa), microtubule binding region or MTBR (245-368aa) and C-terminal region (369-441aa) [21] (*Fig. 2*). The N-terminal region is predominantly negatively charged, which likely induces its projection out of the MT [22]. This mechanism potentially helps keeping MTs separated from each other [23-27]. In addition, this protruding nature makes this domain an ideal “hook” that allows tau to interact with membrane components [28-30]. The PRR binds entirely to the MTs, and it has been found to play critical roles in cell signalling, due to its ability to interact with multiple protein targets [31-40]. In fact, its name comes from the presence of seven PXXP motifs, which allows tau to interact with signalling proteins from the SRC family [34]. Consisting of three or four highly conserved repetitive domains, MTBR is critical for MT binding and its dynamics [41-44]. Additionally, it can bind to actin [45], several phosphatases [46], DNA [47] and RNA [48], among others.

Is important to note the presence of two hexapeptides within the MTBR (^{275}VQJINK^{280} and ^{306}VQJVYK^{311}), which are suggested to be responsible of the formation of β-sheets and subsequent aggregation of tau under pathological conditions [49, 50]. Finally, the C-terminal region, appears crucial for MT polymerization and regulates membrane interactions [31, 39, 51, 52].

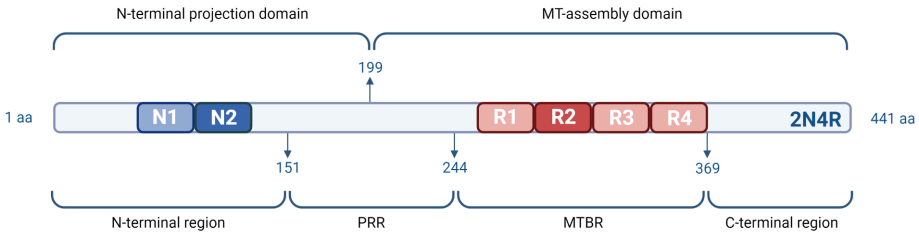


Figure 2. *Tau protein structure and functional domains (2N4R numbering). Created with BioRender.com.*

1.1.2 Function

Tau protein is found primarily in axons bound to tubulin (MT building blocks), where it performs its classic functions: stabilization and regulation of polymerization rates of MTs, in addition to spacing them from one another [1, 27, 53-55] (*Fig. 3*). Thus, tau protein exerts crucial roles helping MTs to act as a mechanical scaffold for the neurons. Furthermore, the dynamic nature of this interaction allows critical cellular processes such as axonal growth, cargo transport, and neuronal plasticity, among others [56-60]. Tau protein can bind to both polymerized and unpolymerized tubulin with equal avidity [42, 61]. The interaction between both proteins takes place at the C-terminal end of tubulin [62, 63] and it is regulated by the phosphorylation status of tau [62, 64]. This regulatory mechanism is highly relevant, as abnormal phosphorylation can lead to the neutralization of the positive charges in tau and the subsequent detachment from the MT [62, 65] (*Fig. 3*). Additionally, tau prevents MT from bundling with each other through the N-terminal projection domain (which is negatively charged), with isoforms 1N and 2N being particularly efficient in this regard [66].

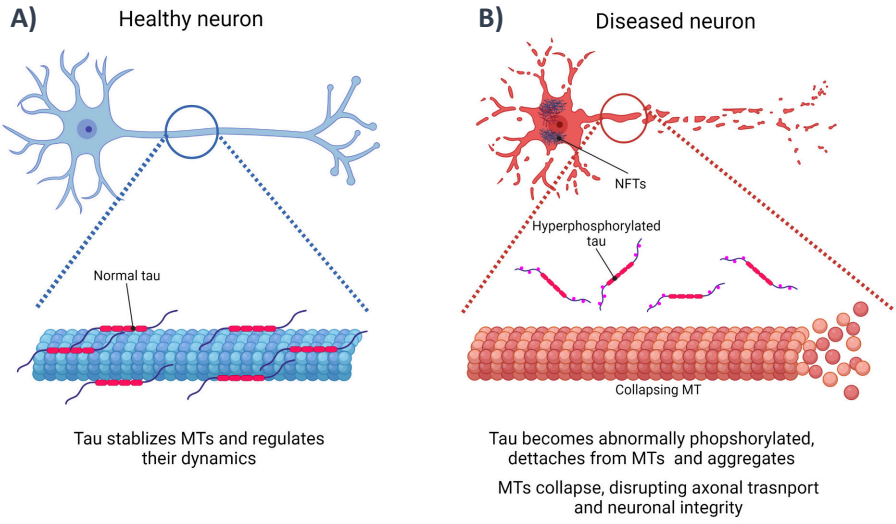


Figure 3. *Tau protein in healthy and diseased neurons.* ***A)*** *In healthy neurons, tau protein is found predominantly attached to the MTs, stabilizing their structure, and regulating their polymerization and depolymerization dynamics.* ***B)*** *In pathological scenarios, tau protein becomes abnormally phosphorylated, detaching from the MTs and start forming aggregates (e.g., NFTs). Consequently, MTs structure and dynamics collapse, which hampers axonal transport and ultimately in the loss of neuronal integrity. Created with BioRender.com.*

It has also been suggested that tau can regulate axonal transport. For neurons to be functional, organelles, lipids and proteins have to be efficiently transport from the cell body into axons and dendrites [67]. In this task, MT act like molecular highways, allowing the cargo transport in both directions. Anterograde and retrograde transport through the MT is carried out by kinesin and dynein respectively [68, 69]. A study published in 2008 explored the differential tau regulatory mechanisms of motor proteins [59], although these results would require *in vivo* validation. One interesting question is why MT-bound tau does not block axonal cargo transport when in fact, under physiological conditions, tau is mostly bound to MTs [70] (which seems to contradict the flexible dynamics of tau-tubulin partnership). An explanation for this has been recently proposed by the so-called kiss-and-hop mechanism, which describes how tau interacts with MTs very shortly (40 milliseconds) to

subsequently jump into a new location [71]. Hence, tau can be almost entirely bound to MTs without hampering axonal transport.

In addition to these classic functions, tau protein has been shown to interact with the actin cytoskeleton [45, 72] and proteins from the plasma membrane [73]. Futhermore, tau has been reported in other locations under physiological conditions, implying further cellular roles. Studies have shown tau presence at pre- and post-synaptic locations [74], where it might play important roles in synaptic plasticity. Tau has also been described in the nucleus, where it has been suggested to play critical roles in maintaining the integrity of both DNA and RNA [12, 13, 75-77]. It is important to highlight that tau is not only found in neurons, but also in oligodendrocytes [14, 78] and astrocytes [79].

1.1.3 Posttranslational modifications

Tau protein functions are tightly regulated by a large variety of posttranslational modifications (PTMs), many of which appear to be dysregulated in disease. Described PTMs affecting tau include glycosylation [80, 81], glycation [82-84], prolyl-isomerization [85, 86], ubiquitination [87, 88], polyamination [89, 90], sumoylation [91, 92] , nitration [93, 94], oxidation [95, 96], acetylation [97, 98], methylation [99, 100], truncation [101, 102] and phosphorylation [17, 103], the latter two gathering the most attention [104-112].

1.1.3.1 Phosphorylation

Phosphorylation is defined in chemistry as the addition of a phosphoryl group, which in proteins can occur at three specific amino acids: threonine (Thr), serine (Ser) and tyrosine (Tyr). 2N4R tau contains 85 potential phosphorylation sites (45 Ser, 35 Thr and 5 Tyr), and among all PTMs, phosphorylation is the most studied [17, 113, 114]. Under physiological conditions, phosphorylation and dephosphorylation tightly regulates the ability of tau to bind MT, being therefore directly implicated in the MT dynamics and stability. There are three main classes of kinases that can

phosphorylate tau: tyrosine kinases (*e.g.*, Fyn), proline-directed kinases (*e.g.*, GSK-3β and Cdk5) and non-proline-directed kinases (*e.g*., DYRK1A and tau tubulin kinase 1 TTBK1) [103]. Similarly, tau can be dephosphorylated by various phosphatases, such as PP2A [115]. Tau phosphorylation has also been associated with the regulation of critical processes such as axonal transport [116], cellular location [117], plasma membrane [51, 118] neurotransmitter receptor binding [119] and cargo delivery [120], among others. Moreover, phosphorylation levels of tau have been described as increased during development [121-123], which might confer MTs the flexibility required for synaptogenesis and synaptic pruning. There are two main reasons which explain why phosphorylation is the most studied PTM. First, aberrant phosphorylation reduces its ability to bind MT. Second, immunohistochemical findings in several neurodegenerative diseases show immunoreactivity in the form of tau inclusions and/or tangles when staining with antibodies targeted against phosphorylated tau (p-tau) residues.

Under pathological conditions, increased overall levels of tau phosphorylation have been observed. When tau becomes abnormally phosphorylated, its ability to bind MTs decreases. This increase in the phosphorylation state of tau is not a defining feature of normal adult brain, and is referred to as hyperphosphorylation [124]. Subsequently, tau detaches from MTs, destabilizing them and leading to their disassembly [110, 125-127]. The collapse of MTs has devastating consequences for the neurons, as all the functions carried out by them become hampered (*Fig 3*). Furthermore, detached hyperphosphorylated species have been shown to mislocate and hijack normal tau and other MAP [128]. Altogether, these events have major repercussions such as impairment of axonal transport, collapse of neuronal integrity and neurotoxicity [126, 129-132]. Increased tau phosphorylation levels represent a signature of neurodegeneration in Alzheimer's disease (AD) and other tauopathies and has been demonstrated to be critical in the formation of pathological aggregates. In AD, tau aberrant hyperphosphorylation occurs prior to aggregation and neurofibrillary tangle (NFT) formation [88, 133]. In late stages, tau within NFTs appears extensively phosphorylated at several threonine and serine residues. Tau phosphorylation levels, like other proteins, are tightly regulated by a battery of phosphatases and kinases, and it is believed that the equilibrium of this system might be hampered under pathological conditions, leading to an imbalance in phosphorylation mechanisms. Hence, effort has been placed on

discovering which enzymes might be down or upregulated in disease [39, 134-136], since this could allow the generation of new therapeutic compounds.

1.1.3.2 Truncation

Under pathological conditions, proteolytic cleavage of tau occurs primarily at both N-terminal and C-terminal ends [137]. As a result, several tau fragments can be found in human brains with underlying tauopathies [138, 139]. Calpains [140], thrombin [141], cathepsins [142], caspases [143] and puromycin-sensitive aminopeptidase (PSA) [144] are some examples of a long list of enzymes capable of cleaving tau [145]. It is well stablished that truncation increments tau ability to form aggregates *in vitro*, by forming seeds and subsequently growing into large fibrils [146-149]. As described previously for enzymes regulating phosphorylation, large efforts have been placed on finding which proteolytic enzymes might be altered in disease scenarios in order to tackle their pathological activities [145, 150-152]. Moreover, tau truncation not only results in a loss of function. The resulting proteolytic fragments have been reported to be capable of inducing neurodegeneration by hampering critical cellular mechanisms (MT dynamics or axonal transport) [153], by having deleterious effects on the synapses [154-156] and by propagating into neighbouring cells [145].

In the last two decades much attention has focused on the proteolytic cleavage of tau protein C-terminus, linking this processing with AD pathogenesis [157-161]. Some early studies have attempted to characterize the main component of paired helical filaments (PHF) by isolating tau assemblies from AD brains [159-161]. When PHFs are extracted and enzymatically digested, a small tau peptide of approximately 12 kDa starting around histidine at position 268 and ending at glutamic acid 391 (Glu391) was found [159-162]. Thus, this fragment has been called the PHF core. An antibody referred to as MN423 can specifically recognise this truncation, which has been observed in NFTs in AD [162]. Moreover, these truncated species have been found to progress in accordance with Braak staging and positively correlate with the severity of dementia [157, 158, 163, 164]. Remarkably, *in vitro* experiments have demonstrated that tau species truncated at this amino acid can polymerize at faster rates than full-length tau [146]. Another interesting C-terminal cleavage that has also gathered

great attention is aspartic acid 421 (Asp421). Immunohistochemistry (IHC) with antibodies specific for truncation at Asp421 has shown that this cleavage is associated with neurofibrillary pathology in AD brains [147]. In a similar way as cleavage Glu391, *in vitro* studies have shown that tau truncated at Asp421 polymerizes at faster rates than the full-length counterpart [147, 148]. Overall, tau truncation at Glu391 and Asp421 have been reported to enhance the ability of tau to aggregate and induce neuronal apoptosis [165-167]. In addition, tau truncated at these residues have been found in AD brain [101, 102, 168] and particularly enriched in insoluble AD brain fractions, further indicating that both truncation are relevant in pathophysiological events [148, 164, 169]. Furthermore, they have been shown to correlate with AD progression [101]. Several studies have confirmed that tau hyperphosphorylation occurs prior to truncation [170, 171], and that these two C-terminal cleavages precede NFT formation [172].

N-terminal truncation is also critical for aggregation and some of the resulting fragments have been shown be related to pathology. For example, a fragment called NH2-tau (26-230aa, 2N4R numbering) has been reported in a mouse model which exhibits an AD-like neurodegeneration with amyloid accumulation [173]. Another relevant example is a tau fragment with sequence 187-441aa identified in 4R tauopathies [174]. Interestingly, a rat model expressing tau with sequence 151-391aa with 3R or 4R have been shown to display neuropathological features similar to those found in human tauopathies [149, 175, 176]. From a biomarker standpoint, N-terminal truncation and the resulting proteolytic N-terminal tau peptides are highly interesting. [177]. These N-terminal proteolytic products, which are soluble and large enough to enable immunoassay development, leak into cerebrospinal fluid (CSF) and blood, thus becoming the focus of tau biomarkers field in the last three decades [178, 179].

1.1.4 Tau oligomers and aggregation

Many neurodegenerative diseases are characterized by tau protein detachment, misfolding and aggregation [180]. Tau aggregation differs among neurodegenerative diseases, leading to a large variety of histopathological tau inclusions depending on the pathological scenario

(comprehensive description of such inclusions can be found in Murray et al., 2014 [181]). The first description of tau aggregates was reported in 1906 when the Bavarian psychiatrist Alois Alzheimer performed a silver staining on brain tissue from his patient, Auguste Deter, who exhibited symptoms during life which we now attribute to AD dementia [182]. At that time, Alzheimer did not know that those inclusions, NFTs, consisted of tau protein. Nowadays, we know that NFTs are composed by highly structured fibrillary accumulations of hyperphosphorylated and at least partially truncated tau proteins in the form of PHFs and straight filaments (SF) [183-186]. NFT formation has become the paradigm of tau aggregation due to a couple of reasons: NFTs are an AD hallmark (together with amyloid (Aβ) plaques) [187] and AD is the major cause of dementia worldwide (60-80% of the cases)[188]. However, and as previously mentioned, tau protein can aggregate into a wide variety of different inclusions.

Tau aggregation seems to occur in a stepwise manner starting with tau detachment from MTs. It is widely believed that the aberrant loss of binding function is due to a phosphorylation dysregulation, which leads to tau hyperphosphorylation [124]. Hyperphosphorylation and MT detachment is then followed by conformational changes [170] resulting in abnormal levels of unbound hyperphosphorylated misfolded tau. Subsequently, this increases cytosolic free tau, which is now prone to aggregation. These free pathological monomers will then bind into dimers, which will grow further by addition of more misfolded and hyperphosphorylated monomers. *In vitro* studies have shown that approximately four to seven dimers can form a nuclei, which acts as a seeding core that enters a rapid elongation phase leading to large filaments [189-191]. As mentioned in the previous section, truncation also increases the ability of tau to polymerize and indeed, NFTs and other tau assemblies found in brain are immunoreactive to truncation-specific antibodies [101, 147, 162, 168]. The exact timing of truncation, *e.g.*, prior to or after aggregation, is still debated. In brain tissue, tau ability to form seeding cores capable of sequestering physiological tau has led many researchers to propose a seeding concept similar to the one described in prion diseases, in which a pathologically misfolded protein acts as a template for other normally folded proteins of the same type to misfold and spread [192-196]. In fact, recent publications support this prion-like behaviour of tau [195, 197-205]

There is a strong relationship between NFTs and neurodegeneration. This is exemplified by a vast number of publications in human brain showing a high temporal and spatial correlation between NFTs and neurodegeneration [206-211]. Despite this, several studies have shown that NFTs might not represent a key toxic entity *per se* under pathological conditions. Neurons bearing NFTs have been found to be functional *in vivo* [212] and moreover, cognitively healthy individuals might present with NFTs [213]. Studies with animal models of tauopathies have shown that NFTs did not affect synaptic function and long-term synaptic plasticity [214]. Other studies in animal models have demonstrated that synaptic dysfunction occurs without NFTs [215-219]. Furthermore, studies have described neuronal loss without existence of NFTs [220]. Additionally, in a mouse model overexpressing tau, tau suppression decreased neuronal loss and memory function was recovered, while NFT formation persisted [221]. Altogether, these studies suggest that cell death may not be fully attributed to NFTs.

On the other hand, intermediate tau species called tau oligomers which precede aggregates and densely packed inclusions, might be responsible for both neurotoxicity and spreading of pathology [216, 218, 222-225]. It is well established that tau hyperphosphorylation promotes its detachment from MTs, and that these tau species gain aggregative capacities building up small oligomers which are detergent soluble. Adding to this hypothesis, studies have reported that oligomers can induce neuronal damage [226-228] and synaptic loss [203, 216, 218]. Some studies have noted that exposing mice brain to tau oligomers lead to cognitive abnormalities and synaptic dysfunction, whereas monomers and fibrils are rather innocuous [229, 230]. Moreover, tau oligomers can induce detachment of normal tau from MTs, altering their native structure. These species can then propagate to non-affected brain regions [200, 231, 232] in a spreading prion-like cycle [222, 229]. However, it is unknown how this prion-like propagation takes place, and most of all, if it indeed occurs. All these compelling evidences have led some scientists to suggest that NFT formation might represent a protective mechanism to prevent neurotoxicity induced by small tau aberrant species such as oligomers [233]. Eventually cellular functions become compromised by the aggregates, leading to cell death and neurodegeneration [233].

1.2 TAU PROTEIN IN PATHOLOGY

Originally, it was thought that neurofibrillary pathology was a typical feature of AD, however, it soon became evident that tau-positive immunohistochemical findings were present in other neurodegenerative disorders. During the 1980s, tau was reported as the main protein component of NFTs in AD brains, and few years later, Pollock and colleagues confirmed the presence of tau-positive accumulations in AD, progressive supranuclear palsy (PSP) and Pick's disease (PiD) [234]. In the late 1990s, Spillantini used for the first time the term tauopathy when describing a pathological entity which she referred as "*familial multiple system tauopathy with presenile dementia*" [235]. In a short time, the term tauopathies become commonly used when referring to both familial and sporadic neurodegenerative disorders characterized by the presence of filamentous aggregations of hyperphosphorylated tau in neurons and/or glial cells [236, 237]. Currently, more than 20 neurodegenerative disorders are comprised under the umbrella term of tauopathies, and they are traditionally classified based on the contribution of tau protein to the overall pathology. Thus, primary tauopathies (*e.g.*, PSP, corticobasal degeneration (CBD) or PiD) incorporate neurodegenerative disorders in which tau protein plays a key role, whereas secondary tauopathies encompass disorders where tau pathology is associated with other proteinopathies (*e.g.*, AD, tramautic brain injury (TBI), chronic traumatic encephalopathy (CTE) or Creutzfeldt-Jakob's disease (CJD)) [153, 238, 239] or occur downstream of the key pathology, *e.g.*, in Niemann-Pick type C, which is due to a defect in intracellular cholesterol transport (*Fig. 4*).

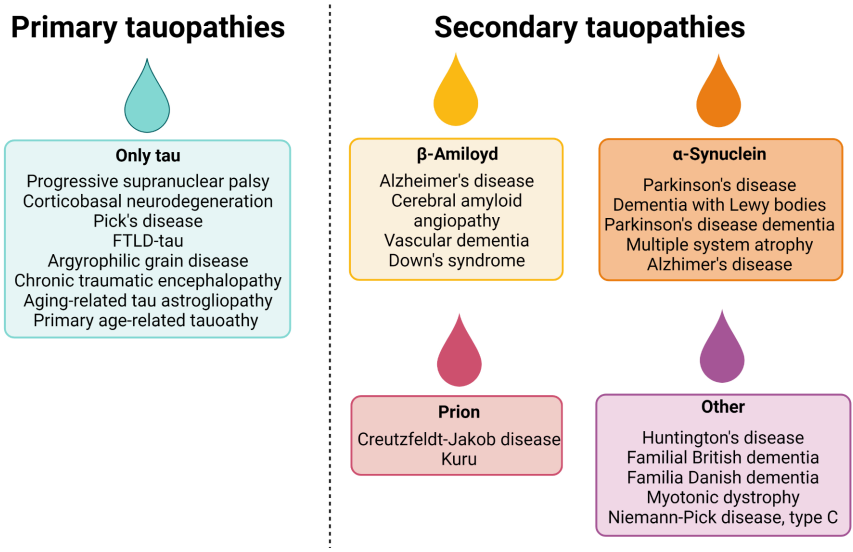


Figure 4. *Classification of some of the most prominent primary and secondary tauopathies. Created with BioRender.com.*

1.2.1 Alzheimer's disease

At the beginning of 1900s, Bielschowsky developed a new technique that allowed the silver staining of neurofibrils [240, 241]. Using this method, he reported for the first time neurofibrillary pathology in patients suffering from senile dementia [242]. This valuable technique promptly become a reliable tool for assessing brains from patients dying from neurodegenerative disorders [243, 244]. As previously mentioned, in 1906, German psychiatrist and neuroanatomist Dr. Alois Alzheimer reported the case of a patient called Auguste Deter afflicted with memory disorder in a congress in Tübingen (Germany) [245]. Using this silver staining technique, Alzheimer reported the first comprehensive description of post-mortem neuropathological findings which we now refer to as amyloid (Aβ) plaques and NFTs [209], which are established as the hallmarks of AD [206, 246, 247] (*Fig. 5A*).

At the neuropathological level, AD is characterized by positive lesions, including Aβ plaques, NFTs, neuropil threads and dystrophic neurites, [248-

251], and negative lesions including neuronal and synaptic loss [182, 252-255]. Aβ plaques are abnormal extracellular accumulations of aggregated Aβ peptides, which are the cleavage product of amyloid precursor protein (APP) [187, 256]. APP is a transmembrane glycoprotein which has been suggested to be involved in a wide variety of neuronal functions, such as neurogenesis, neurite outgrowth, synaptogenesis and neuronal differentiation [257]. APP is sequentially cleaved following two different pathways, referred to as non-amyloidogenic and amyloidogenic [258, 259]. In the non-amyloidogenic pathway, APP is first cleaved by α-secretase, releasing a soluble proteolytic fragment called sAPPα. The remaining fragment, called C83, is further cleaved by γ-secretase complex (including presenilin 1 (PSEN1) and presenilin 2 (PSEN2)), resulting in the p3 peptide [258, 259]. In contrast, in the amyloidogenic pathway, APP is cleaved by β-secretase producing sAPPβ. The remaining C99 fragment is then cleaved by γ-secretase complex releasing Aβ peptides [258, 259], including $A\beta_{1-42}$, which is prone to accumulate and form aggregates [260]. The spreading pattern of Aβ plaques throughout the brain is well defined by Thal staging: firstly, Aβ plaques appear and spread in neocortex, and subsequently, with disease progression, these deposits manifest in subcortical areas such diencephalic nuclei, striatum, brain stem and cerebellum [261] (*Fig. 5B*). On the other hand, NFTs and neuropil threads are composed of intraneuronal accumulations of hyperphosphorylated and truncated tau protein. NFTs appear within the cell soma, whereas neuropil threads are found in neurites [187, 211]. NFTs have gathered much attention within the AD field due to their spatiotemporal progression, which correlates with the disease progression and cognitive decline [262]. In fact, the NFT burden and topography in brain is the basis of Braak staging, which is used for pathological diagnosis of AD [133, 206]. Pathological tau load starts in transentorhinal cortex and hippocampus, from where it further spreads to temporal, frontal and parietal cortex. In the final stages, both sensory and motor areas are affected [133, 206, 263, 264] (*Fig. 5B*).

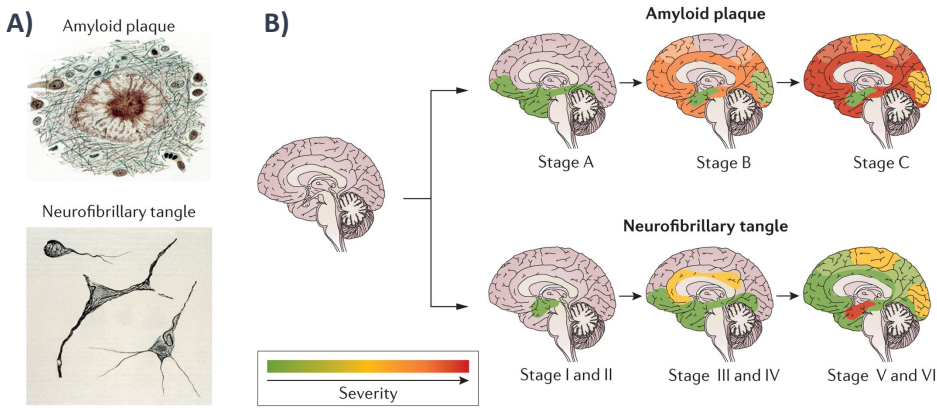


Figure 5. A) *Neuropathological hallmarks of Alzheimer's disease: Aβ plaques and NFTs.* ***B)*** *Aβ plaque and NFT pathology progression in AD. Reprinted with permission from Springer Nature: Nature Reviews, Disease Primers, (Alzheimer's disease, Masters, C. L. et al.), Copyright (2015).*

AD is the major cause of the dementia worldwide, accounting for 60-80% of all dementia cases [265]. With a prevalence of ~5% in people older than 60 years [266], age is the biggest risk factor [267]. A 2018 report estimated that there are 50 million patients worldwide suffering AD, and with an ever-aging population, this number is expected to further increase to approximately 80 and 152 million by the year 2030 and 2050 respectively [268] (*Fig. 6)*. AD is often classified in two subgroups: sporadic AD (sAD) and familial AD (fAD). sAD is the most common type of AD, and it is characterized by a late-onset, generally above 65 years old [269]. While there is no defined cause for sAD, increase susceptibility has been linked to the apolipoprotein E gene (*APOE*) [270]. *APOE* gene has three different alleles, that is ε2, ε3 and ε4. Having homozygous ε4 allele increases the risk of AD by 5-fold, while having heterozygous ε4 allele results in a 2-3 fold increased risk [271]. The ε2 allele on the other hand is considered to be protective against AD [272]. The amino acid changes introduced by the different alleles affect not only the structure of apoE, but also its lipid-binding and receptor-binding abilities [273]. For example, the apoE4 isoform binds less efficiently Aβ than apoE3 and apoE2, which may explain the decrease Aβ clearance and increase amyloid accumulation in *APOE* ε4 carriers [273]. ApoE has also been found to be involved in synaptic repair and cholesterol transport, being apoE4 the least effective in both scenarios [274]. Additionally, apoE4 induces

proinflammatory responses, which might be another leading cause for AD [273]. In contrast, fAD has an estimated prevalence of 1% [275] and it is characterized by an early onset, usually between 40 to 50 years of age, and a faster progression [276, 277]. fAD is caused by mutations in the *APP* and presenilin genes (*PSEN*1, *PSEN*2), which result in an increase Aβ production [278, 279]. In this context, *PSEN*1 mutations are responsible for most fAD cases [280].

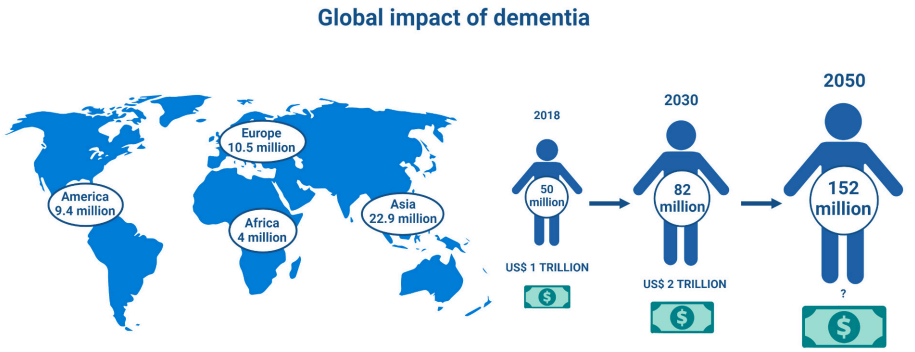


Figure 6. *Global impact of dementia in 2018 and predicted progression both in number of patients and medical expenditure by year 2030 and 2050. Created with BioRender.com.*

AD is an insidious and slowly progressing disease, characterized by a long preclinical asymptomatic phase estimated to start 20 to 30 years prior to the onset of the symptoms [281, 282]. During this phase, the pathology generally progresses finally leading to cognitive decline [283]. A typical clinical presentation includes short-term memory impairment, defined as mild cognitive impairment (MCI). When MCI is caused by AD, the disease will often progress into dementia, defined by memory loss together with behavioural disturbances, changes in personality, apraxia, aphasia and agnosia; disrupting normal daily activities [187, 284]. Unfortunately for patients and clinicians, when the symptoms appear and diagnosis is made, the disease has vastly and irreversibly damaged the brain. As a result, drug development has proven largely unsuccessful, as treatments are more likely to be effective during early stages, when the pathology has not yet widely spread. Therefore, early diagnosis would be a major breakthrough.

The exact cause of the pathology that underpins AD and subsequent symptoms remains a matter of intense debate. The predominant hypothesis, referred to as the "Amyloid Cascade Hypothesis", was firstly proposed in 1991 and according to it, an imbalance in the production and clearance of Aβ results in its extracellular accumulation, which subsequently triggers tau hyperphosphorylation and aggregation, synaptic dysfunction, neuronal death and neurodegeneration, ultimately leading to cognitive decline and dementia [275, 285, 286]. The foremost evidence supporting this hypothesis is related to Down syndrome and fAD. Down syndrome is characterized by an extra copy of chromosome 21, which contains the *APP* gene. This results in an overproduction of Aβ, linked to early-onset and fast progressing AD [287, 288]. On the other hand, fAD is cause by mutations in *PSEN*1 and *PSEN*2 genes, which encode proteins of the γ-secretase complex which cleaves APP. Thus, these mutations result in an increased production in Aβ [289]. Therefore, the unambiguous involvement of mutations related to APP production and cleavage in AD are the main foundation of the amyloid cascade hypothesis. Perhaps the biggest objection to this hypothesis, in addition to lack clinical benefit demonstrated by anti-amyloid trials which are successful in clearing Aβ plaques, is the lack of colocalization of tau deposits in amyloid-rich regions. Initial accumulation of Aβ and tau occurs in very different brain regions, and the spreading pattern of both proteinopathies only minimally overlap over time [206, 261].

In terms of biomarkers, fluid and imaging biomarkers are available. The classic fluid biomarkers for AD in diagnosis and inclusion in clinical trials are CSF $A\beta_{1-42}$, total-tau (t-tau)(the name alludes to the ability to bind all six tau isoforms whether they are phosphorylated or not) and phosphorylated tau a threonine 181 (p-tau181) [290, 291]. $A\beta_{1-42}$ levels in CSF reflect plaque pathology in the brain, particularly in a ratio with $A\beta_{1-40}$ ($A\beta_{1-42/40}$), which normalises for individual variance APP processing. These peptides are sequestered within the plaques, being therefore decreased in preclinical AD, MCI due to AD (MCI-AD) and AD dementia [292-295]. On the other hand, t-tau and p-tau181 are increased in preclinical AD, MCI-AD and AD dementia. According to the biological framework for AD (ATN criteria), t-tau is a biomarker for neurodegeneration, whereas p-tau181 reflects tangle pathology. However, t-tau is increased in only a few disorders (*e.g.*, stroke, CJD) and remains at normal levels in primary tauopathies. Furthermore, while tau PET is clearly related to brain NFT, for p-tau, it is less clear whether it reflects or rather

anticipates early tangle formation. Recent evidence suggests that CSF p-tau species might be indicators of Aβ-induced tau hyperphosphorylation, as they increase with Aβ plaque pathology (indexed by Aβ PET), while NFT pathology (indexed by tau PET) is normal. In fact, recent mouse model data suggests that CSF p-tau increases in response to amyloid deposition in absence of NFT pathology [296]. These tau biomarkers appear increased due to the proteolytic cleavage of tau protein during AD, which in turn releases N-terminal fragments that leak into CSF [297, 298]. The triad of fluid biomarkers (Aβ, t-tau, and p-tau), referred to as the core AD biomarkers, shows high specificity and sensitivity (85-90%) for clinical diagnosis of AD [299]. Since CSF sample collection requires lumbar puncture, much effort has been invested in finding blood biomarkers, which would be more desirable in clinical practice and would allow wider implementation. As mentioned above, there is also imaging techniques available including positron emission tomography (PET) and magnetic resonance imaging (MRI), which allow *in vivo* visualization of the brain, providing valuable molecular, anatomical, and structural information. PET scans use short-living radioactive ligands (C^{11} ~20-minutes; F^{18} ~110-minutes) that can for example bind specific pathological inclusions such as Aβ plaques or NFTs, reflecting respectively the Aβ and tau burden in the brain [300, 301]. Other PET ligands assess brain glucose metabolism (FDG-PET) [302] or synaptic density [303]. On the other hand, MRI allows the determination of grey and white matter brain atrophy but is considered a later event in the AD *continuum* [302].

1.2.2 Progressive supranuclear palsy

The first description of PSP was published in 1964 by Dr. Steele, Dr. Richardson and Dr. Olszewski, which they referred to as a rare combination of pseudobulbar palsy, progressive axial rigidity and dementia [304]. PSP represents the most frequent form of atypical parkinsonism [305], with a prevalence of 1 of 20000 individuals [306], being the most common primary tauopathy. Clinical presentation is characterized by supranuclear vertical gaze palsy, postural instability and cognitive and behavioural disturbances [307]. At a neuropathological level, the most specific immunohistochemical finding in post-mortem PSP brains is tufted astrocytes [308-310]. Diagnosis

criteria also includes presence of NFTs and neuropil threads in several brain regions including substantia nigra, pons, subthalamic nucleus and palladium (*Fig. 7*). Further microscopic findings comprise oligodendroglial coiled bodies, glioisis and neural loss [308, 309, 311]. These tau inclusions are predominantly anti-4R tau immunoreactive. Unfortunately, no treatment capable of tackling this disease has been found so far [312]. However, since PSP is greatly connected to tau protein aberrant functions [313], a large amount of clinical trials have been and are directed to this disease. In terms of CSF biomarkers, total-tau and phospho-tau181 are not increased when compared to healthy controls [314, 315], however, neurofilament light (NfL) is elevated in PSP patients both in CSF and blood [315-318].

1.2.3 Corticobasal degeneration

CBD was firstly described as corticodentatonigral degeneration by Rebeiz and colleagues in 1968 [319]. CBD is a rare progressive neurodegenerative pathology characterized by an age of onset around 63 and fast disease progression (about 6 years ending in demise). Clinically, CBD is diagnosed as corticobasal syndrome (CBS), and the clinical presentation includes heterogeneous symptoms ranging from sensory and motor syndromes, behavioural disturbances and cognitive problems [320-325]. Due to the symptoms heterogeneity and their overlap with other diseases such as PSP, clinical diagnosis is extremely challenging and thus ante-mortem misdiagnosis is rather common [326]. Nevertheless, clinical diagnosis accuracy improves with disease progression [325, 327], although neuropathological post-mortem examination is necessary to confirm diagnosis[328]. Neuropathological examination upon autopsy shows neuronal and glial pathology in white and grey matter in cortex, diencephalon, rostral brainstem and ganglia [329] (*Fig. 7*). These accumulations of tau protein are immunoreactive to anti-4R tau antibodies [329]. The hallmark of CBD patient's brain is astrocytic plaques [330, 331], comprised by abnormal accumulations of tau protein within astrocytes. In addition, numerous neuropil threads can be observed [329]. Besides astrocytic plaques, the main difference between CBD and PSP is that CBD brains have more neuronal tau pathology in forebrain , whereas in PSP its

more abundant in hindbrain [310]. In terms of biomarkers, contradictory studies have shown a marginal tau increase in CSF in patients with CBD when compared with controls [332, 333], whereas others have shown no differences [334, 335]. This is probably due to the fact that approximately half of the patients clinically diagnosed with CBS have CBD, whereas the other half have AD pathology, which would explain the marginal increases in p-tau and t-tau levels reported in some studies.

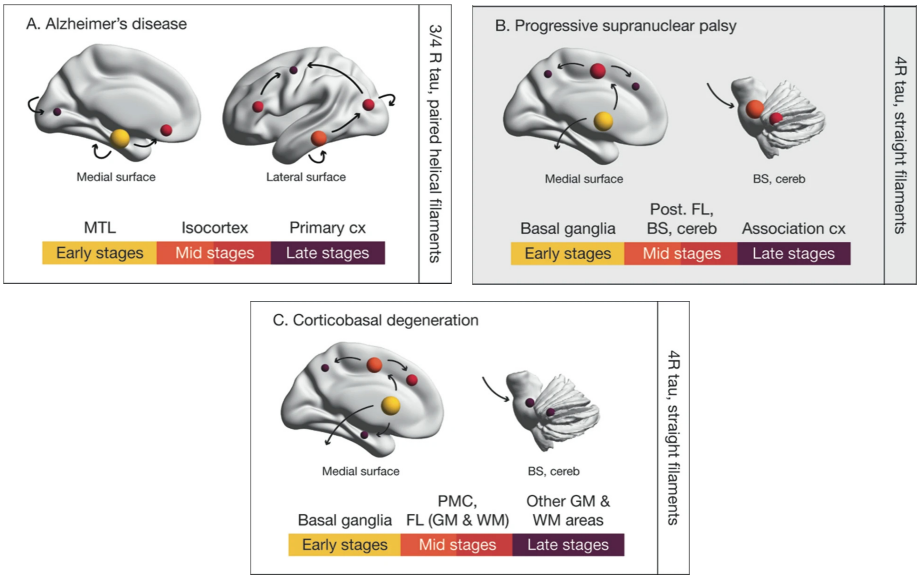


Figure 7. *Tau pathology progression and spreading in* ***A)*** *AD,* ***B)*** *PSP and* ***C)*** *CBD. Colour and sizes of the spheres indicates the areas of the brain affected at early (yellow-large), mid (orange/red-medium) and late (purple-small) stages of disease. Arrows indicate the direction of tau pathology progression and spread. Right panels on each figure indicate the predominant tau isoforms and fibril type found in each pathology. MTL (medial temporal lobe), cx (cortex), BS (brainsteam), Post (frontal lobe), FL (frontal lobe), PMC (primary motor cortex), GM (grey matter), WM (grey matter). Reprinted with permission from Springer Nature: Molecular Psychiatry (Tau PET imaging in neurodegenerative tauopathies—still a challenge, Leuzy, A. et al.), Copyright (2019).*

1.2.4 Pick's disease

From 1892 to 1906, Arnold Pick described uncommon cases of patients suffering from dementia with aphasia and lobar atrophy [336]. In 1911, Alois Alzheimer completed the histological description of Dr. Pick when he examined brains from these patients, highlighting the absence of both NFTs and senile plaques, but the presence of Pick's bodies and ballooned neurons [337]. Fifteen years later, in 1926, Onari and Spatz defined for the first time this pathological entity as "Pick's disease"[338]. PiD is characterized by an onset before 65 years [337], and accounts for 0,4% to 2% of all dementia cases [338], being substantially less frequent than AD [339] . No gender predilection has been reported [340] and disease progression is rather fast. PiD is characterized by behavioural disturbances and language impairment. Behavioural changes are usually characterized by lack of inhibition, impulsiveness, and inappropriate conduct. Other classical symptoms include bradyphrenia and memory deterioration [340, 341]. The classic features of PiD include frontal and temporal lobe degeneration in addition to immunohistochemical findings such as argyrophilic neuronal inclusions called Pick's bodies and swollen achromatic cells referred to as Pick's cells (both showing only 3R tau immunoreactivity) [340, 342-344] (*Fig. 8*). There are currently no biomarkers for PiD, complicating the ante-mortem diagnosis.

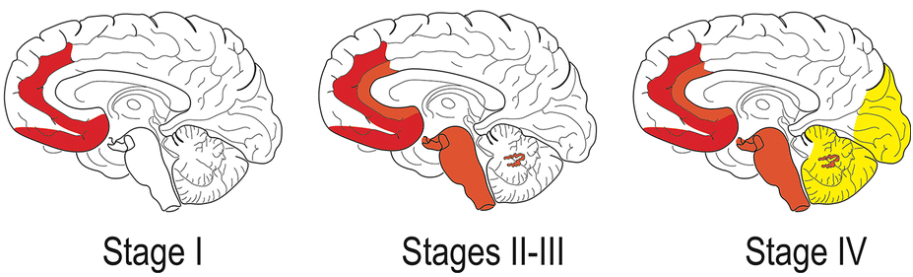


Figure 8. *Tau pathology staging in Pick's disease.* ***Stage I*** *is characterized by tau lesions in limbic and neocortical frontotemporal regions. At* ***stage II-III****, Pick bodies appear prominently at subcortical structures and brainstem. Finally at* ***stage IV****, tau lesions are present in visual cortex and cerebellum. Reprinted with permission from Springer Nature: Acta Neuropathologica (From the prion-like propagation hypothesis to therapeutic strategies of anti-tau immunotherapy, Colin, M. et al.), Copyright (2020).*

1.2.5 Traumatic brain injury and chronic traumatic encephalopathy

TBI means brain trauma caused by external mechanical forces, including both close-head injuries and penetrating injuries [345] (*Fig. 9*). According to a 2007 report, TBI has a worldwide incidence of approximately 10 million people [346]. Clinically, TBI is diagnosed into three groups based on severity: mild, moderate, and severe [347, 348]. On the other hand, TBI can be classified into two distinct categories, acute and chronic [349]. Mild TBI or concussion, is characterized by a plethora of symptoms (*e.g.,* memory and balance alterations, loss of consciousness, dizziness and headaches [350]), which generally resolve in a few days or weeks. However, in some patients, the symptoms may be prolonged for more than a year [351-354]. From a pathological stance, mild TBI does not involve overt lesions, such as haemorrhage or structural brain damage. In contrast, severe TBI is the result of more severe brain trauma, such as internal bleeding and brain tissue damage, which can lead to long-term sequelae and even death [349]. Not surprisingly, acute TBI is common among professional athletes, specifically those that practice full contact sports such as boxing, ice hockey or American football, but also in war veterans [355-358].

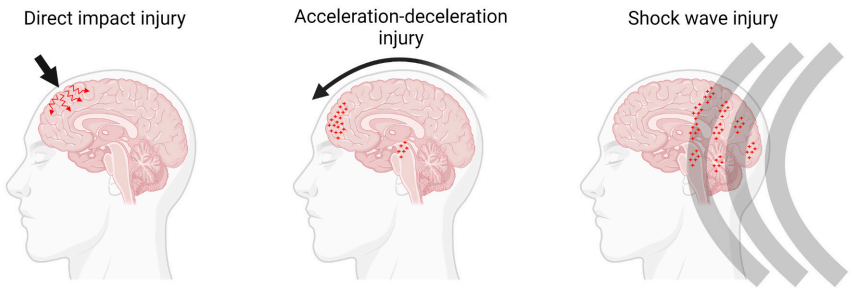


Figure 9*. Common aetiologies of traumatic brain injury (TBI). Created with BioRender.com.*

On the other hand, chronic TBI also referred to as chronic traumatic encephalopathy (CTE) or dementia pugilistica, is the consequence of repetitive brain trauma [359]. In athletes, the clinical onset of CTE normally occurs in midlife, several years after ending their careers. Clinically, CTE is characterized by heterogeneous symptoms and different degrees of severity,

which can range from mild complaints to behavioural alterations, dementia and Parkinson-like symptoms [360, 361].

At a neuropathological level, mild TBI (the most common form of TBI) is characterized multifocal axonal injury and microglia activation [362, 363]. Other immunohistochemical findings include tau aggregates in the form of NFTs and neuropil threads, and TAR DNA-binding protein 43 (TDP43) inclusions [364]. This widely contrasts with CTE, which is characterized by prominent morphological alterations in brain (*Fig. 10*). Microscopically, the hallmark of CTE is the presence of perivascular accumulations of p-tau in both neurons and astrocytes in the cortical sulci [365]. Other abnormalities include Aβ and TDP43 accumulations [366-369].

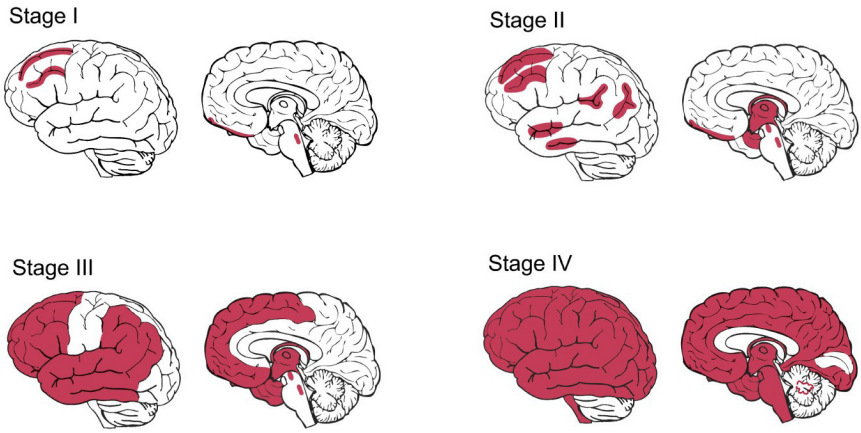


Figure 10. *Schematic representation of tau pathology progression in the four neuropathological stages of traumatic chronic encephalopathy. At* ***stage I****, tau lesions appear at the frontal cortex, prominently in the depths of sulci. At* ***stage II****, tau pathology spreads into parietal and temporal lobes, and diencephalon.* ***Stage III*** *is characterized by positive lesions in basal ganglia, amygdala, and hippocampus, and further tau pathology spread in neocortex. Finally at* ***Stage IV****, medulla, cerebellum, and spinal cord are affected. At this stage tangle pathology is widely spread throughout the cortex, and most brain regions show tau lesions. Reprinted with permission from Springer Nature: Alzheimer's Research & Therapy (Chronic traumatic encephalopathy: a spectrum of neuropathological changes following repetitive brain trauma in athletes and military personnel, Stein, T. D. et al.), Copyright (2014).*

The most studied pathophysiological event in TBI is axonal injury. This is because axonal injury, specifically diffuse axonal injury, is a defining feature of TBI-induced lesions [370, 371]. After axonal injury, MT are disrupted,

leading to the formation of axonal swelling [372]. Interestingly, APP, γ-secretase and β-secretase has been found to accumulate in areas with axonal damage and transport failure [362, 363]. These observations may explain the presence of Aβ plaques in individuals with TBI [373, 374]. In fact, extracellular soluble Aβ has been shown to decrease after TBI [375, 376]. Perhaps the colocalization of APP, γ-secretase and β-secretase in areas with axonal injury might be the result of impaired axonal transport [377], subsequently leading to an aberrant Aβ processing. This in turn would explain the abnormal accumulation of tau protein found in both TBI and CTE. However, in an animal model for TBI, an almost complete reduction in Aβ levels did not impact tau aggregation [378]. An alternative hypothesis has been proposed. After axonal injury, the damage in the neuronal membrane would provoke an increase in intracellular calcium levels, which in turn will damage the mitochondria leading to oxidative stress and abnormal proteolytic activation, which would affect cytoskeletal components and other proteins [379].

Currently, we lack biomarkers that can provide objective answer on whether patients have axonal injury or other type of brain damage, and patient management at clinical settings normally include neurological assessments and CT scans. CT scans can provide relevant information regarding certain treatable life-threatening conditions, such as brain haemorrhage [380], however, they do not detect axonal damage. On the other hand, MRI is more sensitive than CT [380], but less available in clinics, and similarly to CT, MRI does not detect axonal injury. In this scenario, fluid biomarkers might represent a valuable tool for clinical diagnosis of TBI and CTE. Both NfL, t-tau and GFAP concentrations in CSF have been shown to be increased in mild TBI [381, 382]. Interestingly, higher levels of CSF NfL, t-tau and GFAP have been reported with higher number of hits to the head [382]. However, the perceived invasiveness associated with lumbar puncture, has hampered the wide implementation of these CSF biomarkers. Nonetheless, promising results have been obtained in blood when measuring t-tau, p-tau and NfL in cases of TBI [383-386].

1.2.6 Creutzfeldt-Jakob's disease

The term CJD was coined in 1922 by Spielmeyer when he named a condition reported by two German clinicians, Dr. Creutzfeldt and Dr. Jakob [387]. The hallmarks of this disease were established in 1960, and include classic spongiform changes (grey matter vacuolation) and periodic sharp wave complexes in electroencephalography (EEG) [388]. However, post-mortem confirmation remains to be the gold standard for definitive diagnosis. In healthy individuals, a protein expressed predominantly in the nervous system called cellular prion protein (PrP^{C}) is present in lipid rafts on the cellular membrane [389]. The functions of this protein are unknown, and knockout mice do not show any evident abnormalities in brain [390]. The pathological event leading to CJD is the transformation of PrP^{C} into the so-called prion protein scrapie (PrP^{Sc}). While PrP^{C} and PrP^{Sc} have identical amino-acid sequences, PrP^{Sc} has 45% β-sheet conformation (in contrast with PrP^{C} which has 40% α-structure), making PrP^{Sc} highly insoluble and resistant to digestion [193]. This abnormal misfolding causes confers PrP^{Sc} the ability to autocatalytically induce the subsequent misfolding of normal PrP^{C}, using its own structure as a template [193]. This pathological mechanism is highly efficient in hijacking and transforming PrP^{C} into more PrP^{Sc}. As a consequence, PrP^{Sc} accumulates and spreads at high rates, causing neurodegeneration and the aforementioned classic spongiform changes [193] (*Fig. 11*). CJD is classified into three different categories based on the aetiology: sporadic, familial, and acquired (variant and iatrogenic) (*Fig. 11*). Sporadic CJD (sCJD) accounts for the vast majority of all CJD cases (85%) and it is hypothesized that it is caused by either a random misfolding of PrP^{C} or by a somatic mutation in the *PRNP* gene [391]. Familial CJD (fCJD) is the result of mutations in *PRNP* gene, from which more than 50 have been described. Finally, acquired CJD is comprised by variant CJD (vCJD) and iatrogenic CJD. Fistly described in 1996, vCJD is caused by the ingestion of food contaminated with bovine spongiform encephalopathy [392], while iatrogenic CJD is acquired accidentally through medical procedures, and was firstly reported in 1974 [393]. The clinical picture of CJD is a rapidly progressing cognitive decline and dementia of unknown origin, which may be accompanied by atypical neurological symptoms such as myoclonus and chorea [394].

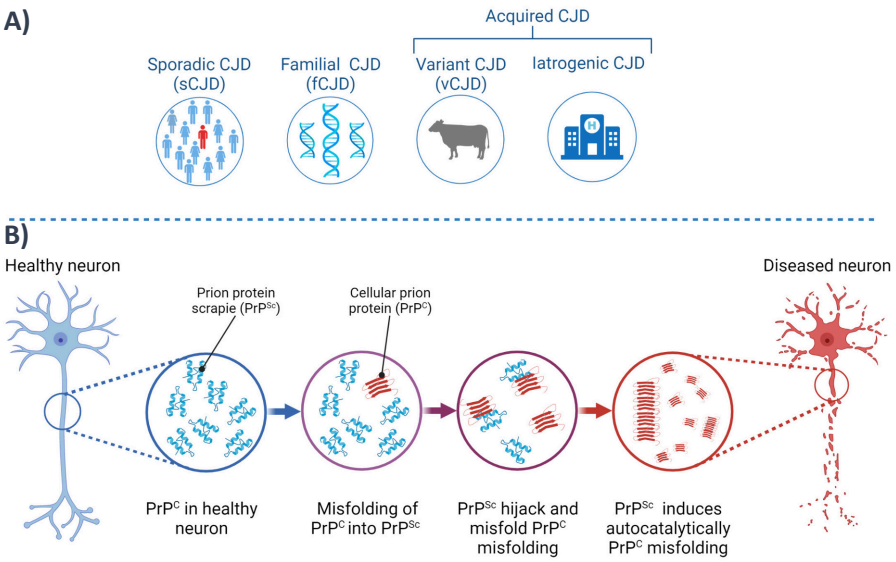


Figure 11. A) *Classification of the four types of Creutzfeldt-Jakob's disease (CJD) based on the aetiology.* ***B)*** *CJD is initiated by the conversion of cellular prion protein (PrP^{C}) into misfolded prion protein scrapie (PrP^{Sc}). Newly formed PrP^{Sc} will then autocatalytically induce the misfolding of more PrP^{C}. This results in PrP^{Sc} spreading and aggregation throughout the brain, causing the intense and fast progressing degeneration which characterizes CJD. Created with BioRender.com.*

Clinical diagnosis is based on EGG, MRI, CSF biomarkers and clinical assessments. While neither EGG nor MRI have shown high diagnostic accuracy to serve alone as a diagnostic test [395-397], CSF biomarkers have shown great promise. The most studied CSF biomarkers for CJD are 14-3-3 protein and t-tau, as both have been shown to reflect neuronal damage [398-400]. While literature regarding 14-3-3 has reported mixed results [398, 399, 401-403], t-tau (and t-tau/p-tau ratio) has shown promising results discriminating CJD from AD and non-AD cases [404-408]. The high performance of t-tau is the consequence of the intense neurodegeneration that characterizes CJD, which results in extremely high levels of this biomarker in CSF [404]. Thus, it seems feasible to think that a combination of clinical examination and CSF t-tau measurements (or t-tau/p-tau ratio) could become a simple and highly sensitive approach to diagnose CJD. With the development of tau biomarkers in blood, the wide implementation of this

proposed approach seems ever closer. In fact, a longitudinal study published in 2021 showed that plasma t-tau is increased in CJD when compared to AD and controls, and that it correlates with progression [409].

1.3 TAU PROFILE IN CSF AND BLOOD

The characterization of tau protein in fluids has been long pursued, and in recent years, with the use of mass spectrometry (MS), our knowledge is increasingly improving. This has resulted in the development of several immunoassays capable of measuring a wide variety of tau biomarkers. Immunoassays have been capable of measuring tau protein in CSF since the early 90s, however, the actual detailed profiling of tau protein (in terms of fragment sequences, phosphorylation and cleavages) in CSF has for long been elusive. Early attempts to profile tau in CSF aimed to address this issue by using western blots techniques. However, these initial studies reported inconsistent and most often than not, mixed results [410-415], likely associated to unspecific binding and other related artefacts [416]. In 2008, Portelius and colleagues reported the first IP-MS method capable of profiling tau in CSF [417]. They identified nineteen tryptic tau peptides, most of which ranged between amino acids 6 and 254 (2N4R tau). They also identified tau fragments belonging to the MTBR and isoform specific peptides from 0N, 1N and 2N [417]. In 2013, Meredith and colleagues published the first study that provided an extensive and comprehensive characterization of tau protein (both tau fragments and p-tau) in CSF from healthy and AD cases [297]. Using reverse phase high performance liquid chromatography (LC) followed by western blotting and immunoassays, the authors were able to demonstrate that tau protein content in CSF is comprised by N-terminal and mid-region tau fragments, but they could not identify fragments expanding into the MTBR and the C-terminus. Moreover, using a wide battery of immunoassays they demonstrated that several tau fragments of various lengths, both phosphorylated and not, were increased in AD [297]. In 2016, Barthelemy and colleagues reported an antibody-free MS method which they used to profile tau protein in CSF from control, AD, PSP and DLB cases [298]. They demonstrated that mid-region tau fragments are substantially more abundant that N-terminal and C-terminal, and that 1N3R is the predominant

tau CSF isoform. They also showed that tau protein content in CSF is much higher in AD than in all other disease groups. This was true for all peptides throughout tau sequence [298]. Another MS study, published in 2016, also confirmed that CSF tau is primarily comprised by mid-region tau fragments expanding from amino acids 156 to 224 [418]. They also reported that 1N tau isoforms are the most abundant in CSF [418]. A major cornerstone for our knowledge regarding tau content in CSF was published in 2018 by Sato and colleagues [419]. In this elegant study, the authors reported that tau content in control CSF is comprised by mid-region and N-terminal tau sequences (99.9%), and that peptide recovery steeply decreased after amino acid 222, finally disappearing after amino acid 256. They also reported the presence of a major cleavage between amino acids 220 and 230 and that tau concentration in CSF increases with age. Most importantly, they demonstrated that both amyloidosis and tau aggregation correlates well with an increased tau production rate in CSF in AD patients [419]. From their results, one can hypothesize that tau protein is cleaved intracellularly, and while the C-terminus and MTBR probably remain inside the cell (where they might be either cleared or form of insoluble aggregates), the mid-region and N-terminal containing fragments are actively released into the extracellular space, from which they presumably leak into the CSF and potentially blood. The next detailed profiling of tau in CSF was published in 2019 by Cicognola and colleagues [178]. Their results using IP-MS with different antibodies and without enzymatic trypsinization demonstrated the presence of two distinct tau pools of endogenous tau peptides: an N-terminal pool expanding from amino acids 2 to 129 and one mid-region pool ranging from amino acid 130 to 224. However, one must take into consideration that these results do not exclude the presence of other longer tau endogenous peptides, which are more challenging to detect and thus were not identified. They addressed this issue by performing a quantitative IP-MS analysis of tryptic peptides using Tau12, HT7 and BT2 (epitopes: 6-18aa, 159-163aa and 194-198aa respectively, using 2N4R numbering). Their results reflected the presence of long tau fragments covering the whole N-terminal and mid-region sequence [178]. Of note, although these results may appear to suggest that some tryptic N-terminal peptides are as high as mid-region ones (contradicting previous reports), this cannot be ultimately concluded as quantification methods using enrichment methods such as immunoprecipitation (IP) are highly depended on antibody properties. One interesting finding from this

study was the presence of two major cleavages, one at amino acid 123 and other at amino acid 224 [178]. The latter truncation aligns well with Sato and colleagues observation of a cleavage between amino acids 220 and 230 [419]. The last scientific addition to our understanding on tau species in CSF was the finding of fragments beyond the mid-region. Two separate publications have reported CSF measurements of tau fragments ending at amino acid 368, which were significantly increased in AD and were shown to be useful tracking tangle pathology [420, 421]. Finally, using a combination of IP and chemical extraction followed by MS, Horie and colleagues demonstrated that both MTBR and C-terminal tau are present in CSF [422]. Most notably, they showed that these fragments are increased in AD, and that they correlate with disease progression [422].

Another important aspect of tau profiling in CSF and blood has been the characterization of phosphorylated residues. As aforementioned, several immunoassays capable of measuring tau in CSF have been available since the early 90's, some of which specifically targeted p-tau residues. The design of these assays was guided by IHC and western blotting with anti-p-tau antibodies in brain samples. Thus, several phosphorylations have been known to exist in CSF for many years, including p-tau181 or p-tau231. However, detailed profiling of p-tau residues in CSF have been only recently become available, primarily thanks to advancements in MS techniques. In 2019 and 2022, Barthelemy and colleagues used an IP-MS method to quantify the relative abundance of different p-tau species in CSF from AD and control, specifically at T111, S113, T153, T175, T181, S199, S202, T205, S208, S214, T217 and T231, many of which appeared increased in AD [423, 424]. The same group reported a MS characterization of tau protein in blood, where they showed that tau protein profiles in CSF and blood are similar: full-length tau was not found, tryptic peptides ranged from amino acid 6 to 254 and a pronounced decrease in abundance was apparent after amino acid 221 [425], matching previous literature [418, 419, 421]. Additionally, they attempted to profile the phosphorylations they previously described in CSF, from which only p-tau181, p-tau202 and p-tau217 were reliably quantifiable. Others such as p-tau205 were detectable but inconsistent, while the rest were simply non-detectable [425]. Although this blood profiling is promising, further developments are still required to achieve higher sensitivities and decrease the large sample volumes required (20mL of plasma).

1.4 FLUID BIOMARKERS OF TAU PATHOLOGY

Before discussing tau biomarkers in detail, it is important to highlight the advantages and disadvantages of PET and fluid biomarkers as biomarkers for neurodegenerative diseases. The main advantage of PET biomarkers is that they allow the visualization of pathological changes in the brains of living patients with spatial information. This makes PET imaging an excellent stand-alone diagnostic tool, with regional information that neither CSF nor blood biomarkers can offer [426, 427]. However, PET has significant drawbacks. The imaging technique and ligand is substantially more expensive than fluid biomarker measurements, as they require from very specialized centres with costly instruments and well-trained personal [428-430] – thus they have very low scalability for widespread use. In term of invasiveness, PET imaging relies on the use of radioactive ligands, which inevitably results on radiation exposure. Determinations by PET focus on one pathology. In the case of AD, tau and amyloid are separate entities, increasing cost and patient time. Future imaging modalities of inflammation, synaptic density and other protein aggregation (TDP-43 or alpha-synuclein) would incur further cost and radioactive exposure. Fluid biomarkers allow for sample biobanking, the measurement of multiple biomarkers (including amyloid, tau, neurodegeneration, inflammation, and alpha-synuclein), and storage for prospective assessment (*Fig. 12)*.

The traditional source of fluid biomarkers in the field of neurodegenerative diseases is CSF. CSF is a colourless fluid generated by the lining cells of the choroid plexus, which are located in the ventricles [431]. From here, CSF flows into the subarachnoid space, circulating around the brain hemispheres and the spinal cord [431]. The CSF assists the brain by clearing metabolic waste, providing nutrients, and offering protection against mechanical injury. Due to this continuity with the brain, CSF is heavily loaded with brain-derived molecules, which provide information about the current state of the central nervous system (CNS), making it ideal for biomarker measurements. On the other hand, brain-derived molecules in blood are highly diluted, with 10 to 100 fold lower concentrations when compared to CSF [432]. From a matrix point of view, CSF is a simpler biofluid to work with when compared to blood. For example, normal healthy CSF has a total protein concentration of around 0.5 g/L [433] while blood values are approximately 60 to 80 g/L [434], which

may result in interference with the analytical methods used [435]. Furthermore, brain-derived molecules in blood are susceptible to degradation by proteases, kidney clearance and liver metabolism [435, 436]. Lastly, some CSF biomarkers (such as tau or Aβ) are also expressed in peripheral tissues (understood as non-cerebral tissues) [437, 438], which can hinder their detection in blood (*Fig. 12)*. Taken together, these limitations have hampered the discovery of reliable plasma biomarkers for a long time. The concept of a blood biomarker for AD is not a new one and there have been several hundred report putative biomarker for AD [439]. However, despite some of these markers clearly having relevance for AD, in terms of genetic association or present in pathological tissue, their abundant peripheral expression and involvement in multiple disease processes lowered disease specificity and reproducibility across cohorts [440-443]. However, in the recent years, major technical developments involving both immunoassays and MS, coupled to detailed characterisation of research cohorts and a newly defined biological definition of AD and related disorders, have begun to show highly accurate and reproducible biomarkers in blood [444].

The undying interest for blood biomarkers is based on several reasons. Firstly, blood is substantially more accessible than CSF. CSF collection requires from lumbar puncture, a technique which is considered to be invasive and it is in fact associated with mild adverse side-effects, such as post-lumbar headaches [445]. Blood sampling, however, is a rather simple and non-invasive procedure. Blood collection via venepuncture can be easily performed at any clinical setting or even at home, whereas CSF sampling must be performed by trained personal at specialized centres (*Fig. 12*). Thus, blood biomarkers are an attractive alternative to both PET and CSF biomarkers, as blood sampling is cost-effective, easy to implement and lacks invasiveness [446, 447]. Due to this, blood biomarkers have emerged as a very suitable diagnostic tool for primary clinical settings, were in combination with cognitive assessments can represent a first-in-line test before referring patients to more specialized centres. From the perspective of clinical trial recruitment, blood biomarkers could be easily implemented as a screening tool for an initial selection of participants. Additionally, blood biomarkers are likely to improve enrolment in clinical or research cohorts, being more appealing for participants and more cost-effective for recruiting institutions [446, 447]. Additionally, they would overcome the economic restrains related to medical care in developing countries (*Fig. 12*).

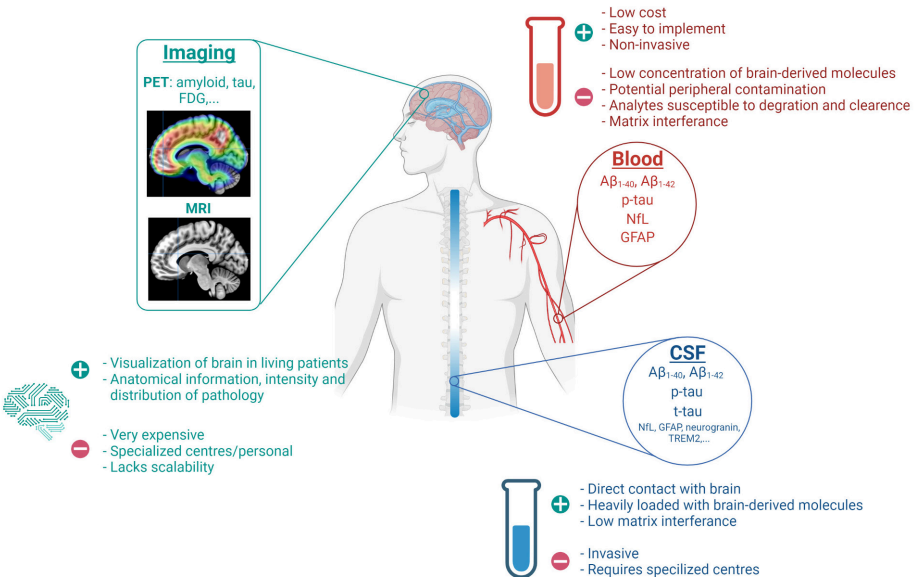


Figure 12. *Pros and cons of fluid and imaging biomarkers. Created with BioRender.com.*

1.4.1 CSF total-tau

The interest on tau protein as a fluid biomarker begun with the discovery of p-tau as the main component of NFT [448]. Not long after, in 1987, the first attempt to quantify tau protein in CSF using western blot revealed that tau was detectable in CSF from AD dementia patients, but not in samples from non-demented individuals [449]. These early results laid the foundation for the development of the first immunoassay measuring tau in CSF, which was published in 1993 [450]. The method used was an enzyme-linked immunosorbent assay (ELISA), composed by a monoclonal antibody targeting the tau mid-region and a polyclonal anti-tau serum. Only two years later, in 1995, Blennow and colleagues developed a novel sandwich ELISA using monoclonal antibodies [451]. Because the antibodies in this ELISA could recognize all 6 CNS tau isoforms regardless of the presence of phosphorylation, this assay was referred as total tau (t-tau).

CSF t-tau is considered a biomarker of neurodegeneration and neuronal/axonal injury and it is one of the three core AD biomarkers [452,

453]. Abnormally high levels of CSF t-tau have been consistently reported over the years both in prodromal AD and AD dementia stages [299], and higher CSF t-tau have been shown to predict a faster AD progression [454-457]. Interestingly, recent studies have suggested that t-tau secretion in AD might be the result of an Aβ-induced response from living neurons, rather than strictly neuronal degeneration and death [419, 458]. AD patients and elderly controls demonstrate tight correlations between CSF levels of p-tau and t-tau, which may suggest that both these biomarkers reflect neurodegeneration. However, in stroke patients, CSF t-tau increases in response to stroke severity, while p-tau does not change [459]. This added support to the view that p-tau is a marker for tau pathology while t-tau reflects neuronal injury. In acute scenarios, CSF t-tau display very dynamic trajectories, normally characterized by a rapid increase after injury followed by a stabilization period that can last weeks, and finally returning to normal levels [381, 459]. In contrast, some chronic neurodegenerative disorders are characterized by permanent high levels of t-tau in CSF. Perhaps the most notable example is CJD, which is characterized by an intense neurodegeneration that results in extremely high levels of t-tau in CSF [404, 407]. Interestingly, in general, CSF t-tau does not change in other neurodegenerative disorders, such as Parkinson's disease and non-AD tauopathies [406]. One common misconception regarding CSF t-tau is that the measured tau fragments leak from injured neurons in a rather passive manner. This idea has been specially challenged in AD, where studies have shown an active tau secretion [419, 460], presumably stimulated by Aβ pathology.

1.4.2 CSF p-tau

The idea behind measuring p-tau emanates from the discovery that NFTs are comprised by highly p-tau [448, 461, 462]. Thus, it was hypothesized that if the phosphorylations found in NFTs were measurable in CSF, such measurement in fluid could reflect the underlying tau pathology in brain. The first method capable of measuring p-tau in CSF was published in 1995 and it consisted on an ELISA targeting simultaneously phosphorylated threonine 181 and 231 [451], which were found to be abundant in NFTs. This early study

also showed that these phosphorylated residues were increased in AD. These findings were further corroborated by Vanmechelen and colleagues in 2000 when using a p-tau181-targeted assay [463]. After this, several studies showed that not only p-tau181 or p-tau231 species were increased in AD CSF, but also the likes of p-tau199, double phosphorylation at p-tau231/235 and p-tau396/404 [415, 464-467]. Not long after, the first study comparing the performance of different CSF p-tau species when discriminating AD from controls and non-AD neurodegenerative disorders was published [466]. This study concluded that CSF p-tau181, p-tau199 and p-tau231 where not just highly correlated with one another, but also that all assays offered similar diagnostic performance [466]. In the following years, other phosphorylations would be explored in CSF, such p-tau202, p-tau205, p-tau212, p-tau214, or p-tau217 [423, 468-470].

CSF p-tau levels have been found to reflect the degree of tau phosphorylation in AD brain [435, 453]. CSF p-tau is a more specific biomarker for AD pathology, and it does not appear abnormally increased in other neurodegenerative disorders if there is no contaminant AD pathology [470-472]. Thus, p-tau does not reflect neurodegeneration, neuronal and axonal injury, neither in acute nor chronic scenarios, but rather the phosphorylation state of tau protein in AD brain. Among all p-tau species studied in CSF, p-tau181 is by far the most studied one and the only clinically validated, being one of three core AD biomarkers [299, 473]. However, in the recent years, other p-tau species have gained increase notoriety, most notably CSF p-tau217 and p-tau231 [469, 470]. Firstly, CSF p-tau217 has been shown to be an excellent biomarker for AD. Using different platforms, specifically ELISA, Simoa and MS, CSF p-tau217 was shown to be highly specific for AD, display larger fold-changes between groups, correlate better with both amyloid and tau PET in AD cases and offer superior cognitive decline prediction when compared with CSF p-tau181 [469, 470, 474-476]. On the other hand, CSF p-tau231 has always being a very promising biomarker but traditional showed no differences from p-tau181 [466, 477-481]. However, the interest on CSF p-tau231 was strongly revived when in 2020 our group measured this phosphorylation together with CSF p-tau217 and p-tau181 in a preclinical AD cohort, demonstrating that p-tau231 is the first p-tau species reaching abnormal values in CSF [469]. This study was followed by yet another comparison of these three p-tau biomarkers, this time in a research cohort comprising participants across the AD *continuum*, controls and non-AD

neurodegenerative disorders, all well-characterized with clinically validated CSF and PET biomarkers [482]. Here, CSF p-tau231 was shown to correlate better with the earliest Aβ fibrils accumulations when compared to CSF p-tau181 and p-tau217 [482]. These results may indicate that p-tau biomarkers might associate with Aβ pathology at the early stages of the disease but later correlate stronger with NFT pathology [483].

1.4.3 Blood total-tau

The first attempt to measure t-tau in plasma goes back to 1999, when Ingelson and colleagues reported no significant differences in ELISA signals between AD dementia and non-AD dementia cases [484]. The first attempts such as this were limited by the lack of sensitivity associated with ELISA. However, the development of ultrasensitive techniques capable of reaching unprecedented limits of quantification soon resulted in encouraging reports [485-488]. One of the major issues found with this biomarker in blood has been the large overlaps observed between groups [486-490], which had led many to question its diagnostic utility. In addition, poor correlations between plasma t-tau and its CSF counterpart [425, 486, 487, 491] and AD signature atrophy measures have been reported [489]. The most plausible reason for this is that available immunoassays measuring plasma t-tau cannot distinguish between peripheral tau (big tau) and CNS-derived tau. Another explanation that has been proposed is that the targeted tau fragment might be vulnerable to protein degradation in blood [492]. Despite this, different studies have showed an increase in plasma t-tau in AD cases [486-488]. Moreover, high plasma t-tau levels have been shown to predict faster decline in cognition and neurodegeneration [488, 490, 493-495]. Blood t-tau measurements have proven very meaningful in cases of acute neurological injury such as stroke, cardiac arrest, or TBI, and in chronic neurodegeneration, such as CJD [385, 496-499]. An interesting development in the recent years has been a novel plasma t-tau immunoassay targeting tau fragments expanding from the N-terminal to the mid-region, which authors referred to a NT1 [500-502]. This novel assay was shown to be specific for AD, but most importantly, capable of predicting cognitive decline and neurodegeneration in cognitively unimpaired individuals [500, 501]. These results using NT1 align

well with our results from paper V, and we believe they open a window of opportunity for further improvements regarding plasma t-tau. Furthermore, the development of CNS tau specific antibodies capable of discriminating brain-derived tau from peripheral tau in blood might propel even further the already promising results with plasma t-tau.

1.4.4 Blood p-tau

As previously mentioned, measuring p-tau species in blood has been long-sought goal in the fluid biomarker field, which remained elusive until very recently. However, from 2014 onwards, several studies have overcome the analytical challenges regarding blood, providing reliable and consistent p-tau measurements. One of the first studies reporting p-tau in blood measured p-tau231 in a TBI cohort [497]. This was quickly followed up by a report in 2016 indicating that serum p-tau181 is increased in AD and MCI when compared to controls [503]. In 2017, Tatebe and colleagues observed an increase in plasma p-tau181 in both AD and Down's syndrome (DS) when compared to controls [504]. Finally in 2018, two publications reported increased plasma p-tau181 measurements in AD cases using immune-magnetic reduction [505] and mesoscale [506] assays. These initial findings were followed by our group in 2020, with the publication of Paper I.

2. AIMS

2.1 GENERAL AIMS

To identify and characterize disease-relevant tau fragments and phosphorylations in the context of neurodegenerative diseases, assessing their potential as biomarkers in CSF and blood.

2.2 SPECIFIC AIMS

Paper I: To develop a novel blood assay for p-tau181 and assess its performance as a biomarker for AD.

Paper II: To investigate if plasma p-tau181 reflects AD pathology in post-mortem confirmed cases, evaluating the diagnostic accuracy and the trajectories of this biomarker in longitudinal samples.

Paper III: To develop a novel blood assay for p-tau231 and assess its performance as a biomarker for AD.

Paper IV: To develop a novel CSF assay for p-tau235 and assess its performance as a biomarker for AD, investigating in depth it's potential as a staging biomarker during preclinical AD.

Paper V: To evaluate the biomarker potential of different N-terminal and MR-region tau fragments in AD and other neurodegenerative disorders in both CSF and blood.

3. MATERIALS AND METHODS

3.1 Brain protein extraction

Brain protein extraction is a method that allows the isolation of proteins with different solubilities and located in distinct cell compartments. This technique usually starts with a conventional tissue homogenization followed by multiple cycles of ultra/centrifugation in combination with different buffers and detergents, resulting in the extraction of different protein fractions. Brain protein extraction protocols widely differ from one another, not only based on the protein of interest, but also based on its location and solubility. For tau protein, there is no standard protocol, and several procedures have been reported and published.

Our extraction protocol is the following: frozen brain tissue is first cut and weighted, obtaining roughly 100mg tissue. Tris-buffered saline or TBS (20 mM Tris HCl, 137 mM NaCl, pH = 7.6, with Complete Protease inhibitor Cocktail) was subsequently added (1mL TBS per 100mg of tissue). This material was then homogenized for 2 min at 200 Hz using the Tissue Lyser II (Qiagen) and later centrifuged for 1 h at 31.000 × *g* at + 4°C. The supernatant, called TBS soluble fraction, was collected, and stored at − 80°C until further analysis. This supernatant contains soluble tau species, partially similar to the ones found in CSF, and was used for MS analysis.

3.2 Immunoprecipitation

Adapted from column affinity chromatography, IP is a technique in which a protein of interest is precipitated from a solution (often a cell lysate or tissue homogenate) using a target-specific immunoglobulin bound to a magnetic bead. This allows the purification and enrichment of an analyte of interest from a complex matrix, for example tissue homogenates, CSF, or blood. The paramagnetic properties of the beads allow them to be retained by using a magnet, so that washes with different buffers can be done. The first step in

an IP consists of the antibody incubation with magnetic beads. After this, excess antibody is washed out and coated beads are blocked with protein, commonly bovine serum albumin (BSA). On some occasions, and extra step referred to as antibody crosslinking is performed prior to blocking using triethanolamine and dimethyl pimelimidate, which minimizes the amount of antibody contamination in the final eluate. After blocking, beads are incubated together with the sample overnight at 4 °C in a tube roller, allowing for the antibody to bind the analyte. The following day, and depending on which method will follow the IP, the analyte, now enriched, is washed with different buffers and finally eluted using for example formic acid when samples are intended to be analysed in a MS, thus completing the IP (resuspension can be performed in other buffers when the intended use is other, such as western blotting) (*Fig. 13*). *Note that these parameters can be optimize.*

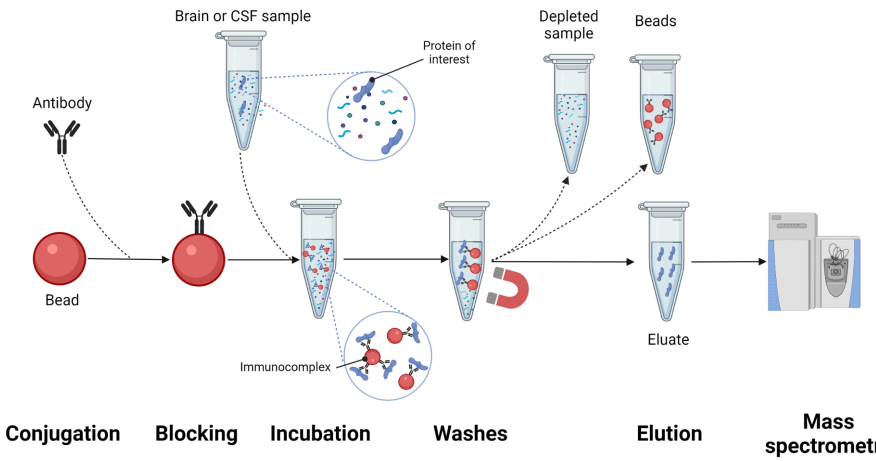


Figure 13. *Schematic representation of immunoprecipitation (IP) followed by mass spectrometry. Firstly, beads are conjugated with the antibody of choice, and subsequently blocked to avoid unspecific binding. Beads are then incubated with the sample (e.g., CSF or TBS). Finally, beads are subjected to a series of washes prior to a last wash in formic acid, which will result in the elution of the captured analyte. Eluate is then dried in a vacuum centrifuge, resuspended and run in the mass spectrometer for quantification. Created with BioRender.com.*

3.3 LC-MS

Reverse phase (RP) liquid chromatography (LC) is commonly coupled online to mass spectrometry (MS). LC is a separation technique that allows a mixture of compounds in solution to be separated using both a stationary and liquid phase in accordance with their physicochemical characteristics. Initially, the sample is loaded onto a column of a certain type (the stationary phase) through which a gradient of hydrophilic-hydrophobic liquid phase (usually water mixed with methanol or acetonitrile) flows. For this to work together with MS the mobile phase from the LC is usually mildly acidic (the acid also improves chromatography by enhancing binding to the column material). The column containing the solid phase is selected depending on the analyte of interest and its properties. Since the different analytes in the mixture will have different affinities for the solid phase, their retention times will differ from one another and thus elute at different times, allowing compound separation. When a compound elutes from the column, it is sprayed into the mass spectrometer.

MS is an analytical method used to determine the mass-to-charge ratio (m/z) of charged species. This physical property allows determining the mass of compounds and (for smaller compounds) their elemental composition. MS has quantitative and qualitative applications, and can be used to identify atoms, molecules, and macromolecules, such as proteins. A mass spectrometer consists of three main elements: ion source, mass analyser (one or more) and detector. First, an ion source, which is the part of the MS capable of transferring the analyte into gas phase ions. The ions will then be transported into the second main component, the mass analyser, which in turn will separate the ions through an electric and/or magnetic field before detection. This way their mass-to-charge ratio will be recorded. Many mass spectrometers are equipped with devices that can fragment ions, *e.g.*, a collision chamber. As a result, fragment ion spectra can be created, so called tandem mass spectrometry or MS/MS, and used for protein identification through subsequent database searching. Different proteins can generate ions of same mass, but if these ions are fragmented, each ion will produce a unique fingerprint.

In Papers I, III and IV LC-MS was utilised. The mass spectrometer (Q Exactive, Thermo Fischer Scientific) had an electrospray ionisation (ESI) ion source, which generates ions by applying high voltage the inlet and the emitter from which the analytes are eluting. The mass analyser employed was a hybrid quadrupole-orbitrap. After the ions are formed in the ion source, ions are either acquired in a non-selective way, a so-called full scan, where a wide m/z range is allowed to pass through the quadrupole and into the orbitrap, or in a way where the quadrupole isolates selected precursor ions acting as a mass filter. These ions are then transferred to and accumulated in a collision cell, where they collide with a neutral gas allowing fragmentation (this is referred as collision-induced dissociation, CID). The resulting fragment ions are then analysed in the orbitrap. The orbitrap is an electrostatic ion trap that allows ions to oscillate around and along a central spindle. The frequency of the latter oscillation is inversely proportional to the square root of the ions' m/z. Detection is obtained through a so-called image current effect; ions that get close to a conducting surface produce an image current that can be amplified, thus allowing detection.

3.4 Single molecule array

Single molecule array or Simoa is a technology developed by Quanterix which shares the same principle as sandwich ELISA. While traditional ELISA requires large sample volumes and has a sensitivity ranging from picograms per millilitre (pg/mL) and above, Simoa on the other hand needs substantially smaller volumes and offers a sensitivity improvement of about 1000-fold (femtogram per millilitre, fg/mL) (*Fig. 14*).

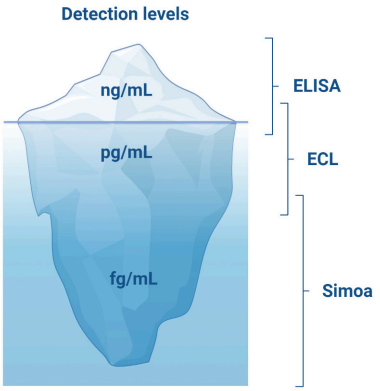


Figure 14. *Representation of the detection levels reached by different immunoassay platforms. Created with BioRender.com.*

Simoa is semi-automated, increasing the precision and consistency while minimizing the variability introduced by manual immunoassays. In Simoa immunoassays, the capturing antibody is conjugated with paramagnetic beads which are incubated with the sample. Since the number of beads added into the sample is much higher than the analyte, beads containing analyte follow a Poisson distribution, meaning that at low concentration of target, each bead will only have one immunocomplex on their surface. In the next step the beads are incubated with biotinylated detection antibody and subsequently B-D-galactopyranoside streptavidin (SBG), so that only the beads that have successfully formed an immunocomplex are able to bind the enzyme. Finally, beads are transferred into a microarray, where each bead fits into a single well. Here, the enzymatic substrate, resofurin B-D-galactopyranoside (RGP), will be added. Therefore, fluorescent will only be generated in wells containing the immunocomplex and hence the enzyme (*Fig. 15*).

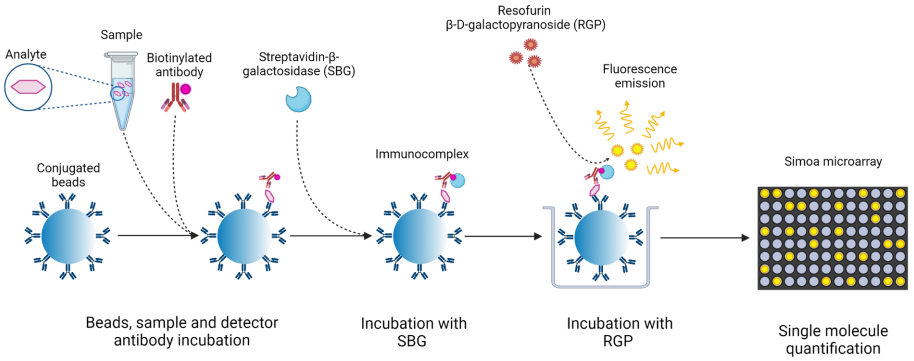


Figure 15. *Schematic representation of the main steps of Simoa immunoassays. First, conjugated beads are incubated with samples and detector, which is subsequently followed by the addition of SBG. After some washes, beads are transferred into a disc microarray in which each bead will be allocated into one single well. Finally, the addition of RGP will result in fluorescence emission by beads with immunocomplexes on their surface, which will be digitally quantified. Created with BioRender.com.*

Protein concentration is calculated by automatic counting the number of wells containing fluorescent beads (that is bead that had successfully form immunocomplexes with the analyte of interest) in relation to the total number of bead-containing wells. This results in digital measurements, in contrast with ELISA, which provides analogue signals. For high-concentration samples, Simoa can quantify in an analogue mode as well, giving the assay a wide dynamic range (*Fig. 16*). Taken altogether, Simoa technology provides a very high sensitivity, low background signal or noise, and robust degrees of reproducibility.

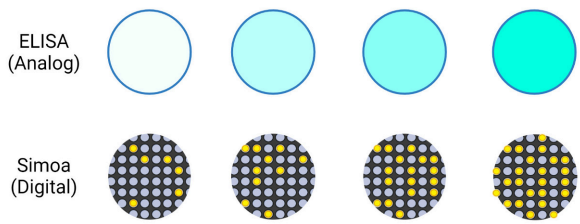


Figure 16. *Comparison between analog and digital quantification in ELISA and Simoa immunoassays respectively. Created with BioRender.com.*

3.4.1 Immunoassay development

Assay development is a set of tests and subsequent optimizations with the purpose of generating research or clinically relevant tools, specifically immunoassays, capable of measuring a target of interest. The following section will describe the main parameters that can be tuned to develop novel immunoassays when using a Simoa platform (note that several of these parameters can also be applied in other immunoassay platforms, such as ELISA or MSD)

3.4.1.1 Antibody selection

Antibody selection is the first step in immunoassay development. The main aspects to take into consideration when choosing antibodies are the following:

- Antibodies must be selected favouring those that display high specificity for the analyte of interest.

- Antibody cross reactivity should be explored. This is particularly important when using antibodies targeting specific PTM's, such as phosphorylation and truncation. This can be tested using dot blot, western blot and IP-MS, but also later on, exposing the novel assay to different analytes.

- Antibodies with high affinity should be used, such as those with equilibrium dissociation constant or K_D lower 10^{-9} M. Stronger binding results in higher signal.

- Monoclonal antibodies are preferable (both as capture and detector) when compared to polyclonal alternatives.

- If possible, selected antibodies should be tested using IP-MS in brain and CSF to demonstrate specificity in biological material. Alternative or complementary assessments may include IHC or western blot.

3.4.1.2 Antibody pair assessment

- Antibodies are to be tested in both directions (as detector and as capture). Some antibodies may perform very well as capture while others may not, and vice versa. This is rather unpredictable, although phosphorylation/truncation-specific antibodies tend to work better as capture, presumably because they introduce a stringent initial filtering on which molecules can bind the beads.

- All possible combinations of antibody pairs should be tested. It is recommended to test even antibodies that from a theoretical point of view may not appear as suitable candidates. This is because (i) less abundant species can be more disease-relevant, (ii) some epitopes might not be available due to analyte conformation or (iii) because there could be knowledge gaps affecting what is known about the sequence and/or structure of the targeted protein in the fluid of interest. Thus, it is encouraged to test as many antibody pairs as possible, even if some of the attempted combinations may seem counterintuitive.

3.4.1.3 Calibrator selection

The calibrator of choice will depend on the type of target of interest. Thus, is important to take into account how the target appears in the fluid of interest: as a full-length protein, as a fragment or as an oligomer. Some proteins, such as tau, are so widely studied that there are several commercial suppliers providing a wide range of recombinant products, including full-length protein (unmodified, phosphorylated, etc) and cleavage specific fragments. If not available, recombinant protein cloning and expression is a viable option, but this requires sequence verification, purification, and validations in order to ensure that the final product is indeed a reliable calibrator. Additionally, recombinant products can be modified *in vitro* if needed, as is commonly done with tau protein to get phosphorylated versions.

Once the calibrator is selected, it is important to evaluate its stability overtime at storage conditions (-80 C). Calibrator should be aliquoted into single use vials to minimize freeze thaw cycles. Different storage buffers should be evaluated to find an optimal buffer that allows the aliquots of calibrator to remain stable and reliable for long periods of time.

3.4.1.4 Bead Conjugation

Simoa is a paramagnetic bead-base immunoassay technology. Thus, bead conjugation, that is the linkage of antibodies to beads, represent one critical element on assay development. Naked beads, that is beads without capture antibody, are carboxylated on their surface. To crosslink the naked beads with the capture antibody, beads have to be "activated". Activation is achieved by incubating the naked bead in a solution containing 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride or EDC, which makes them reactive to the primary amine of the antibody.

Generally, a good starting point is conjugating beads using 0.2 µg/mL of antibody and using EDC at a concentration of 0.3 µg/mL. By measuring the percentage of free antibody before and after bead conjugation, one can get an estimation of the efficiency of the conjugation. However, this is also susceptible of optimization. Bead performance and thus assay performance can be improved by tuning these two parameters. Trying different combinations of both antibody and EDC concentration is therefore recommended to generate the beads with the best signal to noise ratio.

3.4.1.5 Biotinylation

In biochemistry, biotinylation refers to the covalent addition of a biotin (also known as vitamin B7) to another molecule, in our case to antibodies. Commercial sources of biotin are generally link to amine-reactive groups, such as *N*-hydroxysuccinimide ester (NHS) which specifically react with N-terminal and lysine amino groups of proteins forming amide bonds. In our case, the final purpose of linking biotin to antibodies is to bind streptavidin-horseradish peroxidase (HRP) complexes. Biotin displays an extremely high affinity and specificity for streptavidin, and this interaction can sustain high temperatures and extreme pH, making it very suitable for large variety of molecular biology techniques. Additionally, due to the small size of biotin, biotinylation does not interfere with the binding abilities of the antibody, which are preserved intact.

Similarly to bead conjugation, biotinylation of the detector antibody can be optimized in order to improve the immunoassay performance. A good

starting point for antibody biotinylation is an antibody concentration of 1µg/mL and a biotin excess of 40x. The biotin excess can be optimized, decreasing down to 20x in order to reduce background signal or increasing up to 60x if the assay signal to noise is to be increased.

3.4.1.6 Immunoassay definitions

Immunoassays definitions refers to the different incubation protocols and reagent volumes that characterize or define a specific assay. All the following parameters are susceptible of optimization and should be assessed in order to achieve the best performance:

- 2-steps or 3-steps: Simoa immunoassays can be run in 2-step or 3-step mode, in other words, co-incubation or stepwise incubation, respectively. 2-step mode allows the (i) co-incubation of beads, sample and detector, after which (ii) the beads are incubated with SBG. On the other hand, in a 3-step mode, (i) beads and sample are incubated together, followed by the (ii) addition of detector and finally by the (iii) incubation with SBG.

- Incubation times: the time length of all three aforementioned steps can be modified in order to identify the best incubation conditions. For 2-step, a good starting point is 47 min co-incubation of beads, sample and detector followed by a 7 min incubation with SBG. Similarly, a 3-step protocol can be started from a 40 min incubation of beads and sample, followed by a 7 min incubation with detector antibody and finally a 7 min incubation with SBG.

- Reagent volumes: the volume of beads, detector antibody and SBG are also important to optimize. A good reference to begin with when following a 2-step protocol is 25 µL of beads, 20 µL of detector antibody and 100 µL of SBG. In contrast, in a 3-step protocol, 25 µL of beads, 100 µL of detector antibody and 100ul of SBG can generally be used.

- Sample volume and dilution: the volume of plasma, serum, CSF or any other sample can also be modified. Simoa HDx systems provides a range of sample volumes that expands from a minimum of 20 µL to a maximum of 172 µL. Additionally, samples can be mixed with the assay diluent at different dilutions, depending on parameters such as the concentration of the analyte or matrix effects.

3.4.1.7 Reagents titration

This optimization refers to testing combinations of SBG and detector antibody at different concentrations, with the aim of reducing the background signal or noise (which is non-specific) and/or increase the AEB signals. A successful reagent titration experiment often results in a combination of both, which is expressed as signal to noise ratio. Some analytes are so abundant in the fluid of interest, that reagent titration is not necessary, however, several brain-derived molecules, mostly in blood, are present at such low levels that SBG and detector titration are key to achieve successful measurements in all samples. In general, higher concentrations of detector antibody and SBG result in higher signals, while lower concentrations yield lower signals. On the other hand, higher concentrations of detector antibody and SBG result in higher background noise, while lower concentrations yield lower background. Thus, in order effectively find the best signal to noise ratio, different combinations of detector antibody and SBG concentration should be tested.

3.4.1.8 Other optimizations

The buffer used for diluting samples and calibrators is a fundamental aspect of assay development, this having major implications in the overall assay performance. Thus, the optimization of the assay diluent should be investigated early on the development workflow. Some key components to optimize are the buffer pH, blockers (including both general blockers such as BSA and heterophilic blockers), detergents and salt content.

3.4.2 Immunoassay validation

Analytical validation refers to a battery of tests or experiments with the aim of evaluating the performance and quality of an immunoassay. Thus, the outcome of the analytical validation will determine if the assay under investigation fulfils the requirements for measuring the desired analyte in the

biofluid of interest. Validation should be performed immediately after assay development and always before running sample cohorts. It is important to take into consideration that analytical validation would always be more stringent for immunoassays destined for clinical use than those used for research purposes. All the immunoassays described in this thesis are for research purposes, and if eventually progress into clinical use, they will have to successfully undergo further validation. Commonly, analytical validation for research purposes includes the following tests: dilution linearity, spike recovery, quantification limits, precision and accuracy and antibody validation.

- Dilution linearity: the assay is run in a few samples, which are measured undiluted (neat) and also serially diluted following 2-fold series (1:2, 1:4, 1:8, 1:16, etc). This will determine matrix interference and if measurements performed at different dilutions are comparable once adjusted by the dilution factor.

- Spike recovery: two or more samples are analysed diluted 1:2 either unspiked or spiked with known concentrations of analyte. The expected concentration would be the concentration of the unspiked sample plus the concentration of the added spike. The percentage of spike recovery is calculated as the concentration of the spiked samples minus the concentration of unspiked sample, divided by the concentration of the added spike. Spike recovery will help to determine if the matrix is interfering with the measurements by hijacking or absorbing the spikes of analyte.

Quantification limits: limit of detection (LOD) and lower limit of quantification (LLOQ). LOD represents the lowest concentration of analyte that can reliably be distinguished from the blank and is determined by running 16 blanks and adding 3 standard deviations (SD) to the calculated mean value. LLOQ is the lowest concentration of analyte that can be reliable quantified with high precision and is determined by running 16 blanks and adding instead 10 SD to the calculated mean value.

Precision and accuracy: accuracy is how close a measurement is from the true value, while precision indicates how consistent are the different measurements. This can be determined using a few samples of known concentration, which are placed on a plate in replicates of 5, and each well is

run in duplicates. This is repeated during three days. Finally, the CV% of inter- and intra-assay precision and accuracies are calculated.

Antibody validation: the availability in our department of mass spectrometers allows us to verify the specificity of the antibodies of choice in high detail. We generally perform IP-MS in the biofluid of interest to confirm that the antibodies exclusively bind their respective target. However, this can also be assessed via western blot or dot blot, although these techniques do not provide an absolute confirmation such as MS.

4. RESULTS AND DISCUSSION

Note 1: the discussion of each paper uses the nomenclature used in the publication, both for participants groups and statistics.

Note 2: Fig. 17 depicts the immunoassays used in the included publications.

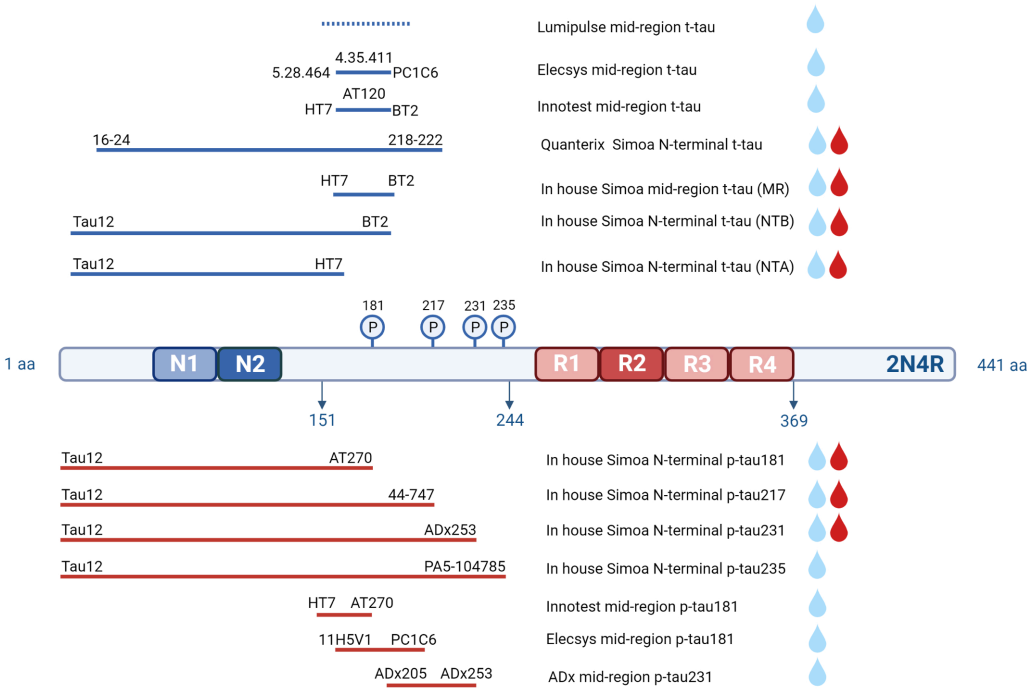


Figure 17. *Schematic representation of the immunoassays used in this thesis, including both in-house developed and commercially available. Minimum peptide length required for detection is represented with a blue/red line. Antibodies comprising each immunoassay are also indicated. Represented with a blue line on the top half, total-tau immunoassays. On the bottom half and with red lines, p-tau assays. Blue droplets indicate immunoassays working in CSF whereas red droplets indicate assays working in blood. Created with BioRender.com.*

4.1 Paper I

Rationale

In vivo detection of amyloid and tau pathology using CSF and PET biomarkers have proven to be highly accurate tools to detect AD [299, 301, 302]. However, both methods present certain drawbacks, such as invasiveness associated to lumbar puncture and radio ligands, the high economic costs or the need of specialized centres, which altogether hamper their broad implementation [430, 445]. The obvious alternative to these two methods would be blood biomarkers, which are generally characterized by cost-effectiveness, scalability, and lack of invasiveness. Therefore, a blood biomarker for AD would pose a great potential to become a widespread diagnostic test in clinical settings and a useful tool for clinical trial screening/recruitment. With this in mind, we developed an ultrasensitive immunoassay capable of measuring p-tau181 in blood, a phosphorylation site known to be highly specific for AD pathology in CSF and a clinically validated test for AD dementia [507]. Blood p-tau181 performance was evaluated in four independent cohorts: (i) the discovery cohort (AD=19, cognitively unimpaired (CU) elders=18), (ii) validation or TRIAD cohort (young adults 27, CU elders=113, MCI=45, AD=33, frontotemporal dementia (FTD)=8), (iii) BioFINDER-2 cohort (CU elders=337, MCI=191, AD=126, behavioural variant frontotemporal dementia (bvFTD)/primary progressive aphasia (PPA)=18, Parkinson's disease (PD)/multiple system atrophy (MSA)=36, vascular dementia (VaD)=12, PSP/CBS=21) and (iv) a primary care clinical cohort (young adults=11, CU elders=72, MCI 12, AD=10).

Results

In the discovery cohort, both plasma and serum p-tau181 were significantly increased in biologically defined AD cases when compared to neurological controls (both $p<0.0001$). Plasma and serum p-tau181 measurements displayed a strong correlation with each other ($r=0.8202$, $p<0.0001$). Similarly, both plasma and serum p-tau181 exhibited high performances for discriminating AD and controls (plasma AUC=90.06%, serum AUC=95.91%).

In the TRIAD cohort, plasma p-tau181 followed an increasing trajectory across the AD *continuum*. Plasma p-tau181 was increased in AD when compared to all other groups (all p<0.0001). No significant differences could be identified between Aβ+ MCI and Aβ- MCI, although it seemed slightly increased in MCI Aβ+ group. Interestingly, plasma p-tau181 was increased in Aβ+ CU group when compared with Aβ- CU (p<0.05), indicating that this biomarker increases during preclinical AD stages (*Fig. 18)*. Finally, we examined the performance of plasma p-tau181 discriminating AD group from all others. Plasma p-tau181 showed high performance in all scenarios, with AUC values ranging between 84.85% to 100%.

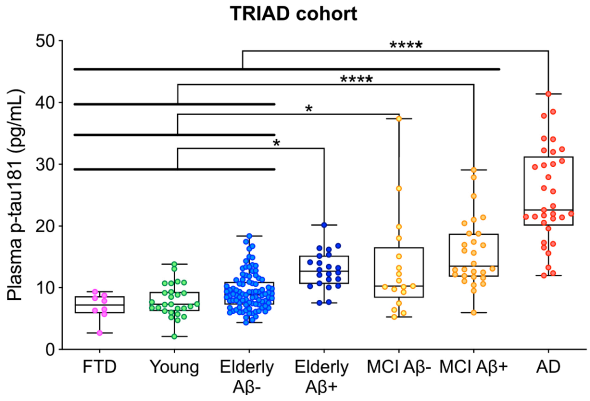


Figure 18. *Plasma p-tau181 levels in TRIAD cohort. Plasma p-tau181 is a highly specific biomarker of AD. Box-and-whisker plots showing the median and the 25th and 75th percentiles. P values are indicated using asterisks: *p<0.05, **p<0.01, *** p<0.001, ****p<0·0001. FTD (frontotemporal dementia), Aβ (amyloid β), MCI (mild cognitive impairment).*

In BioFINDER-2 cohort, plasma p-tau181 also followed an increasing trajectory across the AD *continuum*. Plasma p-tau181 was increased in AD when compared to all other groups (all p<0.001). Plasma p-tau181 was also increased in Aβ+ MCI and Aβ+ CU when compared to both Aβ- MCI and Aβ- CU cases (p<0.0001 and p<0.05 respectively). Additionally, plasma p-tau181 levels were increased in Aβ+ MCI when compared with all Aβ- non-AD groups, excepting bvFTD/PPA group (p<0.05) (*Fig. 19)*. We then investigated the performance of plasma p-tau181 when discriminating AD from groups not belonging to the AD *continuum*. Plasma p-tau181 demonstrated high

performance differentiating AD from Aβ- CU, Aβ- MCI and all individual Aβ- non-AD groups, (AUCs=81.90-92.13%).

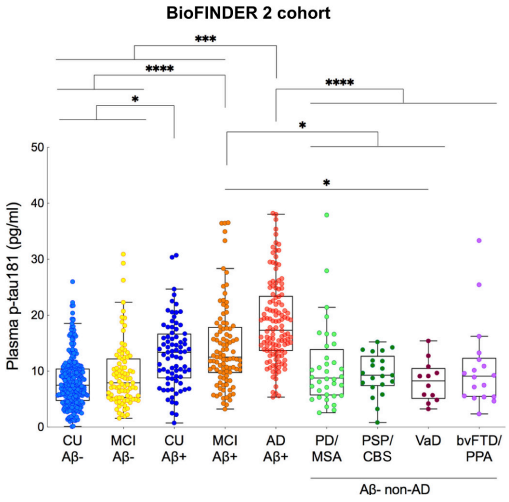


Figure 19. *Plasma p-tau181 levels in BioFINDER-2 cohort. Plasma p-tau181 is a highly specific biomarker of AD. Box-and-whisker plots showing the median and the 25th and 75th percentiles. P values are indicated using asterisks: *p<0.05, **p<0.01, *** p<0.001, ****p<0·0001. Aβ (amyloid β), CU (cognitive unimpaired), MCI (mild cognitive impairment), AD (Alzheimer's disease), PD (Parkinson's disease), MSA (), PSP (progressive supranuclear palsy), CBS (corticobasal syndrome), VaD (vascular dementia), bvFTD (behavioural variant frontotemporal dementia), PPA (primary progressive aphasia).*

In the primary care cohort, once again plasma p-tau181 followed an increasing trajectory across groups. Plasma p-tau181 was found to be increased in participants diagnosed with AD and MCI when compared with older and young adults (p<0.01 and p<0.001 respectively). ROC analysis showed that plasma had a high accuracy distinguishing AD from both older and young adults (AUCs=84.44-100%) but not from MCI cases (AUC=55%).

We examined the association of plasma p-tau181 with Aβ and tau PET in the TRIAD cohort. Plasma p-tau181 showed strong correlations with both Aβ (r=0.6341, p<0.0001) and tau PET Braak I-IV (r=0.5387, p<0.0001) in the whole sample. In Aβ+ cases, p-tau181 was correlated more strongly with tau PET in positive cases (r=0.6280, p<0.0001) compared to tau PET negative cases

(r=0.1636, p=0.0492) (*Fig. 20)*. ROC analysis showed that plasma p-tau181 accurately discriminated Aβ PET-negative from positive cases (AUC=88.09%). The same was true when differentiating tau PET-negative from positive cases (AUC=93.11%). We also examined the levels of plasma p-tau181 across the different Braak stages. Plasma p-tau181 was increased in Braak stages V/VI when compared with Braak stages 0, I/II and III/IV (p<0.001), an also in Braak stages III/IV when compared with Braak stages 0 and I/II (p<0.01). Moreover, plasma p-tau181 was able to discriminate Braak stage 0 Aβ- and Aβ+ (p<0.0001).

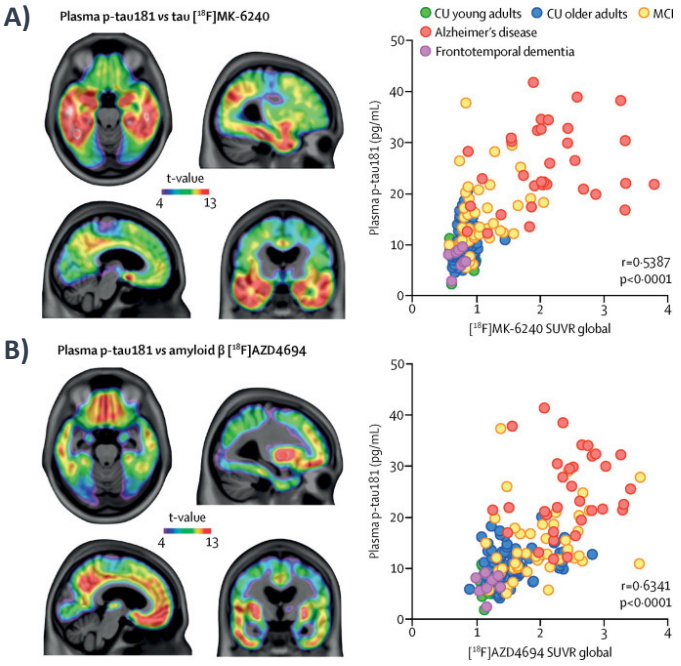


Figure 20. *Plasma p-tau181 association with tau PET ([18F]MK-6240 tau PET)* ***(A)*** *and amyloid PET ([18F]AZD4694 amyloid β PET)* ***(B)*** *in TRIAD cohort. Left graphs depicts the voxel-wise regressions, showing the strength of the association in a color scale from blue (low) to red (high). Right graphs show the Spearman's rank correlation (r) of plasma p-tau181 with the PET tracer in the whole cohort. CU (cognitive unimpaired), MCI (mild cognitive impairment), SUVR (standardized uptake value ratio).*

In the discovery cohort, plasma, and serum p-tau181 strongly correlated with Innotest CSF p-tau181 (r=0.7055, p<0.0001) and $A\beta_{1-42}$ (r=–0.5936, p<0.0001). Similarly, plasma p-tau181 correlated strongly with LUMIPULSE CSF p-tau181 (r=0.7937, p<0.0001) and $A\beta_{1-42}$ (r=–0.6830, p<0.0001) in TRIAD cohort.

We investigated the accuracy of plasma p-tau181 predicting AD diagnosis, elevated Aβ PET and tau PET, in comparison with age, *APOE* ε4 or both combined. Plasma p-tau181 was a superior predictor of AD diagnosis, Aβ PET and tau PET than age, APOE ε4 or both when considering the whole cohort. Additionally, plasma p-tau181 was a better predictor of AD diagnosis, elevated Aβ PET and tau PET than other plasma biomarkers, specifically $A\beta_{1-42}$, $A\beta_{1-42/1-40}$, t-tau and t-tau/$A\beta_{1-42}$.

Finally, we explored the association between plasma p-tau181 with cognitive decline and neurodegeneration in a subset of TRIAD cohort participants that underwent 1-year longitudinal follow-up (n=88). We demonstrated that plasma p-tau181 correlated with baseline (p<0.0001) and 1-year change (p=0.0015) in MMSE values. Similarly, plasma p-tau181 correlated with baseline (p<0.0001) and 1-year change (p=0.015) in grey matter density.

Discussion

The present studied described a MS verified immunoassay capable of measuring p-tau181 in both plasma and serum. We demonstrated that blood p-tau181 is a highly specific biomarker for AD pathology. In both TRIAD and BioFINDER-2 cohorts, plasma p-tau181 increased in a stepwise manner throughout the AD *continuum*. Furthermore, we showed that plasma could discriminate between Aβ- CU and Aβ+ CU, which indicates that plasma p-tau181 increases early within the AD *continuum* prior to symptom onset. This was further confirmed by the significantly higher levels of plasma p-tau181 found in Aβ PET-positive when compared with Aβ PET-negative at Braak 0. Furthermore, these results suggested a link between early Aβ accumulation in AD and plasma p-tau181 secretion prior to the emergence of abnormal tau PET values. Plasma p-tau181 was significantly increased in Aβ+ MCI when compared to Aβ- MCI and in AD when compared to non-AD, which demonstrates that this biomarker has a high performance identifying symptomatic AD.

Another relevant finding was that plasma p-tau181 showed strong correlations with both tau and Aβ PET. The correlation was slightly stronger with the latter, which further strengthened the idea of an Aβ-induced secretion of plasma p-tau181 (aligning well with the Amyloid Cascade Hypothesis [286]). Moreover, we demonstrated that plasma p-tau181 can predict both tau and Aβ PET in two different cohorts and using distinct PET tracers, which indicates that this novel biomarker could represent a non-invasive and easy to implement tool for tracking AD pathophysiology in brain. One interesting finding was that plasma p-tau181 increased very pronouncedly in both Aβ+ CU elders and Aβ+ MCI, which was parallel to increasing tau PET retention values. This however did not occur in AD cases, where the increase in tau PET retention was not followed by plasma p-tau181. This suggests that plasma p-tau181 reflects the overall phosphorylation state of tau in brain, but not tau aggregation.

We also showed that plasma p-tau181 measurements can predict cognitive decline and disease progression in AD, which could be very useful for disease monitoring both in clinical settings and in therapeutic trials. Finally, we demonstrated that plasma-tau181 has a better diagnostic performance than well stablished risk factors for AD (such as age and *APOE* ε4), and also other plasma biomarkers (such as $A\beta_{1\text{-}42}$, $A\beta_{1\text{-}42/40}$ and t-tau). This suggests that a simple plasma p-tau18 blood test could successfully assist the diagnosis of AD without the need of an extended battery of other analysis.

In conclusion, this blood p-tau181 assay has the potential to provide a first-in-line, cost-effective, non-invasive, and scalable test for AD diagnosis and monitoring that could greatly benefit clinical settings and therapeutic trials.

4.2 Paper II

Rationale

In several countries, the in vivo clinical diagnosis of suspected AD is greatly aided by neuropathologically validated CSF and PET biomarkers [283, 473]. Despite the high accuracy of these biomarkers, their broad use has been hindered by their cost and invasiveness, among other reasons [430, 445]. On the other hand, definitive diagnosis of AD still requires post-mortem confirmation of Aβ plaques and NFTs in brain tissue [187, 508]. Thus, a blood biomarker reflecting the ongoing brain pathology would have a major impact not only in clinical settings but also in drug development efforts. At the time of this study, available plasma p-tau181 results were very promising, but they were mainly obtained from research cohorts which were thoroughly characterised and stratified using CSF and PET biomarkers [509]. Therefore, the aim of this paper was the first report to validate plasma p-tau to neuropathological diagnosis of AD. This would add further utility and confirmation of its use in primary care settings in which no biomarker assessments are available. This was achieved by measuring plasma p-tau181 in a unique cohort comprised by 115 participants with longitudinal clinical evaluations and plasma collections at either 8, 4 or 2 years before post-mortem neuropathological assessment (*Fig. 21)*.

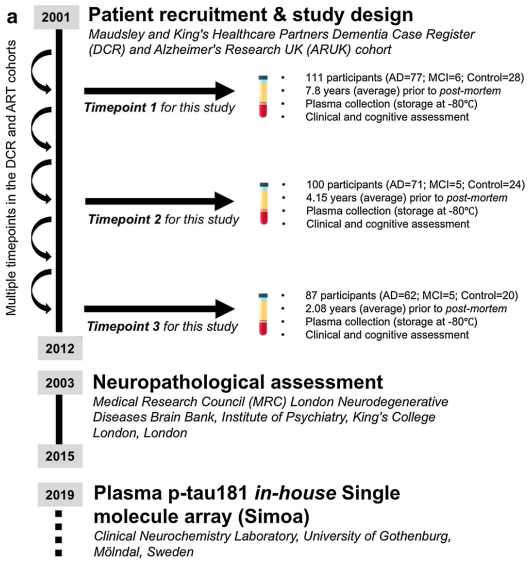


Figure 21. *Schematic representation of the study design. AD (Alzheimer's disease), MCI (mild cognitive impairment).*

Results

We first investigated the levels of plasma p-tau181 in the clinical diagnosis given by physicians without biomarker evidence. At all three timepoints, plasma p-tau181 was significantly increased in AD dementia cases when compared to CU cases (timepoint 1, $p=0.001$; timepoint 2, $p<0.0001$; timepoint 3, $p<0.0001$), however, we could not identify significant differences between AD dementia and MCI cases (*Fig. 22A*). We then investigated the levels of plasma p-tau181 replacing the clinical diagnosis at the time of plasma collection with neuropathological diagnosis at post-mortem (control, non-AD and AD pathology). Interestingly, from all the patients diagnosed with AD dementia by the clinicians, only 75% were confirmed to have AD pathology at post-mortem. A substantial number of AD dementia cases were given another pathological diagnosis and the plasma biomarker clearly confirmed this. Plasma p-tau181 was significantly increased in AD pathology when compared to both controls ($p<0.0001$) and non-AD pathology ($p<0.0001$) at all three

timepoints. No significant differences in plasma p-tau181 were found between controls and non-AD pathology at any timepoint (*Fig. 22B*).

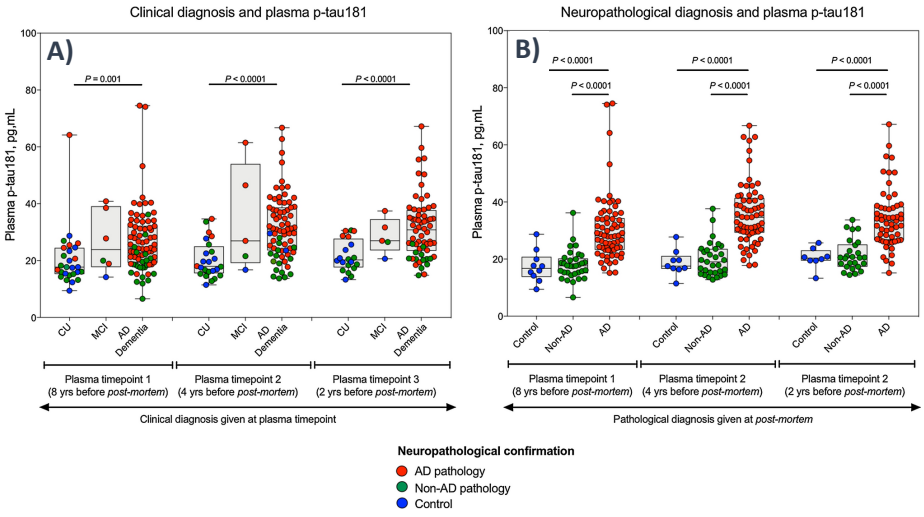


Figure 22. A) *Levels of plasma p-tau181 in participants stratified according to clinical diagnosis at all three timepoints.* ***B)*** *Levels of plasma p-tau181 at all three timepoints in participants stratified according to neuropathological diagnosis at post-mortem.*

We also investigated whether plasma p-tau181 changes in parallel to Braak stages. All samples were stratified based on their post-mortem Braak stage (I/II, III/IV, V/VI) and based on the three different timepoints. At each point, plasma p-tau181 was significantly increased in Braak V/VI when compared to Braak I/II (all, $p<0.0001$). At timepoint 1, that is 8 years prior post-mortem, plasma p-tau181 was increased in Braak V/VI when compared with Braak III/IV ($p=0.035$) but not between Braak I/II and Braak III-IV. At the following timepoints, specifically 4 and 2 years before post-mortem, plasma p-tau181 was increased between Braak I/II and Braak III/IV (timepoint 2, $p=0.006$; timepoint 3, $p=0.004$) but not between Braak III/IV and Braak V/VI.

AD pathology diagnosis group was comprised by four different diagnoses: AD, AD plus cerebral amyloid angiopathy (CAA), AD plus Lewy body and AD plus TDP43 pathology. On the other hand, non-AD pathology included Lewy body, CAA, frontotemporal lobar degeneration (FTLD), 4R tauopathy and VaD

pathologies. Thus, we used this more detailed pathological diagnosis to examine if plasma p-tau181 can indeed discriminate the eventual AD pathology from non-AD pathology. At each point, no significant differences were found between the different AD pathology groups. On the other hand, while no significant differences were observed among non-AD pathologies 8 years prior to post-mortem, increased levels of plasma p-tau181 were observed in Lewy Body group when compared to other non-AD pathologies at timepoints 2 (FTLD, p=0.017; 4R tauopathy, p=0.045) and 3 (FTLD, p=0.035).

We investigated the performance of plasma p-tau181 when discriminating AD "pure" pathology cases from non-AD pathology years before definitive diagnosis at post-mortem. Plasma p-tau181 discriminate AD pathology from non-AD pathology (AUC=97.4%) and controls (AUC=92.2%) with accuracy 8 years before post-mortem. Plasma p-tau181 performed similarly when discriminating AD mixed pathologies from non-AD pathologies (AUC=90.1%) and control (AUC=84.1%) group at timepoint 1. Finally, we observed that plasma p-tau181 could not discriminate between AD pathology and mixed AD pathology (AUC=57.3%), which highlights that the performance of this biomarker identifying AD pathology is not influenced by concomitant pathologies.

Finally, we investigated the longitudinal trajectories of plasma p-tau181 in the different pathological groups. A statistically significant increase in plasma p-tau181 levels was observed between timepoints 1 and 2 for AD pathology ($p<0.0001$) and AD mixed pathology (p=0.022). No significant differences between timepoints 2 and 3 could be found for these pathological groups, and in fact, plasma p-tau181 in both AD pathology and mixed AD pathology seemed to follow a decreasing trajectory. Similarly, we examined the longitudinal changes in plasma p-tau181 in control and non-AD cases. Significant differences could only be observed between timepoints 2 and 3 for both groups (control, p=0.049; non-AD, $p<0.002$), but overall, they appeared to follow a subtle increasing trajectory (*Fig. 23)*.

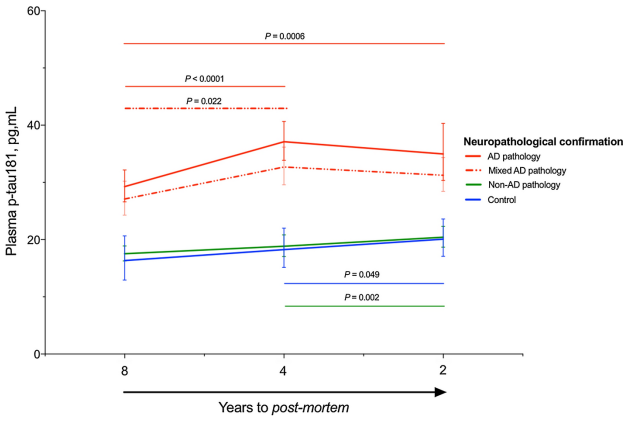


Figure 23. *Longitudinal trajectories of plasma p-tau181 at 8, 4 and 2 years before post-mortem confirmation. AD (Alzheimer's disease).*

Discussion

At the time of the study, this was the first report of plasma p-tau181 in a longitudinal cohort with neuropathological confirmation of the diagnosis. This unique cohort also included the clinical diagnosis, which was determined through clinical assessments and without the assistance of validated CSF or PET biomarkers. Therefore, it provided an ideal scenario to investigate possible discordances between the clinician's diagnosis (in a context of biomarker unavailability) and neuropathology. A clinician's assessment, in the absence of CSF and PET, is the most likely and realistic scenario in primary care, thus adds great validity to the use of plasma biomarkers. Moreover, it allowed to study if plasma p-tau181 levels, measured several years before death, can predict the eventual neuropathological diagnosis and their longitudinal trajectories

We compared plasma p-tau181 levels across all timepoints using two different stratifications: clinical diagnosis (CU, MCI and AD dementia) and pathological diagnosis (control, non-AD and AD). We coloured coded the individual data points in the clinical diagnosis graph with their pathological diagnosis to visualize the discordances between both. Interestingly, several clinical misdiagnoses can be observed across all groups and at all three timepoints. In fact, only 75% of all the reported AD dementia cases were

eventually diagnosed with AD pathology after post-mortem examination. On the other hand, when plasma p-tau181 measurements were stratified across neuropathology confirmed groups, there was very little overlap between the different post-mortem diagnosis, which was reflected in high accuracies to discriminate AD pathology from both controls and non-AD pathology using a blood biomarker. Notably, 8 years before death, plasma p-tau181 exhibited nearly perfect AUC values when discriminating AD pathology from non-AD pathologies (97.4%). These results highlight that regardless of the clinical presentation, plasma p-tau181 is a highly specific biomarker of AD pathology and was not susceptible to the presence of other co-pathologies. Altogether, these results strengthen the idea of using plasma p-tau181 as a first-in-line, cost-effective, accessible, and easy to implement test in primary care settings or non-specialized centres, where CSF and PET biomarker are not available. We also demonstrated that plasma p-tau181 levels associated well with the extend of NFTs deposition. High levels of plasma p-tau181 were found in individuals with late Braak stages. Braak III/IV individuals followed an increasing trajectory across timepoints, while Braak V/VI increased and finally reach a plateau. This contrasted with participants at Braak stage I-II, whose levels remained low and unchanged. This may indicate that plasma p-tau181 usefulness as a predictor of tau pathology burden might be only effective at early stages, when the biomarker follows an increasing trajectory.

Due to the longitudinal nature of the cohort studied, we were able to represent the trajectories of plasma p-tau181 across pathological groups. Both in AD and mixed AD pathology groups, plasma p-tau181 only increased between 8 and 4 years prior to death, after which, the biomarker reached a plateau, perhaps even followed a decreasing trend. These results align well with previous studies reporting a decrease in p-tau181 values in dominantly inherited AD CSF, which might be attributed to a slowdown in the degeneration process, presumably due to high levels of neuronal loss.

In conclusion, these results support the specificity of p-tau181 as a biomarker of AD in blood, even years before neuropathological diagnosis. Most importantly, they further consolidate the idea of plasma p-tau181 as a cost-effective and easy to implement tool for clinicians to determine the underlying cerebral pathology.

4.3 Paper III

Rationale

The first immunoassay capable of measuring p-tau in CSF was an ELISA method directed against phosphorylated threonine 181 and 231 [451]. This was followed by novel immunoassays targeting the individual phosphorylations [463, 464]. Soon after, studies reported that the diagnostic performance of p-tau231 was no different from that of p-tau181 [466]. The belief that both biomarkers offered the same diagnostic performance, together with the clinical validation of CSF p-tau181 as an AD biomarker, resulted on CSF p-tau231 been substantially less studied when compared to CSF p-tau181. However, CSF p-tau231 has been still widely reported as a highly specific AD biomarker [477-480]. In 2020, our group was able to demonstrate that p-tau231 is the earliest emerging p-tau specie in CSF during preclinical AD stages – a critical development given the recent advancements in anti-Aβ therapeutics [469]. This very relevant and distinct characteristic encouraged the development of the first blood p-tau231 assay, and its biomarker potential was investigated in this publication. The novel blood assay was evaluated in four different cohorts: (i) a discovery cohort (CU elders=18, AD=20), (ii) a validation or TRIAD cohort (young adults=32, CU elders=159, MCI=54, AD=42, Non-AD=26), (iii) a primary care cohort (young adults=8, CU elder=131, MCI=17, AD=34) and (iv) a neuropathology confirmed cohort (AD=36, non-AD=11).

Results

We first examined the performance of plasma p-tau231 in the discovery cohort. Here, plasma p-tau231 was significantly increased in biologically defined AD cases when compared to neurological controls ($p<0.0001$), and showed a high accuracy diagnosing AD (AUC=0.9471).

In the TRIAD cohort, plasma p-tau231 was highly increased in AD group when compared to all other groups (p<0.0001) (*Fig. 24*). Plasma p-tau231 displayed similar performance discriminating AD from non-AD Aβ- groups (AUC=0.93) when compared with plasma p-tau181 (AUC=0.94). Plasma p-tau231 was also significantly increased in MCI Aβ+ and CU Aβ+ groups when compared to all other Aβ- groups (p<0.0001) (*Fig. 24*). Plasma p-tau231 display high accuracies discriminating MCI Aβ+ and MCI Aβ- (AUC=80%) and between CU Aβ+ and CU Aβ- (AUC=83%) (similar discriminating accuracy in terms of AUC values than plasma p-tau181: $AUC_{MCIA\beta+vsMCIA\beta-}$=75%, $AUC_{CUA\beta+vsCUA\beta-}$=77%). Interestingly, there were no significant differences between MCI Aβ+ and CU Aβ+, which evidenced the prominent emergence of this biomarker in plasma during preclinical AD stages (*Fig. 24*). In terms of correlation with other plasma biomarker measurements, p-tau231 displayed strong and moderate correlations with both p-tau181 (r=0.654, p<0.0001) and NfL (r=0.414, p<0.0001), respectively.

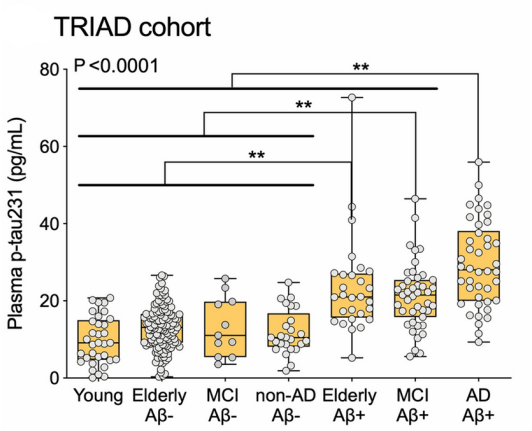


Figure 24. *Plasma p-tau231 levels in TRIAD cohort. Plasma p-tau231 is a highly specific biomarker of AD, increased across the AD continuum. Box-and-whisker plots showing the median and the 25th and 75th percentiles. P values are indicated using asterisks: **p<0.05. Aβ (amyloid β), MCI (mild cognitive impairment), AD (Alzheimer's disease).*

In the primary care cohort, plasma p-tau231 increased across diagnostic groups. Plasma p-tau231 was increased in AD dementia when compared to CU elderly and young groups (p<0.0001) ($AUC_{CU\ elders}$=0.75, AUC_{young}=0.91). In

contrast, plasma p-tau231 was exclusively increased in MCI when compared with CU young participants (p<0.0001).

We also examined plasma p-tau231 levels in a neuropathologically confirmed cohort. Plasma p-tau231 was highly increased in AD pathology when compared to non-AD pathology group (p<0.0001) (*Fig. 25*), and displayed a nearly perfect accuracy discriminating both groups (AUC=99.7%). In this scenario, plasma p-tau231 and plasma p-tau181 exhibited similar performance, although plasma p-tau231 showed a higher AUC value (AUC_{231}=99.7%, AUC_{181}=92.93%) but was not significantly superior. We then stratified the participants into Braak stages. Both plasma p-tau231 and p-tau181 showed a gradual increase throughout the different Braak stages, however, a stepwise increase was more pronounced for plasma p-tau231. When examining in more detailed how these two biomarkers increase across Braak stages, we found that plasma p-tau231 displayed higher AUC values when discriminating Braak I/II from Braak III/IV (AUC=75%) and Braak V/VI (AUC=99%) than plasma p-tau181 ($AUC_{I/II\ vs\ III/IV}$=52%, $AUC_{I/II\ vs\ V/VI}$=84%).

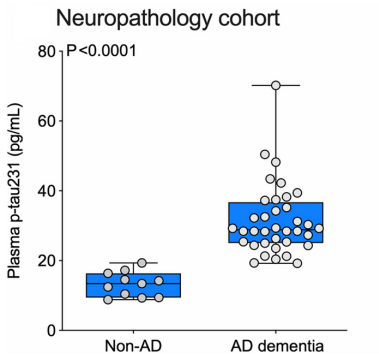


Figure 25. *Plasma p-tau231 levels in neuropathology cohort. Plasma p-tau231 is highly increased in neuropathologically confirmed AD participants when compared to those with non-AD definitive diagnosis. Box-and-whisker plots showing the median and the 25th and 75th percentiles. P values are indicated using asterisks: **p<0.05. AD (Alzheimer's disease).*

We also investigated the associations between plasma p-tau231 with both CSF and PET biomarkers. Plasma p-tau231 demonstrated strong correlations with CSF p-tau231 (r=0.5912, p<0.0001) (*Fig. 26B*) and $A\beta_{1-42}$ (r=-04044, p<0.0001) (*Fig. 26D*), and also with tau PET (r=0.5233, p<0.0001) (*Fig. 26A*) and Aβ PET (r=0.6234, p<0.0001) (*Fig. 26C*). The regional associations of plasma p-tau231 and Aβ PET were refined to earliest changes of Aβ in the medial orbitofrontal, precuneus and posterior cingulate.

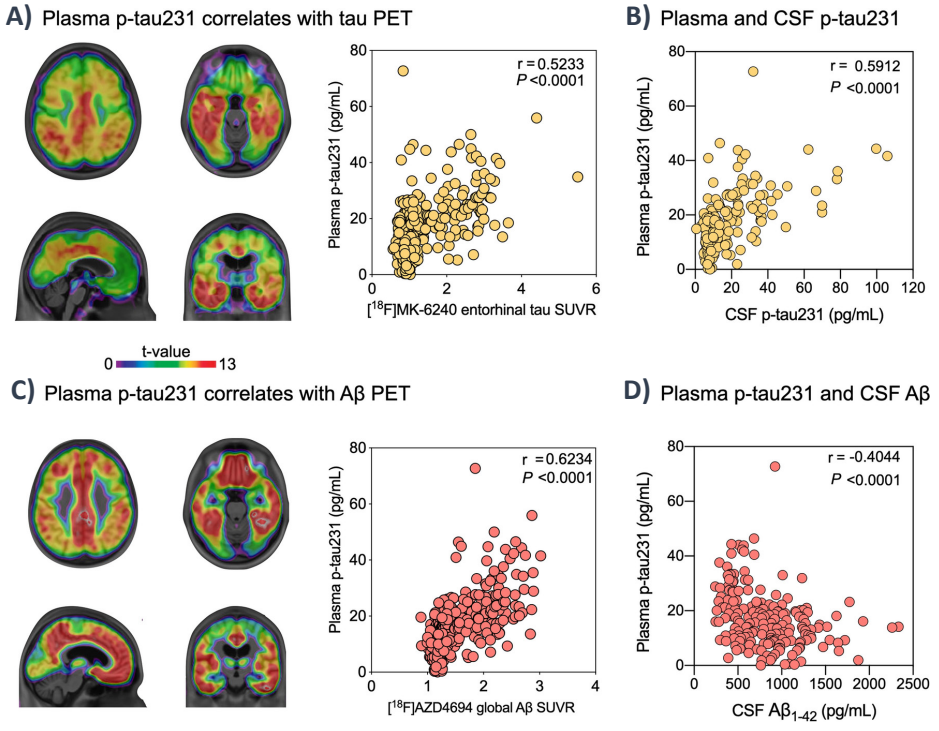


Figure 26. A) *Plasma p-tau231 association with tau PET ([^{18}F]MK-6240 tau PET).* ***B)*** *Correlation between plasma and CSF p-tau231.* ***C)*** *Plasma p-tau231 association with amyloid PET ([^{18}F]AZD4694 amyloid β PET).* ***D)*** *Correlation between plasma p-tau231 and CSF $A\beta_{1-42}$. Brain maps depict the voxel-wise regressions, showing the strength of the association in a color scale from blue (low) to red (strong). Scatterplots show Spearman's rank correlations (r) in in the whole cohort. Aβ (amyloid β), SUVR (standardized uptake value ratio).*

As previously mentioned, plasma p-tau231 was able to discriminate CU Aβ- from CU Aβ+ participants with higher numerical accuracy than that of plasma

p-tau181. In a weighted regression model using biomarkers increases as a function of Aβ burden in centiloids, plasma p-tau231 increased before plasma p-tau181 and before the threshold of Aβ PET positivity was reached. To further examining the increases in biomarkers across Aβ PET, this continuous variable was divided into four quartiles (Q). Plasma p-tau231 showed a stepwise and significant increase from Q1 to Q2, from Q2 to Q3 and from Q3 to Q4 (all $p<0.05$). On the other hand, plasma p-tau181 only increase from Q3 to Q4 ($p<0.05$), whereas CSF p-tau217 did between Q2 to Q3 and from Q3 to Q4 (both $p<0.05$). Lastly, levels of plasma p-tau231 and p-tau181 were assessed in TRIAD participants when stratified according to their Braak stages. While plasma p-tau231 increased in a stepwise manner across the different stages, increases in plasma p-tau181 only occurred among late Braak stages III/IV and V/VI.

Finally, we investigated whether plasma p-tau231 can predict AD progression. Using a subset of participants from TRIAD cohort which underwent 1-year follow up, we demonstrated that plasma p-tau231 predicts longitudinal changes in both cognitive decline ($p=0.0321$) and neurodegeneration ($p=0.0196$), similarly to plasma p-tau181 ($p=0.0452$ and $p=0.0008$ respectively).

Discussion

This is the first study reporting p-tau231 measurements in blood for AD, where we performed an extensive characterization of this novel biomarker in various scenarios, including the AD *continuum* and other neurodegenerative diseases, neuropathologically confirmed AD and non-AD cases, and in a primary care cohort.

In a similar manner to p-tau181, we show that plasma p-tau231 is highly specific biomarker for AD pathology, with high diagnostic accuracy in discriminating AD from non-AD and Aβ+ from Aβ- individuals at different stages of the AD *continuum*. Furthermore, plasma p-tau231 showed a nearly perfect accuracy discriminating neuropathologically confirmed AD from non-AD cases, which highlights the strong specificity of this blood biomarker for AD pathology and its clinical utility.

As previously mentioned, the development of this blood biomarker was motivated by our previous results measuring p-tau231 in CSF. Firstly, in a cohort comprised exclusively by CU individuals including preclinical AD participants (ALFA+), we showed that p-tau231 abnormally emerges earlier that p-tau217 and p-tau181 in CSF, when only very subtle changes in $A\beta_{42/40}$ ratio were detectable (and prior to Aβ PET positivity threshold was crossed) [469]. We further corroborated these findings showing that among these three CSF biomarkers, p-tau231 is the one displaying stronger association with amyloid deposition in Aβ PET-negative individuals [482]. Thus, we hypothesized that these CSF results would be translatable to plasma. Here, we were able to demonstrate that plasma p-tau231 starts increasing prior to Aβ PET positivity threshold is reached in dementia-free individuals, and that this increase preceded that of plasma p-tau181. Notably, we showed that plasma p-tau231 increases in a stepwise manner across Aβ PET quartiles, which was not true for neither plasma p-tau181 nor CSF p-tau217. The fact that plasma p-tau231 outperformed CSF p-tau217 is particularly relevant, as CSF biomarkers usually perform better that their blood counterparts. These findings have major implications for clinical trials. An *in vitro* study published in 2020 showed that targeting Aβ seeds prior to overt amyloid deposition can inhibit Aβ plaque formation [510]. Thus, this study indicates that Aβ-directed therapies might have a better window of success at pre-amyloid phases during preclinical stage. In this context, plasma p-tau231 could prove a very useful biomarker, as it can identify early stages of Aβ pathology prior to Aβ PET positivity threshold is crossed, a scenario in which novel therapeutic compounds would likely stand a better chance to tackle AD as the pathology is not yet widespread.

Plasma p-tau231 also exhibited strong association with tau PET in all regions, being the strongest in the entorhinal cortex, which is known to be one of the earliest regions of tau deposition in AD. Plasma p-tau231 increased in a stepwise manner across Braak stages both when defined by tau PET and by post-mortem assessment. This was not the case for plasma p-tau181, which struggled to differentiate the early stages. Thus, this indicates that plasma p-tau231 could act as an early biomarker of abnormal tau deposition.

In conclusion, plasma p-tau231 appears to be an excellent biomarker of AD pathology, which could prove useful in primary care settings as a first-in-line test together with clinical assessments. Moreover, the remarkably early

emergence of this biomarker in preclinical AD even prior to Aβ PET positivity, highlights the potential of plasma p-tau231 in therapeutic trials, which usually aim their intervention at the earliest possible stages.

4.4 Paper IV

Rationale

AD is characterized by a long asymptomatic phase, which can last up to twenty years and precedes symptom onset. During this preclinical stage, several biomarkers have been shown to display abnormal levels. In 2020, our group investigated the levels of CSF p-tau181, p-tau217 and p-tau231 in a well characterized preclinical AD cohort (ALFA+). Using Aβ PET as proxy of disease progression, we concluded that CSF p-tau231 is the first emerging tau biomarker, followed by CSF p-tau217 and p-tau181 [469]. Although the emergence of these biomarkers might follow a temporal course, it is unclear whether this reflects the actual progression of tau-related pathophysiology in brain. Therefore, despite the major success of these biomarkers in terms of early and accurate detection of AD, new biomarkers capable of reflecting a change or shift of pathophysiological brain process in CSF, such as the sequential phosphorylation of specific amino acid clusters in tau protein [511], would pose a unique opportunity of staging preclinical AD. In this paper, we combined exploratory and targeted phosphoproteomic MS approaches to identify novel p-tau residues with biomarker potential using neuropathologically confirmed brain tissue from control (n=10) and AD cases (n=10). Finally, we translated our brain findings into CSF using an in-house developed Simoa assay targeting p-tau235 and measuring this biomarker in three independent cohorts: (i) discovery cohort (AD=19, controls=21), (ii) validation or TRIAD cohort (CU elders=82, MCI Aβ+ or MCI+ =20, AD=20, non-AD=19) and (iii) a preclinical AD cohort or ALFA+ (A: Aβ pathology, T: tau pathology; A-T- =248, A+T- =104, A+T+ =31).

Results

To investigate novel p-tau residues that could be use as biomarkers, we performed an IP-MS analysis on TBS-soluble fractions of neuropathologically confirmed AD and control cases. For this purpose, we selected HT7 antibody (epitope aa159–163, 2N4R numbering), as this antibody is capable of capturing all 6 tau isoforms and does not discriminate between phosphorylated forms. We started by performing a semi-quantitative

exploratory IP-MS in AD and control TBS pools, where we identified p-tau181, p-tau217, p-tau231 and p-tau235 as the most prominent p-tau residues in AD brain. Interesting, the p-tau species with the highest relative abundance was the double phosphorylation p-tau(231+235). We also observed that p-tau235 was only present together with p-tau231, while p-tau231 was present also without p-tau235 (*Fig. 27A*). We subsequently developed a targeted IP-MS method for p-tau231 and double phosphorylated p-tau(231+235), and applied it on individual TBS fractions. We found that the double-phosphorylated p-tau(231+235), in contrast to single-phosphorylated p-tau231, was highly increased in AD cases when compared to controls (p-tau(231+235): p<0.0001) (*Fig. 27B-C*). Once again, single-phosphorylated p-tau235 could not be found, further corroborating that this p-tau epitope does only appear together with p-tau231, which suggested sequential phosphorylation events involving both residues.

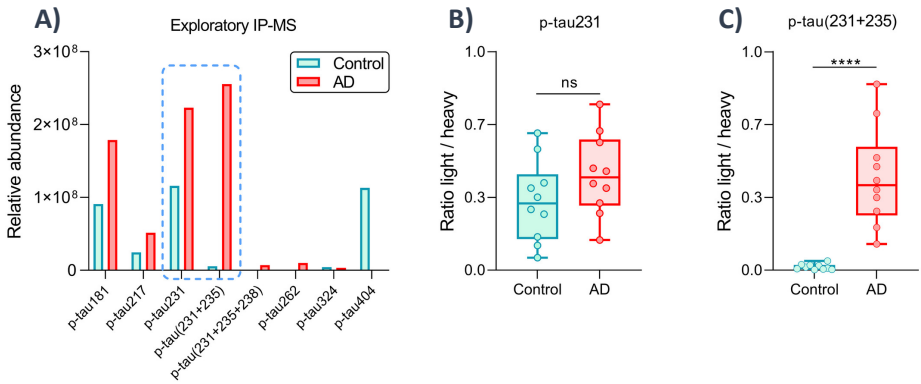


Figure 27. *Exploratory and targeted IP-MS in TBS-soluble brain fractions.* ***A)*** *P-tau(231+235) it's a very prominent tau specie in AD. Bar chart showing the relative abundance of identified p-tau species in control and AD TBS pools.* ***B)*** *Single-phosphorylated p-tau231 is not increased in AD brain. Box-and-whisker plots showing the mono-phosphorylated p-tau231 using targeted IP-MS.* ***C)*** *P-tau(231+235) is highly increased in AD brain. Box-and-whisker plots showing the double-phosphorylated p-tau(231+235) using targeted IP-MS. Box-and-whisker plots show the median and the 25th and 75th percentiles. P values are indicated using asterisks: ****p<0.0001.*

These results encouraged the development of an ultrasensitive immunoassay capable of measuring specifically p-tau235 in CSF. Once developed and validated, we implemented our novel CSF p-tau235 immunoassay on a small pilot cohort comprised by biologically defined AD cases and neurological

controls. We found that CSF p-tau235 was highly increased in AD when compared to control cases (p<0.0001, AUC=96%).

We then further investigated p-tau235 levels in CSF. In a research cohort including participants across the AD *continuum* and including also control and non-AD cases. We further confirmed that CSF p-tau235 is a highly specific biomarker for AD (p<0.0001 with all groups except with MCI+, where no differences were found), and most importantly, that CSF p-tau235 starts increasing early in preclinical AD stages (CU- vs CU+: p<0.05) (*Fig. 28*). CSF p-tau235 also showed excellent discriminatory accuracies between groups ($AUC_{AD\ vs\ Non\text{-}AD}$=0.99, $AUC_{MCI\ vs\ Non\text{-}AD}$=0.96, $AUC_{AD\ vs\ CU\text{-}}$=0.97, $AUC_{MCI+\ vs\ CU\text{-}}$=0.96, $AUC_{AD\ vs\ CU+}$=0.86, $AUC_{MCI+\ vs\ CU+}$=0.84). To contextualize the performance of CSF p-tau235 with other p-tau species, namely CSF p-tau217 and p-tau231, we compared their performance in discriminating cognitively unimpaired Aβ- and Aβ+ cases (based on CSF $Aβ_{1\text{-}42/40}$). While CSF p-tau235 had a lower performance than CSF p-tau231 in this scenario (AUC_{235}=73%, AUC_{231}=88%; $DeLong_{231–235}$ p=0.0004), no significant differences could be stablished with CSF p-tau217 (AUC_{217}=0.81; $DeLong_{217–235}$ p=0.084). We then compared the performance of all three biomarkers when discriminating cognitively impaired Aβ- and Aβ+ cases. CSF p-tau235 displayed the same performance as CSF p-tau217 and p-tau231 in this context (AUC_{235}=0.98, AUC_{217}=1.00, AUC_{231}=0.98, $DeLong_{217–235}$ p=0.12, $DeLong_{231–235}$ p=0.66). Finally we examined the correlation between CSF p-tau235 with CSF p-tau217 and p-tau231, and with Aβ and tau PET. CSF p-tau235 showed strong correlations with both CSF p-tau217 (r_S=0.87, p<0.0001) and p-tau231 (r_S=0.89, p<0.0001). Similarly, CSF p-tau235 exhibit high correlation with both Aβ (r_S=0.66 p<0.0001) and tau PET (r_S=0.66, p<0.0001).

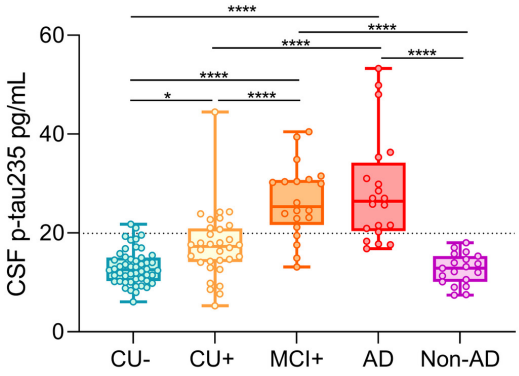


Figure 28. *CSF p-tau235 levels across the AD continuum in TRIAD cohort. Box-and-whisker plot showing the median and the 25th and 75th percentiles. P values are indicated using asterisk: *p < 0.05, ****p < 0.0001. Dash line indicates the cut-off value for CSF p-tau235 positivity calculated as the mean ± 2 SD of the A−T− group in ALFA+ cohort (19.92 pg/mL).*

One of the main findings in the TRIAD cohort was the increased of CSF p-tau235 in preclinical AD cases. Thus, we decided to further investigate CSF p-tau235, this time in a cohort exclusively comprised by cognitively unimpaired cases, stratified in three groups based on the presence of Aβ pathology (A+, CSF $A\beta_{1\text{-}42/40}$) and tau pathology (T+, CSF p-tau181): A-T-, A+T- and A+T+. Our results in ALFA+ cohort indicated that CSF p-tau235 increases very early during preclinical AD stage, when only subtle abnormalities in CSF $A\beta_{1\text{-}42/40}$ are detectable (A-T- vs A+T-: p<0.0001) (*Fig. 29*). As previously shown in TRIAD, we wanted to compare the performance of CSF p-tau235 in preclinical AD with CSF p-tau217 and p-tau231. CSF p-tau235 had a lower performance than both CSF p-tau217 and p-tau231 when discriminating A-T- cases from A+T- cases (AUC_{235}=0.64, AUC_{217}=0.75, AUC_{231}=0.78; $DeLong_{217–235}$ p=0.0001, $DeLong_{231–235}$ p=0.0001). However, CSF p-tau235 displayed same performance as CSF p-tau217 and p-tau231 when distinguishing A-T- from A+T+ (AUC_{235}=0.98, AUC_{217}=0.97, AUC_{231}=0.99, $DeLong_{217–235}$ p=0.33, $DeLong_{231–235}$ p=0.073). Notably, CSF p-tau235 outperformed CSF p-tau217 when discriminating early and late preclinical AD stages, that is A+T- and A+T+ (AUC_{235}=0.95, AUC_{217}=0.89, $DeLong_{217–235}$ p=0.021) and matched CSF p-tau231 (AUC_{231}=0.93, $DeLong_{231–235}$ p=0.18). In this cohort, CSF p-tau235 showed stronger associations with CSF p-tau231 (r_S=0.80, p<0.0001) than with CSF p-

tau217 (r_S=0.67, p<0.0001). On the other hand, CSF p-tau235 displayed a weak correlation with Aβ PET in this cohort (r_S=0.32, p<0.0001).

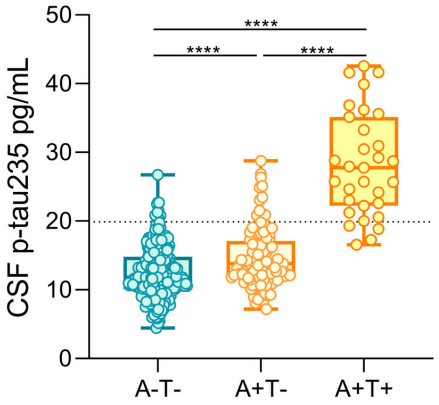


***Figure 29**. CSF p-tau235 levels across preclinical AD in ALFA+ cohort. Box-and-whisker plot showing the median and the 25th and 75th percentiles. P values are indicated using asterisk: ****p < 0.0001. Dash line indicates the cut-off value for CSF p-tau235 positivity calculated as the mean ± 2 SD of the A−T− group (19.92 pg/mL).*

We also modelled the trajectories of the different tau biomarkers in preclinical AD using Aβ PET as a proxy of disease progression. CSF p-tau231 was the first tau biomarker reaching abnormal levels, followed by CSF p-tau217 and CSF p-tau235 (*Fig. 30*).

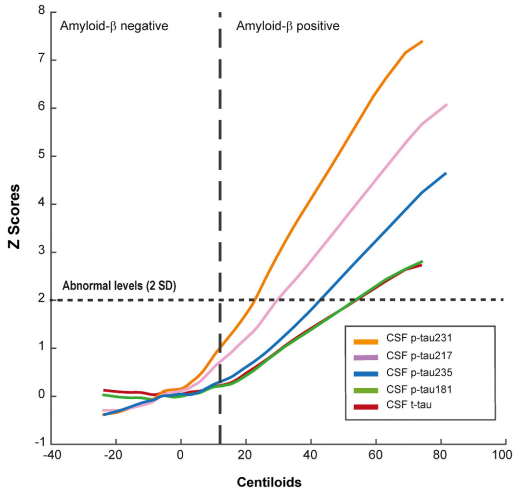
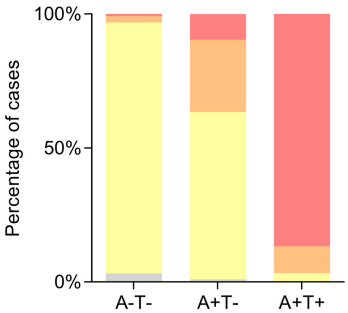


Figure 30. *CSF tau biomarker trajectories in preclinical AD (ALFA+ cohort). Trajectories were modelled using a local weighted regression method. Biomarker changes are displayed as z-scores using Aβ PET as a proxy of disease progression. Cutoff for biomarker abnormality was stablished as levels higher than two SD of the mean. Aβ PET positivity cutoff was stablished as centiloids (CT) values higher than CL 12.*

Finally, we investigated if the sequential phosphorylation observed in brain by others and us involving p-tau231 and p-tau235 is translatable into CSF in preclinical AD, as this could have interesting staging implications. Using two different cut-off methods, we determined negative and positive biomarker values for both CSF p-tau biomarkers. Our results show indeed high concordance with the sequential phosphorylation hypothesis. In A-T- cases, almost all participants were negative for both biomarkers ([231-/-235]=93.57%), whereas in A+T- cases, the sequential increase on the biomarkers started to begin ([231-/-235]=62.50%, [231+/-235]=26.92%, [231+/+235]=9.62%). In A+T+, this transition was almost completed, with almost all cases being double-positive for p-tau231 and p-tau235 ([231+/+235]=86.67%). Moreover, discordant cases with the sequential phosphorylation hypothesis, that is single-positive p-tau235 cases, were almost non-existing in the whole cohort ([231-/+235]=2.35%) (*Fig. 31*).



	A-T-	A+T-	A+T+	Total
[231+/+235]	0.80 (2)	9.62 (10)	86.67 (26)	9.92 (38)
[231+/-235]	2.41 (6)	26.92 (28)	10.00 (3)	9.66 (37)
[231-/-235]	93.57 (233)	62.50 (65)	3.33 (1)	78.07 (299)
[231-/+235]	3.21 (8)	0.96 (1)	0.00 (0)	2.35 (9)

Figure 31. *Sequential phosphorylation of p-tau231 and p-tau235 in CSF during preclinical AD. Stacked bar chart showing the percentages of [231+/+235], [231+/235], [231−/-235] and [231−/+235] cases in the three ALFA+ groups. Table includes the detailed composition of participants in percentages (and exact numbers) for each group. Discordant cases with the sequential phosphorylation hypothesis (that is [231−/+235]) are color-coded in grey.*

Discussion

This study is a combination of biomarker discovery in brain and a successful translation of neuropathological findings to clinical utility in CSF. We began with an exploratory IP-MS approach using brain to identify p-tau residues with biomarker potential. These initial findings suggested TBS soluble brain extracts reflect p-tau content in CSF and that p-tau235 is a highly specific phosphorylation in AD. Using a targeted IP-MS method, we confirmed these results and most importantly, we observed that p-tau235 only appears in combination with p-tau231, while the latter was detectable and quantifiable without p-tau235. These findings aligned well with previous reports suggesting a sequential phosphorylation of tau in the threonine and serine cluster located between amino acids 230 to 240 (2N4R numbering) [511]. Considering that CSF p-tau231 appears to be the earliest p-tau specie emerging in preclinical AD [469], we decided to investigate whether this sequential phosphorylation event in brain was translatable to CSF and if it had staging potential.

For this purpose, we developed and validated a novel immunoassay targeting specifically p-tau235 in CSF. CSF p-tau235 was found to be highly specific

biomarker for AD, and also to increase across the AD *continuum*. Furthermore, we demonstrated that CSF p-tau235 had a similar ability to identify symptomatic AD than CSF p-tau217 and CSF p-tau231. Interestingly, we found that CSF p-tau235 was increased early during preclinical stages in the TRIAD cohort. As this cohort focused on the full spectrum of AD, we decided to next investigate a larger preclinical cohort in ALFA+. In this cohort, we confirmed our previous finding that CSF p-tau235 begins increasing during preclinical AD stages. Moreover, we were able to demonstrate that this increase occurs very early during preclinical AD, when Aβ pathology starts to be detectable in CSF but not tau pathology as measured by CSF p-tau181. We also demonstrated that while both CSF p-tau217 and CSF p-tau231 discriminate better between A-T- and A+T-, CSF p-tau235 outperformed CSF p-tau217 and matched CSF p-tau231 when discriminating early and late preclinical participants (A+T- and A+T+ respectively). Finally, we demonstrated that the sequential phosphorylation event reported in AD brain translates well into CSF during preclinical AD stages, where the abnormal emergence of CSF p-tau231 is followed by a later increase in CSF p-tau235. Overall, these results indicate that the combined use of p-tau231 and p-tau235 could provide with a useful tool to discriminate between incipient AD from later preclinical AD cases in an imminent progression into cognitive decline. With clinical trials starting to focus more in targeting AD pathology at the earliest cases, such characterization of preclinical AD into early and late could help identifying the best window of opportunity for novel drugs and also serve as a tool for determining if AD progression is successfully tackled.

4.5 Paper V

Rationale

In the recent years, the field of fluid biomarkers in neurodegeneration has experienced a major expansion, particularly in regards to tau protein. Several research groups and pharmaceutical vendors have contributed to the development of a large variety of immunoassays targeting different p-tau and total tau species. One of the main differences between all these assays has to do with the "length" of the tau fragments measured, a characteristic that can be used to dichotomize tau assays into two categories: N-terminal and mid-region directed tau immunoassays. Interestingly, it appears that targeting N-terminal tau fragments, both in p-tau and total-tau assays, might pose a distinct advantage in terms of early detection and availability in blood. However, extracting a definitive conclusion in this regard is challenging, as no direct head-to-head comparison between N-terminal and mid-region targeted assays using same platform and same sample cohort has been ever performed. To address this, we developed three different t-tau immunoassays using a Simoa platform, two of them targeting N-terminal fragments of different lengths and one directed against tau mid-region. Subsequently, the CSF levels of these novel t-tau biomarkers and their performance was evaluated in comparison with (i) clinically validated mid-region p-tau and t-tau, and (ii) N-terminal p-tau assays (N-ptau181 and N-ptau217), using various cohorts: a pilot CSF cohort (n=44), a clinical cohort comprised by participants across the AD *continuum* (n=276) and other neurological conditions (including those characterized by high and normal levels of t-tau) (n=68). Finally, the novel t-tau assays developed were investigated in two plasma cohorts (pilot plasma cohort (n=44) and clinical cohort (n=50)), in order to assess their biomarker potential in blood.

Results

We began by developing and validating three novel Simoa CSF t-total assays: NTA (6-18aa to 159-163aa, 2N4R numbering), NTB (6-18aa to 194-198aa) and MR (159-163aa to 194-198aa) (*Fig. 32*).

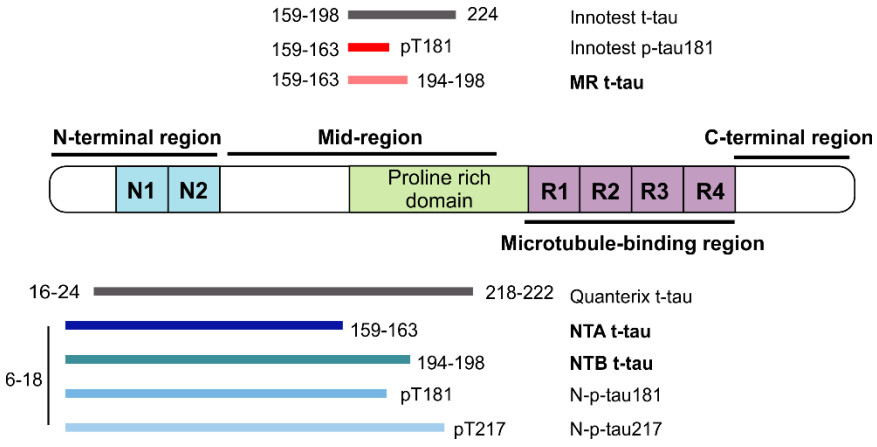


Figure 32. *Schematic representation of the different immunoassays (both t-tau and p-tau assays) included in the study. In bold letters, the newly developed t-tau immunoassays. The lines represent the minimum peptide length require for detection by each immunoassay.*

After this, we proceeded to evaluate their ability to identify AD in a pilot CSF cohort comprised by biologically defined AD cases and neurological controls. All three novel t-tau were significantly increased in AD cases ($p \leq 0.001$). We then compared their performance to discriminate between AD and control, finding that NTA and MR assays had statistically higher AUC values than NTB (AUC_{NTA}=88%, AUC_{MR}=88%, AUC_{NTB}=78%; $DeLong_{NTA\text{-}NTB}$ p=0.043, $DeLong_{MR\text{-}NTB}$ p=0.038). All in-house developed and clinically validated t-tau assay displayed strong correlations with each other ($r_S \geq 0.64$, $p<0.0001$).

We measured the in-house t-tau assays in a clinical CSF cohort comprising cases across de AD *continuum* as well as controls, Aβ- MCI and non-AD dementia cases. All three novel t-tau were increased in AD dementia cases when compared to controls, Aβ- MCI and non-AD dementia ($p<0.001$) (*Fig. 33*). In contrast, only NTA and NTB were able to differentiate Aβ+MCI from controls and Aβ- MCI (NTA p=0.0006; NTB p=0.0013) (*Fig. 33C-D*).

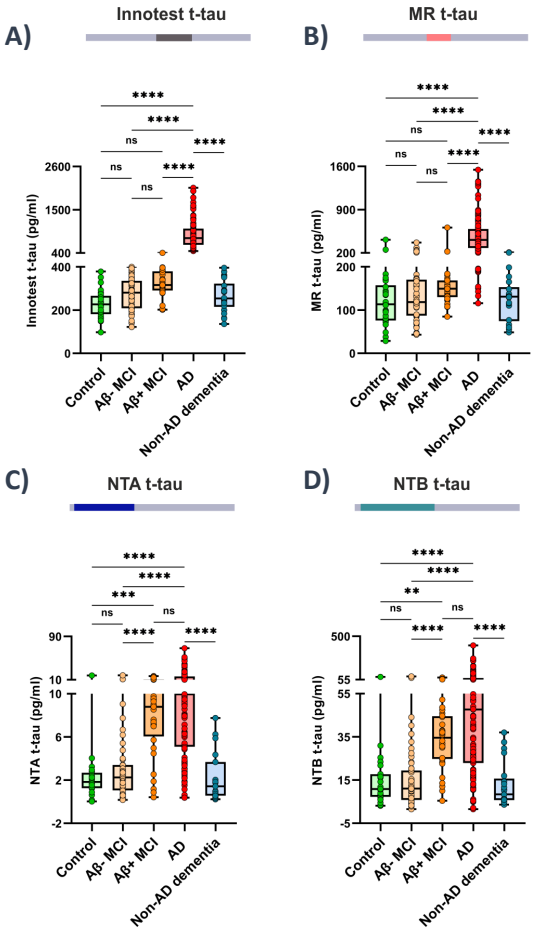


Figure 33. *T-tau biomarker (Innotest, MR, NTA and NTB) levels in the CSF across the AD continuum.* ***A)*** *Innotest t-tau was highly increased exclusively in AD,* ***B)*** *similarly to MR t-tau. On the other hand, both* ***C)*** *NTA and* ***D)*** *NTB were increased in Aβ+ MCI and AD groups. P values are indicated using asterisks: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, non-significant. Box-and-whisker plots showing the median and the 25th and 75th percentiles. Aβ (amyloid Aβ), MCI (mild cognitive impairment), AD (Alzheimer's disease).*

Subsequently, we compared the performance of the three novel t-tau assays with clinically validated Innotest p-tau181 and Innotest t-tau, and with in-house developed N-ptau181 and N-ptau217. Both NTA and NTB were able to accurately distinguished Aβ- MCI from Aβ+ MCI (AUC_{NTA}=84%, AUC_{NTB}=82%), with similar performances to N-p-tau217 (AUC=83%, $DeLong_{Np217\text{-}NTA}$ p=0.93;

$DeLong_{Np217\text{-}NTB}$ p=0.88) and N-p-tau181 (AUC=75%; $DeLong_{Np181\text{-}NTA}$p=0.20; $DeLong_{Np181\text{-}NTB}$ p=0.30). In addition, NTA and NTB t-tau assays outperformed MR t-tau (AUC 59%; $DeLong_{MR\text{-}NTA}$ p=0.0015, $DeLong_{MR\text{-}NTB}$, p=0.0012), Innotest t-tau (AUC 70%; $DeLong_{Innot\text{-}tau\text{-}NTA}$, p=0.046, $DeLong_{Innot\text{-}tau\text{-}NTB}$, p=0.056) and Innotest p-tau181 (AUC=65%; $DeLong_{Innop\text{-}181\text{-}NTA}$, p=0.016; $DeLong_{Innop181\text{-}NTB}$, p=0.024). On the other hand, when discriminating AD vs non-AD dementia cases, MR tau showed a nearly 100% AUC value (AUC=98%), performing significantly better than NTA and NTB (AUCs=90-91%; $DeLong_{MR\text{-}NTA}$, p=0.013, $DeLong_{MR\text{-}NTA}$, p=0.012), and matched the accuracies of N-ptau181 and N-ptau217 (AUCs=98-95%). We also examined the correlation between all tau biomarkers in the whole cohort, finding moderate to strong correlation among all tau assays (all $r_S \geq 0.63$, p<0.0001).

We continued the study by investigating the novel t-tau assays in CSF from CJD and acute neurological disorders (AND). All in-house t-tau biomarkers were increased in CJD and AND when compared to both AD dementia and control cases (all p<0.0001). Interestingly, larger overlap between CJD and AND was observed with NTA and NTB assays. Additionally, we observed larger fold changes (versus controls) for NTA and NTB when compared with MR t-tau and Innotest t-tau in both CJD (fold changes: MR=42, NTA=57, NTB=133) and AND (fold changes: MR=11, NTA=45, NTB=61). NTA and NTB displayed nearly perfect performance discriminating CJD and AD (AUCs=99%), similar to mid-region tau assays (all AUCs=90-98%; DeLong p>0.089), but better than N-ptau181 and N-ptau217 (AUCs 67-81%; $DeLong_{NTA\text{-}Np217}$, p<0.0001; $DeLong_{NTA\text{-}Np181}$, p=0.0006; $DeLong_{NTB\text{-}Np217}$, p<0.0001; $DeLong_{NTB\text{-}Np181}$, p=0.0003). On the other hand, MR t-tau, Innotest t-tau and N-ptau assays (all AUCs=82-74%) outperformed NTA, NTB and Innotest p-tau181 (all AUCs=60-51%) when differentiating CJD from AND.

We also investigated if our novel t-tau assays could discriminate PSP cases from control cases, however, neither our in-house t-tau nor in house N-p-tau assays or Innotest assays could successfully differentiate these groups.

Finally, we evaluated the performance of the three novel t-tau assays in a plasma pilot cohort and compared them with Quanterix t-tau (*Fig. 34A*). NTA showed the best accuracy differentiating AD and controls (p=0.0056, AUC=75%) (*Fig. 34B*), and thus, this assay was subsequently assessed in a plasma clinical cohort consisting of patients across the AD *continuum* and non-AD cases. In contrast with Quanterix t-tau (*Fig. 34C*), NTA was

significantly increased in AD when compared to MCI Aβ- (p=0.027) and controls (p=0.0033) (*Fig. 34D*). Most notably, the performance of NTA when differentiating AD and controls (AUC=94%) matched that of N-p-tau181 (AUC=97%) and N-p-tau231 (AUC=95%). Plasma NTA also displayed strong correlations with both plasma N-p-tau181 (r_S=0.68, p<0.0001) and N-p-tau231 (r_S=0.69, p<0.0001).

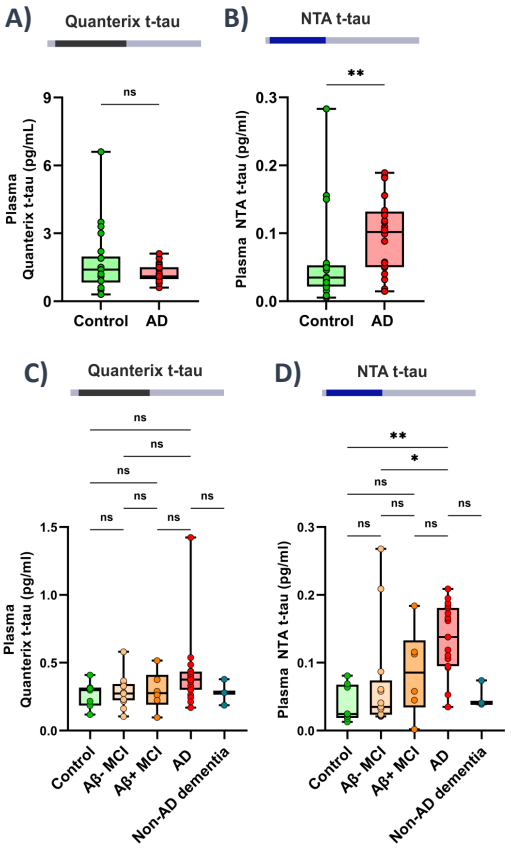


Figure 34. *Plasma Quanterix and NTA t-tau levels in pilot plasma pilot cohort and plasma clinical cohort. Quanterix t-tau assays unsuccessfully discriminated AD from other groups in neither* ***A)*** *the pilot nor* ***C)*** *the clinical plasma cohorts. Contrary to this, NTA assay was significantly increased in AD cases in both* ***B)*** *the pilot and* ***D)*** *the clinical plasma cohorts. P values are indicated using asterisks: *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; ns, non-significant. Box-and-whisker plots showing the median and the 25th and 75th percentiles. AD (Alzheimer's disease), Aβ (amyloid β), MCI (mild cognitive impairment).*

Discussion

It is generally agreed that tau protein in CSF is primarily comprised by fragments of different length between amino acids 1 to 254 [178, 418, 419]. It is also well documented that these fragments, regardless of the presence of phosphorylations or not, appear increased as a result of underlying AD pathology in brain (but not exclusively, such as t-tau in acute brain injuries and some chronic neurodegeneration) [299, 382, 404, 406]. With several immunoassays available targeting N-terminal and mid-region tau epitopes, but also phosphorylated and non-phosphorylated versions of the protein, is hard to pinpoint the actual benefits and drawbacks of targeting these species in different clinical scenarios. In consequence, is also hard to reach conclusions about their biomarker potential. Further adding complexity, these biomarkers can be and are measured in various platforms. Therefore, we tried to shed some light on this issue by developing three novel t-tau assays targeting different tau fragments using a Simoa platform, an comparing their performance with N-ptau181 and N-ptau217 (also Simoa assays) and gold standard Innotest p-tau181 and Innotest t-tau across AD *continuum* and in neurological disorders.

Our findings suggest that indeed different t-tau assays have different biomarker potential in CSF and plasma. One of our main findings was that N-terminal t-tau assays, that is NTA and NTB (but also N-ptau181 and N-p-tau181), were capable of successfully discriminating Aβ+ MCI cases from Aβ- MCI, which contrasted with mid-region t-tau assays (both in MR t-tau and Innotest t-tau). While at first glance this might appear to contradict the multitude of studies reporting CSF Innotest t-tau increases in Aβ+ MCI or MCI due to AD [299, 512, 513], it is important to take into consideration that the Aβ+ MCI cases in this cohort are A+T-N-, meaning that they represent the earliest cases of the MCI due to AD spectrum. This further highlights that N-terminal directed assays might have a distinct advantage when it comes to early detection of AD. Based on these results and MS reports [298], we hypothesized that N-terminal fragments are substantially less abundant than mid-region tau fragments in CSF, and that their subtle changes are highly meaningful for early AD detection. Therefore, while both mid-region t-tau assays studied here should be able to capture the N-terminal fragments measured by NTA and NTB, only the latter assays can exploit their early increase in AD, due to the N-terminal fragments being “diluted” in a large mid-

region pool of tau fragments. Altogether, these findings align well with previous reports indicating that N-terminal tau fragments are secreted early during AD pathology due to neuronal exposure to Aβ [419]. In contrast, MR t-tau assay performed significantly better than NTA and NTB when discriminating AD dementia from non-AD dementia cases, suggesting that MR t-tau assay are more suitable for diagnosing patients already at dementia stages. Another novel finding was that N-terminal targeted t-tau assays displayed higher fold changes in CJD and AND than mid-region t-tau assays, although this does not appear to have major clinical implications, as the levels of t-tau are severely increase for both type of assays.

Finally, we investigated the performance of the three novel t-tau assays in plasma and compared it with commercially available Quanterix plasma t-tau. NTA (6-18aa to 159-163aa) outperformed the other two N-terminal directed t-tau assays, that is NTB (6-18aa to 194-198aa) and Quanterix t-tau (16-24aa to 218-222aa), but also MR t-tau (159-163aa to 194-198aa), while it showed similar accuracies as N-p-tau181 and N-p-tau231. Previous reports have suggested that N-terminal directed t-tau assays can successfully discriminate AD from control cases in plasma [500-502] . Interestingly, their best performing assay, which they refer as NT1, uses the same antibodies as our NTB assay. They also reported that shorter N-terminal assays, specifically NT1, perform better than longer N-terminal counterparts [500, 502]. Although our NTB did not show a good performance in blood, our findings also suggest that targeting shorter N-terminal t-tau fragments results in a superior performance identifying AD in blood when compared with assays directed against longer N-terminal and mid-region tau fragments.

5. BLOOD P-TAU: A DEVELOPING TOPIC

From the publication of Paper I onwards, our group and several groups around the world have further corroborated and expanded these early findings. This has resulted in the development of various assays capable of measuring different p-tau residues in blood. At the moment, the most promising p-tau species in blood are p-tau181, p-tau217 and p-tau231, all of which have demonstrated high diagnostic potential. Therefore, the following discussion will focus exclusively on them.

Results included in this thesis, and also the following literature has demonstrated increases of blood p-tau181, p-tau217 and p-tau231 across the AD *continuum* using multiple analytical platforms [428, 509, 514, 515]. Similarly to CSF [469], these p-tau species appear to abnormally emerge early within the AD *continuum*, specifically during preclinical stages [509, 514, 515]. This is evidenced by the fact that all p-tau biomarkers can discriminate CU Aβ- from CU Aβ+ individuals but with no overt tau tangle pathology. Their main difference in preclinical AD stages is that p-tau231 seems to be the earliest p-tau abnormally emerging in blood [514], in a similar fashion to what Suarez-Calvet and colleagues previously reported in CSF [469]. The early emergence of p-tau181 and p-tau217 species has been elegantly shown in familial AD cohorts, where blood p-tau181 and p-tau217 started increasing approximately two decades before symptom onset [515-517]. Blood p-tau181, p-tau217 and p-tau231 have also been shown to accurately discriminate between AD and non-AD cases, not only when diagnosed clinically but also when neuropathologically confirmed [514, 515, 518]. Of note, nearly perfect discriminatory accuracies between AD and non-AD confirmed cases have been described for blood p-tau181, even 8 years prior to post-mortem examination [518]. These confirms their high specificity and association with AD pathophysiology. Some studies have compared the performance of blood p-tau181 and p-tau217 discriminating AD from non-AD, showing similar accuracies [490, 519, 520]. Similarly, this has been shown when comparing blood p-tau181 and p-tau231 [514]. Another important aspect to discuss is the association of blood p-tau species with Aβ and tau accumulation in brain. All three blood p-tau species have been demonstrated to strongly associate with Aβ deposition, correlating better in Aβ PET-positive

individuals [509, 514, 515]. In 2021, Mielke and colleagues compared the ability of blood p-tau181, p-tau217 and p-tau231 predicting Aβ PET, finding no differences between the three biomarkers [521]. However, subtle but relevant differences in this regard have been reported by other groups. For example, it has been shown that plasma p-tau231 correlates with Aβ PET in CU Aβ- individuals with very subtle Aβ accumulations [514]. This highlights yet again the very early nature of plasma p-tau231 as biomarker of incipient AD pathology. Similarly, all blood p-tau species display strong correlations with tau PET [509, 514, 515]. Interestingly, when looking at their association with tau PET and neuropathological NFTs across the AD *continuum*, this is stronger at early phases of tau accumulation, and once late stages are reached, this seems to plateau [509, 514, 515, 518]. While no differences between all three blood p-tau biomarkers have been found in terms of tau-PET prediction [521], plasma p-tau231 levels appear to increase in a more gradual manner across Braak stages when compared to plasma p-tau181 [514].

6. CONCLUSIONS

The studies included in this thesis, and subsequently the wider literature, which was published as a consequence of these findings, explored the value of a single protein, tau, as a biomarker primarily, but not exclusively, for AD (see paper V with CJD and acute neurological diseases). In the early 90s, pioneering assays capable of measuring p-tau and t-tau in CSF were developed. These assays demonstrated a strong correlation, suggesting that they reflected the same pathophysiological events in brain. While this in principle is true, our knowledge of the development of AD has subsequently increased and subtle but meaningful differences in p-tau has come to light. Likewise, investigating the potential biomarker value of different tau fragments now has tremendous value, possible given the detailed characterisation of AD *continuum* via molecular imaging of amyloid and tau aggregates. The clinical validation of CSF mid-region p-tau181 and t-tau, which now are emended in the diagnostic criteria for AD, only depict AD at the symptomatic phase stage of the disease.

First and foremost, the work in this thesis, specifically Papers I, II and III has contributed to achieve the long-sought goal of dementia evaluation in primary care – a blood test for AD by p-tau (p-tau181 and p-tau231). The results presented in paper II demonstrated the first neuropathological validation of blood p-tau and demonstrated the first insight into longitudinal trajectories in MCI and AD dementia patients. While CSF and imaging biomarkers have shown, and continue to show, excellent clinical utility, their worldwide implementation has always been hampered by their invasiveness, high costs and the need of specialized centres. Our group and others have demonstrated that blood p-tau is a highly specific biomarker of AD, consistently showing very high clinical performance (even at preclinical AD stages) and a useful tool of differential diagnosis of AD from other neurodegenerative diseases (nearly perfect discrimination in neuropathologically confirmed cases) and high association with AD progression (determined through MRI and amyloid/tau PET). Thus, the work by us and others in this regard opens a new chapter in the field of fluid biomarkers of neurodegeneration, making ever closer the worldwide access to non-invasive, cost-effective and simple blood testing. Furthermore, blood

p-tau species have the potential to become highly relevant tools in clinical trials, both in terms of patient recruitment, target engagement and outcome evaluation.

Another important achievement of this thesis was the development of a biomarker capable of staging preclinical AD. This is especially important when considering that this asymptomatic phase can last up to 20 years, and thus preclinical AD patients can be at very different stages of disease progression. While we have biomarkers capable of detecting preclinical AD with high accuracy, these biomarkers cannot determine whether an individual is at the beginning of the asymptomatic phase (A+T-) or approaching symptom onset (A+T+). Thus, Paper IV describes a novel CSF biomarker, p-tau235, capable of staging preclinical AD in fluid on the basis of the sequential phosphorylation of tau in AD brain at the threonine/serine cluster found between amino acids 230 and 240. Hence, this paper highlights the potential use of p-tau235 in combination with p-tau231 to identify and enrich clinical trials with participants at earliest phases of preclinical AD, in which pathology is not yet overt and in which novel drugs would stand a better chance of successfully tackling the pathology. Moreover, the longitudinal trajectories of p-tau235 and p-tau231 biomarkers could also help evaluating the performance of novel therapeutic compounds.

Finally, this thesis also addressed another important question of tau species (both p-tau and t-tau) as biomarkers: does targeting N-terminal or mid-region tau fragments pose a distinct biomarker advantage? The recent expansion in the field of fluid biomarkers in neurodegeneration, particularly in tau biomarkers, has resulted in the development of several p-tau and t-tau immunoassays targeting tau species of various peptide lengths and using a wide array of analytical platforms. Based on our successful development of immunoassays capable of measuring p-tau species in blood, we hypothesized that N-terminal containing tau species might possess a distinct advantage in terms of early detection and blood availability. We addressed this knowledge gap in Paper V, where we explored the biomarker potential of three newly developed in-house t-tau assays targeting different peptide lengths and comparing their performance with various commercially available and in-house developed p-tau and t-tau assays. This work indicates that targeting N-terminal containing tau species may indeed provide a distinct advantage when it comes to early AD detection. Most importantly, this allowed the

successful transfer of CSF NTA t-tau into blood, adding yet another tool to the blood biomarker list.

In conclusion, this thesis highlights the distinct roles of various tau species (both phosphorylated and non-phosphorylated) as biomarkers in fluid, and their ability to reflect a wide variety of meaningful aspects of brain pathology, spanning from asymptomatic to symptomatic AD, as well as primary tauopathies and acute neurological disorders.

7. FUTURE PERSPECTIVES

Altogether, the blood p-tau biomarkers presented in this work, p-tau181 and p-tau231, and others reported by other research groups *e.g.,* p-tau217 are showing great promise. However, the future is no exempted of challenges. At the moment, these novel biomarkers are generally measured in well-characterized research cohorts. This makes the direct translatability of these results into real-world clinics, that exhibit a heterogeneous population exempt from exclusion criteria, a major focus of further research. Also, there is limited information of these novel biomarkers in diverse populations, both in terms of social-economic backgrounds and ethnicities. Further, how pre-analytical procedures and common comorbidities in the ageing population (*e.g.,* renal function or cardiovascular disease) affect the levels of blood biomarkers is currently not known. Another important aspect is the direct comparison of all these novel biomarkers. Currently, there are several tau biomarkers targeting different phosphorylations and fragments, but also several assays using different analytical platforms (*e.g.,* Simoa and Meso Scale Discovery [MSD]), antibodies, reagents, and more. Therefore, head-to-head comparisons and standardization efforts would be an outstanding task in the coming years. It can be expected that pharmaceutical and biomedical companies, together with research institutions around the world, will play a major role in achieving these objectives, and in fact several of them have already set in motion plans to address these upcoming challenges.

Like other CSF biomarkers, the next step to follow with p-tau235 will be to assess its utility in blood. Unlike p-tau181 or p-tau231, the abundance of p-tau235 in CSF is comparatively low, and with an estimated 100-fold decrease between CSF and blood, this might be quite challenging. However, the rapid development of new analytical platforms in terms of sensitivity suggests that this will be possible soon. Meanwhile, our immediate goal is to (i) further assess the performance of this novel biomarker in clinical cohorts, comparing its diagnostic performance with other p-tau biomarkers, and (ii) confirm its preclinical AD staging ability in cohorts comprised by genetic AD, such as fAD and Down's syndrome.

When it comes to the novel NTA t-tau, our goal is to further investigate this biomarker in blood. The promising preliminary results described in Paper V were assessed in small but well characterised cohorts. Therefore, our aim now is to expand these findings by analysing larger cohorts, with special focus on those with longitudinal measures of neurodegeneration (*e.g.,* MRI atrophy or brain glucose metabolism), to determine whether this novel blood biomarker can provide a cost-effective and accessible reflection of accumulating neural injury. Moreover, as t-tau is a biomarker of neurodegeneration, this opens the possibility of exploring NTA in various scenarios such as TBI, CTE, cardiac arrest and non-AD tauopathies, potentially extending its utility outside of AD.

Last, but not least, there is an important aspect that requires further research, and that this thesis has highlighted: the need for more research into biomarkers for non-AD tauopathies (*e.g.,* PSP, CBD, FTD or MSA). In primary care, in combination with a detailed clinical assessment, our work has shown the low p-tau result is indicative of a non-AD dementia. This can also be combined with a high NfL for more accuracy. However, these results do not reveal the underlying pathology. As tau pathology is a common pathophysiological element in these diseases, is not hard to speculate that the biomarker potential of tau protein is not yet fully uncovered. Furthermore, FTD syndromes exhibit protein aggregates and pathology outside of tau (*e.g.,* TDP-43). Two possible alternatives to address this might be: (i) biomarker discovery in brain and translation into fluid, such as in Paper IV, and (ii) biomarker discovery in fluid. The first alternative has the advantage of neuropathological confirmation and the great abundance of tau or other protein aggregates in brain. However, the latter advantage also can be viewed as a double-edge sword, since pathology-relevant tau species could be diluted by the excess of other non-disease-relevant tau species. On the other hand, working with fluid is a more direct approach for biomarker discovery. However, big challenges surround this alternative. First, tau in fluid is present at low concentrations, and CSF, where is more concentrated, is very precious due to the limitations associated with lumbar puncture. Secondly, clinical diagnosis of non-AD tauopathies, even if supported by biomarker measurements, rarely matches definitive post-mortem diagnosis. This could be bypassed if one would use a neuropathology confirmed CSF cohort, however, this kind of cohorts are very limited and precious. Finally, non-AD tauopathies cases are substantially lower in numbers when compared to AD

cases, and most often than not, neuropathological assessment reveals the presence of mixed pathologies, further reducing the numbers of pure non-AD tauopathies cases. While all the aforementioned may complicate a biomarker breakthrough, the incredibly fast development in MS techniques in terms of throughput and sensitivity may soon discover disease specific PTM's, finally providing targets for successfully developing novel biomarkers specific for non-AD tauopathies.

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